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A Mouse Model of Ischemic Spinal Cord Injury with Delayed Paralysis Caused by Aortic Cross-clamping

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ABSTRACT

Background: Spinal cord ischemia and paralysis are devastating perioperative complications that can accompany open or endovascular repair surgery for aortic aneurysms. Here, we report on the development of a new mouse model of spinal cord ischemia with delayed paralysis induced by cross-clamping the descending aorta.

Methods: Transient aortic occlusion was produced in mice by cross-clamping the descending aorta through a lateral thoracotomy. To establish an optimal surgical procedure with limited mortality, variable cross-clamp times and core temperatures were tested between experiments.

Results: The onset of paresis or paralysis and postsurgical mortality varied as a function of cross-clamp time and core temperature that was maintained during the period of cross-clamp. Using optimal surgical parameters (7.5-min cross-clamp duration at 33°C core temperature), the onset of paralysis is delayed 24–36 h after reperfusion, and more than 95% of mice survive through 9 weeks after surgery. These mice are further stratified into two groups, 70% (n = 19/27) of mice developing severe hind limb paralysis and the remaining mice showing mild, though still permanent, behavioral deficits.

Conclusion: This new model should prove useful as a preclinical tool for screening neuroprotective therapeutics and for defining the basic biologic mechanisms that cause delayed paralysis and neurodegeneration after transient spinal cord ischemia.

What We Already Know about This Topic

- Spinal cord ischemia and paralysis are devastating perioperative complications that can accompany open or endovascular repair surgery for aortic aneurysms.

What This Article Tells Us That Is New

- A new mouse model of spinal cord ischemia with delayed paralysis induced by cross-clamping the descending thoracic aorta may prove useful for defining mechanisms of delayed paralysis and as a screening tool for neuroprotective therapeutics after transient cord ischemia.

SPINAL cord ischemia with paralysis is a devastating perioperative complication of surgical repair of aortic aneurysms.[1] Paralysis can be immediate but is often delayed, with the reported incidence (0–40%) varying among centers and as a function of the surgery and Crawford classification. Recent reviews focusing on high-volume centers indicate that 5–11% of cases are accompanied by delayed postoperative paresis or paralysis.[2–4]

To understand the mechanisms responsible for ischemic spinal cord injury (ISCI), a number of experimental models have been developed.[5–9] Rat, rabbit, dog, sheep, and pig models are used regularly; however, none is ideal for testing preclinical therapies or understanding mechanisms of injury. They are either too expensive (*e.g.*, pig, dog), associated with high incidence of morbidity and mortality, or lack reproducibility. In 2000, a mouse model of ISCI was developed to take advantage of a growing number of gene knock-in and knockout mice.[10] Indeed, with the mouse genome defined, it is now possible to study the effects of spinal ischemia on specific genes and molecular signaling pathways. Although it

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Table 1. Summary of Surgical Parameters and Group Sizes

Cross-clamp Location	Temperature, °C	Cross-clamp Time, min	n
Aortic Arch, LSA	35–36	8–11	14
Descending Thoracic Aorta	35	3–7.5	11
Descending Thoracic Aorta	35	8–11	8
Descending Thoracic Aorta	33	3–7	11
Descending Thoracic Aorta	33	7.5	29
Descending Thoracic Aorta	33	8–10	3
Descending Thoracic Aorta	33	7.5 (acute time points)	24
Descending Thoracic Aorta	33	7.5 (MAP and blood gases)	6

LSA = left subclavian artery; MAP = mean arterial pressure.

has been ~10 yr since the first ISCI mouse model was described, it has been used sparingly, probably because the surgical approach is difficult and postsurgical mortality is high.[10] For animals that do survive, survival is limited to less than 1 week and paralysis is usually immediate.[10–13] Our goal was to create a new clinically relevant model of mouse ISCI in which these various outcomes could be controlled more consistently.

Here, we describe a simple and reproducible preclinical model of murine ISCI caused by transient cross-clamp of the descending aorta through a lateral thoracotomy. Using a battery of physiologic, behavioral, and anatomical assays, we show that this ISCI model causes reproducible intraspinal inflammation and neuropathology accompanied by profound neurologic impairment. The onset and magnitude of paralysis varies as a function of aortic cross-clamp time, and the intraoperative core temperature that was maintained during the period of ischemia. By changing these parameters, mice can be consistently stratified into two groups: mice in which paralysis is immediate and severe and those in which paralysis is delayed, thereby mimicking the phenomenon that occurs in a subset of humans undergoing similar surgery. This new model will facilitate the screening of neuroprotective therapeutics and will help reveal basic mechanisms underlying postischemia/reperfusion pathologic conditions and functional impairment caused by aortic cross-clamp.

Materials and Methods

Mice

Adult C57BL/6 mice (male and female, 7–12 weeks old; 17–22 g) were purchased from Harlan (Indianapolis, IN) or The Jackson Laboratory (Bar Harbor, ME). All procedures were performed using aseptic technique, and all mice were housed in high-efficiency particulate air-filtered Bio-clean units. All procedures were approved by and performed in accordance with The Ohio State University's Institutional Lab Animal Care and Use Committee (Columbus, Ohio).

Anesthesia and Surgical Preparation

For all surgical procedures, mice were maintained at predefined core temperatures (33, 35, or 36°C) on a temperature-controlled surgical platform (World Precision Instruments, Sarasota, FL). Mice were anesthetized by inhalation of 3% isoflurane driven by 100% O_2 for induction, then maintained at 2% isoflurane (100 ml/min O_2). Ventilation was achieved using an endotracheal cannula and a mouse ventilator [Hugo Sachs-Harvard Apparatus Minivent (Hollinston, MA); tidal/stroke volume, 250 μl; rate, 230 ventilations/min]. For tracheal intubation, mice were anesthetized, and a ventral midline incision was made between the ears and extending slightly past the anterior-aspect of the pinna. The underlying musculature and submaxillary glands were retracted laterally to expose the larynx and trachea and a lubricated mouse tracheal intubation cannula was inserted into the trachea (Hugo Sachs VK32). The throat incision was closed with surgical glue and a single suture. Before thoracotomy, mice were injected subcutaneously with heparin (200 IU/kg), xylazine (0.1 mg/mouse in 0.02 ml), and gentamicin (0.1 mg/mouse in 0.1 ml).

Spinal cord ischemia was induced by transient aortic cross-clamp at the level of either the aortic arch or descending aorta, as described below. Numbers of animals in the different experimental groups are provided in table 1.

Transient Cross-clamp at the Aortic Arch and Left Subclavian Artery

Initial studies used the methods described by Lang-Lazdunski *et al.*[10] In brief, this method uses an anterior sternotomy with transient aortic occlusion produced by aneurysm clips placed at the aortic arch (between the left common carotid and left subclavian arteries [LSA]) and on the LSA (at its origin). Core temperature was maintained at 35–36°C.

