# **Original** Article

# Increased production of reactive oxygen species contributes to motor neuron death in a compression mouse model of spinal cord injury

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**Study design:** Experimental laboratory investigation of the role and pathways of reactive oxygen species (ROS)-mediated motor neuron cell death in a mouse model of compression spinal cord injury.

**Objectives:** To analyze ROS-mediated oxidative stress propagation and signal transduction leading to motor neuron apoptosis induced by compression spinal cord injury.

Setting: University of Louisville Health Science Center.

**Methods:** Adult C57BL/6J mice and transgenic mice overexpressing SOD1 were severely lesioned at the lumbar region by compression spinal cord injury approach. Fluorescent oxidation, oxidative response gene expression and oxidative stress damage markers were used to assay spinal cord injury-mediated ROS generation and oxidative stress propagation. Biochemical and immunohistochemical analyses were applied to define the ROS-mediated motor neuron apoptosis resulted from compression spinal cord injury.

**Results:** ROS production was shown to be elevated in the lesioned spinal cord as detected by fluorescent oxidation assays. The early oxidative stress response markers, NF- $\kappa$ B transcriptional activation and c-Fos gene expression, were significantly increased after spinal cord injury. Lipid peroxidation and nucleic acid oxidation were also elevated in the lesioned spinal cord and motor neurons. Cytochrome *c* release, caspase-3 activation and apoptotic cell death were increased in the spinal cord motor neuron cells after spinal cord injury. On the other hand, transgenic mice overexpressing SOD1 showed lower levels of steady-state ROS production and reduction of motor neuron apoptosis compared to that of control mice after spinal cord injury.

**Conclusion:** These data together provide direct evidence to demonstrate that the increased production of ROS is an early and likely causal event that contributes to the spinal cord motor neuron death following spinal cord injury. Thus, antioxidants/antioxidant enzyme intervention combined with other therapy may provide an effective approach to alleviate spinal cord injury-induced motor neuron damage and motor dysfunction.

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**Keywords:** spinal cord injury; reactive oxygen species; cytochrome *c*; caspase-3; apoptosis; motor neuron

# Introduction

Traumatic spinal cord injury (SCI) consists of primary insult and secondary damage leading to neurological dysfunction. The primary injury results from direct mechanical tissue disruption and acute cell necrosis.<sup>1</sup> The secondary effects of cell damage results from a cascade of biological processes that include increased oxidative stress, calcium mobilization, glutamate toxicity and inflammatory factors. The enhanced production of reactive oxygen species (ROS) during SCI appears to play an important role in neuronal cell death and neurological dysfunction.<sup>2,3</sup> ROS are molecules containing oxygen but with higher reactivity than the ground state of oxygen. These include oxygen-free radicals, for example superoxide, hydroxyl radicals, lipid radicals

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and nonradicals such as hydrogen peroxide, lipid peroxide and peroxynitrite. Owing to the chemical and biochemical natures of the reactive activity, ROS can directly interact with proteins, lipids and nucleic acids, leading to cellular and molecular damage, and consequently neurological dysfunction.<sup>4</sup> On the other hand, ROS may act as an intracellular messenger and modulate cellular responses, toxicity and cell death.<sup>5,6</sup> Inhibition and scavenging of ROS production have been demonstrated to increase functional recovery following traumatic CNS injuries, thereby suggesting that enhanced ROS production contributes to SCI-mediated neuronal cell damage and neurological dysfunction.<sup>7,8</sup>

Neurons are the most oxidative stress-susceptible cell type in the CNS. Both necrosis and apoptotic neuronal cell death have been detected in mouse and rat models following traumatic brain injury and/or SCI.<sup>9</sup> Excess levels of ROS initiate oxidative chain reactions, damage cellular molecules and ultimately lead to cell death.<sup>2,8-10</sup> Neurological and behavioral functional assays coupled with oxidative stress assay suggest that the enhanced ROS production plays an active role in neuronal cell death by secondary effects of SCI.<sup>4,8,11</sup> Nevertheless, the specific cellular compartments of ROS generation, oxidative stress propagation and biochemical actions leading to neuronal cell, particularly motor neuron cell death remain largely unknown. In this study, we applied a severe mouse spinal cord compression injury model combined with transgenic mouse systems to analyze the role of ROS in SCI-mediated motor neuron cell death. We demonstrated that enhanced ROS production is an early and likely causal event that contributes, at least partially, to motor neuron cell death after SCI.

