Sporotaneous cord injuries (SCIs) lead to immediate and usually permanent lifestyle alterations. Most patients require lifelong medical care, and some patients cannot live independently. They suffer from frequent complications, such as ulcers, urinary tract infections, pneumonia, and fractures. The individual lifetime financial burden can exceed $2 million, and in the United States, $7 billion is spent annually to care for people with SCIs. Previous studies have shown that the initial trauma to the spinal cord can sever neurons, which leads to a loss of signal from a certain part of the body to the brain; however, the full SCI occurs in 2 phases. The initial trauma is followed by inflammation that leads to fibroblastic scar tissue, glial scarring, and cavity formation. Scarring causes further axon death around and above the injury. A reduction in secondary injury could lead to functional improvement. In this study, hyaluronic acid (HA) hydrogels were implanted into the gap formed in the hemisected spinal cord of Sprague-Dawley rats in an attempt to attenuate damage and regenerate tissue.

**METHODS** A T-10 hemisection spinal cord injury was created in adult male Sprague-Dawley rats; the rats were assigned to a sham, control (phosphate-buffered saline), or HA hydrogel–treated group. One cohort of 23 animals was followed for 12 weeks and underwent weekly behavioral assessments. At 12 weeks, retrograde tracing was performed by injecting Fluoro-Gold in the left L-2 gray matter. At 14 weeks, the animals were killed. The volume of the lesion and the number of cells labeled from retrograde tracing were calculated. Animals in a separate cohort were killed at 8 or 16 weeks and perfused for immunohistochemical analysis and transmission electron microscopy. Samples were stained using H & E, neurofilament stain (neurons and axons), silver stain (disrupted axons), glial fibrillary acidic protein stain (astrocytes), and Iba1 stain (mononuclear cells).

**RESULTS** The lesions were significantly smaller in size and there were more retrograde-labeled cells in the red nuclei of the HA hydrogel–treated rats than in those of the controls; however, the behavioral assessments revealed no differences between the groups. The immunohistochemical analyses revealed decreased fibrous scarring and increased retention of organized intact axonal tissue in the HA hydrogel–treated group. There was a decreased presence of inflammatory cells in the HA hydrogel–treated group. No axonal or neuronal regeneration was observed.

**CONCLUSIONS** The results of these experiments show that HA hydrogel had a neuroprotective effect on the spinal cord by decreasing the magnitude of secondary injury after a lacerating spinal cord injury. Although regeneration and behavioral improvement were not observed, the reduction in disorganized scar tissue and the retention of neurons near and above the lesion are important for future regenerative efforts. In addition, this gel would be useful as the base substrate in the development of a more complex scaffold.
become reactive and start to form a glial scar composed of dense astrocytes and altered extracellular matrix, such as increased chondroitin sulfate. The glial scar seals the blood-brain barrier and blocks infiltrating mononuclear cells and fibroblasts but also inhibits axon regeneration. The fibrotic and glial scars are incorporated together in a region of amorphous dense tissue. Furthermore, phagocytosis of dying cells and debris can form cavities inside the spinal cord. In addition to the inflammatory pathways, nonpathological pathways inhibit the ability of the central nervous system to regenerate, such as by expressing the neurite outgrowth inhibitor proteins. Therefore, to regenerate after injury in the central nervous system, axons must overcome several formidable barriers, including dense scar tissue, fluid-filled gaps in nervous tissue, and nonpathological inhibitory pathways.

Spinal cord injury involves some combination of contusion, crush, pinch, twist and torsion, partial tearing, and vascular injury. Although all types of SCI induce a secondary-phase scarring process, there are some differences seen clinically in the rates of recovery, depending on the specific injury type.

Clinically, SCI is managed by stabilizing the spine, decompressing the spinal cord, and repairing the dura mater where necessary. Although clinical trials on SCI are currently underway, there are no devices or treatments approved by the US Food and Drug Administration that are available for the reduction of secondary injury or restoration of function after SCI.