Transient Cross-clamp at the Descending Thoracic Aorta

Each intubated mouse was placed on its right side (horizontal lateral position) and the left forelimb was positioned laterally beneath the mandible and secured to the surgical platform. A small transverse (dorsal to ventral) incision was made below the left forelimb and shoulder to expose the underlying rib cage. Using scissors, muscle between the second and third rib was cut, exposing the lateral pleura. Retractors were used to open the incision and lateral hooks were used to completely expose the inferior vena cava, thymus, left atrium, and de-

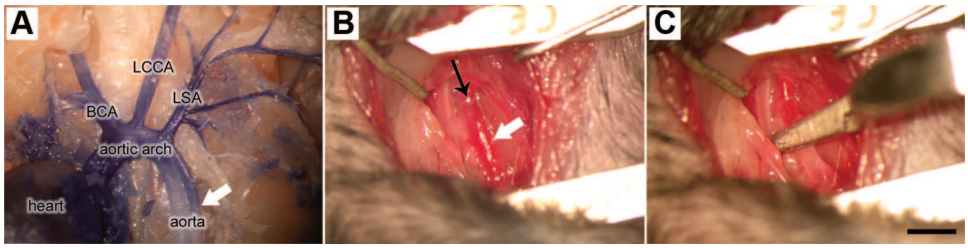

Fig. 1. Visualizing the surgical site and region of aortic cross-clamp. (*A*) Post mortem dissection of the heart and thoracic vascular tree showing the location of descending aorta cross-clamp (*arrow*). Intravascular perfusion with 1% Evans Blue dye was used to visualize the heart and vasculature. (*B*) Example of intraoperative exposure of the descending thoracic aorta (*white arrow*). *Thin black arrow* indicates direction of blood flow. *White arrow* indicates location of aneurysm clip. (*C*) The same surgical site as in *B* but after placement of the aneurysm clip. Note blanching of the aorta distal to the clip, indicating lack of blood flow. Scale bar in *C* = 1.6 mm and applies to *B* as well. BCA = brachiocephalic artery; LCCA = left common carotid artery; LSA = left subclavian artery.

scending thoracic aorta. Forceps were used to blunt dissect ~2 mm of the descending thoracic aorta beginning 1 mm distal to the LSA artery. To ensure completeness of the cross-clamp, the aorta was elevated slightly using a stainless steel hook and then a small aneurysm clip was placed across the vessel (fig. 1A–C). Aortic cross-clamp was maintained for 3–11 min, after which the clip was removed and surgical sponges were used to absorb fluid overlying the surgical site. The rib cage, muscle, and overlying skin were closed using 6-0 polypropylene and 5-0 Dermalon/nylon sutures.

Postsurgical Care

After reperfusion, mice spontaneously recovered from anesthesia on the surgical platform while maintaining their core temperature. After ~15 min, mice were extubated and placed into an oxygen-supplemented intensive care unit (oxygen flow rate ~1 l/min) where they were maintained at ambient room temperature for 4–5 days. Bladders were manually expressed twice daily for the duration of the experiment. Mice also were given 1.0–1.5 ml lactated Ringer's solution (subcutaneously) twice per day and prophylactic antibiotics once per day through the first 7 days after surgery. Some mice developed seizures 12–48 h after surgery (see Results). To quiet seizure-like activity, mice were injected intraperitoneally with ketamine and xylazine diluted to ~33% of a normal anesthetic dose.[14–16]

Behavioral Evaluation

Bilateral hind limb function was monitored daily (in some cases up to three times per day) for the first week, then once a week thereafter using the Basso Mouse Locomotor Rating Scale. The Basso Mouse Scale is a 10-point scale (0–9) that uses operational definitions to quantify the magnitude and rate recovery of hind limb movements, forelimb-hind limb coordination, and trunk stability in spinal cord-injured mice.[17]

Hemodynamic Monitoring and Measurements of Blood Gases

Small superficial incisions were made over the left carotid and left femoral arteries in a small cohort (n = 6) of intubated mice. To cannulate the femoral artery, the artery was isolated by blunt dissection, and proximal blood flow was briefly interrupted by placing a small aneurysm clip proximal to the surgery site. Then, a small transverse incision was made on the artery using fine Vannas-style vascular scissors (Fine Science Tools, Foster City, CA). The cannula, heat-stretched polyethylene-10 tubing (0.2 mm OD), with the tip cut to a bevel, was threaded through the incision into the proximal artery and secured with a single silk suture. The carotid artery was cannulated by threading a 0.3-mm diameter cannula 5–6 mm toward the heart through a guide hole made using a 26-gauge needle. The cannula was then secured using a silk suture. Both cannulas were attached to a two-channel blood pressure/HR monitor (Columbus Instruments Physiomex, Columbus, OH).

Tissue Harvest and Processing

At different times after aortic cross-clamp, mice were anesthetized with ketamine/xylazine then transcardially perfused with 25 ml 0.1 M phosphate-buffered saline, followed by 100 ml paraformaldehyde, 4%, in phosphate-buffered saline. Spinal cords were removed, postfixed for 2 h, and then incubated in 0.2 M phosphate buffer for 18 h at 4°C. The next day, tissues were placed in 30% sucrose in 0.2 M phosphate buffer and stored for 48–72 h. Sucrose-infiltrated tissues spanning the low-cervical to sacral cord were blocked every 1.5 mm, embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Torrance, CA), and then rapidly frozen on powdered dry ice. From these blocks, 10-μm cross-sections were cut on a cryostat and collected onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA). Tissues were collected as 20 sets of serial sections (200 μm between adjacent sections) and then stored at −20°C.

Histology and Immunohistochemistry

Adjacent sets of tissue sections were used for histologic and immunohistochemical analyses. Sections were stained with a cocktail of antimouse 200-kDa neurofilament (chicken antineurofilament heavy chain) and biotinylated antihuman HuC/HuD with and without eriochrome cyanine (to visu-

alize myelin), as described previously.[18,19] When primary antibodies were derived from mice, nonspecific staining was blocked using a cocktail of bovine serum albumin and complete horse and mouse serum for 1 h at room temperature. After blocking solution was removed, sections were overlaid with preconjugated primary and secondary antibody cocktail (*e.g.*, containing mouse anti-X plus biotinylated horse antimouse immunoglobulin G diluted in blocking solution) for 18 h at room temperature or 4°C. The primary antibodies and their final concentrations were as follows: chicken antimouse neurofilament heavy chain (axons/dendrites, 1 μg/ml; Aves Labs, Tigard, OR), biotinylated mouse antihuman HuC/HuD (neuronal somas, 1 μg/ml; Invitrogen, Carlsbad, CA), rat antimouse CD11b (macrophages/microglia, clone MAC-1, 1 μg/ml; Abcam Inc., Cambridge, MA), rabbit anticow glial fibrillary acidic protein (glial fibrillary acidic protein: astrocytes, antiserum, 1:20,000 dilution; Dako North America, Inc., Carpinteria, CA).