# Materials and methods

## Animals

Normal adult (80–90-day-old with an average body weight of 25–30 g) female C57BL/6J mice (Stock # 000664, Jackson Laboratory, Bar Harbor, ME, USA), age and body weight matched SOD1-transgenic mice (Stock #002629, Jackson Laboratory, Bar Harbor, ME, USA) and NF- $\kappa$ B promoter-driven LacZ reporter transgenic mice<sup>12</sup> were used to study the role of ROS in SCI-mediated motor neuron death. All mice were handled in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the suffering that might be experienced by the mice and to reduce the number of animals used in the mouse compression SCI model.

## Experimental model for mouse compression SCI

The experimental model for mouse severe compression SCI was essentially similar to that previously described with minor modifications.<sup>13</sup> Briefly, animals were deeply anesthetized with pentobarbital in a dose 20 mg/kg body weight by i.p. After skin decontamination, a 15–20 mm midline incision was made, and a laminectomy of T12 to L3 vertebra was performed under a dissection micro-

scope. Animals were then placed in a modified stereotaxic apparatus, and a 20 g of weight was applied to the spinal cord for 5 min with a  $1 \times 2$ -mm rectangular plastic plate. After injury, skin was sutured and mice were kept under a heating lamp for recovery.

At different time points following SCI, mice were killed and spinal cords were harvested for fluorescent, biochemical and immunohistochemical analyses of ROS production and motor neuronal cell death.

# Assays of ROS production in the lesioned spinal cord and motor neurons

To determine whether ROS are involved in SCImediated motor neuron cell death in the severe compression model, we intraperitoneally (i.p.) administered 0.2 ml saline buffer containing 30% of DMSO alone (vehicle control) or saline buffer containing 2',7'dicholorfluorescein diacetate (DCF, 25 mg/kg) and hydroethidine (HEt, 15 mg/kg) dissolved in DMSO to adult C57BL/6J mice and transgenic mice overexpressing wild-type SOD1 (WT-SOD1) (80-90 days old, 25–30 g body weights, four mice per group).<sup>10</sup> Mice were then kept at room temperature conditions for 30 min followed by compression SCI. At 6h after SCI, mice were then perfused with 10 ml of 0.9% saline buffer followed by 10 ml of 4% paraformaldehyde (PFA). Spinal cord sections  $(12 \,\mu\text{m})$  were examined under a fluorescent microscope for DCF and HEt oxidation.

## Spinal cord tissue preparation

Spinal cord tissue preparation for biochemical analysis At different time points (1, 6, 12, 24 h) after SCI, mice were reanesthetized and perfused with 20 ml of 0.9% saline buffer. The epicenter and 3 mm of adjacent (rostral and caudal) regions were excised and homogenized on ice in  $1 \times \text{RIPA}$  lysis buffer (150 mM NaCl, 1% Nonidet P-40, 12 mM sodium deoxycolate, 0.1% SDS, 50 mM Tris-HCl, pH 7.2) supplemented with protease inhibitors (1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin). After centrifugation and determination of protein concentration (Bio-Rad, Hercules, CA, USA), the spinal cord lysates were used for a variety of biochemical analyses.