Hyaluronic acid (HA) is a linear glycosaminoglycan extracellular matrix component found in all tissues. It has been used in many medical applications and is frequently investigated as a potential biomaterial for tissue engineering.

Hyaluronic acid is highly biocompatible, immunologically and chemically inert, and nontoxic; thus, using it prevents the problem of neural tissue rejection. Furthermore, high-molecular-weight HA promotes neuroprotective cell-signaling cascades. Hyaluronic acid is often used in a modified hydrogel because native HA is not mechanically durable. These HA hydrogels can adhere firmly to host tissue and bridge the whole spinal cord lesion. We previously reported that the use of a cross-linked HA scaffold promotes robust neurite outgrowth in vitro.

Khaing et al. implanted an HA hydrogel in a rat hemisection model and found that the hydrogel decreased the number of reactive astrocytes at short and long time points and decreased a glial scar component (chondroitin sulfate proteoglycan) at a short time point. They also found that HA hydrogels were used to fill the hemisection spinal cord lesions of adult rats to evaluate their neuroprotective and neuroregenerative potential at time points up to 16 weeks.

Methods

Hyaluronic acid (molecular mass 110 kD) was purchased from Genzyme. Poly(ethylene glycol)-diacylate (PEGDA) (molecular mass 3400 Da) was purchased from Nektar Therapeutics. Phosphate-buffered saline (PBS), syringe filters (0.2 μm), syringes, and sodium hydroxide were purchased from VWR International LLC. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide, 3,3'-dithiobis (propionic acid) (DTDP), diithothreitol (DTT), and hydrazine hydrate were obtained from Sigma Chemical Co. Dextran microruby (D7162), wheat germ agglutinin-Oregon green 488 (W6748), and Prolong gold antifade reagents were purchased from Invitrogen Corp. Hamilton syringes and needles were purchased from Hamilton Company. Sprague-Dawley rats were supplied by Charles River Laboratories International, Inc.

Hydrogel Preparation

The hydrogel was prepared according to the method outlined by Shu et al. In brief, this method created a 2-liquid component system that, when mixed together, formed a hydrogel. In this experiment, the components remained separated until the time of surgery, when the gels were allowed to form in situ. The first component was a thiolated HA created by adding DTP to the glucuronate carboxyl group of HA and removing the disulfide bond using DTT. The second component was a PEGDA solution, which cross-links the thiol groups of HA.

The thiolation of HA was performed in a 2-step reaction. First, we performed a 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide–mediated reaction in which DTP and HA reacted at pH 4.75 for 1 hour. The reaction was terminated by raising the pH to 7.0. The disulfide bond in DTP was broken, which formed a thiol group by adding the reducer DTT at pH 8.5. The solution was dialyzed against a buffer (100 mM NaCl, 0.3 mM HCl) at pH 3.5 to remove reactants and by-products.

Solutions of 0.52%, 0.64%, 0.75%, 1%, and 1.25% (w/v) thiolated HA and a 2.25% (w/v) solution of PEGDA were prepared in PBS at pH 7.4. All solutions were filtered through a 0.2-μm syringe filter to sterilize them. At the time of surgery, the HA and PEGDA solutions were mixed together at a 0.45 PEGDA/thiolated HA ratio. After mixing, the PEGDA acted as a bifunctional electrophilic cross-linker, which reacted with the nucleophilic thiol groups in a Michael-type addition. The cross-linking created a stable hydrogel.

Hydrogel Analysis

The morphologies of the HA hydrogels were visualized with a scanning electron microscope (SEM). Fully cross-linked HA gels of 0.64%, 0.75%, 1%, and 1.25% (w/v) thiolated HA were flash frozen by immersion in liquid nitrogen, fractured with a scalpel, and placed immediately on a lyophillizer for freeze-drying. The HA gels were then gold coated (10 μm), and images were acquired with the SEM.
(XL30 ESEM-FEG, FEI Co.) at 15 kV and a spot size of 2. The SEM images were analyzed for pore size by using MATLAB (MathWorks, Inc.).

