Transplantation of viable mitochondria attenuates neurologic injury after spinal cord ischemia

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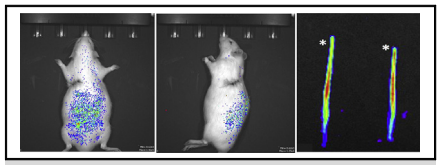
ABSTRACT

Objectives: Spinal cord ischemia (SCI) is one of the major concerns of postoperative paraplegia during major vascular or aortic surgery. Since mitochondrial dysfunction develops at the early stage of SCI, this study tested the neuronal protective effect of transplantation of viable mitochondria to the ischemic cord in rats.

Methods: SCI was induced by crossclamping of thoracic aorta at T6 level for 25 minutes, followed by release of vascular clip to restore aortic blood flow in the anesthetized rats. Mitochondria (100 μg) were isolated from freshly harvested soleus muscle and delivered via the internal jugular vein before releasing of vascular clip. The motor function was assessed independently up to 7 days after reperfusion. Spinal cords were harvested and analyzed for molecular and histological changes.

Results: Whole-body in vivo images acquired by an in vivo imaging system confirmed the enhancement of MitoTracker fluorescence at the regions below crossclamping and in the ischemic cord. Compared with control vehicles, transplantation of mitochondria significantly improved the lower-limb locomotor function of rats subjected to cord ischemia up to 7 days after surgery. Mitochondrial transplantation suppressed the regional endoplasmic reticulum stress in the ischemic cord by attenuating CCAAT-enhancer-binding protein homologous protein expression and restoring binding immunoglobulin protein levels. In accordance, tissue levels of interleukin-6, tumor necrosis factor-α, and caspase-3 were attenuated in the mitochondrial transplanted group. Histologic examination also showed significant increase in numbers of Nissls bodies in the neurons at the ventral horn of ischemic cord following mitochondrial transplantation.

Conclusions: Our study showed that transplantation of freshly isolated mitochondria during the early stage of spinal cord ischemia–reperfusion injury suppressed the oxidative stress in endoplasmic reticulum of the injured cord, thereby reducing neuroapoptosis and improving locomotor function of rats with SCI. (J Thorac Cardiovasc Surg 2021;161:e337-47)



Systemic delivery of mitochondria selectively distributed to ischemic spinal cord.

CENTRAL MESSAGE

Transplantation of mitochondria at the early stage of spinal cord ischemia–reperfusion injury attenuates neuronal apoptosis and improves motor function recovery.

PERSPECTIVE

Spinal cord ischemia is a major concern after aortic surgery due to postoperative paraplegia with an incidence of up to 20%. The improvement in hindlimb locomotor function following mitochondrial delivery during early phase of cord ischemia suggests that transplantation of mitochondria might potentially provide therapeutic effect in preservation of the neurologic function of spinal cord during major aortic surgery.

See Commentaries on pages e349 and e350.

Spinal cord ischemia (SCI) is one of the major concerns after aortic surgery due to the development of postoperative paraplegia, with an overall incidence of 0.5% to 1.5% for coarctation repair, 10% for thoracic aneurysm repair, and up to 20% for thoracoabdominal aorta repair.[1] Although the improvement in surgical techniques has significantly reduced the risk of SCI after aortic repair, the incidence of postoperative neurologic deficit remains as high as 9% to 16%.[2,3] Furthermore, 1.2% to 2.6% of these patients suffered from permanent paraplegia after endovascular procedures.[2-4]

Mitochondrial dysfunction has been recognized as the initial step of neuronal injury during cord ischemia and is also crucial for the amplification of secondary injury and

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Abbreviations and Acronyms

BAX = Bcl-2-associated X protein
BBB = Basso–Beattie–Bresnahan
Bcl-2 = B-cell lymphoma-2
Bcl-XL = B-cell lymphoma extra large
CHOP = CCAAT-enhancer-binding protein homologous protein
ER = endoplasmic reticulum
GFAP = glial fibrillary acidic protein
GRP78 = binding immunoglobulin protein
Iba-1 = ionized calcium-binding adapter molecule-1
IL-6 = interleukin-6
IVIS = in vivo imaging system
PBS = phosphate buffer solution
POD = postoperative day
SCI = spinal cord ischemia
TNF-α = tumor necrosis factor-α
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling

subsequent neuronal cell death by increase in mitochondrial oxidative damage.[5] Within minutes after neuronal ischemic injury, mitochondrial dysfunction in the neurons is induced by deprivation of oxygen and glucose, followed by depletion of adenosine triphosphate production and substantial production of reactive oxygen and nitrogen species upon reintroduction of oxygen after reperfusion, and may subsequently lead to neuronal apoptosis, autophagy, and necroptosis.[6,7] Therefore, mitochondria-targeting therapy has recently been proposed in the treatment of spinal cord injury, including combinatorial therapies, such as pharmacologically increasing antioxidant activity and decreasing mitochondrial fission.[8] Unfortunately, many of these pharmacologic agents remain to be assessed in humans, and none of these have yet proven successful for the treatment of spinal cord injury. Since transplantation of viable mitochondria is a clinically feasible approach in ischemia–reperfusion injury,[9-11] this study hypothesized that transplantation of exogenous viable mitochondria restored the dysfunctional mitochondria in the neuronal cells during the early phase of SCI and improved neuroapoptosis and functional recovery in a rat model of ischemic cord.

METHODS

Rat Model of Spinal Cord Ischemia

All experimental procedures were approved by the Institutional Animal Care and Use Committee (The National Cheng Kung University, Tainan, Taiwan; IACUC approval number 107177). SCI was induced in adult Sprague–Dawley male rats anesthetized with 1.5 v/v% isoflurane in oxygen, as previously characterized by our group.[12] The anesthetized rats were placed at right decubitus position and the thoracic cavity was exposed by incision of the left T5 to T6 intercostal space. Blood flow in thoracic–lumbar spinal cord was temporarily occluded by application of a microvascular clamp to the thoracic aorta at the T6 level. According to our previous study, near-complete abolishment of blood flow into the lumbar spine was confirmed by absence of dye staining following intravenous infusion of Evans blue during the period of aortic crossclamping.[12] Twenty-five minutes after crossclamping, the vascular clip was released to restore blood flow to the spinal cord. The animals were recovered from anesthesia under a warm blanket, and they were returned to cages after regained of upper limb activity.