Spinal cord tissue preparation for analysis of cytochrome c release At different time points (1, 6, 12, 24h) after SCI, mice were reanesthetized and perfused with 20 ml of 0.9% saline buffer. The epicenter and 3 mm of the adjacent (rostral and caudal) regions (total about 6–8 mm) of the lesioned spinal cord were excised and homogenized in 50 mM Tris-HCl buffer (pH 7.6) containing 100 mM NaCl and 1 mM EDTA and supplemented with protease inhibitors (1 mM PMSF,  $10 \mu g/ml$  leupeptin and  $10 \mu g/ml$  aprotinin) in a Dounce homogenizer. Once tissues and cells were disrupted, lysates were centrifuged at 12 000g at 4°C for 10 min. Cytosolic fraction (supernatant) was transferred to a

new tube, and the pellet was resuspended in 1/5 of original volume of the same buffer, homogenized on ice briefly and centrifuged under the same conditions. The supernatant from this centrifugation was then transferred to the cytosolic fraction-containing tube. The pellet was then lysed in  $1 \times \text{RIPA}$  buffer and sonicated for 15 s twice on ice. After centrifugation at 12000g at 4°C for 10 mins, the supernatant was transferred to a new tube (mitochondrial/ organelle fraction). Analysis of cytochrome *c* redistribution in cytosolic and mitochondrial fractions was performed by Western blotting as described below.

Spinal cord tissue preparation for immunohistochemical analysis At different time points (1, 6, 12, 24 h) after SCI, mice were reanesthetized and perfused with 20 ml of 0.9% saline buffer at room temperature followed by 40 ml 4% PFA in 1 × PBS (pH 7.5). The spinal cord was excised and placed in the same fixative buffer (4% PFA) overnight, followed by incubation in 20% sucrose in 1 × PBS (pH 7.5). After embedding in OCT, the spinal cord was sectioned at 12  $\mu$ m on a Leica cryostat (Model 3050S) and collected on Superfrost Plus slides. Once tissue sections dried completely, slides were stored in -80°C freezer until immunohistochemical analysis.

# Western blot analysis

Protein equivalents ( $20 \mu g$  of protein/well) from control and each time point after SCI were loaded to the wells and electrophoresed on a 12.5% SDS polyacrylamide gel (SDS-PAGE), blotted to an Immobilon-P membrane (Millipore, Bedford, MA, USA), probed with specific antibodies, and visualized by enhanced chemiluminescence (ECL) method.

# Lipid peroxidation assay

Lipid peroxidation measured by malondialdehyde (MDA) production in the samples was assessed using LPO-586 kit (OxisResearch, Portland, OR, USA)<sup>14,15</sup> with minor modifications.<sup>16</sup> Briefly, spinal cords from control and lesioned mice were dissected out, frozen in liquid nitrogen immediately and stored in -80°C until assay the following day. Spinal cord tissues were homogenized in 20 mM phosphate buffer (pH 7.4) containing 0.5 M butylated hydroxytolene to prevent sample oxidation. After protein concentration determination, equal amounts of protein (usually 1.5 to 2.0 mg protein from each sample) in triplicates were used to react with the chromogenic reagents at  $45^{\circ}$ C in  $500 \,\mu$ l buffer for 1–2 h. The samples were then centrifuged and supernatants were measured spectrophotometrically at 586 nm. The levels of MDA production were calculated with the standard curve according to the manufacturer's instructions (OxisResearch, Portland, OR, USA).

Lipid peroxidation was also assayed by immunohistochemistry using anti-MDA antibodies (Alpha Diagnostics Inc., San Antonio, TX, USA) as described in the following section.<sup>11</sup>

# Nucleic acid oxidation

The nucleic acid oxidation in the spinal cords was measured by the level of hydroxyguanosine using an 8-hydroxyguanosine (8-OHG) antibody (QED Bioscience, San Diego, CA, USA) as described previously.<sup>17,18</sup> A DAB approach was adopted following the manufacturer's instruction (Vector Laboratories, Burlingame, CA, USA).