Surgical Procedure

A total of 89 adult male Sprague-Dawley rats (weighing 250–350 g) were used in 2 separate cohorts of animals. The experimental design of each cohort is shown in Fig. 1 and described below; in brief, each cohort contained a sham surgery group (negative control), a positive control group, and an HA hydrogel–treated group.

Each animal was anesthetized with an intramuscular injection of ketamine/xylazine/acepromazine (50/7/1 mg/g of body weight). Fluoroscopy was used to identify the T9–10 level, which was marked with a gold bead inserted subcutaneously. Under sterile conditions, a T-10 laminectomy was performed, and the dura was opened. In each rat in the control and HA hydrogel–treated groups, a lateral hemisection of the left spinal cord was performed at T9–10 using an operating microscope (Zeiss OPMI Pentero, Carl Zeiss Meditec, Inc.). The hemisection was carefully verified by placing a dissector in the gap. Figure 2 shows the spinal cord before, during, and after the hemisection through the operating microscope. Adequacy of the hemisection was confirmed by 3 independent observers, including the surgeon using the operating microscope. High-magnification digital images of each completed hemisection were obtained. Next, either the experimental HA hydrogel or PBS was injected into the hemisection lesion until the lesion was filled but not expanded. This methodology led to a variable HA hydrogel volume that was approximately 20–30 μl in all cases. The surgeon was blinded to the choice of material for injections, although he could possibly feel and see the difference between the PBS and HA hydrogel, depending on the viscosity of the substances. Finally, the muscular fascia was sutured with Vicryl 4.0 (Ethicon, Inc.), and the skin was sutured with Ethilon 3.0 (Ethicon). The dura was not closed. The surgeries were identical in the sham group except that hemisection of the cord was not performed.

Each animal received ceftriaxone for 3 days after surgery. Manual bladder expression was performed until the animals recovered bladder control. All animal experiments were performed with approval from the Institution.
al Animal Care and Use Committee at St. Joseph's Hospita-

tal and Medical Center.

Retrograde Tracing

Twenty-three adult male Sprague-Dawley rats (weigh-
ing 250–350 g) were randomly assigned to 1 of 3 groups
using Excel (Microsoft Corp.): sham (n = 3), control (n = 5),
or HA hydrogel treated (n = 15). Twelve weeks after
surgery, each animal received a retrograde tracer injec-
tion caudal to the lesion site. The tracer traveled up the
axons and labeled cells in the corresponding area of the
red nucleus.

For the retrograde tracer injection, each rat first under-
went a laminectomy at L-2, localized using fluoroscopy.
Surficeom was placed around the injection site to prevent
any leakage of the tracer. An incision was made in the pia
matter using a 32-gauge needle. An automatic nanojector
(World Precision Instruments, Inc.) with a 33-gauge
Hamilton needle was positioned in the spinal cord using a
Kopf stereotaxic system. Care was taken to avoid damag-
ing the spinal cord with excess pressure from the injec-
tion. For this reason, the 33-gauge needle was first posi-
tioned 2 mm deep in the left paramedian gray matter and
then was retracted 0.5 mm to allow space for the tracer
to be injected. Over a period of 60 seconds, 100 nl of 2%
Fluoro-Gold (Fluochrome, LLC), a retrograde tracer,
in PBS was injected to allow for pressure equilibration in
the spinal cord. Over the next 60 seconds, the needle was
retracted 0.5 mm. Then, a second 100 nl of Fluoro-Gold
was injected over 60 seconds. After a 60-second delay,
the needle was removed from the spinal cord.