Hindlimb Locomotor Function Assessment

The Basso–Beattie–Bresnahan (BBB) scale (range 0-21) was used to assess the hindlimb locomotor function of the rats once daily up to 7 days after SCI.[13] The BBB scale measures the functional recovery of spinal cord by assessing hindlimb joint movements, stepping, forelimb and hindlimb coordination, trunk position and stability, paw placement, and tail position.[14] A score of "0" indicates paralysis and "21" indicates normal locomotion. The locomotor function was assessed independently by an experienced research assistant who was blinded to the treatment groups.

Isolation and Administration of Mitochondria

Healthy naïve rats were asphyxiated with CO_2 and bilateral soleus muscles were harvested for isolation of viable mitochondria using a mitochondria isolation kit (Thermo Scientific, Waltham, Mass). After being washed with phosphate buffer solution (PBS, containing 0.1 M sodium phosphate and 0.15 M sodium chloride at pH 7.2), the soleus muscle tissue (approximately 5 mg) was cut into small pieces and incubated with the tryptase solution (0.3 mg/mL) for 3 minutes. The proteolytic activity of tryptase was quenched by bovine serum albumin and the tissue was homogenized using a grinder. The tissue sample was centrifuged at 11,752*g* for 3 minutes at 4°C to remove the supernatant. The mitochondrial pellets were eventually obtained after 3 repeats of suspension and centrifugation to discard the cytosolic fraction using the mitochondria isolation reagents (A to C solutions). All mitochondria used for transplantation were isolated within 1 hour before administration. At 5 minutes before release of aortic clamping, the freshly isolated mitochondria (100 μg) were resuspended in 0.2 mL of PBS and intravenously transplanted via the jugular vein of the recipient rat. The dosage of mitochondria was directly derived from the dose-dependent assays characterized by Gollihue and colleagues.[15]

In Vivo Imaging System (IVIS) to Localize the Distribution of Transplanted Mitochondria

In some experiments, the isolated mitochondria were stained with a red fluorescence (MitoTracker Deep Red; Invitrogen/Molecular Probes, Inc, Eugene, Ore) or green fluorescence dyne (MitoTracker Green; Invitrogen) for the in vivo or ex vivo imaging, respectively. The whole-body scan was performed at 2 hours and 24 hours after intravenous delivery of MitoTracker Deep Red fluorescence-labeled mitochondria under an IVIS (Caliper IVIS Spectrum System, PerkinElmer, Waltham, Mass). Rats that received MitoTracker Green fluorescence-labeled mitochondrial were sacrificed by CO_2 asphyxia at 2 hours after delivery. The whole spinal cord was removed en-bloc immediately after sacrifice and scanned under the IVIS for the fluorescence expression. IVIS is a noninvasive spectrum system used to illuminate the fluorescent sources or bioluminescent reporters in living animals or tissues with a 2-dimensional or 3-dimensional tomography.[16]

Allocation of Experimental Groups and Study Protocol

Animals were randomly allocated to receive sham operation (thoracotomy only, sham group) and spinal cord ischemia (thoracotomy and aortic

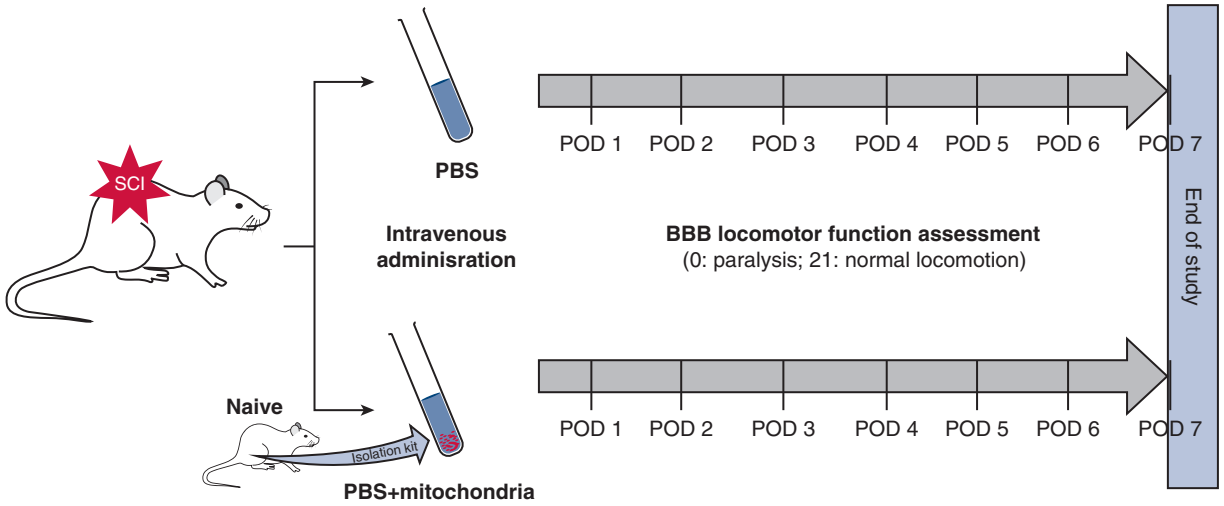

FIGURE 1. Diagram of study design and experimental flowchart. The primary study endpoint was the changes of locomotor function from day 1 to day 7 after operation. *SCI*, Spinal cord ischemia; *PBS*, phosphate buffer solution; *BBB*, Basso–Beattie–Bresnahan scale; *POD*, postoperative day.

crossclamping, SCI group). Rats with SCI were then assigned to receive intravenous administration of plain PBS or skeletal muscle-derived mitochondria in PBS, as the control or mitochondrial treatment group, respectively. Naïve rats were defined as the healthy aged-matched rats that used for skeletal muscle harvesting and served as controls for the whole-body IVIS studies. A total of 37 rats received SCI treatment (14 controls and 23 mitochondrial transplantation), 8 rats received sham operation, and 6 rats served as naive. The study protocol is shown as Figure 1.

Harvest of Spinal Cord Tissues

Rats were sacrificed by CO_2 asphyxiation and decapitation at postoperative day 7. Following thoracotomy, the T13 rib (the lowest rib) was identified to trace the corresponding T13 vertebral body. Spinal cord tissues below T11 vertebral body (considered as lumbar cord) were harvested for analysis.[12]

Western Blot

Lumbar spinal cord was minced and homogenized in lysis buffer. Equal amount of proteins (100 μg) were loaded into polyacrylamide gels (9%-12%) and transferred to nitrocellulose membranes by the wet-transferring method. The membranes will be incubated overnight with primary antibodies of appropriate dilutions at 4°C. After washing with PBS, the membranes were incubated with appropriate dilution horseradish peroxidase-linked secondary antibodies for 1 hour at room temperature. Bands were visualized using enhanced chemiluminescence and quantified by scanning densitometry (the ImageJ; 1.48v, National Institutes of Health, Bethesda, Md).

