A murine model of hypertonic saline as a treatment for acute spinal cord injury: effects on autonomic outcome

Laboratory investigation

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Object. Spinal cord injury (SCI) continues to be a problem without a definitive cure. Research based on improved understanding of the immunological aspects of SCI has revealed targets for treating and ameliorating the extent of secondary injury. Hypertonic saline (HTS), a substance both easy to create and to transport, has been investigated as an immunologically active material that can be used in a clinically relevant interval after injury. In this pilot study, HTS was investigated in a murine model for its abilities to ameliorate secondary injury after a severe spinal cord contusion.

Methods. Female C57Bl/6 mice with severe T8–10 contusion injuries were used as the model subjects. A group of 41 mice were studied in a blinded fashion. Mice received treatments with HTS (HTS, 7.5%) or normal saline solution (NSS, 0.9%) at 2 discreet time points (3 and 24 hours after injury.) A separate group of 9 untreated animals were also used as controls. Animals were assessed for autonomic outcome (bladder function). In a group of 33 mice, histological assessment (cellular infiltration) was also measured.

Results. Bladder function was found to be improved significantly in those treated with HTS compared with those who received NSS and also at later treatment times (24 hours) than at earlier treatment times (3 hours). Decreased cellular infiltration in each group correlated with bladder recovery.

Conclusions. The increased effectiveness of later administration time of the more osmotically active and immunomodulatory substance (HTS) suggests that interaction with events occurring around 24 hours after injury is critical. These events may be related to the invasion of leukocytes peaking at 8–24 hours postinjury and/or the peak benefit time of subject rehydration. (DOI: 10.3171/2010.9.SPINE08314)

KEY WORDS • spinal cord injury • hypertonic saline • murine • mouse • bladder • secondary injury

Although chronic SCI may not be the most prevalent disease affecting humans, the economic and emotional costs are significant, especially given that more than half of people sustaining SCI are 15–29 years old (unpublished data, Centers for Disease Control and Prevention). In fact, according to Centers for Disease Control and Prevention data, approximately 11,000 Americans sustain an SCI each year, and there are approximately 200,000 people in the US living with the sequelae of SCI.

Currently, there is no cure for SCI. In a survey by Anderson,3 members of the SCI community would find their lives positively affected even by modest improvements in function. People with quadriplegia most wished for return of arm/hand function and people with paraplegia most desired improvement in bowel/bladder and sexual function. One can infer from this that during the course of a new SCI minimizing secondary injuries may prevent functional losses that would radically improve the life of the injured individual. Secondary injury, believed to arise from multiple causes. These include ischemia, free radical formation, toxic metabolite accumulation, and others.6,49,75,90

Abbreviations used in this paper: BBB = Basso-Beattie-Bresnahan; HTS = hypertonic saline; NSS = normal saline solution; SCI = spinal cord injury.
In experiments we conducted, HTS was used as a therapeutic agent to ameliorate secondary injury. It was chosen for its many positive attributes. In previous experiments from our laboratory, rodents (mice and rats) treated with HTS had improvements in motor scores, as well as in bladder function.57,58,77,78,80,84,86 Other researchers have also been exploring the effects of HTS, noting its effects on adhesion molecules and leukocyte adhesion, as well as noting the effects of blocking adhesion molecules involved in the same pathway.4,5,12,37–40,61,65,69,70,81,87 Hypertonic saline has also been extensively studied in the general surgery and trauma literature. It is favored for many abilities, including its role as a volume expander in shock and trauma, as a cheap and efficient fluid to transport, and as a fluid with favorable effects on the inflammatory cascade.15,20,21,23–25,47,62,76,83,86

Factors such as the time of administration and the type of fluid for treatment have been studied as they relate to outcome measures for bladder recovery and cellular infiltration. Mice received 7.5% hypertonic (5 ml/kg) saline or normal saline (5 ml/kg) at either 3 or 24 hours intravenously after the initial injury.

Methods

Spinal Cord Injury

All procedures and protocols were approved by the Temple University Institutional Animal Care and Use Committee (No. 1319). The animals used were female C57Bl/6 mice (Taconic Inc.) weighing approximately 15–20 g. Procedures for acclimation, anesthesia, surgery, intravenous access, and care have been detailed.58,59 A group of 41 mice received a 60-kdyne force using an IH Impac-credé maneuvers (manual bladder emptying). The emp-tied urine was weighed and followed as a total daily mass of expressed urine in milligrams. Animals that produced daily totals of less than 500 mg for 3 consecutive days (6 consecutive bladder emptying events and measurements) were considered to have recovered a functional bladder. The animals that were considered to have recovered bladder function continued to have their bladders palpated twice daily and were assigned sizes (small, ≤ 200 mg; medium, 300 mg; and large, ≥ 400 mg) corresponding to measured, expelled urine masses. With practice, a handler can accurately estimate bladder volume from palpated bladder size. Animals with large bladders (≥ 400 mg) for 3 consecutive measurements (1.5 days) were considered as having been erroneously assigned a functional bladder or possibly having lost a functional bladder and were returned to twice daily credé maneuver emptying. (This occurred only once over the course of the experiments, including instances in previously published data.)

