Inhibition of x-irradiation–enhanced locomotor recovery after spinal cord injury by hyperbaric oxygen or the antioxidant nitroxide tempol

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Object. Hyperbaric oxygen (HBO), the nitroxide antioxidant tempol, and x-irradiation have been used to promote locomotor recovery in experimental models of spinal cord injury. The authors used x-irradiation of the injury site together with either HBO or tempol to determine whether combined therapy offers greater benefit to rats.

Methods. Contusion injury was produced with a weight-drop device in rats at the T-10 level, and recovery was determined using the 21-point Basso-Beattie-Bresnahan (BBB) locomotor scale. Locomotor function recovered progressively during the 6-week postinjury observation period and was significantly greater after x-irradiation (20 Gy) of the injury site or treatment with tempol (275 mg/kg intraperitoneally) than in untreated rats (final BBB Scores 10.6 [x-irradiation treated] and 9.1 [tempol treated] compared with 6.4 [untreated], p < 0.05). Recovery was not significantly improved by HBO (2 atm for 1 hour [BBB Score 8.2, p > 0.05]). Interestingly, the improved recovery of locomotor function after x-irradiation, in contrast with antiproliferative radiotherapy for neoplasia, was inhibited when used together with either HBO or tempol (BBB Scores 8.2 and 8.3, respectively). The ability of tempol to block enhanced locomotor recovery by x-irradiation was accompanied by prevention of alopecia at the irradiation site. The extent of locomotor recovery following treatment with tempol, HBO, and x-irradiation correlated with measurements of spared spinal cord tissue at the contusion epicenter.

Conclusions. These results suggest that these treatments, when used alone, can activate neuroprotective mechanisms but, in combination, may result in neurotoxicity.

Key words • spinal cord injury • contusion • locomotor function • irradiation • hyperbaric oxygen • tempol • rat

In several experimental models of SCI, x-irradiation of the injured spinal cord has been shown to promote recovery of locomotor function. Previously, x-irradiation was found to induce partial recovery of locomotor function following transection of the spinal cord that was associated with reduced cavitation and loss of spinal cord tissue.¹⁸–²⁰ More recently, x-irradiation improved locomotor function and spared spinal cord tissue after experimental compression injury²⁰ as well as contusion injury,²⁰ the most common type of SCI. The effects of x-irradiation are thought to be due largely to the production of ROS. These ROS, for example, O$_2^-$, H$_2$O$_2$, and OH$^-$, in turn react with biomolecules,²⁵ although the cellular targets within spinal cord tissue are unknown. It has also been proposed that the antiproliferative effect of irradiation may reduce populations of reactive astrocytes or other cell types, such as microglia and endothelial cells, that can promote neurodegeneration.²⁷ Another possibility is that irradiated tissue produces factors that are neuroprotective. For example, x-irradiation of the spinal cord can increase expression of vascular endothelial growth factor,¹ which can improve locomotor function following contusion injury.²⁰

Treatment with HBO²⁰ or the antioxidant nitroxide tempol²⁰ can also improve recovery from contusion injury in a model of SCI. These agents can penetrate spinal cord tissue and alter levels of ROS as well. Inspired HBO increases tissue O$_2$ tension, resulting in increased superoxide and H$_2$O$_2$ production.²¹,²² On the other hand, tempol affects tissue levels of ROS by acting as a superoxide dismutase mimic converting superoxide to H$_2$O$_2$ and by inhibiting conversion of H$_2$O$_2$ to hydroxyl radicals via abe-Weiess-Fenton reactions.²³ Treatment with a soluble form of superoxide dismutase also improves locomotor function after contusion injury and has been shown to enhance expression of other neuroprotective factors, including nerve growth factor, ciliary neurotrophic factor, and neurotrophin-3, as well as hypoxia inducible factor–1α, which regulates vascular endothelial growth factor expression.²⁵,²⁶