Mitochondrial apoptosis and was determined by the protein levels of Bcl-2-associated X protein (BAX; Santa Cruz Biotechnology Inc, Santa Cruz, Calif), B-cell lymphoma-2 (Bcl-2; Abcam, Cambridge, Mass), B-cell lymphoma extra large (Bcl-XL; Cell Signaling, Danvers, Mass) and caspase-3 (Cell Signaling). Mitochondrial or endoplasmic reticulum (ER) stress was determined by the protein expressions of CCAAT-enhancer-binding protein homologous protein (CHOP; GeneTex, Irvine, Calif) and binding immunoglobulin protein (GRP78; GeneTex). Neuroinflammation was determined by the protein expressions of glial fibrillary acidic protein (GFAP; GeneTex), ionized calcium-binding adapter molecule-1 (Iba-1; BD Biosciences, San Jose, Calif), interleukin-6 (IL-6; GeneTex), and tumor necrosis factor-α (TNF-α; Abcam).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Apoptotic cells in the optimal cutting temperature–embedded spinal cord sections were investigated by TUNEL assay (ApopTag In Situ Apoptosis Detection Kits; Sigma-Aldrich, St Louis, Mo). Tissue sections were washed with PBS and incubated with TUNEL working strength TdT enzyme reaction mixture. Tissue sections were then incubated with anti-digoxigenin conjugate (rhodamine) for 30 minutes, and TUNEL-positive nuclei were observed under a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Histologic and Immunostaining Examinations

Lumbar spinal cord was fixed in 10% buffered formalin saline for 24 hours. The tissue biopsies were processed through increasing grades of alcohol and embedded in paraffin wax. Sections of spinal cord were stained by the hematoxylin and eosin and Nissl staining methods and were examined under a light microscope. Numbers of viable neurons and the containing Nissl bodies in the ventral horn of lumbar cord were computed under high-power fields (200-400×) by a researcher (S.Y.F.) who was blinded to the treatment groups.

Statistics

The primary study endpoint of this experiment was the change in hindlimb locomotor function up to 7 days after spinal cord ischemia–reperfusion injury. A pretest sample size estimation determined that 8 rats in each group would able to detect a mean difference in BBB scores of 2 (standard deviation of 1.3) with an α value of 0.05.[12] All data sets were tested for normality assumption using the Shapiro–Wilk test before statistical procedures. The values of continuous variables were compared by an unpaired *t* test when the normality assumptions were met; otherwise. the Mann–Whitney *U* test was used. Two-way repeated-measures analysis