Experimental Design and Statistical Analysis

Animals were assessed to 28 days and euthanized. Those that had injuries to their lower extremities (such as flank wound or toe injury) were excluded. The model was that of a randomized blinded study. In this experiment, 4 groups of mice were involved: 11 mice treated at 3 hours with NSS, 10 mice treated at 24 hours with NSS, 10 mice treated at 3 hours with HTS, and 10 mice treated at 24 hours with HTS. Five mice from each group were euthanized at 14 days for histological analysis. An additional group of 9 mice with injury but no intravenous intervention was also included for comparison. Bladder recovery days were analyzed using a Cox proportional hazard model. A 2-way ANOVA with factors group × time was used to analyze the cellular infiltrates. Post hoc analyses were carried out using Bonferroni tests. All significance values were set at p < 0.05. A Spearman rank correlation was used to analyze the relationship between bladder recovery and cellular infiltrates.

Tissue Collection, Histology, and Quantification

Thirty-three of 41 mice were used for histological analysis.89 (8 specimens were lost in histological processing). Spinal cord tissue samples were preserved as in previously published reports. Briefly, spinal cord segments were removed and the dorsal roots marked with an indelible marker for segmental identification later. The spinal cord was then incubated for 3 days in 30% sucrose (at 4°C) before being cryo-sectioned into 12-μm cross-sections. Subsequently, spinal cord sections were stained with H & E for visualization of cellular infiltrates.

To quantify changes in inflammatory cell infiltration within the spinal cord, H & E–stained slides were analyzed with an image analysis system (Bioquant II) using similar video-count thresholding methods as described by Elliott et al.29 Video-count area refers to the number of pixels in a designated field that meet a user-defined criterion multiplied by the area of a pixel at the selected magnification (20 × objective and 350 magnification factor for this analysis). Threshold criteria for infiltrating immune cells included dark purple (hematoxylin) stained cells...
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and excluded the lighter purple stained cells believed to be resident glia (Fig. 1). The mean area fraction of thresholded reactive product in a selected region of interest, which included the dorsal column white matter, was determined by dividing the video-count area of pixels above background thresholds by the total number of pixels in the entire chosen image field. An irregular region of interest tool was used for each measurement. An EXFO X-Cite 120 Fluorescent illuminator (EXFO America) and a Retiga EXI cooled camera (QImaging) were used to maintain consistent illumination throughout the project. Group means and SEM (3–5 per group) are expressed as mean percentage of the area of immunoreactivity. All assessments and image analyses were performed blinded.

Results

Autonomic Testing (Bladder Functional Recovery)

Bladder functional recovery was significantly improved by both increased tonicity in treatment solution (HTS vs NSS) and in increased time interval to treatment (Fig. 2 upper). All groups treated at 24 hours after injury had a higher likelihood of bladder function recovery than groups treated at 3 hours after injury. When the time of treatment is controlled, animals receiving HTS treatments were more likely to have functional bladder recovery than those receiving NSS. The percentage of animals with recovered bladder function in the HTS-treated group at 24 hours compared with the animals receiving no intravenous therapy (no treatment) was statistically significant (p < 0.05, Bonferroni posttests). It is interesting to note that the average day of recovery does not change across the treated groups, but is different from the untreated group (Fig. 2 lower).

Histological Outcomes

The 33 mice were divided into 4 groups, each with 2 time periods. Groups consisted of 3 to 5 subjects. Mice received either NSS (0.9%, 5 ml/kg) or HTS (7.5%, 5 ml/kg) at either 3 hours or 24 hours after a 60-kdyne SCI. Mice were then killed at 14 days or 28 days. The SCI produced significant cellular infiltration within the injury zone, as well as in affected gray and white matter areas in spinal cord segments in the region of the injury.

Cellular infiltration was measured in the spinal cord dorsal columns because, unlike humans, the cortical spinal tract in rodents is located in proximity to the dorsal

Fig. 1. Photomicrographs of spinal cord sections stained with H & E showing leukocyte infiltration (small, darker stained cells) and green outlined region of interest. There is less infiltration for the HTS group (upper) compared with NSS at 3 hours (lower). Black arrows indicate the region most proximal to the lesion.