Abbreviations used in this paper: ANOVA = analysis of variance; BBB = Basso-Beattie-Bresnahan; HBO = hyperbaric oxygen; LSD = least significant difference; ROS = reactive oxygen species; SCI = spinal cord injury.
The extent of tissue oxygenation has been found to be related to the effectiveness of x-irradiation as an antiproliferative treatment for neoplastic cells in cancer. Conditions such as anemia or tumor hypoxia reduce efficacy.\textsuperscript{13} Consistent with this, HBO and erythropoietin were shown to increase a radiation-induced delay in animal models of tumor growth.\textsuperscript{4,22,31} The resulting radiosensitization can be attributed to increased ROS production derived from higher tissue O\textsubscript{2} levels during irradiation.\textsuperscript{24} Treatment with x-irradiation has also been used together with tempol in experimental studies as a radioprotective agent\textsuperscript{12} for tissue surrounding the tumor without affecting loss of the tumor itself.\textsuperscript{6,9,11,33} This protective ability is thought to be due to the predominance of the reduced and inactive form of tempol resulting from hypoxia within the tumor.

In our experiments, treatment with x-irradiation of the injured spinal cord was used concurrently with either HBO or tempol to determine whether there is greater benefit from combined therapy.

Materials and Methods

Adult female (~ 240 g) Wistar rats were obtained from Charles River Breeding Laboratories and housed in a temperature-regulated (23°C) animal facility. All of the procedures involving vertebrate animals were approved by the Institutional Animal Care and Use Committee of New York Medical College.

As in our previous studies,\textsuperscript{15,37–39} the spinal cords of the rats were contused at the level of T-10 with a weight-drop apparatus. The rats were anesthetized with an injection of pentobarbital sodium (60 mg/kg intraperitoneally). A laminectomy was performed aseptically at T9–10 to expose the spinal cord. The sinus processes at T-8 and T-11 were fixed with wound clips, and the rats received 0.02% amoxicillin in a 2 ml saline. Approximately 20 minutes after injury, with a dose of 275 mg/kg in 0.5 ml saline. Approximately 20 minutes after injury, the rats receiving HBO were placed into the hyperbaric chamber that had been flushed with 100% O\textsubscript{2}. Following sealing of the chamber the pressure of O\textsubscript{2} was increased gradually for 5 minutes to 2 atm for a period of 1 hour, after which the chamber was decompressed over a 5-minute period.

The x-irradiation of the SCI site was performed according to the methods of Kalderon and Fuks.\textsuperscript{2,10} Approximately 20 minutes after contusion, the spinal cord of each control animal was exposed to a single dose of 20 Gy of x-irradiation from a MaximA-R-100 (C.P. Type 1, General Electric) x-ray unit with a 2-mm aluminum filter at 90 kVp and 5 mA. The distance from the source to the dorsal surface was 10 cm, with 71% of the surface dose delivered at 5 mm depth to the spinal cord. The dose of irradiation was delivered dorsally to a 2 × 2.5–cm radiation field centered at the site of the lesion during a 9-minute period. For rats receiving combined treatment with HBO or tempol, x-irradiation was started 15 minutes after exposure to HBO or the administration of tempol. In all there were six groups of rats: x-irradiated (23 rats), tempol-treated (21 rats), HBO-treated (10 rats), x-irradiated and tempol-treated (13 rats), x-irradiated and HBO-treated (10 rats), and untreated control (20 rats).

Behavioral Analysis

Recovery of locomotor function was determined using the BBB locomotor scale.\textsuperscript{23} Briefly, the scale ranges from 0 (total paralysis) to 21 (normal locomotion). Scores 1 to 8 are assigned for small or large movements of the three joints of the hindlimb, a score of 9 indicates weight-bearing status or dorsal stepping, and scores of 10 to 21 indicate progressive improvements in coordinated walking ability. The rats were acclimatized daily to a circular observation area 3 ft in diameter for a week before surgery. For 3 consecutive days following contusion and at 1-week intervals thereafter up to 6 weeks postinjury, each rat was scored for locomotor function according to the BBB scale. To facilitate scoring, training materials, including a videotape of locomotor behavior corresponding to the levels of the BBB scale, were kindly provided by Dr. D. Michele Basso (Ohio State University). The score for each animal was assigned by two observers who were blinded to the treatment regimen during each 4-minute session of open-field testing. The scores for both hindlimbs were averaged to obtain the score for each session.