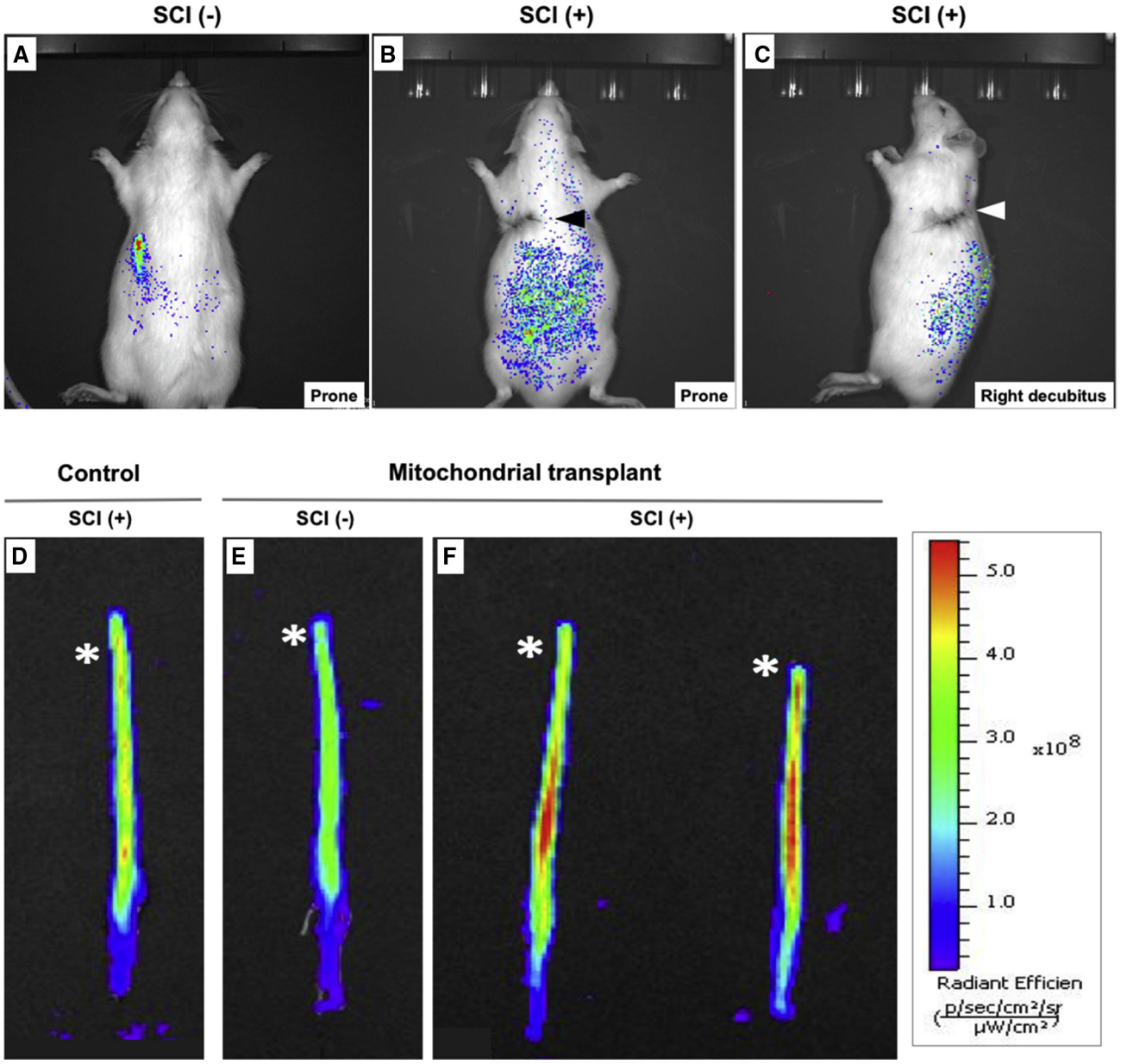

FIGURE 2. Representative images acquired by an in vivo imaging system (IVIS) (Caliper IVIS Spectrum System) showing the whole-body in vivo images (A-C) stained by a red fluorescence dyne (MitoTracker Deep Red; Invitrogen), or ex vivo images (D-F) stained by a green fluorescence dyne (MitoTracker Green; Invitrogen). MitoTracker Deep Red was specifically localized at the left upper abdominal quadrant after transplantation of mitochondria in naïve rats (A). In rats with spinal cord ischemia (SCI) injury, the fluorescence was highly expressed at the dorsal regions below the levels of aortic crossclamping (*arrowheads*) (B and C). The ex vivo images showed low MitoTracker Green fluorescence uptake in isolated spinal cord harvested from SCI rats receiving only fluorescence dyne (D) and naïve rats receiving mitochondria labelled with MitoTracker Green fluorescence (E). High MitoTracker Green fluorescence expression was detected in the lumbar cord isolated from SCI rats receiving mitochondrial transplantation (F). *Indicates the thoracic spinal cord level 6 (T6) where the vascular clip was applied to the thoracic aorta. Experiments were performed in 3 naïve rats and 5 rats with SCI.

of variance was used to compare the differences in BBB scales between the control and mitochondrial transplantation groups at the different study point times. A Bonferroni post hoc procedure was used for multiple comparisons. Results are presented as the median and interquartile range. Statistical significance was accepted at a level of $P < .05$. All of the statistical analyses were performed using the SigmaPlot 14.0 (Systat Software Inc, San Jose, Calif).

RESULTS

Distribution of Transplanted Mitochondria After SCI

In the naïve rats, the fluorescence of MitoTracker Deep Red was specifically localized at the left upper quadrant of the abdomen at 2 hours after transplantation of

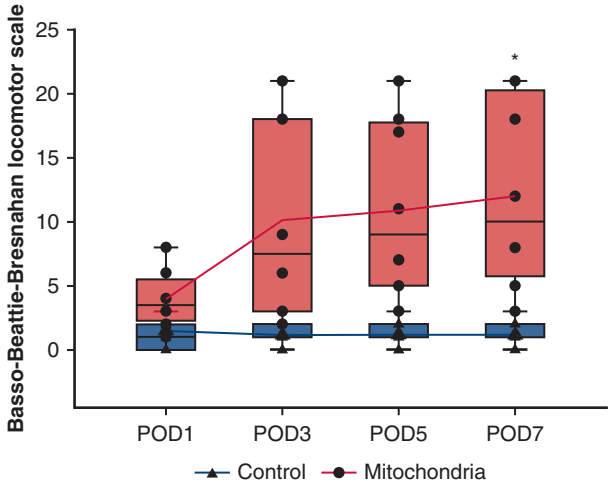

FIGURE 3. The Basso-Beattie-Bresnahan (BBB) scale (range 0-21) was used to assess the hindlimb locomotor function of rats once daily from day 1 to day 7 after spinal reperfusion by an experienced research assistant who were blinded to treatment groups. The BBB scales were significantly improved in the animals that received mitochondrial transplantation. Results were analyzed using 2-way repeated-measures analysis of variance. *$P < .05$ control (n = 7, *blue box* and *triangular plots*) vs mitochondrial (n = 8, *red box* and *circle plots*) groups. Data are presented as *box-and-whisker plots*, in which the horizontal lines of color boxes indicate the 75th percentile, median, and 25th percentile of the distribution and the *upper* and *lower whiskers* indicate the maximal and minimal values. *POD*, Postoperative day.

mitochondria (Figure 2, *A*). At 2 hours after intravenous administration of mitochondria in rats with cord ischemia and reperfusion, in vivo images showed that the fluorescence was highly expressed at the dorsal regions below the levels of aortic crossclamping (Figure 2, *B* and *C*). The isolated spinal cord was also scanned under IVIS, and the ex vivo images confirmed that the MitoTracker Green fluorescence was highly stained in the lumbar cord subjected to ischemic injury (Figure 2, *D-F*).

Hindlimb Locomotor Function Assessment

The hindlimb BBB locomotor rating scales were assessed through postoperative day (POD) 1 to POD 7 in rats with SCI (Figure 3). The BBB scales were significantly reduced to extremely low levels at POD 1 in both groups, suggesting the presence of motor dysfunction due to ischemic cord syndrome. Compared with controls, the hindlimb locomotor function was significantly improved in the rats that received mitochondrial transplant from POD 1 to POD 7 and was returned to a significantly greater BBB rating scale score at 7 days after operation (1.2 ± 0.7 vs 12.0 ± 7.2, control vs mitochondria groups; mean difference 10.8, 95% confidence interval, 3.8-17.9; $P = .006$; Figure 3).

Mitochondrial Dysfunction and ER Stress in the Ischemic Cord

Ischemia–reperfusion injury in the lumbar spinal cord induced mitochondrial dysfunction (increased BAX-to-Bcl-2 ratio) and ER stress (suppressed GRP78 and increased CHOP) (Figure 4, *A* and *B*). The expression of Bcl-XL was also significantly enhanced following SCI (Figure 4, *A*). Expression of cleaved caspase-3 and TUNEL assay was used as biomarkers for assessing neuroapoptosis in the ischemic cord. Spinal cord ischemic injury significantly increased the protein expression of CHOP, cleaved caspase-3, and number of apoptotic cells in the cord (Figure 4, *B* and *C*). Following transplantation of mitochondria, expression of CHOP and GRP78, the BAX-to-Bcl-2 ratio, and Bcl-XL were restored in the ischemic cord (Figure 4, *A* and *B*). Furthermore, expression of cleaved caspase-3 and formation of apoptotic cells in the cord tissue of rats with SCI were also reduced in the mitochondrial treated rats (Figure 4, *B* and *C*).

Neuroinflammation in the Ischemic Cord

The neuroinflammatory reactions in the ischemic cord were determined by the tissue levels of inflammatory cytokines (IL-6 and TNF-α)[17] and cell-mediated immunoreactivity (GFAP and Iba-1).[18] The enhanced expressions of IL-6 and TNF-α in the ischemic cord were significantly suppressed by mitochondrial transplant (Figure 5), but changes in the protein levels of GFAP and Iba-1 were not significantly different at POD 7 (Figure 5).

Histologic Examinations

There were significant central chromatolysis and reactive gliosis in the gray matter of ischemic spinal cord (Figure 6). Numbers of neurons in ventral horns of the gray matter of lumbar cord were reduced in animals subjected to cord ischemia–reperfusion injury with or without mitochondrial transplant (Figure 6). Compared with controls, the numbers of Nissl bodies in the ventral horn neurons were significantly increased in the mitochondrial group (Figure 6).