# Immunohistochemical staining

For immunohistochemical staining, sections collected on Superfrost Plus slides were rehydrated in  $1 \times PBS$ (pH 7.4). After permeabilization with 0.2% Triton X-100 and blocking with 10% goat serum in  $1 \times PBS$ , sections were incubated overnight with specific antibodies at 4°C as follows: anti-active caspase-3 (Cell Signaling): 1:400; anti-NeuN (Chemicon): 1:400; anti-c-Fos (Santa Cruz): 1:250; anti-c-Jun (Santa Cruz): 1:250; anti-single-stranded DNA (Chemicon): 1:150. Sections were washed 6 times with 0.2% Triton X-100 in  $1 \times PBS$ for 5–10 min each, and then incubated with secondary antibody conjugated to either fluorescein or rhodamine for 2h at room temperature. After extensive washes, sections were mounted with antifade medium and examined under a fluorescent microscope. All images were collected and analyzed with a Nikon E800 fluorescent microscope equipped with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and Photoshop software (Adobe Systems, San Jose, CA, USA).

# Reporter gene LacZ ( $\beta$ -Gal) activity assay

For the wholemount  $\beta$ -Gal activity staining, control and lesioned spinal cords were fixed in the 2% PFA in  $1 \times PBS$  for 20 min at 4°C. After washing twice with  $1 \times PBS$ , the spinal cords were incubated in the X-gal solution (4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl<sub>2</sub> and 400 µg/ml X-gal) at room temperature for 6–8 h. Afterward, the spinal cords were washed several times with 1 × PBS and postfixed with 70% ethanol. The spinal cords were then photographed using a Leica microscope attached to a video camera.

For the histological  $\beta$ -Gal activity staining,  $12 \mu m$  sections were cut using a cryostat and collected on Superfrost Plus slides. The sections were then stained in the X-gal solution as described above.

For the  $\beta$ -Gal activity assay, spinal cord samples were harvested without PFA fixation. After lysis and protein concentration determination, the measurement of  $\beta$ -Gal activity was carried out according to the manufacturer's instruction using the activity assay kit (Promega Inc., Madison, WI, USA).

#### Apoptotic cell death analysis

Apoptotic cell death was analyzed by immunohistochemical staining of sections with TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) kit (Roche Biochemicals, Indianapolis, IN, USA) and anti-single-stranded DNA antibody (Chemicon, CA, USA) respectively, according to the manufacturer's instructions. The identification of neuronal cells undergoing apoptosis was achieved by double labeling with anti-NeuN and TUNEL or antisingle-stranded DNA antibodies, respectively.

#### Cell counting analysis of ventral motor neuron apoptosis in the lesioned spinal cord

The criteria established by Grossman *et al*<sup>19</sup> for determination and quantification of ventral motor neuron were used to analyze motor neuron apoptosis after SCI. Four spinal cord sections  $(100-150 \,\mu\text{m})$  interval, the lumbar region, caudal to the epicenter) from the lesioned center of each spinal cord (total three spinal cords (mice) were used in each group) were used to count TUNEL (or single-stranded DNA) positive motor neuron cells. The average numbers of TUNEL (or single-stranded DNA) positive motor neuron cells in the ventral horn of the lesioned spinal cord were quantified and compared to that of control and SOD1-transgneic mice.

#### Statistical analysis

At least three mice per group were used in each assay of the SCI experiment. Values were presented as mean  $\pm$  SE. Statistical analyses of lipid peroxidation and motor neuron cell death between lesioned spinal cords and that of controls were established with a twotailed Student's *t*-test and significance was accepted at P < 0.05.