Quantitative Lesion Analysis

Spinal cord lesion volumes at 7 days after ischemia were analyzed using the Cavalieri method as described previously.[18,20] Images of antineurofilamant-heavy+HuC/D double–stained sections were digitized using a Zeiss Axioplan II Imaging microscope (Carl Zeiss, Thornwood, NY) and an MCID 6.0 Elite system (InterFocus Imaging, Cambridge, UK). Low-power digitized sections were printed at 30× magnification, and areas of total tissue, spared gray matter, and lesion were manually circumscribed. Spared gray matter was defined as tissue containing normal gray matter cytoarchitecture with visibly healthy neuron profiles present at a normal density. Frank lesion was defined as necrotic tissue or tissue lacking normal cytoarchitecture with few or no neuronal somas. Pathologic condition of white matter (diminished density of transversely oriented axon profiles) was not included in the lesion volume analysis. Uniform point grids were placed randomly onto printed hard copies of digitized images, and points falling within each area of interest were tallied. Reference areas for each section (*e.g.*, tissue area) and total tissue volume were estimated in the same manner. Point tallies were converted into volume estimates using the formula: volume $= T \times a/p \times {}^{11}\sum_{i-1}P_i$, where T equals the slice spacing, a/p equals the calculated area per point, and ${}^{11}\sum_{i-1}P_i$ equals the sum of the points sampled. Areas per section for each region were calculated using the same formula with omission of the T multiplier.

Statistical Analyses

Statistical analyses were performed using Prism 5.0 (GraphPad Software, La Jolla, CA). In all cases, P values less than 0.05 were considered significant, using two-tailed statistical comparisons. Kaplan–Meier survival curves (fig. 2 and Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/A626) were compared using Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests (only Supplemental Digital Content 1, fig. 1 http://links.lww.com/ALN/A626). Behavioral scores (Basso Mouse Scale) are presented as mean ± SEM. Lesion volumes at 7 days after ischemia/reperfusion were analyzed in one representative cohort using one-way analysis of variance with Tukey multiple comparison test.

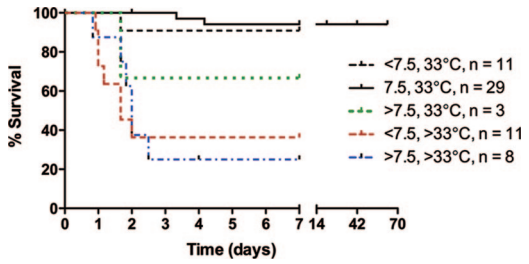

Fig. 2. Transient aortic cross-clamp (up to 7.5 min.) with systemic hypothermia (33°C) markedly improves survival. Kaplan-Meier survival curves illustrate the effects of varying intraoperative core temperature and duration of aortic cross-clamping. Survival curves were significantly different (*P* less than 0.0001), according to the Log-rank (Mantel-Cox) test.

Results

Transient Cross-clamp of the Descending Thoracic Aorta Is a Simplified Surgical Approach That Decreases Mortality

To test therapeutics and define the biologic mechanisms underlying paralysis caused by spinal cord ischemia subsequent to aortic cross-clamp, a model system is needed in which experimental subjects can survive for weeks or months after surgery. Our initial goal was to use the mouse model developed by Lang-Lazdunski *et al.*,[10] then maintain mice for a minimum of 7 days after surgery. In the Lang-Lazdunski *et al.*[10] model, mice receive an anterior sternotomy with transient (9–11 min) cross-clamp at the aortic arch and the LSA while maintaining core temperature at 35–36°C.

In our hands, this procedure caused immediate hind limb paralysis in 80% of cases (n = 12/15), and most mice (n = 13/15) died 1 h to 4 days after ischemia–reperfusion (mean survival time = 39 h; Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/A626, Kaplan–Meier survival curves of mice held at 35°C during aortic cross-clamp at either the aortic arch/LSA or descending aorta). Mice that died or were preemptively euthanized showed labored breathing and seizure-like activity. Seizure-like activities occurred in 67% of mice (n = 10/15), usually by 9 h after surgery (range 1–40 h). Only n = 2 of 15 mice survived to our goal of 7 days after surgery.

To ensure complete cessation of blood flow at the level of the cross-clamp, one must be able to isolate the aorta and LSA from surrounding tissues and then place the aneurysm clip across the entire circumference of the vessels. This is difficult to do in the mouse using the ventral approach described by Lang-Lazdunski *et al.*[10] In addition, because the vagus nerve passes near the LSA and is difficult to separate from the vessel, it can become inadvertently damaged during LSA cross-clamp. This may explain the high mortality rate

using the ventral approach. In an effort to increase survival, we modified our surgical approach such that a left lateral thoracotomy was used to position a single aneurysm clip on the thoracic aorta distal to the LSA (see fig. 1). The lateral thoracotomy facilitates access to the descending aorta, making it easier to isolate the vessel and ensure complete occlusion. Moreover, this approach is clinically relevant as it is often used to (surgically) repair descending thoracic and thoracoabdominal aortic aneurysms in humans.

Using the lateral approach for aortic cross-clamp (35°C), mice developed immediate paralysis (n = 5/8) with a marginal, albeit statistically significant, increase in survival (mean survival = 68 h after surgery; $P = 0.0250$ *vs.* cross-clamp at aortic arch/LSA; see Supplemental Digital Content 1, fig. 1, http://links.lww.com/ALN/A626). Unfortunately, only n = 1 of the 8 mice survived to our target of 7 days after occlusion. Still, because of the improved survival and the relative ease of cross-clamping the descending aorta *via* the lateral surgical approach, all subsequent studies used this surgical approach.

Intraoperative Core Temperature and Duration of Aortic Cross-clamp Affect Postischemia/Reperfusion Morbidity and Mortality

To meet the goal of improving survival to at least 7 days, we experimented with changing intraoperative core temperature and duration of cross-clamp (table 1). In general, the survival and health of the animals varied as a function of core temperature and aortic cross-clamp duration (fig. 2; see also Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A626, which depicts survival of all animals as a function of cross-clamp time and intraoperative core temperature). When core temperature was maintained at 35°C, survival was increased slightly by reducing cross-clamp times to less than 7.5 min. In contrast, > 88% of mice survived to the target survival time of 7 days (n = 38/43) when core temperature was maintained at 33°C. In general, this improved survival was independent of cross-clamp time; however, more than 93% (n = 27/29) of mice survived for at least 7 days (up to 63 days; see fig. 2 and Supplemental Digital Content 1, fig. 2, http://links.lww.com/ALN/A626) when cross-clamp was applied for 7.5 min at 33°C. Approximately 50% of these mice still developed seizure-like activity, but the frequency and intensity of these events was noticeably less than that observed in mice subjected to longer cross-clamp times at higher intraoperative core temperatures.