Fig. 2. Upper: Bladder functional recovery. The percentage of mice recovering bladder function improved with increased tonicity of treatment fluid (HTS vs NSS) as well as in increased time interval to treat (24 vs 3 hours). The mice treated at 24 hours with HTS reached statistical significance compared those receiving no treatment (no tx) (*p < 0.05). Lower: Average day of bladder recovery. Mice that recovered function tended to recover by about the same day regardless of the tonicity of treatment. Note that untreated mice recovered at a later time on average.
columns. With cellular infiltration into the dorsal column measured at 14 and 28 days after injury across the 4 groups, a pattern again is noted. All HTS-treated groups improved over time in terms of cellular infiltration (Fig. 3). All groups that received NSS had increasing cellular infiltration from Day 14 to 28 postinjury.

After grouping similar outcomes, there is a weak, but significant, correlation ($r = 0.3663$ and $p = 0.0392$) using a Spearman rank correlation (with 1 outlier excluded) between the likelihood of bladder recovery and the presence of reduced cellular infiltration.

**Discussion**

The pathophysiology of SCI remains an active field of research. While the mechanisms involved in secondary SCI continue to be investigated, researchers have been able to demonstrate that direct manipulation of adhesion molecules and cellular infiltration are able to create measurable changes in clinical outcome. A variety of substances (steroids, antibodies, and nutrients) have been employed.

The treatment for acute SCI is generally agreed to be centered on secondary injury. In a review, Hulsebosch highlights 11 targets for SCI intervention. On the list, the author includes reduction of edema and free radical production, rescue of susceptible neural tissue, control of inflammation, and rescue of at-risk neurons/glial cells. In this pilot project, we suggest that HTS, administered at the correct time, may help in these areas.

Hypertonic saline, as a therapeutic agent, is already in use as a valuable adjunct to general and trauma surgery. Essentially a solution of sodium chloride and water, it has been used as a volume expander and as a modulator of leukocyte activity. Additional effects of HTS on ICAM, CD11/CD18, and other integrins have been providing insight into mechanisms that may be directly affected by HTS administration.

Previous work in our laboratory on murine species (mice and rats) has demonstrated benefits of administering HTS in both head and spine trauma situations. Using a mouse model of SCI, we were able to demonstrate a significant improvement in motor outcomes as a result of treatment with HTS at 24 hours after injury. We observed that these mice appeared to have improved bladder recovery as well. One can conclude from these previous experiments that there is a relationship between HTS administration and improved neurological outcome in the mouse model.

In the current study, an interesting outcome involves bladder function. There were 5 treatment groups (no treatment, NSS at 3 hours, NSS at 24 hours, HTS at 3 hours, and HTS at 24 hours). These 5 groups were compared using a Cox proportional hazards model, rating the percentage of mice recovering over the course of the experiment (Fig. 2 upper, Table 1). As one moves from each treatment modality, the chance of bladder recovery increases by 66% ($p < 0.05$). This means that treatment with HTS compared with NSS at equivalent times always resulted in greater likelihood of bladder recovery. All mice that recovered bladder function tended to do so at approximately 7–9 days without significant difference between each group. Later times of treatment yielded greater likelihood of recovery. Interestingly, this holds true for animals receiving normal saline treatment as well. This suggests that there may be an aspect of volume restoration that may also be protective for SCI. This is consistent with previous work performed in our laboratory in rats. For example, rats receiving HTS after ischemic SCI have increased spinal cord blood flow and preservation of somatosensory evoked potentials. Higher spinal cord blood flow is noted than in cases treated with equivalent levels of isotonic saline.

At 14 days, animals receiving HTS at 3 hours and NSS at 24 hours had the least infiltration of cells. It is surprising that HTS at 24 hours did not have the least cellular infiltration. This would have been the expected result, given the superior bladder recovery of the group, but the small sample size may be responsible for the results.

However, by 28 days, each group receiving HTS had a decreased cellular count while NSS-treated groups had an increased cellular count. Overall, there is a correla-

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**TABLE 1: Group listing for the Cox proportional hazards model**

<table>
<thead>
<tr>
<th>Group</th>
<th>Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment</td>
<td>0</td>
</tr>
<tr>
<td>NSS at 3 hrs</td>
<td>1</td>
</tr>
<tr>
<td>HTS at 3 hrs</td>
<td>2</td>
</tr>
<tr>
<td>NSS at 24 hrs</td>
<td>3</td>
</tr>
<tr>
<td>HTS at 24 hrs</td>
<td>4</td>
</tr>
</tbody>
</table>

*Groups are arranged from no treatment through 24 hours treatment. The likelihood of recovery is increased by 66% in animals that progress from one treatment modality to the next.*
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tion, albeit weak, with cellular count and the likelihood of bladder recovery. This supports the idea that controlling cellular infiltration augments tissue preservation, leading to improved recovery. The invading leukocytes are known to arrive by 8–24 hours after injury, which would support why treatments coincident in timing with leukocyte arrival may be more effective.