DISCUSSION

This study showed that ischemia–reperfusion injury induced ER stress and mitochondrial apoptosis in the lumbar spinal cord, leading to neurodegenerative and impaired locomotor function in the hindlimbs of experimental rats. Transplantation of exogenous viable mitochondria at the acute phase of spinal cord reperfusion injury significantly improved the hindlimb locomotor function by attenuating ER stress and mitochondrial injury, as shown by the restoration of expressions of GRP78/CHOP and Bcl-2 family proteins (BAX/Bcl-2 ratio and Bcl-XL) in the injured spinal cord (Figure 7). Mitochondrial treatment also significantly reduced the regional proinflammatory reaction (IL-6 and

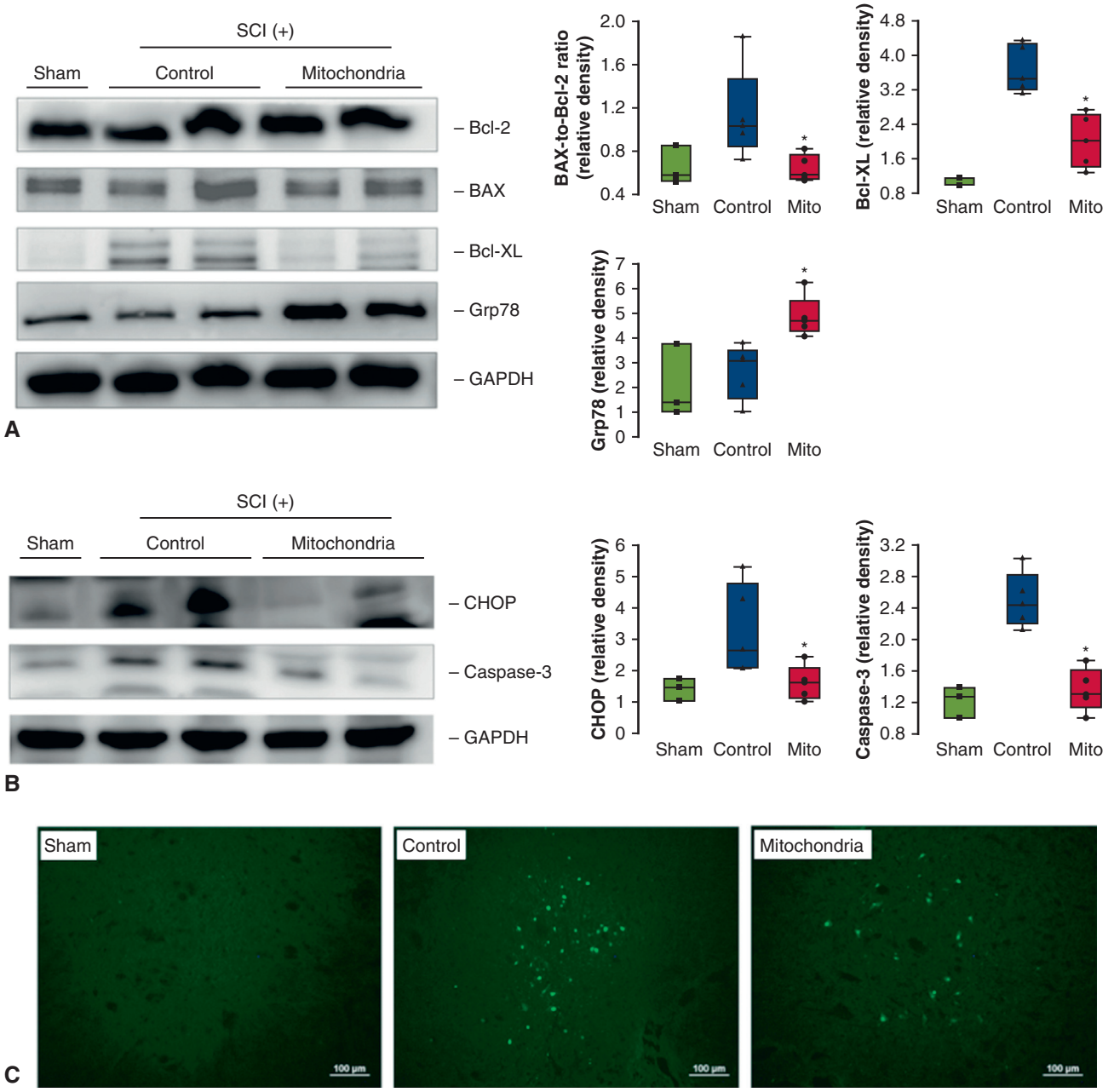

FIGURE 4. Protein expressions of markers for mitochondrial-apoptosis and endoplasmic reticulum stress (A and B). An in situ apoptosis detection kit was used to visualize the apoptotic neuronal cells (green fluorescence) in the immunofluorescence sections of spinal cord (C). ApopTag-fluorescent densities were suppressed in the ischemic spinal cord harvested from animals that received mitochondrial transplantation. Experiments were performed in 3 controls and 5 rats in the mitochondrial group. Protein expressions were analyzed using Mann–Whitney *U* test. **P* < .05 versus controls, n = 3 in the sham operation group, and n = 5 different animals in the control and mitochondrial groups. Data are presented as *box-and-whisker plots*, in which the *horizontal lines of color boxes* indicate the 75th percentile, median and 25th percentile of the distribution, and the upper and lower whiskers indicate the maximal and minimal values. *SCI*, Spinal cord ischemia; *Bcl-2*, B-cell lymphoma-2; *BAX*, Bcl-2-associated X protein; *Bcl-XL*, B-cell lymphoma extra large; *Grp78*, binding immunoglobulin protein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *CHOP*, CCAAT-enhancer-binding protein homologous protein.

TNF-α levels) (Figure 7). The improvement in mitochondrial function and neuroinflammation suppressed cell apoptosis and neuronal chromatolysis in the spinal cord.