Transient Occlusion at the Descending Thoracic Aorta Predictably Alters Mean Arterial Blood Pressure and Acid-Base Balance

To verify that the optimal protocol (7.5 min of aortic cross-clamp with core temperature held at 33°C) affected hemodynamic and physiologic parameters as predicted, n = 6 mice were instrumented with indwelling carotid and femoral cannulas. Before aortic cross-clamp, mean arterial pressure was 30–38 mmHg in the carotid artery and 24–34 mmHg in the femoral artery (see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A626, which contains graphs depicting changes in mean arterial pressures caused by aortic cross-clamp as measured in the carotid and femoral arteries in three separate mice). During the period of cross-clamp, mean arterial pressure in the femoral artery dropped to ~10 mmHg. Once the aneurysm clip was removed, femoral mean arterial pressure returned to baseline. Conversely, carotid mean arterial pressure (proximal to the site of occlusion) increased to 60–80 mmHg during cross-clamp and then returned to baseline after clip removal.

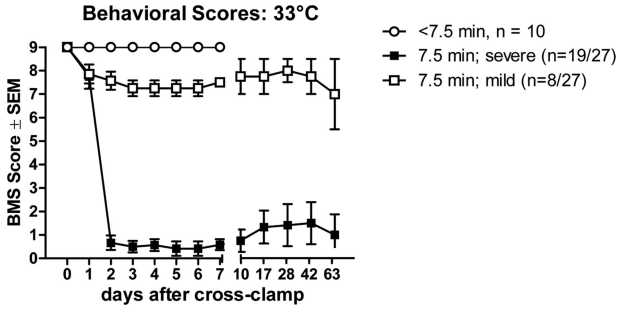

Fig. 3. Transient aortic occlusion (7.5 min) with mild systemic hypothermia (33°C) causes consistent but delayed hind limb locomotor deficits. Analysis of open-field locomotor function using the Basso Mouse Scale (BMS) reveals two functionally distinct cohorts of mice (*i.e.*, those that develop mild *vs.* severe neurological impairment). See Results for detailed description of the functional attributes that define these cohorts. Data are presented as mean (± SEM) BMS score as a function of days after ischemia/reperfusion.

Before cross-clamp, po_2, pco_2, [HCO_3], base deficit, pH, and lactate levels were normal in femoral arterial blood (table 2). Transient (7.5 min) aortic cross-clamp (with ischemia) predictably disrupted these physiologic parameters, causing hypercapnia and acid/base imbalances (increased base deficit and decreased HCO_3) with acidosis (decreased pH and increased lactate).

The Onset and Severity of Paralysis Varies As a Function of Intraoperative Core Temperature and Aortic Cross-clamp Time

Supplemental Digital Content 1, figure 2, http://linkw.lww.com/ALN/A626, provides a summary of the relationship between intraoperative core temperature, aortic cross-clamp time, placement of cross-clamp (*e.g.*, aortic arch *vs.* descending aorta), survival, and paralysis. Below is a descriptive account of these data.

35°C. Survival was poor in this group (see Supplemental Digital Content 1, figs. 1 and 2, http://links.lww.com/ALN/A626), making a detailed analysis of locomotor behavior impossible. Only the onset and severity of functional deficits were recorded. Of those mice subjected to 8–11 min of cross-clamp at 35°C (arch + LSA or descending aorta) that survived for a minimum of 4 h after surgery, 72% (n = 18/25) were paralyzed immediately after recovery from anesthesia, showing flaccid hind limbs without the ability to support weight or step. Of the remaining seven mice, two developed paralysis within 24 h of reperfusion,

Table 2. Physiologic Parameters and Blood Gases before and after Aortic Cross-clamp

	pH		po_2, mmHg		pco_2, mmHg		Beecf, mM		HCO_3, mM		Lactate, mM	
Mouse	Pre-CC	10 min Reper	Pre-CC	10 min Reper	Pre-CC	10 min Reper	Pre-CC	10 min Reper	Pre-CC	10 min Reper	Pre-CC	10 min Reper
1	7.345	7.003	463	150	34.7	36.2	−7	−22	19	9	4.7	10.85
2	7.452	7.007	333	148	28.5	34.7	−4	−22	19.9	8.7	5.05	11.04
3	7.374	7.053	219	97	27.9	28	−9	−23	16.3	7.8	6.86	13.2

Beecf = base deficit; CC = cross-clamp; Reper = reperfusion.

and the other five initially maintained the ability to step. These latter mice subsequently died before a detailed behavioral assessment could be completed.

33°C. Aortic cross-clamp times of 3–7 min failed to produce neurologic impairment in n = 10 of 11 mice that were evaluated up to 7 days after surgery (fig. 3). In one mouse, aortic cross-clamp for 4 min caused immediate hind limb paralysis and severe seizures. This mouse was euthanized 40 h after surgery. By extending the cross-clamp time to 7.5 min at 33°C, we reached a critical threshold at which it was possible to reproducibly cause paralysis without killing the mice. In fact, mice invariably awoke from anesthesia without signs of morbidity or neurologic impairment (fig. 3). After ~24 h, gait abnormalities manifested as toe-drags, occasional missed steps, loss of forelimb–hind limb coordination, a wide base of hind limb support, external hind paw rotation during the lift-off phase of the step cycle, and/or trunk instability (mean onset of deficits, 40.5 h; range, 20–49 h. Mice that survived for at least 7 days could be stratified into two groups based on severity of their hind limb deficits (fig. 3). In most cases (n = 19/27 mice), mice showed a progressive decline in hind limb function that occurred over a period of 6–12 h, with permanent hind limb paralysis being evident by ~48 h (severe; fig. 3). Conversely, a smaller subset of mice (n = 8 of 27) was able to step with only mild deficits in forelimb–hind limb coordination or paw rotation (Basso Mouse Scale scores, 6–8). Regardless of the severity of impairment, none of the mice recovered normal locomotor function during the period of analysis (up to 63 days after surgery; fig. 3). There was no evidence of gross forelimb deficits in any mice.