# Results

#### Mouse spinal cord compression injury model

The severe compression injury model was selected to analyze the role of ROS in SCI-mediated motor neuron cell death according to a similar procedure described by Farooque.<sup>13</sup> After SCI, the mouse hind limbs remained completely paralyzed within 24 h, while the control mice had minimum alteration in walking behaviors. The morphological and pathological changes within the epicenter and adjacent areas of the control and lesioned spinal cords are shown in Figure 1a-c. There was extensive swelling, hemorrhage and degeneration as shown in the epicenter sections stained with hematoxylin and eosin (HE) (Figure 1b) and cresyl violet (Figure 1c). Tissue degeneration in the lesioned spinal cord 12 and 24 h after SCI is more severe than that of 1 and 6 h (Figure 1b and c). Apparently, some molecular processes may be responsible for the tissue and cell damage in addition to the initial mechanical injury.



Figure 1 Mouse model of the compression spinal cord injury. (a) Gross morphological changes of the lesioned spinal cord at different time points after surgery. (b) Cellular morphological changes of the lesioned spinal cord at different time points after surgery detected by HE staining. (c) Cellular morphological changes of the lesioned spinal cord at different time points after surgery detected by cresyl violet staining

# ROS production and oxidative stress propagation after SCI

*Increased ROS production after SCI* The fluorescent intensity of oxidized HEt represents the relative levels of superoxide production.<sup>20,21</sup> Similarly, oxidation of DCF reflects the general ROS production, particularly the peroxide production.<sup>22,23</sup> After a series of preliminary tests using different doses and different lengths of time of fluorescent dye exposure, we selected DCF dose at 25 mg/kg body weight, HEt dose at 15 mg/kg body weight and 6 h after SCI to analyze the relative levels of ROS production. As shown in Figure 2a, the oxidation of HEt and DCF was increased, indicating there was an increased ROS production in the lesioned spinal cord following SCI. On the other hand, transgenic mice overexpressing SOD1 gene exhibited decreased HEt and DCF oxidation compared to that of normal mice, confirming there is an enhanced ROS generation after SCI (Figure 2a).

SCI induces NF- $\kappa$ B transcriptional activation and c-Fos expression To study whether increased ROS production after SCI modulates oxidative stress marker gene expression, we examined NF- $\kappa$ B transcriptional activation and c-Fos expression in the mouse models. As shown in Figure 2b, transcriptional activation of NF- $\kappa$ B was markedly increased in mouse spinal cord after SCI compared to that of controls. Similarly, SCI also induced c-Fos (Figure 2c) and c-Jun (data not shown) expression in the spinal cord detected by immunohistochemical analyses. The increased oxidative stress response gene expression after SCI further indicates that there is an increase of ROS production.

Lipid peroxidation and nucleic acid oxidation are elevated in the lesioned spinal cords and motor neurons after SCI We further applied three different oxidation markers, MDA for lipid peroxidation, carbonyl protein for protein oxidation (data not shown) and 8-hydroxyl 208



Figure 2 SCI induces ROS generation and oxidative stress response gene expression in the lesioned spinal cord. (A) Steady state of superoxide and ROS generation in the lesioned mouse spinal cord compared to that of the surgical control and SOD1-transgenic mice as detected by hydroethidine (HEt) and DCF assays respectively. (B) Spinal cord injury induces NF- $\kappa$ B transcriptional activation in the  $\beta$ -gal reporter transgenic mouse model. (b1) Whole mount  $\beta$ -gal staining of the lesioned spinal cord compared to that of the surgical control. (b2) Betagal staining of the ventral horn region in the lesioned spinal cord compared to that of the surgical control. (b3) Beta-gal activity assay of the lesioned spinal cord compared to that of the surgical control (\* indicates P < 0.05). (c) Immunohistochemical analysis of c-Fos expression in the lesioned spinal cord compared to that of the surgical control and SOD1transgenic mice

guanosine (8-OH-G) for nucleic acid oxidation to analyze oxidative stress propagation in the spinal cords and motor neurons after SCI. Lipid peroxidation as detected by MDA levels was modestly but significantly increased starting at 6h after SCI (Figure 3a). The increases in lipid peroxidation were also detected in motor neuron cells after SCI by immunohistochemical staining (Figure 3a). Similarly, the increases of nucleic acid oxidation in spinal cord and motor neurons after SCI were also observed (Figure 3b). While the transgenic mice overexpressing SOD1 showed significantly less lipid oxidation (Figure 3a) and nucleic acid oxidation (data not shown) compared to that of normal mice under identical SCI conditions. The increased oxidative damage in lipids, proteins and nucleic acids in the lesioned spinal cord and motor neurons further suggests that there is increased ROS production and oxidative propagation.

