Spinal cord injury often leads to permanent paralysis, which may be the result of the initial injury or delayed damage. Paralysis occurs because the neural tissue necessary for locomotor and sensory function is irreversibly lost as the injury evolves over time during the acute, subacute, and chronic phases. The most common type of spinal cord injury is contusion injury, in which initial mechanical disruption of the cord is followed by multiple deleterious sequelae that cause cell death and ultimately a reduction in viable tissue volume.12

Traumatic injury to the spinal cord often leads to reduced blood flow,6,11,13,15,16,23,25–27 which can increase tissue damage and interfere with functional recovery. A particularly critical but vulnerable component of nervous tissue is the vasculature, which normally supplies sufficient blood to meet the high metabolic requirements of the CNS. Because CNS tissue is very compliant, the vasculature is easily disrupted, leading to widespread hemorrhaging in the tissue surrounding the contusion site.1,21,24 As a compensatory response to reduce bleeding, blood vessels undergo vasoconstriction through a variety of mechanisms, including vasospasm and vasomotor paralysis.1,15 However, the short-term benefit of vasoconstriction may not outweigh the possibility of longer-term ischemic damage to viable tissue. One study revealed that vasospasm alone could account for a loss of up to 80% of cord blood supply in an experimental model of spinal cord injury.1 The maintenance of postinjury spinal cord perfusion appears to be of primary importance for preserving viability and functionality since nervous tissue has a high metabolic demand and cannot survive in the absence of adequate perfusion. Despite the intuitive appeal of this idea, a re-

Perfusion imaging of spinal cord contusion: injury-induced blockade and partial reversal by β2-agonist treatment in rats

Laboratory investigation

ABRAHAM BROWN, PH.D.,1 ANNA NABEL, M.S.,1 WILLIAM OH, M.D.,1 JOSEPH D. ETLINGER, PH.D.,1,2 AND RICHARD J. ZEMAN, PH.D.1,2

1Department of Cell Biology and Anatomy, New York Medical College, Valhalla; and 2MotoGen Inc., Mount Kisco, New York

Object. Traumatic injury to the spinal cord results in considerable delayed tissue loss. The authors investigated the extent to which ischemia occurs following contusion-induced spinal cord injury and whether ischemia exacerbates tissue damage that leads to the loss of locomotor function. They also determined if ischemia is reversed with β2-adrenoceptor agonist treatment, which has been established to be neuroprotective following contusion injury.

Methods. The extent and role of circulation loss in spinal cord injury was determined in an established experimental model of contusion injury. The spinal cord dura mater of Wistar rats was exposed by performing a laminectomy at T-8 to T-11. Laser Doppler perfusion imaging was then used to measure microcirculation in the exposed spinal cord. After imaging, a moderately severe contusion injury was produced using a weight-drop device unto the exposed dura at T-10. Perfusion imaging was again performed, scans were quantitated, and integrated intensities were compared.

Results. Postinjury imaging revealed an 18%–27% reduction in perfusion in regions rostral and caudal to the injury site, and a 68% reduction was observed at the contusion epicenter. These perfusion losses persisted for at least 48 hours. At 24 hours after injury, some rats were intraperitoneally injected with 2 mg/kg of the β2-adrenoceptor agonist clenbuterol, which has been shown to promote the partial recovery of locomotor function and spare spinal cord tissue when administered within 2 days after contusion injury. Clenbuterol injection caused a gradual increase in perfusion, which was detectable at 30 minutes postinjection and continued over time, resulting in an 127% overall increase in perfusion at the epicenter 24 hours after treatment.

Conclusions. These results suggest that the occurrence of chronic perfusion loss after contusion contributes to delayed damage and tissue loss. In contrast, β2-adrenoceptor agonist treatment may exert neuroprotection by restoring perfusion, thereby preventing ischemic neurodegeneration. The ability of laser Doppler imaging to measure the loss of perfusion and its restoration upon treatment suggests that it may have clinical utility in the assessment and treatment of spinal cord injury.