Spinal Cord Histomorphometry

Immediately after the final behavioral evaluation, the spinal cords were fixed by transcardial perfusion in the anesthetized rats (pentobarbital sodium 60 mg/kg intraperitoneally) with phosphate-buffered saline (pH 7.4) containing 1% glutaraldehyde and 4% paraformaldehyde, dissected, and embedded in paraffin for serial sectioning with a microtome. Spinal cords were sectioned transversely from T-9 to T-11 in 10-mm blocks including the contusion site, which could be visualized externally. The contusion site was sectioned transversely throughout at a thickness of 15 μm and stained for myelin with Luxol fast blue and counterstained with cresyl violet as previously described.\textsuperscript{4} Quantification of the cross-sectional area of spared spinal cord tissue either absolutely or relative to total spinal cord cross-sectional area was performed with planimetry software (SigmaScan) from digitized images (Photometrics). The digitized images of the stained section exhibiting the largest lesion, that is, the contusion epicenter, of each spinal cord were obtained without knowledge of the treatment condition.

Statistical Analysis

The statistical significance of the effects of treatment with x-irradiation, HBO, and tempol on locomotor scoring was determined by mixed factorial ANOVA with repeated measures and one-way ANOVA and by the LSD or Duncan multiple range test post hoc (SPSS, Inc.). The statistical significance of the effects of these treatments on sparing of spinal cord tissue was determined with the LSD multiple range test. Linear regression analysis was performed with Excel 4.0 (Microsoft). Statistical significance was determined at a probability level less than 0.05.

Results

Behavioral Recovery

Contusion injury decreased locomotor function, as determined by BBB locomotor scores, to a level of complete paralysis for several days following injury (Fig. 1A). Partial recovery of locomotor function then occurred within 1 to 4 weeks of injury to levels that were maintained for the remainder of the 6-week observation period.

The x-irradiation at the spinal cord contusion site administered 20 minutes after injury significantly increased locomotor recovery during the 6-week observation period relative to the unirradiated-contused treatment group (Fig. 1). Significant increases in BBB scores were found beginning at the 2nd week after irradiation, with further increases during the following several weeks to plateau levels. These results were similar to previous results obtained with x-irradiation.\textsuperscript{38} Tempol (275 mg/kg) administered to rats at 20 minutes after injury improved locomotor recovery relative to the untreated rats with significant increases in BBB scores, ob-
served at the 3rd through 6th weeks after injury (Fig. 1) as previously reported. On the other hand, treatment with HBO 20 minutes after injury did not significantly improve locomotor recovery relative to the untreated injured group (Fig. 1).

In contrast to treatment with x-irradiation alone, x-irradiation combined with prior treatment with either HBO or tempol did not significantly improve locomotor recovery relative to the untreated injured group (Fig. 1).

Histological Study

Weight-drop contusion resulted in the appearance of a centrally located lesion within the spinal cord and sparing of a peripheral rim of tissue at the contusion epicenter (Fig. 2 left) as previously described. The central lesion consisted mostly of areas of gliosis and cyst formation or cavitation and included most or all of the area previously occupied by gray matter as well as contiguous regions of white matter. The peripheral rim of spared tissue consisted of remaining white matter and in some cases small areas of gray matter including the dorsal horn. Within the spared white matter, staining with Luxol fast blue indicated the presence of myelinated fibers, although scattered microcysts indicating axonal injury were also observed.

Treatment of the contusion site with x-irradiation or with tempol significantly increased the amount of spinal cord tissue spared compared with untreated contused spinal cords (Fig. 2 right) without altering the histological appearance of the centrally located area of cavitation and peripheral rim of white matter (Fig. 2 left). In contrast, x-irradiation together with tempol, HBO alone, or HBO together with x-irradiation did not significantly increase the amount of spinal cord tissue spared compared with untreated contused spinal cords.