In this study, spinal cord ischemic injury was induced by temporary occlusion of aortic blood flow at T6 level, which mimicking the crossclamp of thoracic aorta during

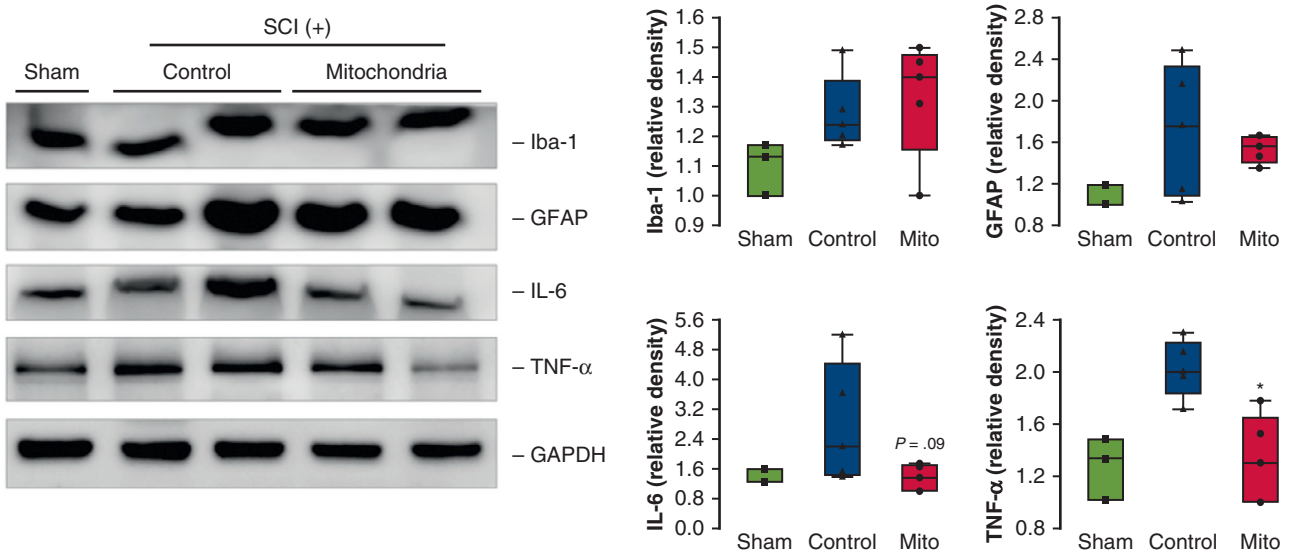

FIGURE 5. Protein expressions of inflammatory cytokines (IL-6 and TNF-α) and cell-mediated immunoreactivity (GFAP and Iba-1) in the spinal cord after ischemia–reperfusion injury. Results were analyzed using the Mann–Whitney *U* test. *$P < .05$ vs controls, n = 3 in the sham operation group, and n = 5 different animals in the control and mitochondrial groups. Data are presented as *box-and-whisker plots*, in which the *horizontal lines of color boxes* indicate the 75th percentile, median and 25th percentile of the distribution, and the upper and lower whiskers indicate the maximal and minimal values. *SCI*, Spinal cord ischemia; *Iba-1*, ionized calcium-binding adapter molecule-1; *GFAP*, glial fibrillary acidic protein; *IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor-α; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

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thoracolumbar aortic repair surgery.[12] Paraplegia of lower limbs developed in rats after the operation, and the locomotor functions was not significantly improved up to 7 days after reperfusion of aortic blood flow, indicating the permanent neurologic damage in the ischemic cord. Mitochondrial injury in the cord tissue was detected by the enhanced expression of BAX-to-Bcl-2 ratio and Bcl-XL and suppression of GRP78. The BCL-2 protein family tightly regulates the permeability of outer mitochondrial membrane and plays a key role in the intrinsic apoptotic pathway during ischemic neuronal injury, as Bcl-2 is antiapoptotic and BAX is proapoptotic.[19,20] The increased BAX/Bcl-2 ratio represents a sensitive biomarker for proapoptotic activity during neuronal ischemic injury.[21] Previous studies demonstrated that protein levels of Bcl-XL in the neuronal mitochondria decreased from 2 hours to 24 hours after spinal cord injury[22] but were significantly enhanced from 2 days to 7 days after neuron ischemia.[20] Although Bcl-XL is generally considered as an antiapoptotic member in the Bcl-2 family, the phosphorylation of Bcl-XL at serine 73 or interaction with BAD (ie, Bcl-2 associated death promoter) during ischemic injury may promote cell death.[22,23] Therefore, the enhanced BAX/Bcl-2 ratio and Bcl-XL expression in this study may simply suggest the delayed phase of mitochondrial dysfunction or progressive neuronal death in the ischemic spinal cord.

GRP78 is a major ER chaperone protein that regulates the unfolded protein response, as well as mediates antiapoptotic properties.[24] Furthermore, CHOP is a multifunctional transcription factor in the ER stress response.[25] Our results showed that GRP78 was significantly suppressed and CHOP was significantly upregulated in the spinal cord subjected to ischemia–reperfusion injury, suggesting a greater level of ER stress in the injured neuronal tissue.[24] SCI also enhanced the expressions of neurodegenerative markers (GFAP and Iba-1) and fragmentation of Nissl bodies in the neurons. Nissl bodies are the protein synthesis infrastructure of a neuron, and fragmentation of Nissl bodies (chromatolysis) usually represents degeneration of the injured neurons.[26] Collectively, our results showed that ischemia–reperfusion injury in lumbar spinal cord induced ER stress and mitochondrial dysfunction and led to increased tissue expression of cell apoptosis marker (ie, cleaved caspase-3) and neuroinflammatory cytokines (ie, IL-6 and TNF-α).

Current therapeutic interest for spinal cord injury has been focused on the restoration of mitochondrial function after injury by targeting on inhibition of the mitochondrial membrane leak, use of alternate energy sources, enhanced endogenous antioxidant activity, and maintenance of mitochondrial morphology.[8] Transplantation of exogenous viable mitochondria was recently showed to maintain the