(http://thejns.org/doi/abs/10.3171/2013.10.SPINE13113)

Key Words • spinal cord injury • contusion • perfusion • laser Doppler imaging • clenbuterol
Blockade of perfusion after spinal cord injury

cent review concluded that “there is little evidence linking hemodynamic support and neurological outcome after [spinal cord injury].”

The effects of spinal cord injury and various treatments on perfusion have been examined through a variety of techniques in experimental models of spinal cord injury. However, these studies generally followed perfusion for only 2–5 hours postinjury with limited spatial and/or temporal resolution. Because spinal cord injury evolves over time, alterations in perfusion measured acutely may not be relevant to longer-term changes in circulation and tissue viability. In addition, in the above studies, measurements of perfusion were performed at isolated loci that may not have been representative of the secondary spreading of spinal cord injury that is known to occur over time.

To address these investigational concerns, we designed experiments to comprehensively measure spinal cord perfusion with greater spatial and temporal resolution following injury. Laser Doppler perfusion imaging provides 2D measurements of tissue perfusion and can be used to monitor the injured spinal cord without physical contact. The technique yields a quantitative measure of perfusion that has been extensively validated and is in use clinically for assessing temporospatial loss of circulation in a variety of tissues and conditions, including diabetes, burns, and Raynaud’s syndrome. This imaging method involves the use of a scanning laser beam to determine the average flux of erythrocytes up to a depth of several hundred micrometers within the spinal cord by detecting the relative Doppler shift in the frequency of backscattered photons.

In the following experiments, perfusion imaging of the spinal cord was performed at postinjury intervals spanning several days. We also examined perfusion of the injured spinal cord both before and after treatment with clenbuterol, an agent that is neuroprotective for spinal cord injury. Data in the present study demonstrated that spinal cord contusion results in a persistent reduction in perfusion centered at the contusion site and that can account for later tissue loss. We also present data indicating that clenbuterol treatment results in rapid and continuing partial reversal of perfusion loss, which is consistent with previously observed neuroprotection by clenbuterol.

Methods

The Institutional Animal Care and Use Committee of New York Medical College approved all procedures involving the vertebrate animals.

Eight adult female Wistar rats (approximately 240 g) were obtained from Charles River Breeding Laboratories and housed in a temperature-regulated (23°C) animal facility. The rats received 0.02% amoxicillin in the drinking water for the duration of the experiment to prevent infection, and their bladders were manually expressed twice daily. Prior to surgery, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). During the period of anesthesia, body temperature was maintained at 37°C with a temperature-controlled heating pad. In all 8 rats laminectomy was performed aseptically to expose the spinal cord dura mater from T-8 to T-11. Laser Doppler perfusion imaging of the dorsal spinal cord was performed using a PeriScan PIM II system (Perimed AB) during a 16-minute observation period at a rate of 0.5 scans/minute beginning immediately following laminectomy. As in our previous studies, the rat spinal cord was then contused (8 rats) at the level of T-10 with a weight-drop apparatus similar to the NYU impactor. The spinous processes at T-7 and T-12 were fixed with clamps to prevent movement of the spine during contusion. Moderately severe contusion injury was produced by dropping a 10-g rod with a tip diameter of 2.5 mm from a height of 25 mm onto the exposed dura. Following contusion, the spinal cord was scanned again for 15 minutes, and the incisions were closed with wound clips. At 24 hours after contusion, all 8 rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and the incisions were opened for scanning (16 minutes). Some of the contused rats (n = 5) were then intraperitoneally injected with clenbuterol (2 mg/kg in a 0.5-ml saline vehicle; a kind gift from Eric Hagsater of Chino Pharmaceeval Products) and immediately scanned for 2 consecutive 16-minute periods. After another 24 hours after contusion, clenbuterol-treated (n = 5) and untreated (n = 3) rats were again anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and the incisions were opened for a final scanning (16 minutes).