To determine the relationship between tissue sparing and locomotor recovery, as demonstrated in previous studies, regression analysis was performed between the percentage of spared tissue and final BBB scores for each treatment group. A significant linear relationship was found between the mean values of the final BBB score and the percentage of spared tissue (Fig. 3) for each treatment group ($r^2 = 0.70$, slope $0.27/1000$, $p < 0.05$).

Radiation-Induced Alopecia

To determine whether tempol conferred protection against radiation-induced alopecia as in previous studies, the $2 \times 2.5$–cm area of skin at the irradiation site was examined for hair loss 6 weeks after irradiation. Hair loss was extensive in the rats that were exposed only to x-irradiation, so that the underlying epidermis was visible in the irradiated area of skin (not shown). In contrast, in rats in which tempol was administered before x-irradiation, hair abundance appeared similar to surrounding nonirradiated skin where hair loss did not occur.

Discussion

In this study we have demonstrated that treatment with either HBO or the antioxidant nitroxide tempol can inhibit the effect of x-irradiation of the injured spinal cord in enhancing recovery of locomotor function following spinal cord contusion injury. Because each of these agents has previously been shown to enhance locomotor recovery following contusion injury, it was hypothesized that there would be greater benefit from combined therapy. Contrary to this expectation, however, combined treatment with x-irradiation and tempol did not enhance—but rather inhibit-

Fig. 1. Graphs showing the effects of x-irradiation (X-ray), tempol, and HBO on the time course of recovery of locomotor function following spinal cord contusion injury. A: Values are the means ± standard errors of the mean (SEMs) of determinations of locomotor recovery according to the BBB locomotor scale. *$p < 0.05$, mixed factorial ANOVA with repeated measures (group $F(5,91) = 2.5$, $p < 0.05$; session $F(5,455) = 253.8$, $p < 0.0005$; group–session interaction $F(25,455) = 1.9$, $p < 0.01$) and LSD post hoc. There was a significant effect of treatment with x-irradiation or tempol compared with untreated rats receiving the same injury. B: Mean final BBB scores ± SEMs from panel A. *$p < 0.05$, one-way ANOVA ($F(35,546) = 13.1$, $p < 0.0005$) and Duncan multiple range test post hoc. There was a significant effect of treatment with x-irradiation or tempol compared with untreated rats receiving the same injury.
ed—improved locomotor function relative to that observed when tempol or irradiation was administered alone. In the case of HBO treatment, although HBO administered alone did not significantly improve recovery in our experiments, hyperoxia was sufficient to prevent significant improvement in recovery seen in rats exposed to x-irradiation. The lack of additivity in the effectiveness of these treatments in improving locomotor recovery by combined treatment with these agents suggest that a similar neuroprotective mechanism is activated by x-irradiation, HBO, and tempol.

A consistent feature of studies in which the contusion model of SCI is used is that the magnitude of locomotor recovery is closely paralleled by the extent of tissue sparing at the contusion epicenter over a range of injury severities.\(^2,3,15,27,37–39\) In agreement with this, we found that the degree of improved locomotor recovery according to the BB8 score with either separate (x-irradiation, HBO, or tempol) or combined treatment was directly related to the amount of spinal cord tissue sparing at the epicenter. The degree of improvement was greatest in x-irradiated rats and least in untreated rats. The slope of the linear regression, 0.27 (BBB score/percentage of tissue sparing), was similar to values of 0.22 and 0.20 found in several previous studies.\(^37–39\) This relationship is likely to be due to loss of myelinated axons at the contusion epicenter that are necessary to support locomotion. The amount of sparing at the epicenter where tissue loss is greatest thus puts an upper limit on the number of functional axons that traverse the epicenter to coordinate lumbar pattern generators.