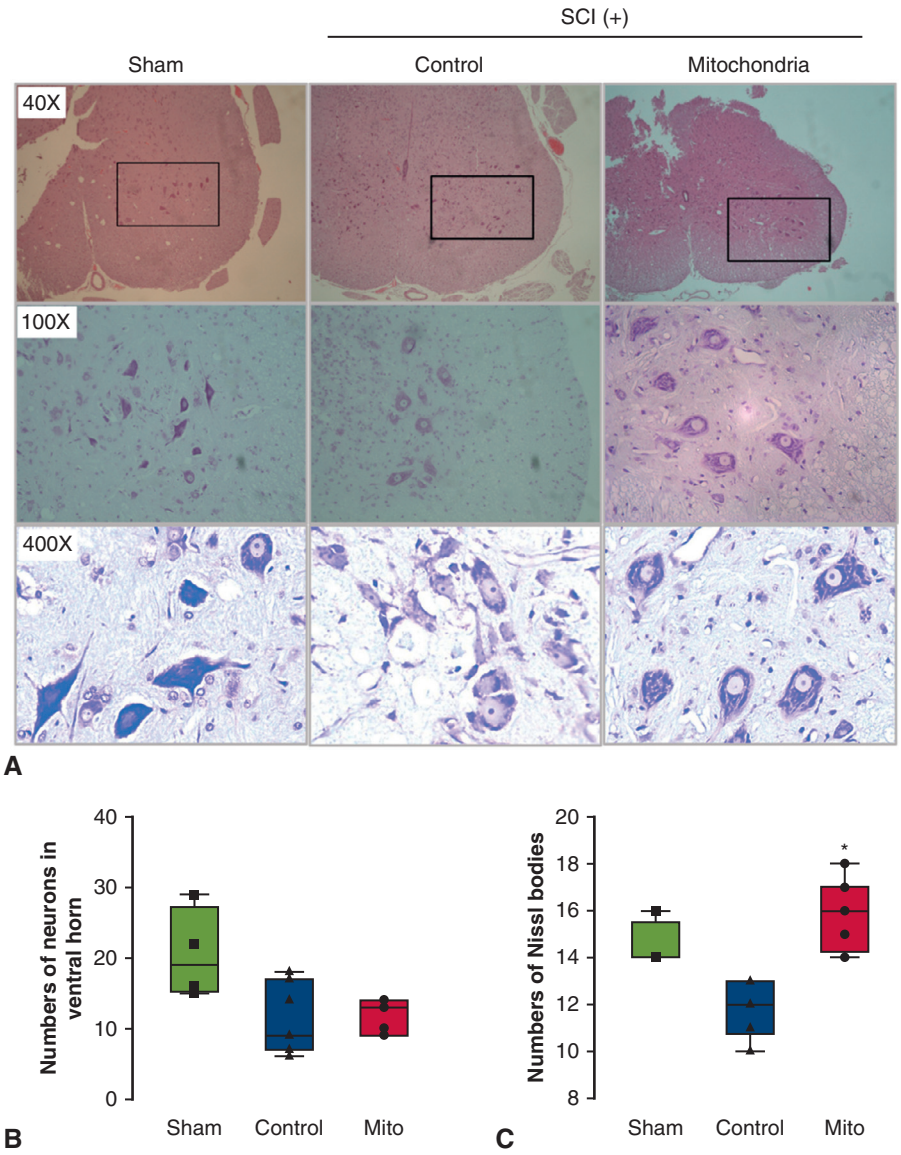

FIGURE 6. Representative histologic sections of spinal cord obtained from rats with spinal cord ischemia-reperfusion injury (A). The *upper 2 panels* are spinal cord sections stained using the hematoxylin and eosin stain, and the *lowest panels* are tissue stained by the Nissl staining method. There were significant central chromatolysis and reactive gliosis in the grey matter of ischemic spinal cord (B). Numbers of neurons in ventral horns of the gray matter of lumbar cord were reduced in animals subjected to cord ischemia–reperfusion injury with or without mitochondrial transplant (C). Compared with controls, the numbers of Nissl bodies in the ventral horn neurons were significantly increased in the mitochondrial group. Results were analyzed using the Mann–Whitney *U* test. **P* < .05 controls (n = 6) versus mitochondrial group (n = 8). Data are presented as *box-and-whisker plots*, in which the *horizontal lines of color boxes* indicate the 75th percentile, median and 25th percentile of the distribution, and the upper and lower whiskers indicate the maximal and minimal values. *SCI*, Spinal cord ischemia.

acute bioenergetics of the injured spinal cord15 and other central nervous system trauma.[27] However, no previous study has reported the administration of viable mitochondria in the acute phase of spinal cord ischemia–reperfusion injury.

In this study, we labeled the mitochondria harvested from the calf muscle using MitoTracker fluorescence to localize the distribution of mitochondria following systemic delivery via the jugular veins. In the naïve rats, the in vivo MitoTracker red fluorescence was detected mainly in the left upper quadrat of abdominal cavity at 2 hours after transplantation, which suggested entrapment of exogenous mitochondria in the spleen. However, the MitoTracker red fluorescence-labeled mitochondria were disseminated in