Perfusion scans were quantitated by generating perfusion intensity curves along the axis of the spinal cord, which were averaged over 6–8 consecutive traces. Integrated intensities of contiguous regions rostral (6–7 mm), caudal (6–7 mm), and centered at the contusion site (3 mm) at different times were compared. The statistical significance of the effects of contusion injury and clenbuterol treatment on perfusion intensity normalized to preinjury levels was determined using mixed-factorial ANOVA with repeated-measures and pairwise comparisons post hoc and paired Student t-tests (SPSS Inc.). The statistical significance of increased perfusion at the contusion site 48 hours postinjury in clenbuterol-treated compared with untreated spinal cords was determined with an unpaired Student t-test (SPSS Inc.). Statistical significance was determined at the p < 0.05 level.

Results

To determine the extent and magnitude of circulation loss due to injury, measurements of spinal cord perfusion were performed in an established experimental model of contusion injury. After laminectomy, laser Doppler perfusion imaging was used to measure microcirculation flux throughout the length and width of the exposed spinal cord (Fig. 1 upper). Repeated perfusion scans were obtained over a period of 16 minutes (2 minutes per scan) to verify the stability of perfusion over time. Other than random fluctuations due to respiratory motions, no systematic trend or drift in the perfusion signal was detected during a contiguous scan sequence. Comparison of panels A and B in Fig. 1 illustrates that the spinal cord was much more abundantly perfused than surrounding bone, muscle,
and connective tissue. This finding is consistent with the high metabolic demand of the CNS. The observation period was followed by a contusion injury to the exposed dura at T-10 (Fig. 1 upper), and perfusion imaging of the spinal cord was then repeated for a period of 16 minutes. Contusion injury caused an immediate loss of perfusion signal that was most pronounced at the contusion site (Fig. 1 upper and C). The extent of reduced perfusion remained at 24 hours postinjury (Fig. 1D).

Images were quantitated by extracting intensity profiles along a line centered on the spinal cord axis (Fig. 2A). Analysis of the postinjury images in the animal featured

**Fig. 1. Upper:** Contusion injury rapidly reduced overall spinal cord perfusion. The rat received moderately severe contusion injury from a 10-g weight with a 2.5-mm tip dropped 25 mm onto the exposed spinal cord dura at the level of T-10. A laser Doppler perfusion imager (PeriScan PIM II) was then used to measure microcirculation throughout the length and width of the exposed spinal cord (from T-8 to T-11). Comparison of perfusion images taken before and after contusion indicated rapid and extensive loss of perfusion at the contusion site as well as rostrally and caudally. Bar = 4 mm. **Lower:** Perfusion loss was persistent and restored by clenbuterol. Dorsal view of rat spinal cord before contusion injury (A). Perfusion image of spinal cord before contusion injury (B). Fifteen minutes after injury (C). Twenty-four hours after injury (D). Thirty minutes after 2 mg/kg intraperitoneal clenbuterol (E). Twenty-four hours after clenbuterol (F). Fifteen minutes after a lethal dose of pentobarbital (G). arb units = arbitrary units.
in Fig. 2 revealed immediate perfusion reduction at the contusion site and neighboring regions (Fig. 2). Quantitative analysis confirmed that reduced perfusion was maintained to a similar extent 24 hours later (Figs. 1D and 2C).

The rat was then injected with clenbuterol, a β₂-agonist with vasodilator activity,7 which in our previous studies was shown to promote partial recovery of locomotor function and was associated with commensurate sparing of spinal cord tissue.28,29 At 30 minutes post–clenbuterol injection, perfusion was increased in and near the contusion site (Figs. 1E and 2C). Perfusion recovery was enhanced over time due to clenbuterol, since further increases in perfusion were observed only in clenbuterol-treated animals 24 hours postinjection (Figs. 1F and 2C). As a final control to establish the relationship between the Doppler signal and perfusion, a lethal dose of pentobarbital (150 mg/kg, intraperitoneal injection) was administered. The perfusion signal was abolished with the cessation of heart function (Figs. 1G and 2C).