It is also known that the blood–spinal cord barrier (analogous to the blood-brain barrier) is disrupted by injury and its reformation interacts with the delivery time of therapeutics. Bhardwaj et al. studied cerebral infract volume after treatment with HTS. In their study, rats receiving 7.5% HTS (10 ml/kg) immediately on reperfusion had an increase in cortical infarct volume after middle cerebral artery occlusion. Considering the possibility of damage from osmotically active sodium and water crossing a disrupted blood-brain barrier, the authors do suggest that the therapy may have different effects if administered later, when the blood-brain barrier has stabilized. There is evidence, however, that HTS solutions given after cortical impact injuries may improve outcome. Related work with head contusion in rats by Elliott et al. also reveals that increased preservation of these descending tracts by HTS administration at a time coincident with leukocyte migration explains the improvement in bladder function. Tract preservation was not measured directly in this pilot study, but would be a valuable histological outcome measure for future studies.

The timing of treatment may also relate to important clinical practices. The hydration protocol for the SCI mice is based on the protocol used at the University of California, Irvine, laboratories for the experimental treatment of SCI (unpublished data, Reeve-Irvine Research Center SCI Techniques Lab Manual, 2005). Perhaps the intravenous injection of volume, either HTS or NSS at 24 hours after injury, is protective by way of hydrating at a time when the animal is most dehydrated. This suggests that the protocols used, while previously thought to be adequate for hydration (subcutaneous infiltration of NSS), may be inadequate for murine SCI. The HTS, while given in the same physical volume (5 ml/kg) as the normal saline, has enhanced volume restoration abilities. Note that the average mouse blood volume is 80 ml/kg. An injection of 5 ml/kg represents 1/16th of the animal’s total intravascular volume. Because HTS effectively serves to expand circulating volume as much as 2 or 3 times the equivalent dose of NSS, this would represent a 1/8 to nearly 1/4 the animal’s blood volume. As such, some of the effect of the HTS may be as a volume expander. This would also explain how subjects receiving NSS at 24 hours could still show benefit, possibly by treating dehydration.

Kølsen-Petersen has raised doubts as to the clinical application of HTS. After a thorough review of the literature, the author originally stated that despite suggestive reports from cellular and animal studies with HTS, there are too few large clinical trials to draw a conclusion. However, in more recent papers he suggested that there may be a modest beneficial effect. Certainly, more large-scale clinical trials are required to evaluate the utility of HTS as a treatment modality.

Other groups working with HTS have also reported positive results. In work by Derec and associates, the addition of pentoxifylline, a nonspecific phosphodiesterase inhibitor, to HTS resuscitation has been observed to improve results in gut injury. Pentoxifylline is known to attenuate the neutrophil oxidative burst and CD11b expression, both thought to cause damage to surrounding tissue in SCI. Some authors have noted improvement with HTS treatment, but treatment with both substances together was even more effective. Similarly, work by both Gris and Weaver has shown that modulation of neutrophil function through CD11 expression can improve functional outcome in rats. This is the same pathway that is affected by HTS in modulating neutrophil function. In fact, many authors are actively investigating the role of CD11 and the role of neutrophils in the pathophysiology of injury.

This pilot study provides further evidence suggesting that HTS may be an effective therapeutic agent for the treatment of SCI and the timing of administration appears to be critical. Treatment with HTS after SCI was found to result consistently in improved bladder recovery both at 3 and 24 hours postinjury. At equivalent administration intervals, HTS, compared with NSS, improved the likelihood of bladder recovery. The treatment points chosen of 3 to 24 hours postinjury are reasonable in clinical practice. This suggests that HTS administration is a clinically relevant treatment for SCI suitable for future clinical trials. Multiple mechanisms are possible, including volume expansion, treatment of dehydration, amelioration of immunological response, and ultimately preservation of neural tissue. Future work is planned to further investigate the role of HTS and therapy administration time on the modulation of neutrophil invasion in the mouse model of SCI. Additional studies testing additional or multiple treatment times would help target the ideal treatment time for HTS. Larger studies with larger animal models, including clinical studies, would be necessary to fully determine the ideal use of this beneficial agent.

Disclosure

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