How then would x-irradiation spare spinal cord tissue and improve locomotor recovery following SCI in a manner that would be inhibited by elevated O\(_2\) tension or the presence of the nitroxide tempol? Tissue x-irradiation has multiple effects, including inhibition of cell proliferation, increased apoptosis, and altered redox signaling. The main therapeutic use for x-irradiation is to prevent cell proliferation responsible for neoplasia by inhibiting cell cycling or promoting apoptosis via denaturation of DNA. It has been proposed that this antiproliferative effect of irradiation may also be used to reduce populations of reactive astrocytes or other cell types such as microglia and endothelial cells that, in turn, promote neurodegeneration following SCI.\(^17\) Reactive astrocytes and microglia may produce abundant levels of ROS as well as reactive nitrogen species, such as peroxynitrite, that are injurious to oligodendrocytes and axons. Endothelial cells generated as a consequence of injury-induced angiogenesis may produce vessels that compromise the blood–spinal cord barrier so that harmful blood

![Fig. 2. Effects of x-irradiation, tempol, and HBO on sparing of spinal cord tissue following spinal cord contusion injury.](image-url)
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components are no longer excluded, thereby causing greater secondary destruction of the spinal cord tissue. The finding that treatment with an antiangiogenic agent, CM101, improved recovery in an experimental model of SCI suggests a negative role for cell proliferation involved in angiogenesis. Interestingly, recent evidence suggests that proliferating endothelial cells are a cellular target in radiotherapy for cancer.

The therapeutic efficacy of the combined use of either HBO or tempol together with x-irradiation for SCI differs from previous results with radiotherapy for cancer, suggesting that other non–antiproliferative actions of x-irradiation are involved. The effectiveness of x-irradiation in preventing cell proliferation as a treatment for cancer has been found to be directly related to the level of tissue oxygenation, which can increase the generation of superoxide due to ionizing radiation. Conditions such as anemia or tumor hypoxia reduce efficacy so that efforts are made to increase O2 delivery to the cancerous tissue to be treated. Consistent with increased sensitivity to radiation, HBO was shown to increase the delay in tumor growth induced by x-irradiation in an experimental animal tumor model. The effect was achieved only when x-irradiation was administered within 5 minutes of HBO treatment, in agreement with a requirement for tissue hypoxia. The resulting increase in antiproliferative activity can be attributed to increased ROS production derived from higher tissue O2 levels during irradiation. In contrast, when the same pressure and duration of HBO treatment followed closely by x-irradiation was used in the present experiments to treat spinal cord–contused rats, reduced, rather than greater, efficacy was observed. Thus, desensitization rather than sensitization to radiation results from HBO, even though SCI leads to hypoxia that would be at least in part reversed by HBO. It is possible that lower doses of irradiation could be used in combination with either HBO or tempol to produce optimal levels of ROS for neuroprotection. Such radiosensitization might reduce the dose of irradiation required for efficacy, which would be desirable for treating spinal cord–injured patients.

On the other hand, tempol, which acts as a radioprotective agent, also caused desensitization to x-irradiation for treating SCI that was similar to the effect of treatment with HBO. In studies of radiotoxicity in different tissues, tempol prevented radiation-induced death due to bone marrow failure after whole-body irradiation, loss of salivary gland tissue due to irradiation of the neck, as well as alopecia following skin irradiation. These effects can be attributed to the ability of tempol to oppose the antiproliferative action of radiation on pools of stem cells within these tissues. Although tempol has radioprotective activity, the antioxidant did not affect the ability of radiation to delay tumor growth. This is thought to be due to the predominance of the reduced and inactive form of tempol, hydroxylamine, resulting from hypoxia within the tumor, a condition that exists at the SCI site. Consistent with the radioprotective properties of tempol, in these experiments tempol prevented radiation-induced alopecia at the irradiation site while simultaneously inhibiting enhanced recovery of locomotor function caused by irradiation. It appears contradictory, however, that HBO, which sensitizes, and tempol, which desensitizes tissue to the antiproliferative effect of x-irradiation, would cause similar decreases in efficacy for treating SCI.