Averaged Results and Statistical Analysis

Quantitation of pre- and postinjury images for a group of clenbuterol-treated rats (n = 5) was normalized and revealed an 18%–27% (p < 0.001) reduction in perfusion in regions rostral and caudal to the injury, whereas a larger 68% (p < 0.0005) reduction was observed at the contusion epicenter (Fig. 3 upper). As a final control to establish the relationship between the Doppler signal and perfusion, a lethal dose of pentobarbital (150 mg/kg, intraperitoneal injection) was administered. The perfusion signal was abolished with the cessation of heart function (Figs. 1G and 2C).

At 24 hours postcontusion, some of the rats were intraperitoneally injected with 2 mg/kg of clenbuterol. At 15–30 minutes postinjection, 8%–28% increases in perfusion were observed in all regions, which reached statistical significance (p < 0.015; Fig. 3 upper). Twenty-four hours postinjection, perfusion was enhanced overall due to clenbuterol (p < 0.001) with increases of 21%–54% rostrally and caudal to the injury as well as a 127% increase at the epicenter, where perfusion was initially impaired to the greatest extent.

Figure 3 (lower) shows the outcome of spinal cord perfusion in a group of rats (n = 3) following contusion in the absence of pharmacological intervention. Perfusion was reduced by contusion (p < 0.025) and remained unchanged at 1 and 2 days postinjury in the untreated animals (p > 0.05). Thus, the observed perfusion increase after clenbuterol treatment cannot be attributed to spontaneous recovery.

Comparison of perfusion at 48 hours postinjury showed that perfusion was elevated 73% at the contusion site by clenbuterol treatment compared with untreated contused spinal cords (p < 0.05; Fig. 3).

Discussion

The main findings of this study are that contusion causes a long-lasting reduction in perfusion centered at the contusion site and that a fraction of the perfusion loss is rapidly and persistently reversed by treatment with clenbuterol, a neuroprotective agent in spinal cord injury. A previous study found that delayed tissue loss (> 24 hours)
Fig. 3. Upper: Averaged perfusion values for clenbuterol-treated animals. Values are the means of perfusion normalized to the pre-contusion mean (± standard error) at the contusion site and adjacent rostral and caudal regions of the spinal cord in a group of rats (n = 5) before and after contusion injury and after clenbuterol treatment during a 48-hour observation period. There was a significant reduction in perfusion (a, p < 0.0005, paired t-test) as a result of contusion of the spinal cord. There was a significant reduction in perfusion (b, p < 0.0005, mixed-factorial ANOVA with repeated measures [F(2,30) = 34.54, p < 0.0005] and pairwise comparisons) at the contusion site relative to adjacent rostral and caudal regions of the spinal cord at 0.3–48 hours postinjury. There was a significant increase in perfusion (c, p < 0.015) of the spinal cord at 15–30 minutes after clenbuterol injection compared with perfusion at 24 hours after contusion. There was a significant increase in perfusion (d, p < 0.001, mixed-factorial ANOVA with repeated measures [F(3,30) = 13.84, p < 0.0005] and pairwise comparisons) of the spinal cord at 24 hours after clenbuterol injection. Lower: Averaged perfusion values for untreated animals. Values are the means of normalized perfusion (± standard error) at the contusion site and adjacent rostral and caudal regions of the spinal cord in a group of rats (n = 3) before and after contusion injury treatment during a 48-hour observation period. There was a significant reduction (a, p < 0.025, paired t-test) in perfusion due to contusion of the spinal cord. There was a significant reduction in perfusion (b, p < 0.015, mixed-factorial ANOVA with repeated measures [F(2,12) = 10.67, p < 0.0025] and pairwise comparisons) at the contusion site relative to adjacent rostral and caudal regions of the spinal cord at 0.3–48 hours postinjury. Perfusion of the spinal cord at 48 hours after contusion was not significantly different (NS; p > 0.05, mixed-factorial ANOVA with repeated measures [F(2,12) = 2.48, p > 0.05] and pairwise comparison) from perfusion at 24 hours after contusion.
Blockade of perfusion after spinal cord injury