# SCI enhances cytochrome c release, caspase-3 activation and motor neuronal cell death in lesioned spinal cord

We employed Western blotting and immunohistochemical staining approaches to analyze intracellular cytochrome c redistribution in the lesioned spinal cord, particularly in the spinal cord motor neurons. As shown in Figure 4a–d, cytochrome c release to the cytoplasmic fraction is increased upon SCI, while reciprocal decreases in cytochrome c residing in mitochondria occurs over time after injury. The fold-decrease of mitochondrial cytochrome c and the fold-increase of cytochrome c in cytosol compared with the respective controls after COXIV and  $\beta$ -actin calibration are shown in Figure 4b and d. Immunohistochemical staining further showed that cytochrome *c* immunoreactivity is distributed in a perinuclear pattern in control spinal cord motor neurons, while it is an increased release from mitochondria to cytosol in a perikaryal pattern in the lesioned motor neurons (Figure 4b).

Activation of caspase-3 activity contributes to apoptotic cell death in many systems including CNS.<sup>24</sup> Severe compression SCI increased caspase-3 activation as assayed by immunohistochemical staining (Figure 5a). Double immunolabeling with anti-NeuN and anticaspase-3 antibodies showed that active capase-3 is colocalized with some motor neuron cells in the lesioned spinal cord compared to that of surgical control (Figure 5a). Transgenic mice overexpressing SOD1 showed substantial reductions in SCI-mediated caspase-3 activation under identical lesion conditions (Figure 5a and b). To further analyze SCI-mediated motor neuron cell apoptosis, immunohistochemical staining of lesioned spinal cord sections was carried out with anti-NeuN antibody and TUNEL together. There was a significant increase of motor cell apoptosis in the lesioned spinal cord compared to that of surgical control (Figure 5c and d). Transgenic mice overexpressing SOD1 gene exhibited reduced SCI-mediated TU-NEL staining (Figure 5c and d). Immunohistochemical assessments with anti-single-stranded DNA antibody and anti-NeuN antibody further confirmed these findings of increased motor neuron apoptosis in the lesioned spinal cord (Figure 5c and d). Finally, a cell counting on the caudal region to the epicenter (lumbar region) demonstrated that there is a significant increase of



Figure 3 SCI-induced oxidative stress propagation in lesioned spinal cord. (A) Lipid peroxidation in the lesioned spinal cord and ventral horn motor neuron cells. (a1) Lipid peroxidation in the lesioned mouse spinal cord compared to that of the surgical control and SOD1-transgenic mice as detected by MDA levels in the spinal cord tissue. MDA production was modestly but significantly increased from 6 h and up after injury (\* indicates P < 0.05). (a2) Lipid peroxidation in lesioned spinal cord motor neurons compared to that of the surgical control and SOD1-transgenic mice by immunohistochemical analysis using anti-MDA antibody. (B) Nucleic acid oxidation in the lesioned spinal cord and ventral horn cells. (b1) Nucleic acid oxidation in the lesioned mouse spinal cord compared to that of the surgical control by immunohistochemical analysis using an anti-hydroxyguandine antibody. (b2) Nucleic acid oxidation in the ventral horn cells of the lesioned mouse spinal cord compared to that of the surgical control by immunohistochemical analysis using an anti-hydroxyguandine antibody.

SCI-mediated motor neuron cell apoptosis in the C57BL/6J mice compared to that of surgical control (Figure 5e). On the other hand, there is a significant reduction of apoptotic motor neurons in transgenic mice overexpressing SOD1 that underwent identical conditions of compression SCI (Figure 5e).