Spinal Cord Pathology Varies As a Function of Intraoperative Core Temperature and Aortic Cross-clamp Time

Using routine histology and immunohistochemistry, we qualitatively and quantitatively analyzed the temporal sequence of intraspinal pathology and neuroinflammation that occurs subsequent to cross-clamp of the descending aorta for 7.5 min at 33°C. Spinal cords were analyzed at 12 h or 1, 2, 3, 5, 7, 21, 42, or 63 days after reperfusion (n = 4–6/group). In all cases, spinal cord pathology was limited to mid-thoracic (~ T6 spinal level) levels and below. Representative lumbar spinal cord pathology at 21 days after surgery is shown in figures 4–6. Qualitatively similar pathologic conditions are seen at 42 and 63 days (not shown) and in the thoracic spinal cord (see Supplemental Digital Content 1, fig. 4, http://links.lww.com/ALN/A626, which shows the temporal progression of neuropathologic lesions at the level of the mid-thoracic spinal cord). In general, the mild or severe functional deficits described in figure 3 predicted the onset, magnitude, and rostrocaudal extent of intraspinal pathology, gliosis, and neuroinflammation. Figures 4 and 5 illustrate the spatiotemporal progression of pathologic lesions in mice that developed severe neurologic impairment, whereas figures 6 and 7 illustrate quantitative differences between mild and severely affected mice.

In most mice, overt pathologic changes were not evident until 24 h after surgery (figs. 4 and 5), a time when hind limb deficits were beginning to appear (see fig. 3). Pathologic lesions were evident first as small isolated pockets of necrosis within gray matter, usually in Rexed lamina VII lateral to the central canal (fig. 5). Subtle changes in astrocytic and microglial morphology were seen around areas of necrosis and in intact gray matter throughout lamina V and VI and in the ventral horns surrounding neurons (figs. 4 and 5).

Necrosis and lesion expansion progressed between 2 and 7 days after surgery, predominantly within the spinal cord gray matter. This pathologic condition was characterized first by loss of ventral horn neurons and then by the formation of a contiguous necrotic lesion dominated by large phagocytic microglia/macrophages. By 7 days, these lesions extended over multiple segments of the mid-thoracic and lumbar spinal cord (fig. 6). Reactive CD11b-positive microglia and glial-fibrillary acidic protein-positive astrocytes were clearly visible within and surrounding regions of necrosis; activated glia also highlighted white matter tracts in which axons were undergoing anterograde and retrograde degeneration (see figs. 4 and 7). The progressive loss of gray and white matter probably caused the pronounced spinal cord atrophy that was visible by 21 days after surgery (see fig. 4).

Discussion

During surgical repair of thoracic and thoracoabdominal aortic aneurysms, blood flow to the spinal cord is interrupted for minutes or hours. Throughout this time and reperfusion,

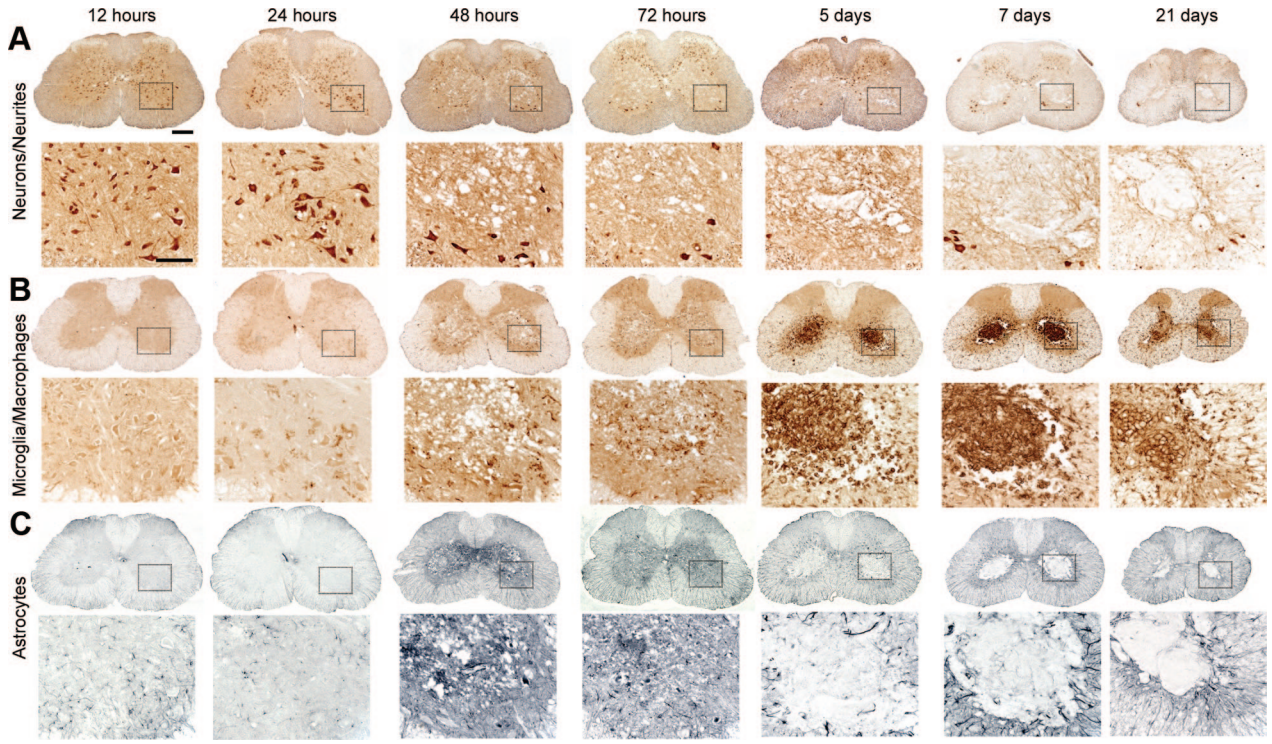

Fig. 4. Temporal sequence of intraspinal pathologic lesions in the lumbar spinal cord caused by two transient aortic cross-clamps (7.5 min) at 33°C. Representative cross-sections in series reveal time-dependent changes in neurons (*A*), microglia/macrophages (*B*), and reactive astrocytes (*C*). Note the significant spinal cord atrophy at 21 days. All sections were sampled from the ~L3–L4 spinal cord and were collected at the times indicated from a severely affected animal. An enlargement of the boxed regions is provided below each cross-section. Scale bar = 200 μm in low-magnification images and 100 μm in high-power images.

irreversible damage to the spinal cord can occur. Although it is well recognized that neurologic deficits, including paralysis, are devastating and all-too-common consequences of aortic aneurysm repair,[1,21] there is no adjunct therapy that effectively eliminates these adverse reactions. In addition, there is no way to predict *a priori* whether paralysis will be immediate or delayed in onset or whether specific mechanisms can explain these unique pathologic presentations.