Two weeks after labeling, each animal underwent
induction of anesthesia and was killed by transcardial
infusion of 200 ml of saline followed by 250 ml of 4%
paraformaldehyde in 0.1 M PBS, pH 7.4. The brains and
spinal cords were dissected and placed in 4% parafor-
maldehyde for 24 hours and then in 30% sucrose for 24
hours. Thereafter, the samples were sent to NeuroScience
Associates for cryoprotection (overnight in 20% glycerol
and 2% dimethyl sulfoxide), gelatin embedding, and sec-
tioning to a 40-μm thickness. Every other section was
mounted. Slides were studied under a fluorescent micro-
scope (Olympus BX61, Olympus America, Inc., Scientific
Equipment Group) with a chroma Fluoro-Gold filter set
(excitation/emission 350/515 nm) and a 400-nm dichroic
beam splitter (50 nm wide with 80% transmission).

Cells present on each side of the red nucleus were
counted in every slide and multiplied by 2 because only
every other slide was mounted. The cells in the right red
nucleus corresponded to the lesion side because the rubro-
spinal tracts cross.

Lesion Size

Microscope images of each of the histological slides
from the retrograde tracing experiment were collected.
The lesions were assumed to be rectangular in shape. Le-
sion sizes were approximated using the following equa-
tion:

\[ V_{\text{lesion}} = N \times L \times W \times (T + S), \]

where N is the number of histological sections, L is the
vertical length of the lesion, W is one-half the width of the
thoracic spinal cord, T is the histological slice thickness,
and S is the distance between histological slices (which
is the same as T). The volume of the HA hydrogel was
excluded from lesion-size calculations.

Behavioral Testing

The motor function of each hind limb was assessed by
using the Basso, Beattie, and Bresnahan (BBB) scale. In
brief, during 4 minutes of free activity, we used a 21-point
scale to evaluate joint movements, paw placement, weight
support, and forelimb-to–hind limb coordination. As-
essments were performed weekly for 12 weeks.5 Scores
were averaged between 2 independent observers who were
blinded to the treatment group.

Histochemical Analysis

Sixty-six adult male Sprague-Dawley rats (weighting
250–350 g) were assigned to 1 of 3 groups: sham (n = 12),
control (n = 12), or HA hydrogel treated (n = 42). Either 8 or
16 weeks after surgery, the animals were split evenly into
1 of the 2 time point groups and anesthetized and killed
as described above (Fig. 1). Samples were cryo-protected
in 30% sucrose for 24 hours before extraction of a 1.0-cm
length of spinal cord at the injury site. The section of spi-

nal cord was embedded in paraffin and serially sectioned
parasagittally in multiple 20-μm slices. The sections were
stained with H & E for general morphology. Immunohis-
tochemistry was performed to study specific biomarkers.
Samples were stained with a neurofilament stain to study
neurons and axons (mouse antineurofilament 200, 1:500;
Sigma), the Nauta method of silver staining to selectively
stain disrupted axons (performed by NeuroScience As-

FIG. 2. Pictures of the hemisection surgery through the operative microscope showing a dissector applied to the anterior aspect of the spinal cord to prevent damage of the anterior dural venous plexus during hemisectioning (A), a hemisection performed with a scalpel (B), and instruments removed after the hemisection (C).
sociates), an astrocyte stain (mouse antiglial fibrillary acidic protein, 1:200; Boehringer-Mannheim Chemicon), and an Iba1 stain for mononuclear cells such as macrophages and microglia (antibody against ionized calcium-binding adaptor molecule 1, performed by NeuroScience Associates). All microscopic analyses were performed by a professional pathologist.