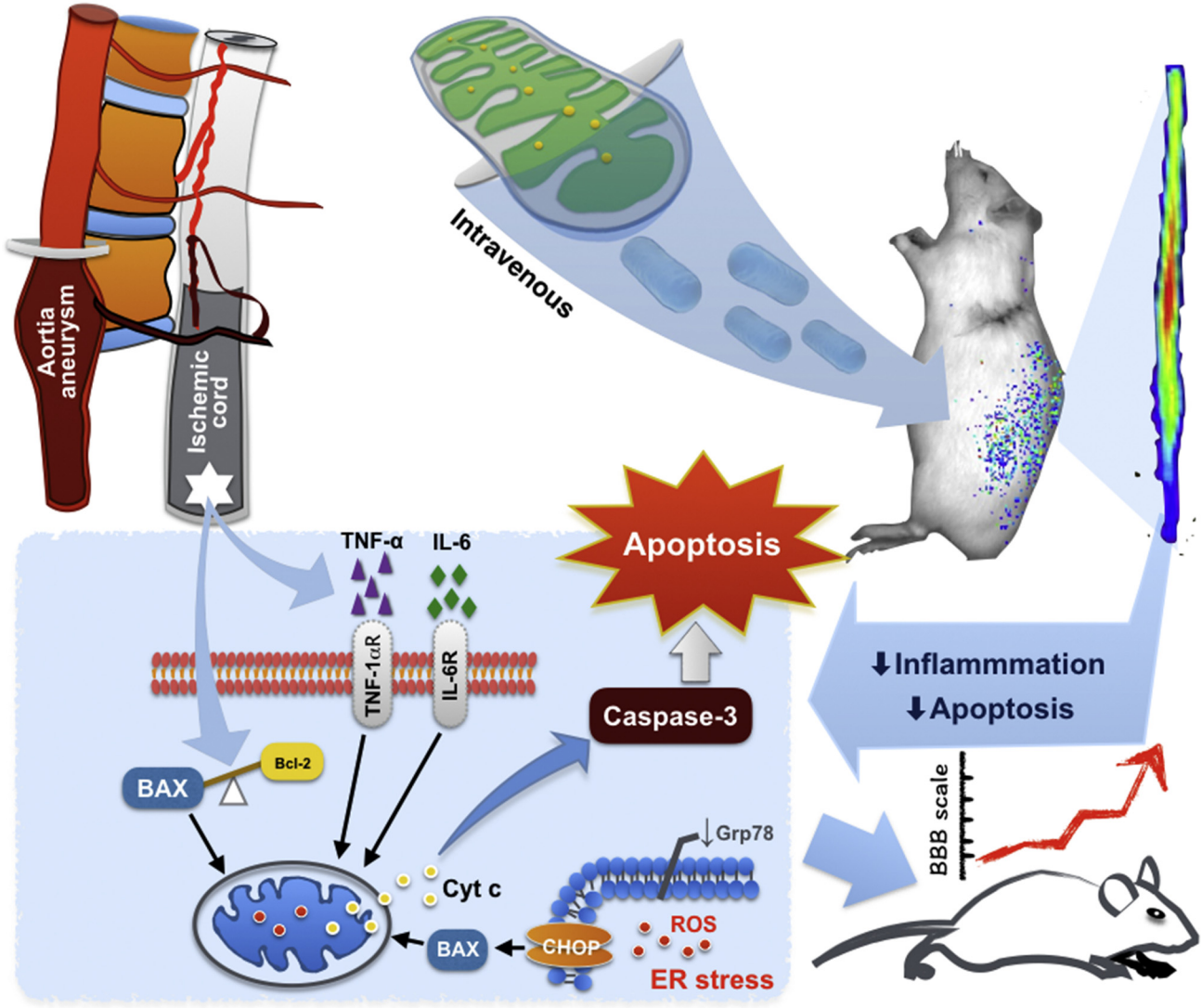

FIGURE 7. Ischemia–reperfusion injury during aortic surgery generates inflammatory responses (activation of interleukin [*IL*]-6 and tumor necrosis factor [*TNF*]-α) and induces mitochondrial dysfunction (increased BAX-to-Bcl-2 ratio) in the lumbar spinal cord, leading to caspase-3–mediated neuroapoptosis and impaired locomotor function in the hindlimbs of experimental rats. Transplantation of exogenous viable mitochondria at the reperfusion phase of spinal cord ischemic injury significantly improves the hindlimb locomtor function by attenuating endoplasmic reticulum (*ER*) stress and mitochondrial injury, as shown by the restoration of expressions of GRP78/CHOP and Bcl-2 family proteins (BAX/Bcl-2 ratio) in the injured spinal cord. Mitochondrial treatment also significantly reduces the regional pro-inflammatory reaction (IL-6 and TNF-α). The improvement in mitochondrial function and neuroinflammation suppresses cell apoptosis in the spinal cord. *BAX*, Bcl-2-associated X protein; *Bcl-2*, B-cell lymphoma-2; *TNFR-1*, tumor necrosis factor receptor-1; *IL-6R*, interleukin-6 receptor; *Cyt c*, cytochrome c; *CHOP*, CCAAT-enhancer-binding protein homologous protein; *Grp78*, binding immunoglobulin protein; *ROS*, reactive oxygen species; *BBB scale*, Basso-Beattie-Bresnahan scale.

the body parts below T6 levels where the aortic clip was applied. The ex vivo MitoTracker green fluorescence also confirmed that the transplanted mitochondria were only localized in the thoracolumbar cord that subjected to ischemia–reperfusion injury. Although the mechanisms for ''end-organ homing'' and ''subcellular internalization'' where the transplanted mitochondria are retained in the injured down-stream organs remained to be determined,[11] our imaging measurements clearly demonstrate the selective distribution of mitochondria in the ischemic tissues following intravenous administration.

In animals that received mitochondrial transplantation, the expressions of GRP78/CHOP and Bcl-2 family proteins (BAX/Bcl-2 ratio and Bcl-XL) were significantly restored,

indicating the attenuation of ER stress and mitochondrial function in the ischemic cord. The tissue levels of IL-6 and TNF-α in the spinal cord were also suppressed by mitochondrial treatment. The improvement in mitochondrial function and neuroinflammation in the mitochondrial-treated animals thus resulted in suppression of cell apoptosis (cleaved caspase-3 and TUNEL cells) and neuronal chromatolysis (Nissl bodies) in the spinal cord. Assessment of the hindlimbs further verified the significant functional improvement in locomotor activities in rats treated with mitochondria at the early reperfusion stage of SCI.

The first clinical application of mitochondrial transplantation was reported by McCully group in 2017.[9] Their results showed that intramyocardial injection of viable mitochondria in 5 pediatric patients with ischemia–reperfusion-associated myocardial dysfunction resulted in improvement of ventricular function in 4 of these patients, and they were then successfully separated from extracorporeal mechanical support. Therefore, mitochondrial transplantation has been highlighted as a revolutionary approach for tissue regeneration in which conventional therapies are unsuccessful.[28] With regard to the other advantages in regenerative medicine, mitochondrial transplantation can be rapidly isolated and purified within 30 minutes to meet the clinical needs,[29] such as at the acute phase of cord ischemia–reperfusion injury during aortic repair surgery. In addition, absence of alloreactivity and damage-associated molecular pattern molecules reaction following single or serial injections of syngeneic or allogeneic mitochondria might potentiate the clinical applications of mitochondrial transplantation.[30] Our study also highlights that intravenous administration of mitochondria is a more convenient route of transplantation than regional injection.

There are several limitations in this study. Although we demonstrated that the exogenously transplanted mitochondria distributed into the ischemic cord under IVIS images, the molecular pathways of uptake and internalization of these viable mitochondria into the neurons of spinal cord should be further characterized. In fact, the transmigration of exogenous mitochondria through the vascular walls and uptake to the ischemic tissue following systemic vascular delivery are undetermined.[11] Second, the optimal number of mitochondria transplanted was not characterized in this study. The quantity of mitochondria used for transplantation in our experiment was derived from the study reported by Gollihue and colleagues, in which the neuroregenerative effect of mitochondrial transplantation was tested in a rat model of contusive cord injury.[15] Third, molecular changes in the injured spinal cord were not measured at the early phase after cord ischemia. Nevertheless, the locomotor function of lower limbs was continuously assessed from immediately after injury to 7 days later. The progressive improvement in locomotor activities over the observational period reflected the restoration of subcellular function of the ventral neurons following mitochondrial transplantation. Fourth, the recovery of other neurologic functions of spinal cord (such as sensory, neuropathic pain, and autonomic responses) should be analyzed in our future experiments. Fifth, this study used plain PBS rather than the mitochondrial incubation medium as the placebo solution in the control group. Since the incubation time of the freshly isolated mitochondria in PBS was considerably short (within 1 hour), the potential paracrine effects mediated by the incubation medium were less likely to affect the recovery of ischemic cord.

In conclusion, our study demonstrated that transplantation of freshly isolated mitochondria at the early stage of spinal cord ischemia–reperfusion injury significantly suppresses the ER stress and mitochondrial dysfunction in the ischemic cord, leading to attenuation in the inflammatory and neuroapoptotic reactions, and improvement in the motor function recovery. Transplantation of viable mitochondria might potentially provide therapeutic effect in preservation of the neurologic function of spinal cord during major aortic surgery.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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