# Discussion

The present study has taken the advantage of a wellcharacterized experimental SCI model<sup>13</sup> and focused on the potential role of ROS in SCI-mediated neuronal, particularly motor neuron cell death. The data presented in this study have confirmed and extended previous observations regarding the role of oxidative stress in SCI-mediated neuronal cell damage and neurological

dysfunction: (1) increased ROS production is an early and possibly a causal event of SCI-mediated mouse spinal cord motor neuron cell death (Figure 2); (2) oxidative stress propagations including lipid peroxidation (Figure 3), protein oxidation (data not shown) and nucleic acid oxidation (Figure 3) may collectively promote motor neuron cell damage; (3) cytochrome crelease from mitochondria (Figure 4) and caspase-3 activation (Figure 5) contribute, at least partially, to the SCI-mediated motor neuron cell apoptosis; (4) the extent of ROS production, oxidant stress propagation and apoptosis were attenuated in transgenic mice overexpressing SOD1 gene (Figures 2-5), suggesting and reinforcing the concept that the cascade of oxidative damage contributes to SCI-mediated motor neuron death. Thus, antioxidant (antioxidant enzyme)-targeted





Figure 4 SCI induces cytochrome c redistribution in intracellular compartments. (a) Western analysis of cytochrome c in mitochondrial fraction of lesioned spinal cord after SCI compared to that of surgical control. (b) Relative levels of cytochrome c in mitochondrial fraction upon SCI compared to that of surgical control calibrated with cytochrome c oxidase IV (\* indicates P < 0.05). (c) Western analysis of cytochrome c released to cytosol in the lesioned spinal cord compared to that of surgical control. (d) Relative levels of cytochrome c released to cytosolic fraction upon SCI compared to that of surgical control calibrated with  $\beta$ -actin (\* indicates P < 0.05). (e) Immunohistochemical analysis of cytochrome c release from mitochondria to cytosol in the motor neurons of the lesioned spinal cord compared to that of the surgical control and SOD1 transgenic mice

interventions in conjunction with other therapeutic approaches may effectively reduce the neuronal damage from secondary effects of SCI, particularly those involving motor neuron cell death after SCI.<sup>27</sup>

## Mouse SCI model

Selection of mouse SCI model in the current study was primarily based on the availability of transgenic<sup>28,29</sup> and knockout<sup>30,31</sup> mouse systems as the useful tools to investigate the secondary effects of SCI on neuronal cell vulnerability. The transgenic (knockout) mouse model systems can be potentially applied to define the specific role of specific genetic element in protection/facilitation of neuronal vulnerability.<sup>28–31</sup> Several mouse SCI models, such as weight-drop, contusion and compression have been well established and applied to study SCI-mediated neuropathophysiological functions.<sup>1,32</sup> The reason that we adopted the compression approach to induce spinal cord injury is based on its reproducibility and simplicity.<sup>13</sup> As shown in Figure 1, the morphological, histological and pathological alterations in SCI compared to that of surgical control provided in this study reproduced and validated previous observations in the compression and other SCI models.<sup>13</sup> To a large extent, the combinations of the compression SCI approach with the transgenic mouse model of SOD1 overexpression have allowed us to identify and validate the molecular process of oxidative stress-mediated motor neuron death.

# Enhanced ROS production and motor neuron cell apoptosis in the compression SCI model

The roles of ROS in alteration of cellular function have been widely studied in different models of CNS injury.<sup>8,4,21</sup> Hall *et al*<sup>8,11</sup> have demonstrated that neuronal oxygen radical-induced lipid peroxidation was associated with neuronal cell vulnerability. Luo *et al*<sup>20,21</sup> have shown that there was an extensive increase





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*P*<0.05)