due to contusion injury is centered at the contusion site.20 Moreover, the extent of tissue loss and the loss of locomotor function were correlated with impact severity but could not be attributed to the consequences of hemorrhage or edema. Our current observation of perfusion loss centered at the contusion site suggested that the persistent loss of local perfusion may have contributed to delayed ischemic tissue loss. A second correlation between tissue loss and perfusion is that the region of delayed loss extends beyond the contusion site,20 as does the region that suffers reduced perfusion (Fig. 2B). Clenbuterol has been shown to partially reverse the loss of tissue and locomotor function,28,29 and we have now demonstrated that this β2-agonist restores perfusion at the contusion site and its surround. Therefore, we propose that an important neuroprotective effect of clenbuterol may be the promotion of nervous tissue survival through the restoration of perfusion.

The effects of spinal cord injury and various treatments on perfusion have been examined by numerous investigators using a variety of techniques in experimental models of spinal cord injury. Reduced spinal cord blood flow was measured 2 hours after contusion injury in cats using the tracer 14C-antipyrine.9 Perfusion was partially restored during this period via the administration of either isoproterenol or aminophylline, both of which were suggested to have therapeutic potential for spinal cord injury.

The steroid methylprednisolone, which is used therapeutically in spinal cord–injured humans, was also found to partially reverse acute loss of perfusion as measured by hydrogen clearance in experimental spinal cord injury in cats.12,27 although the improvement in circulation was lost when steroid administration was delayed for several hours postinjury.13 Note, however, that some investigators were unable to demonstrate an increase in blood flow due to methylprednisolone treatment in spinal cord injured rats.16 Others found that methylprednisolone induced acute increases in rat spinal cord blood flow that, nonetheless, did not lead to increased locomotor recovery or sparing of spinal cord tissue.11

Claims of enhanced spinal cord perfusion have been made in association with spinal cord injury treatment with various agents including naloxone,7 fibroblast growth factor,13 hypertonic saline,23 insulin,25 and glutathione monoethyl ester.11 However, because perfusion measurements were performed either acutely and/or at spinal cord loci removed from the injury site, they may not be indicative of the overall temporal and spatial development of spinal cord injury.

We addressed these methodological concerns by measuring perfusion over an extended area that included the contused region and surrounding tissue. Furthermore, perfusion status was repeatedly examined over a more extended period (48 hours), and a well-characterized treatment paradigm was used. The interval for the experiments was chosen to correspond to the entire therapeutic window that we had already established for clenbuterol treatment.29 This approach allowed us to examine the correlation of perfusion changes during the first 48 hours postcontusion with the previously established behavioral and histological long-term effects of clenbuterol treatment.

Since the extent of tissue penetration by laser Doppler imaging is at least several hundred micrometers, it is sufficient to penetrate the spinal cord dura and detect the microcirculation of dorsal and lateral white matter tracts whose blood supply arises from deeper-lying vessels. These white matter tracts are necessary for conducting ascending and descending activity required for locomotor function and are partially spared by clenbuterol treatment.28,29 The observed changes in perfusion provide a measure that is representative of white matter perfusion, although perfusion throughout the entire cross-section of the spinal cord was not measured by Doppler imaging.

Our previous studies of spinal cord contusion injury have shown that clenbuterol improved locomotor recovery that was quantitatively associated with the sparing of spinal cord tissue.28,29 The therapeutic time window for clenbuterol to enhance tissue sparing and locomotor recovery was found to have occurred within 48 hours postinjury and was ineffective thereafter.29 This finding suggested the possibility that a reversal of some unidentified process, such as ischemia, within this period spares white matter.