Alternatively, these agents may act within the spinal cord by a mechanism that does not involve cell killing, but instead upregulates neuroprotective factors as a result of elevated levels of ROS. These agents could act like preconditioning agents except that instead of acting prior to injury, they would be protective for neurodegenerative events that occur with delay following SCI. Numerous agents exert protective action in the central nervous system when administered before injury. For example, treatment with tumor necrosis factor–α, brief ischemia, or repeated HBO, which leads to increased generation of ROS, can all induce tolerance to neurodegeneration caused by ischemic injury to the brain.

In the present experiments we have demonstrated, as in our previous studies, that x-irradiation improves recovery of locomotor function following spinal cord contusion injury. The use of x-irradiation has also been shown to enhance locomotor recovery after spinal cord compression and transection injury. Recently, authors of several reports also examined the effects of x-irradiation in spinal cord contusion injury. Kalderon and coworkers (unpublished data) have shown that a 20-Gy dose of x-irradiation fractionated into 10 daily doses starting 8 days after injury improved tissue degeneration after spinal cord contusion in rats, although a procedure for reducing intraspinal fluid pressure was required. In another study, contrary to the present results, 22 Gy of x-irradiation was ineffective for improving locomotor recovery after contusion injury. Differences in the conditions of irradiation, such as greater dose rate and energy of irradiation, may account for this discrepancy. For example, for a given total dose of irradiation, delivery at higher dose rates can increase cell killing, whereas lower dose rates were shown to be more effective
in stimulating expression of several protooncogenes that are involved in redox signaling, including c-jun, jun-B, and c-fox. In addition, the total dose of irradiation delivered to the spinal cord may be difficult to control when falloff due to tissue penetration is high. Although x-irradiation did not improve locomotor scores, reactive astrogliosis was greatly reduced, indicating sufficient irradiation for killing astrocytes. Conversely, authors of another study showed that 25 Gy of x-irradiation delivered to the injured spinal cord did not reduce populations of astrocytes, but microglia were less abundant. To resolve these differences in the effects of x-irradiation, systematic examination of variation in dose rate, total dose, and spectral energy should be performed to optimize the effectiveness of irradiation.

Hyperbaric O₂ did not significantly improve locomotor recovery in the present experiments, although a similar dose was effective in previous studies. Again, several differences in experimental procedures between these studies, including injury severity and O₂ level, may explain this disparity. In the older study, HBO was administered for 1 hour at 2.8 atm, whereas a level of 2 atm was used in the previous experiments. This somewhat lower O₂ level might result in diminished improvement in locomotor recovery, although 2 atm has been shown to be sufficient to elevate tissue O₂ and improve recovery in another study. Another factor is that mild injury rather than the moderately severe injury produced with a greater weight-drop distance (25 mm compared with 6.25 mm) was used in the present studies. Greater injury severity may compromise delivery of O₂ to the tissue, making HBO treatment less effective. Authors of previous experiments have also shown a reduction in the effectiveness of HBO for more severe injuries.

Conclusions

We report here that outcome following radiotherapy for spinal cord contusion injury was not improved by combination therapy with either HBO or tempol. However, findings from the present study do not exclude the possibility that radiosensitization by an agent such as HBO might reduce the dose of x-irradiation required for optimal efficacy. Although numerous agents are claimed to have some benefit for SCI, an advantage of x-irradiation, as opposed to injected or inhaled neuroprotective agents, is that x-ray beams can completely penetrate injured spinal cord tissue without requiring compromised circulation for delivery of the therapeutic agent. Further optimization with regard to dosage, dose rate, and radiation energy, as well as stereotactic targeting could improve the efficacy of x-irradiation for use in spinal cord–injured patients.

Acknowledgment

We thank Kristin Kraus for her editorial assistance during the preparation of this manuscript.

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Accepted December 20, 2006.
This work was supported by grants from the National Institutes of Health (Grant No. R43 NS047760) and the US Department of Defense (Grant No. PR043366) to Dr. Zeman.
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