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Figure 6 Molecular pathways of ROS-mediated motor neuron cell apoptosis (death) resulting from the compression SCI. SCI-mediated ROS production leads to mitochondrial damage, cytochrome c release, caspase-3 activation and consequently motor neuron apoptosis (refer to context). Enhanced ROS production may also activate other molecular factors, such as calcium mobilization, glutamate toxicity and inflammation activation, which subsequently induce cell death. On the other hand, SCI-mediated activation of molecular factors, for example, calcium, glutamate and inflammatory cytokines may also induce ROS generation, and consequently cause motor neuron cell death

of ROS production analyzed by flow cytometry assay after SCI. More recently, studies by Sugawara et al<sup>10</sup> have demonstrated that enhanced ROS, particularly superoxide production, contributed to motor neuron death in the normal, but not transgenic rats overexpressing SOD1 gene. Nevertheless, the specific location and the specific effects of ROS production on SCI-mediated motor neuron death remain largely unknown. In the present study, we determined superoxide production by HEt assay and ROS production by DCF assay. Such measurements demonstrated that the increased cellular production of ROS including superoxide indeed occurs in mouse spinal cord and motor neuron cells upon SCI, particularly in view of the attenuated ROS production observed in SOD1 transgenic mouse spinal cords (Figure 2). In addition, the activation of oxidative response markers NF-kB, c-Fos (Figure 2) and c-Jun expression (data not shown) suggest that the increased generation of ROS is likely to contribute to the cascades of oxidative damage. The formation of highly reactive ROS was further shown to induce lipid peroxidation (Figure 3), protein oxidation (data not shown) and nucleic acid oxidation (Figure 3) in the lesioned spinal cord and motor neurons. These data together provide direct evidence to show that the increased ROS production and oxidative stress propagation contribute to SCI-mediated motor neuron death.

Biochemically, the increased ROS production can initiate a cascade of intracellular signal transduction leading to cell death.<sup>5,25</sup> Previous studies have demonstrated that cytochrome c release and caspase activation play pivotal roles in cell apoptosis after SCI.<sup>5,25,29</sup> More recently, Sugawara *et al*<sup>10</sup> have shown that release of cytochrome c from mitochondria is responsible for SCIinduced motor neuron apoptosis in the rat model. For this reason, we focused our attention on the potential role of mitochondria in motor neuron apoptosis after SCI. Our findings indicate that cytochrome c redistribution (Figure 4) and caspase-3 activation (Figure 5) underlie spinal cord motor neuron cell apoptosis. Oxidative stress-dependent release of mitochondrial cytochrome c is now well documented in traumatic brain and spinal cord injury.<sup>10,33</sup> Our results confirm and extend these previous findings and further demonstrate that the release of cytochrome c coupled with the activation of caspase-3 contribute to motor neuron apoptosis after SCI.<sup>10,24,25</sup> Since motor neuron apoptosis in SOD1 transgenic mice was reduced (Figure 5), it is more likely that the enhanced ROS production is a causal event that is responsible for SCI-induced motor neuron cell death.

It should also be pointed out that besides oxidative stress, other molecular processes, such as calcium mobilization, inflammatory factor production and excitatory amino acid release, may also contribute to SCI-induced motor neuron cell death. More likely, these molecular events may be coupled with ROS production and oxidative stress propagation to facilitate SCIinduced motor neuron cell death (Figure 6). Thus, the current compression SCI approach in conjunction with specific transgenic and/or knockout mouse lines will offer a model system to define the mechanisms of molecular interactions leading to the SCI-induced motor neuron cell death.

In summary, using a well-characterized compression mouse SCI model, we showed that enhanced ROS production is an early, and likely a causal event of motor neuron cell death (Figure 6). On the other hand, we demonstrated that modifications of the antioxidant/ antioxidant enzyme capacity, such as elicited by transgenic mice overexpressing SOD1, reduced motor neuron death. Thus, our study provides direct evidence to support the use of antioxidants/antioxidant enzyme systems in the pharmacotherapy of SCI-induced motor neuron damage and motor dysfunction.

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