The need to minimize paresis and paralysis caused by ISCI subsequent to aortic cross-clamp has stimulated the development of pig, canine, rabbit, and rat models.[2,5–9] More recently, a mouse model was developed, making it possible to use transgenic and knock-out mice to reveal the effects of discrete genes and signaling pathways on recovery from ISCI.[10] In that model, an anterior thoracotomy was performed, then aneurysm clips were used to cross-clamp the aortic arch and LSA. The core temperature was maintained at 35–36°C. With this approach, 60–80% of mice developed hind limb paralysis but there was limited survival beyond 48 h of reperfusion. Despite our best efforts using that model, we were able to extend survival to 7 days in only a few mice; most mice died within 72 h of reperfusion. This is a significant shortcoming of the model and limits its use for testing therapeutic interventions or understanding mechanisms that regulate chronic changes in spinal cord structure or function caused by ischemia/reperfusion injury.

Here, we present a simplified mouse model of ISCI in which reproducible behavioral deficits and intraspinal pathologic lesions can be quantified by manipulating the duration of aortic cross-clamp and the core temperature. In addition, the effects of ischemia/reperfusion on hemodynamic and blood gas parameters are similar to those measured in humans undergoing aortic repair surgery.[21] When optimal surgical parameters are used (*i.e.*, cross-clamp for 7.5 min at 33°C), mice develop paralysis, and more than 90% of mice survive indefinitely. In our studies, we maintained mice for as long as 9 weeks after occlusion. The induction and maintenance of systemic hypothermia during the period of aortic cross-clamping and reperfusion seems to be key to ensure this survival. Our model maintains mice at 33°C, a temperature commonly used in the clinic as an adjunct neuroprotective measure during aortic repair and other cardiac surgeries.[22–25] Future studies will determine the optimal parameters for neuroprotective hypothermia, including the magnitude and duration of cooling and the rate of rewarming. Each of these variables is recognized as a critical determinant for the successful translation of hypothermia as a treatment or adjunct to surgical intervention for spinal cord/brain ischemia or traumatic central nervous system injury.[26,27]

An interesting and unexpected consequence of our new model was the delay in onset of intraspinal pathology and hind limb dysfunction or paralysis. Indeed, all mice exhib-

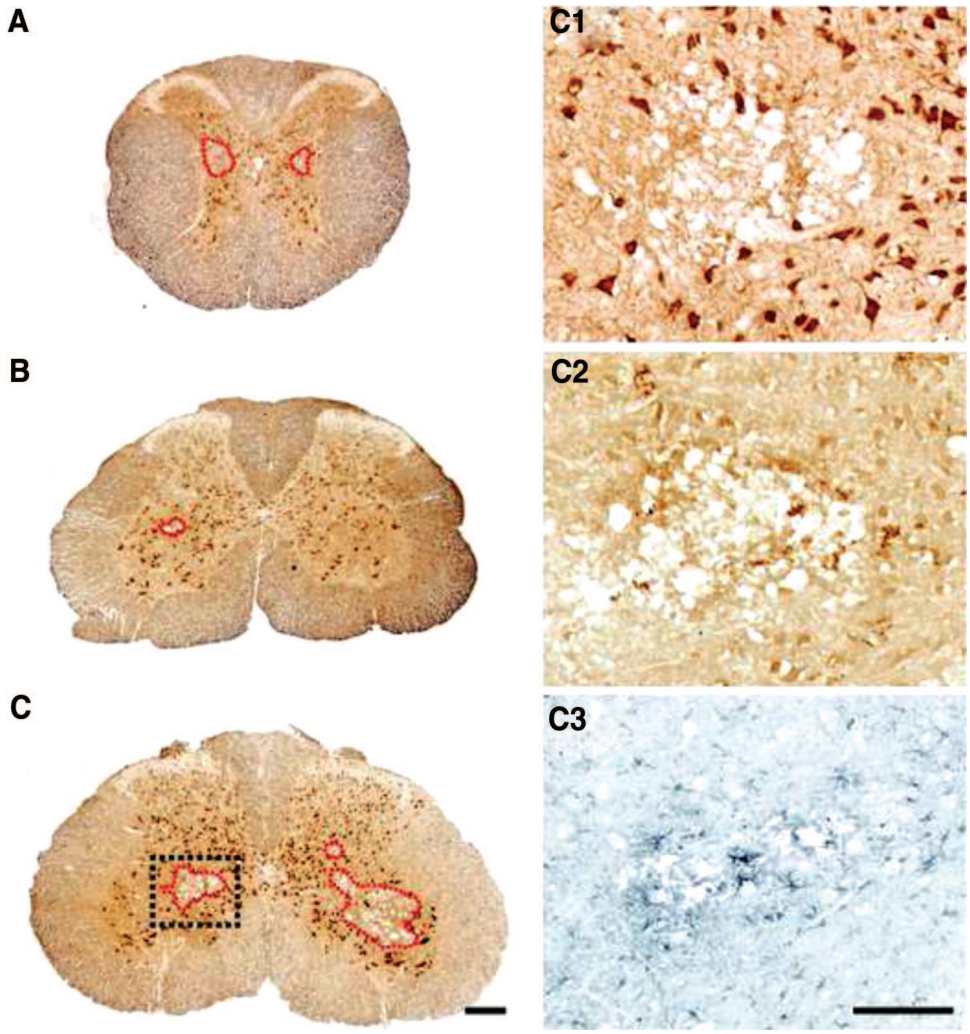

Fig. 5. Cross-sections from independent mice 24 h after aortic cross-clamp with reperfusion illustrate the subtle and heterogeneous nature of the early pathologic lesions caused by transient spinal ischemia. (*A–C*) sections from three different mice stained to reveal neurons and axons. *Dashed red lines* circumscribe regions of necrosis. No other lesions were seen in these three animals. Note that pathologic lesions exist in thoracic and lumbar spinal cord that are heterogeneous in size and can be found on either or both sides of the spinal cord. (*C1–C3*) High-power views of the boxed region in *C* shows neurons (*C1*), microglia/macrophages (*C2*), and astrocytes (*C3*). Images in *C2* and *C3* were collected from sections immediately adjacent to (*C/C1*). Note the mild glial reaction surrounding regions of necrosis and neuron loss at this early time point. Scale bars = 200 μm.

ited normal locomotor function for at least 24 h after ischemia/reperfusion, after which they all developed mild or severe hind limb deficits. The onset and magnitude of intraspinal pathologic lesions correlated with degree of functional impairment. In nearly all other models of ischemic stroke and spinal cord injury, the onset of neurologic impairment is immediate.[28,29] However, delayed onset of paralysis subsequent to aortic repair surgery in humans is becoming increasingly more common.[4] The reasons for this are not clear, but our new model seems to be an ideal tool for defining the mechanisms underlying discrete forms of mild or severe spinal cord pathologic lesions with associated changes in neurological function.