Transmission Electron Microscopy

Regenerating axons were identified by preparing the spinal cord from 1 rat in each histochemical analysis group for transmission electron microscopy (TEM). Tissues were fixed overnight in 3.0% glutaraldehyde buffered to a pH of 7.4 with Millonig phosphate buffer and post-fixed for 2 hours with 2.0% osmium tetroxide buffered to a pH of 7.4 with Millonig phosphate buffer. The samples were then dehydrated with ethanol and propylene oxide and embedded in Epon substitute (LX-112). Blocks were sectioned to 80 nm using a diamond knife (DiATOME Ltd), mounted on 150-mesh copper grids (Ted Pella, Inc.), and stained with lead citrate and uranyl acetate. Transmission electron microscopy images were taken with a Philips CM100 transmission electron microscope (FEI Co.). Images were captured with a Gatan 697 or Gatan Orius (SC1000 1) camera. Image contrast and brightness were adjusted using Adobe Photoshop.

Statistical Analysis

All parametric data were analyzed with an ANOVA test in Statistica (StatSoft, Inc.). The differences in number of cells were analyzed using repeated-measures ANOVA. All other data were analyzed with 1-way ANOVA. In all cases, the normality assumption was checked with the normal probability plot. Post hoc t-tests were performed in Excel (Microsoft Corp.) to determine specific differences between groups; p values less than 0.05 were considered significant. Error bars shown in the figures represent the SD. In Results, mean values are presented ± SD.

Results

Properties of the HA Hydrogels

Pore size and channel formation are important for cell infiltration. The SEM images showed that the pores formed interconnected channels in all the HA hydrogels (Fig. 3). Furthermore, the pore sizes (mean 131 ± 87 to 210 ± 59 μm) were not significantly different between the various weight-percent gels listed in “Hydrogel Preparation” (Fig. 4). Because there were no apparent differences between the different weight-percent gels, data from all HA hydrogel–treated groups were analyzed together for the remainder of the experiment.

Exclusions Due to Surgery

A total of 5 animals from the retrograde tracing cohort were excluded from analysis. Two animals in the HA hydrogel–treated group died, and 1 animal in the control group was killed because of extensive chewing of a hind paw during the course of the study. Two other animals were excluded from the study after the complete follow-up period: 1 in the sham group that showed signs of a vascular lesion and 1 in the control group that seemed to have an incomplete microscopic hemisection. Therefore, the study included data from 18 rats: 2 in the sham group, 3 in the control group, and 13 in the HA hydrogel–treated group. A total of 5 animals in the histology cohort died. One animal was in the control group, and the other 4 were in the HA hydrogel–treated group. Therefore, the study included histology data from 61 animals: 12 in the sham group, 11 in the control group, and 38 in the HA hydrogel–treated group.

FIG. 3. Scanning electron microscope images of HA hydrogels at concentrations of 0.64% (A), 0.75% (B), 1% (C), and 1.25% (D) HA (w/v). Bar = 50 μm.

FIG. 4. Quantification of pore size in different HA weight-percent gels using SEM images.
Retrograde Tracing

The right red nucleus was contralateral and corresponded to the left spinal hemisection. The total numbers of labeled cells in the red nuclei did not vary among the HA hydrogel–treated, control, and sham groups (p = 0.16); however, the numbers of cells on the right side were significantly lower than the numbers of cells on the left side in both the control group (mean 267 ± 108 cells on the left, 106 ± 125 cells on the right; p = 0.006) and the HA hydrogel–treated group (mean 698 ± 259 cells on the left, 568 ± 250 cells on the right; p < 0.0001) (Fig. 5A). Furthermore, the ratio of number of cells on the right to the number of cells on the left (R/L) was significantly lower (p = 0.0005) in the control group (mean 0.32 ± 0.28) than in the HA hydrogel–treated group (0.78 ± 0.12) (Fig. 5B). The improved ratio in the HA hydrogel–treated group indicated a greater percentage of functioning neurons in that group. The number of labeled cells in the sham group was not significantly different between sides (p = 0.70), and the R/L ratio (mean 0.89 ± 0.04) was not significantly different from either the control group (p = 0.07) or the HA hydrogel–treated group (p = 0.33). These results indicate that there were more functional axons in the HA hydrogel–treated group than in the control group. In a fully regenerated spinal cord, the number or ratio of axons in the treated group would not be different from that in the sham surgery group; however, the sham group did not differ from the control group, so the significance of this result is unclear.