Results observed in the current study led us to propose that the restoration of ischemia-induced perfusion loss associated with spinal cord injury may explain at least part of the action of clenbuterol to spare tissue loss. One unexpected but positive observation was the sustained increase in perfusion induced by clenbuterol treatment. Perfusion remained elevated 24 hours after treatment, which is long after the drug has been cleared from circulation. This suggests that acute β2-agonist treatment may “reset” the vascular tone and thus may explain the long-term functional benefit that was observed from a single administration of clenbuterol.29

Spinal cord injury results in the loss of perfusion, which may be attributable to a variety of vascular events, including vessel rupture, occlusive thromboses, loss of vascular autoregulation, and vasospasm. Vasospasm following injury may be initiated by several mechanisms including mechanically induced vasoconstriction or release of vasoconstrictive neurotransmitters (serotonin, endothelin) or hemoglobin.7 Oxidative stress resulting from spinal cord injury can reduce availability of the vasorelaxant nitric oxide by promoting peroxynitrite formation.14 Another consequence of spinal cord injury is depletion of the endogenous antioxidant glutathione, which can cause long-lasting vasoconstriction.11,30 Although loss of circulation due to vessel rupture or thrombosis may not be readily reversible after spinal cord injury, vessels that undergo vasospasm represent a potentially treatable loss of circulation that may be therapeutic for spinal cord injury.

How then does the β2-adrenoceptor agonist clenbuterol enhance perfusion after injury? Clenbuterol has been shown to directly act as a vasodilator on CNS resistance vessels,7 which may overcome the effect of vasospasm leading to enhanced perfusion. β2-receptor–dependent mechanisms have also been shown to sustain cortical microvascular perfusion under conditions of hypoxia.22 Additionally, clenbuterol may promote vasodilation via activation of nitrergic neurons,16 which would also enhance perfusion. The neuroprotective effect of clenbuterol in spinal cord injury has been shown to require synthesis
of glutathione, which could also have a role in reversing vasospasm since glutathione loss leads to vasoconstriction. Consistent with this hypothesis, treatments that increase glutathione in the injured spinal cord promote the return of perfusion and enhance locomotor recovery. Another possible explanation for the activity of clenbuterol is that it increases perfusion by stimulating β2-receptors that are abundant in astrocytes, which form a network that is in contact with the vasculature and is known to regulate blood flow. Interestingly, hyperperfusion of white matter occurs in multiple sclerosis and has been attributed to a lack of astrocytic β2-receptors that is associated with this condition.

The current study also illustrates the potential value of laser Doppler perfusion imaging in the evaluation of spinal cord injury. This modality may prove to be valuable for assessing the severity of spinal cord injury and the extent of surrounding tissue involvement. Further studies are needed to determine if it can be used prognostically or to optimize interventions aimed at restoring spinal cord perfusion. Finally, laser Doppler perfusion imaging can be productively used to determine the mode of action of neuroprotective treatments and in the screening for agents that are more effective in restoring spinal cord perfusion.

Conclusions

We have presented evidence of a chronic loss of perfusion after spinal cord contusion, which suggests that ischemia in and around the contusion site contributes to delayed neuronal dysfunction and tissue loss. The results of this study also suggest that β2-adrenoceptor agonists may exert neuroprotection by reversing chronic loss of perfusion at the injury site, thereby preventing ischemic neurodegeneration. The ability of laser Doppler imaging to measure the loss of perfusion and its reversal with treatment indicates that the modality may have clinical utility in the assessment and treatment of spinal cord injury.

Disclosure

The authors gratefully acknowledge the funding support of Grant No. W81XWH-05-1-0076 from the Department of Defense (R.J.Z.).

Author contributions to the study and manuscript preparation include the following. Conception and design: Zeman, Brown. Acquisition of data: Nabel, Oh. Analysis and interpretation of data: Zeman, Brown, Etlinger. Drafting the article: Zeman, Brown, Etlinger. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Zeman. Statistical analysis: Zeman. Administrative/technical/material support: Zeman, Brown. Study supervision: Zeman, Brown.

References

Blockade of perfusion after spinal cord injury


Manuscript submitted February 1, 2013.
Accepted October 29, 2013.
Please include this information when citing this paper: published online December 6, 2013; DOI: 10.3171/2013.10.SPINE13113.
Address correspondence to: Richard J. Zeman, Ph.D., Department of Cell Biology and Anatomy, New York Medical College, Valhalla, NY 10595. email: zeman@nymc.edu.