Our optimal surgical parameters (7.5 min of aortic cross-clamp at 33°C) consistently produced two functionally distinct groups of mice; approximately two thirds were severely affected by ischemia/reperfusion and the remaining third were relatively resistant to ischemia/reperfusion injury. Lang-Lazdunski *et al.*[10] made a similar observation in their model, ~60% of mice developing complete paralysis and the remaining mice exhibiting milder deficits but with limited survival beyond 48 h. Thus, it is unlikely that these distinct cohorts are a result of variability in the cross-clamp procedure. Indeed, Lang-Lazdunski *et al.*[10] confirmed cessation of blood flow using laser Doppler velocimetry. We also used laser Doppler velocimetry (not shown) and real-time monitoring of blood pressure to ensure cessation of blood flow in the femoral artery. However, such detailed monitoring is not essential when the aorta is approached *via* a lateral thoracotomy (see fig. 1). Indeed, it is easy to visualize and then lift the aorta by inserting a wire hook around the vessel. This ensures that an aneurysm clip can be placed across the full diameter

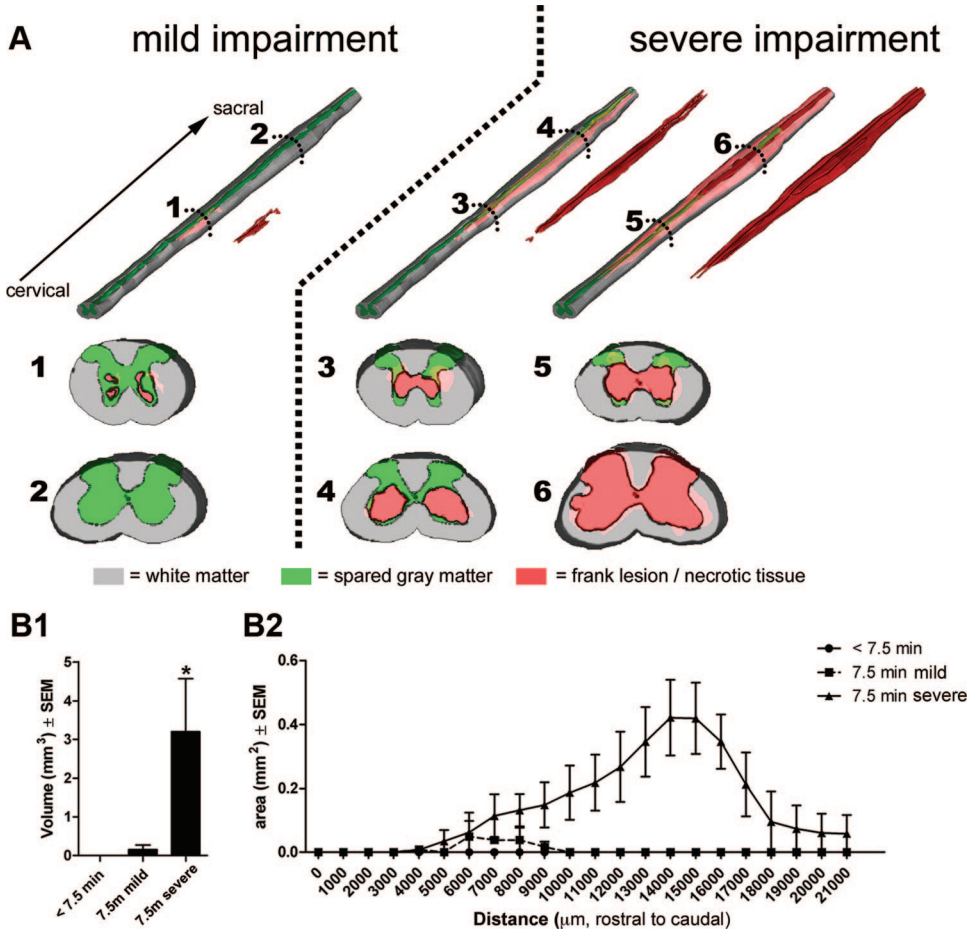

Fig. 6. Three-dimensional (3D) lesion analysis of mouse spinal cords 7 days after aortic cross-clamp with reperfusion (7.5 min at 33°C). (*A*) 3D reconstructions of representative spinal cords taken from mice that developed mild (top left; *A1* and *A2*) or severe hind limb deficits (*A3–A6*). The 3D image in the top center (*A3* and *A4*) is representative of a spinal cord with a median lesion size with severe impairment, whereas that at the top right (*A5* and *A6*) represents the largest lesion that we observed. Entire reconstructions and lesion only (3D red image) reconstructions are provided for the rostrocaudal extent of the lesion or in the coronal plane (*e.g.*, *1*, *2*; *3*, *4*; or *5*, *6*) at defined areas within the lesion. (*B1*) Quantitative analysis of lesion volume (n = 5, 2, and 3 mice, moving left-to-right in graph. (*B2*) Rostral-caudal distribution of lesion areas shown in *B1*. These data were used to generate 3D reconstructions in *A* and calculate lesion volumes in *B1.* Lesion areas are expressed as a function of hind limb deficit (mild *vs.* severe). No pathologic conditions were observed in mice with cross-clamp times less than 7.5 min. *p = 0.0250, one-way analysis of variance with Tukey multiple comparison test. Scale bars represent SEM.

of the aorta. We also found that when a second aneurysm clip was placed distal to the first clip (to further ensure complete blockage of perfusion below the clip), a subset of mice still developed only mild hind limb deficits (not shown). Rather than incomplete occlusion, a more likely explanation for the functionally distinct cohorts of mice is interanimal variation in vascular anatomy, specifically, collateral branching from the arch vessels and descending aorta above the location of the aneurysm clip.[30] Ongoing studies will use our new model to characterize the relationship between vascular anatomy and the magnitude of functional impairment caused by ISCI.

Because it is not currently possible to predict *a priori* whether a mouse will be severely or mildly affected by the aortic cross-clamp procedure, larger groups of animals will be required to detect statistically significant decreases in the ratio of severely to mildly affected mice. Such a change would indicate that a given drug has therapeutic effects. However, as our understanding of the mechanisms that regulate the natural stratification of neurologic outcome improves, this new mouse model would become ideal for studying therapeutics that target only mild or severely affected mice. Indeed, the application of a "magic bullet" therapy to all forms of central nervous system ischemia/reperfusion injury (or traumatic injury) is impractical. Some drugs may only be effective in a subset of individuals who are less susceptible to the effects of ischemia/reperfusion or trauma. Our model lends itself to comparative analyses of this type, because long-term functional variability is limited to only two cohorts (mild *vs.* severe) and can be predicted within 48 h.