Lesion Size

The lesions had a significantly lower (p = 0.03) mean volume in the HA hydrogel–treated group (1292 ± 334 mm³) than in the control group (2884 ± 1753 mm³) (Fig. 5C). The significant difference in lesion size might indicate either retention of axons or axon regeneration. Data from the sham group were not included in the analysis because the lesion volume was 0.

Behavioral Testing

All the groups showed some recovery of motor function, assessed according to the BBB scale, during the 12-week follow-up period (Fig. 5D). Compared with the left side, the right side in both the control and HA hydrogel–treated groups had decreased function (p = 0.002), but there was no significant difference between the control and HA hydrogel-treated groups (p = 0.95).

Microscopic Analysis

The H & E stain from the control samples revealed dense scar tissue filling the lesions and disorganization of the tissue cranial to the lesion site (Fig. 6A). The HA hydrogel–treated group not only lacked scar tissue in the lesion but also retained more structural alignment above the lesion site (Fig. 6B). The HA hydrogel in the lesion site showed slight degradation 8 weeks after injection (Fig. 6B). The Nauta silver stain revealed more disrupted axons...
above the lesion site in the control group (Fig. 6C) than in the HA hydrogel–treated group (Fig. 6D). Similarly, the neurofilament stains in the controls (Fig. 6E) revealed a pronounced loss of axons cranial to the lesion site, which was absent in the HA hydrogel–treated group (Fig. 6F). The neurofilament stain adjacent to the lesion showed that axons in the control group were poorly aligned in the lesion scar (Fig. 6G), whereas the HA hydrogel–treated group maintained axon alignment along the edge of the lesion (Fig. 6H). Together, these results show that there was less scar tissue and subsequently more axons in the HA hydrogel–treated group than in the control group, as evident by the neurofilament stain, retrograde tracing, and decreased mean lesion size.

Inflammation was present in both the HA hydrogel–treated and control groups, although it appeared qualitatively better in the HA hydrogel–treated group (Fig. 6D). The Iba1 stain (Fig. 7A and B) revealed the presence of mononuclear cells in both the control and HA hydrogel–treated groups, respectively, although there were fewer mononuclear cells in the HA hydrogel–treated group. Reactive astrocytes were noted near the lesion site (Fig. 7C). Transmission electron microscopy confirmed the presence of inflammatory cells, including both mononuclear macrophages, some of which had lysosomes filled with myelin debris, and multinucleated macrophages (Fig. 7D and E, respectively). Neither neutrophils nor necrosis was found with TEM in the HA hydrogel–treated group.

Transmission electron microscopy also showed peripheral-type myelinated nerve fibers in the spinal cord tissue above the lesion site in the control group (Fig. 6C) than in the HA hydrogel–treated group (Fig. 6D). Similarly, the neurofilament stains in the controls (Fig. 6E) revealed a pronounced loss of axons cranial to the lesion site, which was absent in the HA hydrogel–treated group (Fig. 6F). The neurofilament stain adjacent to the lesion showed that axons in the control group were poorly aligned in the lesion scar (Fig. 6G), whereas the HA hydrogel–treated group maintained axon alignment along the edge of the lesion (Fig. 6H). Together, these results show that there was less scar tissue and subsequently more axons in the HA hydrogel–treated group than in the control group, as evident by the neurofilament stain, retrograde tracing, and decreased mean lesion size.

Discussion

Multiple aspects of SCI have been studied in terms of pathophysiology and treatment strategies. Despite the identification of multiple approaches for decreasing posttraumatic ischemia and inflammation, and for boosting the initial neurite outgrowth in the central nervous system, the prevailing cavitation limits the possibility of bridging the gap. Therefore, multiple scaffolding strategies have been explored. Autotransplantation of stem cells, Schwann cells, and olfactory ensheathing cells in the damaged spinal cord, at both short and long time periods after injury, and implantation of artificial materials have been performed with various results. Hyaluronic acid is especially interesting in light of the fact that not only is it a natural part of the extracellular matrix and is nonimmunogenic, but also it has been used commercially in the skin and joints and intraabdominally. It has also shown strong neurite-promoting characteristics in vitro. In vivo, HA has been shown to have neuroprotective
Hyaluronic acid hydrogel is neuroprotective in spinal cord injury

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and antiinflammatory functions. Khaing et al. found an antiinflammatory effect of an HA hydrogel in a hemisection model, and Austin et al. found that a composite hydrogel that included HA had a neuroprotective effect in a crush-injury model.

In this study, HA hydrogels were placed in a hemisectioned spinal cord lesion to promote regeneration. Structural changes in the spinal cord were studied using histochemistry, functional changes were assessed using a retrograde tracer, and the effects of those changes on motor function were observed. The hemisection model is not as rigorous as a full transection model; more often, human SCI involves some combination of contusion, crush, pinch, twist and torsion, partial tearing, and vascular injury. We chose a hemisection model because of the relatively controlled injury environment in which only part of the spinal cord was damaged. This model had the added benefit of making the animal model much easier to maintain than other models, which can include extensive morbidity, such as seen in full transection or crush-injury models, specifically with bladder function and ability to feed and groom. Furthermore, the hemisection model had the advantage of easy implantation of the HA hydrogel; in contrast, in crush-injury models, the spinal cord is closed, when not torn by the impact, and the injection site is difficult to determine. In addition, crush injuries are highly variable in terms of the characteristics and features of injury. Therefore, the controlled injury environment of the hemisection model made the HA effect more easily assessed in a small number of animals than the crush-injury models, in which there is greater animal-to-animal variability. The choice of a hemisection model limited the study in that hemisection injuries are far less common than contusion injuries; however, the previous success of an HA crush-injury model that includes hydrogel makes it likely that the HA hydrogels are neuroprotective in multiple types of SCI. The lesions were significantly smaller in the HA hydrogel–treated group than in the control group; in addition, there were noticeable changes between the HA hydrogel–treated and control groups in the quality of the lesions. The HA hydrogel–treated group had increased longitudinal alignment of the tissue above and adjacent to the lesion site. Furthermore, there was increased retention of intact axons immediately cranial to the lesion site and decreased mononuclear cells present in the treated groups. A major function of the neuroinflammatory pathway is to restore the blood–brain barrier. In this experiment, the blood–brain barrier was partially restored by the HA hydrogel as a result of simple mechanical blocking of exogenous inflammatory cells. The artificial barrier might have led to the decreased inflammatory response by the spinal cord and, subsequently, to decreased scar tissue formation in the spinal cord and lesion site. The decreased scar tissue correlated with the retention of functional axons adjacent to the lesion in the HA hydrogel–treated group, as indicated by the increased retrograde tracer uptake. There was no neural tissue visible in the hydrogel, indicating that the hydrogel was not functioning as a scaffold; therefore, it is probable that the increased function was a result of the neuroprotection.

There are multiple ways in which gel formation in situ could provide neuroprotection. Because the gel forms a...
A tight seal over the lesion site, one possible neuroprotective mechanism is the mechanical blocking of exogenous inflammatory cells and fibroblasts. This mechanical blocking would prevent scar tissue from forming in the lesion site and decrease infiltration of cells into the spinal cord. HA itself has also been shown to have neuroprotective properties; therefore, the neuroprotective effect could be a result of molecular interaction of the HA with the surrounding cells. However, the gel might also have neuroprotective effects not directly tested in this experiment, such as prevention of hematoma formation and a corresponding decrease in toxic effect from hemoglobin degradation, prevention of cerebrospinal fluid leakage, and mechanical support of the spinal cord near the lesion.