The lack of obvious pathologic conditions within 12 h of ischemia/reperfusion, with a further delay in the onset of functional impairments, suggests that the metabolic derangements in neurons and glia caused by ischemia/reperfusion collaborate with other pathogenic cascades to cause cell

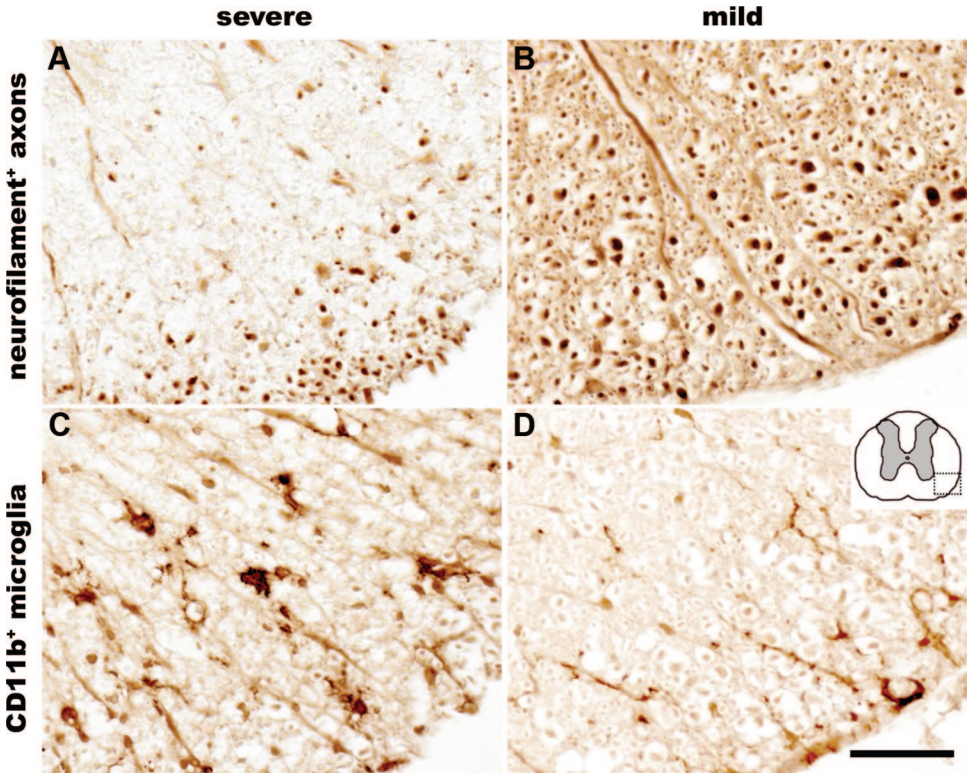

Fig. 7. High-magnification images showing ventrolateral spinal cord at the cervical enlargement 21 days after aortic occlusion in a representative mouse with severe (*A* and *C*) or mild neurological impairment (*B* and *D*). Adjacent sections were stained to reveal axons (*top panels*) and activated microglia (*bottom panels*). Note the paucity of axons and the presence of reactive microglia in mouse with severe impairment. Schematic in *D* shows approximate region from which image was obtained (*box*). Scale bar = 50 μm. CD11b$^+$ = CD11b-positive.

injury and lesion expansion. Indeed, progressive necrosis, axonal degeneration, and intraspinal inflammation occur over multiple segments of the spinal cord between 2 and 7 days of reperfusion. This delay means that it might be possible to minimize tissue damage and preserve neurologic function by inhibiting cellular and molecular pathways activated downstream of ischemia and reperfusion. Several cascades, including neuroinflammation, excitotoxicity, oxidative stress, channelopathy, etc., have been implicated and can be explored with improved resolution with the mouse ISCI model.[1,9,31]

Recognizing subclinical pathologic conditions during or immediately after aortic repair surgery in humans is difficult because neither electromyography nor strength/sensory tests are performed routinely,[32] and most patients are discharged without reporting overt neurologic complications. This does not mean, however, that the spinal cord is not damaged or that neurologic impairment will not develop. In fact, during surgery it is not uncommon to see red blood cells and activated lymphocytes accumulate and turbidity increase in cerebrospinal fluid drained from the lumbosacral spinal cord of these individuals (Dr. Awad, unpublished observations, 2007). It is not known whether this or other signs of intraspinal damage can be used to predict subclinical pathologic lesions or neurologic impairment; however, this should be possible using perioperative electromyography, rigorous postoperative neurologic exams, or biomarker analysis of cerebrospinal fluid samples obtained during routine cerebrospinal fluid drainage. Our new mouse model may be useful for establishing these correlations early (within a few days) or at later times post-surgery.

Although our new model of ISCI recapitulates many aspects of the clinical condition in humans, a consequence of the ischemia/reperfusion insult that is unique to the mouse model is the acute but transient development of mild seizure-like events. These were seen in ~50% of the mice when cross-clamp was applied at 33°C. It is not clear why this happens, but it seems logical that dramatic changes in cerebral blood pressure (as measured in the carotid arteries proximal to the site of occlusion; see Supplemental Digital Content 1, fig. 3, http://links.lww.com/ALN/A626) or systemic pathologic lesions caused by impaired acid-base balance could contribute (see table 2). In humans, complete or partial cardiopulmonary bypass during cross-clamp alleviates cerebral hypertension and facilitates maintenance of acid–base balance before blood is returned to the circulation. Regardless of mechanism, these events were transient; once they passed, they did not adversely affect long-term survival. At this time, we also do not know whether a truly postictal state exists in these mice. This is an important consideration because it could influence behavioral evaluation. Although the seizure-like events are responsive to ketamine (known to in-

hibit spontaneous neuronal depolarization and cortical spreading depression),[14–16] more sophisticated electrophysiological and behavioral analyses would be needed to define these postischemia/reperfusion events as conventional seizures. Both analyses would be difficult given the unpredictable nature of the seizure-like events. Even if the mice were truly postictal, preservation and recovery of spontaneous motor function in mice with seizure-like events was indistinguishable from that of mice that did not experience these events.

Our new model of mouse ISCI offers several advantages over existing models. First, mice are relatively inexpensive compared with pigs, dogs, rabbits, or rats. Second, by using transgenic/knockout mice, it will be possible to manipulate specific genes and cell signaling pathways that are known to cause or contribute to secondary central nervous system injury or repair. Third, the surgical approach that we used and the changes in hemodynamics and blood gases that occur with this model of aortic cross-clamp mimic what occurs in humans. Finally, because mice survive indefinitely with our optimized protocol, the consequences of ischemia/reperfusion can be studied for weeks or months after injury. Together, these features of the model should simplify the preclinical development and testing of neuroprotective interventions for ISCI.

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