The neuroprotective effects of the HA hydrogel will need to be tested for their time-dependent effect. In this study, the HA hydrogel was placed in the surgical site immediately after injury for ease of the surgical procedure; however, clinically, the hydrogel would not be placed until hours after an injury. The time delay could hinder the neuroprotective effect of the HA hydrogel.

Although neuroprotection led to a decreased lesion volume and greater retention of functional axons, motor function was not significantly improved according to the BBB scale scores. In hemisection models, especially in rats, there is expected recovery due to collateral recruitment. Therefore, with a larger sample size or a different animal model, an HA hydrogel–treated group could show some functional motor improvement compared to that of control animals.

One limitation of this study was the small number of animals in the behavioral testing group, with 18 animals included in total and only 2 animals in the sham group. Furthermore, no data on sensation or bowel and urinary function were collected, so it is unknown if there were nonmotor functional improvements. However, even if the HA hydrogel did not cause any motor or sensory retention, it could serve an important function in future or concurrent regenerative efforts. The retention of functioning axons near the edge of the lesion, decreased scar tissue, and decreased cavitation all serve to decrease the gap that regenerating nerves need to cross.

Even in its basic form, the HA hydrogel shows potentially useful neuroprotective effects, but this study could also be considered a basis for further development. For instance, results could be improved by adding growth-promoting signaling factors (i.e., neural growth factor), growth-inhibitory signaling suppressors (i.e., neurite outgrowth inhibitor 66 receptor antibody), cell-attachment moieties, and various types of progenitor cells. Much of this work has already been performed or is underway by other research groups; however, the work development.
scribed here highlights the importance of base material choices in the development of complex scaffolding systems.

**Conclusions**

Hyaluronic acid hydrogel protected the spinal cord from inflammation and reduced secondary injury in an animal model. The HA hydrogel–treated group had decreased lesion sizes and significant retrograde uptake of the tracer. Although these results could indicate regeneration, they more likely indicate decreased scar tissue formation. The immunohistochemistry results showing decreased inflammatory cell presence, decreased scarring, and increased axon retention in the HA hydrogel–treated group support this theory. Although results of the behavior assessment in this experiment did not indicate functional improvement in the treated group, the decrease in lesion size and the retention of structural alignment of the spinal cord near the lesion could be important for future regenerative efforts.

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Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Preul, Kushchayev, Hom Eng, Martirosyan, Mortazavi, Theodore, Panitch. Acquisition of data: Kushchayev, Hom Eng, Martirosyan, Mortazavi. Analysis and interpretation of data: all authors. Drafting the article: Preul, Kushchayev, Giers, Hom Eng, Martirosyan, Mortazavi, Panitch. Critically revising the article: Preul, Kushchayev, Giers, Eschbacher, Mortazavi. Reviewed submitted version of manuscript: all authors. Statistical analysis: Giers. Administrative/technical/material support: Preul, Panitch. Study supervision: Preul, Panitch.

Supplemental Information
Previous Presentations
 Portions of this work were presented at annual meetings of the American Association of Neurological Surgeons, San Diego, CA, May 6, 2009, and Washington, DC, May 4, 2015. Portions of this work were also published in 2009 in the doctoral dissertation “Hyaluronan hydrogels for spinal cord regeneration” by Doris Hom Eng, Arizona State University.

Current Affiliations
Dr. Kushchayev: Department of Radiology, Mercy Catholic Medical Center, Philadelphia, PA; Dr. Hom Eng: Ventana Medical Systems, Inc., Tucson, AZ; Dr. Mortazavi: California Neurological Institute, Los Robles Hospital and Medical Center, Thousand Oaks, CA.

Correspondence
Mark C. Preul, c/o Neuroscience Publications, Barrow Neurological Institute, St. Joseph’s Hospital and Medical Center, 350 W. Thomas Rd., Phoenix, AZ 85013. email: neuropub@dignityhealth.org.