Nanobubble technology to treat spinal cord ischemic injury

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ABSTRACT

Background: Spinal cord ischemic injury is a severe complication of aortic surgery. We hypothesized that cerebrospinal fluid (CSF) oxygenation with nanobubbles after reperfusion could ameliorate spinal cord ischemic injury.

Methods: Twenty white Japanese rabbits were categorized into 4 groups of 5 rabbits each: sham group, with balloon catheter insertion into the aorta; ischemia group, with spinal cord ischemic injury by abdominal aortic occlusion; nonoxygenated group, with nonoxygenated artificial CSF irrigation after spinal cord ischemic injury; and oxygenated group, with oxygenated artificial CSF irrigation after spinal cord ischemic injury. At 48 hours after spinal cord ischemic injury, the modified Tarlov score to reflect hind limb movement was evaluated. The spinal cord was histopathologically examined by counting anterior horn cells, and microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses were performed.

Results: The oxygenated group showed improved neurologic function compared with the ischemia and nonoxygenated groups ($P < .01$ and $P = .019$, respectively). Anterior horn neuron prevention in the sham, nonoxygenated, and oxygenated groups was confirmed (mean modified Tarlov score: sham, 9.2 ± 1.9; nonoxygenated, 10.2 ± 2.2; oxygenated, 10.4 ± 2.2; ischemia, 2.7 ± 2.7). Microarray analysis identified 644 genes with twofold or greater increased signals between the ischemia and sham groups. Thirty-three genes related to inflammatory response were enriched among genes differentially expressed between the oxygenated and ischemia groups. Interleukin (IL)-6 and tumor necrosis factor (TNF) expression levels were significantly lower in the oxygenated group compared with the ischemia group, while qRT-PCR showed lower IL-6 and TNF expression levels in the oxygenated group compared with the ischemia group ($P < .05$).

Conclusions: CSF oxygenation with nanobubbles after reperfusion can ameliorate spinal cord ischemic injury and suppress inflammatory responses in the spinal cord. (JTCVS Open 2020;3:1-11)

Cerebrospinal fluid oxygenation mitigated ischemia-reperfusion injury to the spinal cord.
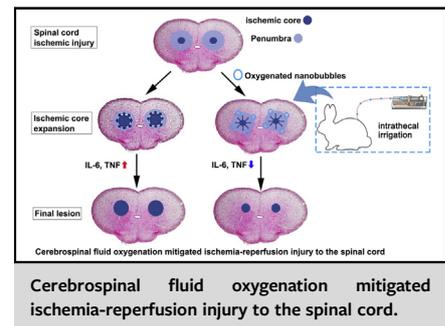
CENTRAL MESSAGE

Cerebrospinal fluid oxygenation with nanobubbles after reperfusion can ameliorate spinal cord ischemia-reperfusion injury in rabbits and suppress inflammatory responses in the spinal cord.

PERSPECTIVE

Cerebrospinal fluid drainage is a widely used option to prevent and treat spinal cord ischemia-reperfusion injury with limited efficacy as a therapeutic modality. Cerebrospinal fluid oxygenation may enable symptom-driven postoperative intervention and serve as a bridging method to supply oxygen to the spinal cord tissues until a collateral network develops.

▶ Video clip is available online.

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Spinal cord ischemic injury after surgery for thoracic aortic aneurysm is a severe and unpredictable complication. The reported prevalence of paraplegia ranges from 3.8% to 13.2% after thoracoabdominal aortic aneurysm repair[1-3] and from 2.5% to 9.4% after thoracic endovascular aortic repair.[4,5] Several spinal cord protective maneuvers have been documented, including the maintenance of a relatively high mean perfusion pressure during and after reparative surgery,[6] use of systemic[7] and/or local hypothermia around

the spinal cord with epidural perfusion cooling,[8,9] selective hypothermic intercostal artery perfusion,[10] cerebrospinal fluid (CSF) drainage,[11,12] reattachment of the intercostal and lumbar arteries,[13,14] and preconditioning of the paraspinal collateral network by segmental artery coil embolization.[15] Among these spinal cord protection methods, CSF drainage is the sole validated method for preventing and treating spinal cord ischemic injury[16]; its protective effect is limited, however.

We previously demonstrated that preischemic CSF oxygenation with the combined use of artificial CSF and nanobubble technology can ameliorate spinal cord ischemic injury induced by infrarenal abdominal aortic occlusion in a rabbit model[17] and described a possible alternative method to supply oxygen to the spinal cord via the intrathecal fluid. However, in that previous study we did not investigate the subcellular mechanism of oxygenated CSF with nanobubble technology.

Recently, the importance of the penumbra, clinically defined as the hypoperfused area surrounding the ischemic core, has been advocated in stroke research. A sustained inflammatory reaction in the ischemic penumbra can exacerbate deleterious metabolic processes via microvasculature impairment and matrix degradation, with resultant expansion of the ischemic core peripherally in the course of ischemia-reperfusion injury.[18] With regard to spinal cord ischemic injury, a deterioration in neurologic functional correlates with increases in inflammatory chemokine release and microglial activation.[19,20] Moreover, manipulation of the molecular pathogenesis related to ischemic injury will be a possible future intervention for spinal cord protection.[19,20] Therefore, we speculated that the oxygenated CSF prevents the ischemic core from expanding to the penumbra via inflammatory reactions in the spinal cord. However, whether postischemic CSF oxygenation can mitigate ischemia-reperfusion injury as a postischemic therapy remains unclear. In this study, we aimed to examine the effect of CSF oxygenation with nanobubbles after ischemia-reperfusion and to elucidate the subcellular mechanism of this treatment modality.

METHODS
Artificial CSF Oxygenation Protocol
Oxygenated artificial CSF was produced using a nanobubble-generating device (MA3FS; ASUPU, Shizuoka, Japan) as described previously.[17]

Using artificial CSF (ARTCEREB; Otsuka, Tokushima, Japan), the irrigation solution typically used during cerebrospinal surgery and composed with similar composition of human CSF, we measured the serial changes in partial pressure of oxygen (PO₂) in CSF.

Animal Model, Surgical Procedure, and CSF Oxygenation Protocol
Twenty white male Japanese rabbits were categorized into 4 groups of 5 rabbits each. In the sham group, a microcatheter was inserted in the intrathecal space, and the infrarenal abdominal aorta was occluded for 5 seconds only. In the ischemia, nonoxygenated, and oxygenated groups, a microcatheter was inserted in the intrathecal space, and the infrarenal abdominal aorta was occluded for 15 minutes. In the nonoxygenated and oxygenated groups, 15 minutes later, nonoxygenated and oxygenated artificial CSF, respectively, was injected into the intrathecal space through a microcatheter at 5 mL/h for 60 minutes, and the CSF pressure through the catheter was monitored during the injection. If the pressure of the intrathecal cavity exceeded 30 mm Hg, we drained 0.1 mL of CSF to decompress the cavity.

The animal procedures have been described previously.[17] In brief, all rabbits were anesthetized with 5.0% isoflurane added to mixed gas. The inspired oxygen fraction was regulated to 0.5, and 50 mg/kg of ketamine hydrochloride was injected intramuscularly. Anesthesia was maintained with inhalation of 2.0% to 3.0% isoflurane. The animals breathed spontaneously with face masks. Ringer's lactate solution (Lactec; Otsuka) was infused as maintenance fluid at a rate of 7 mL/kg/h.

The rabbits were placed in the prone position, and a skin incision was made at the level of the first lumbar vertebra (L1). The intrathecal space was punctured with an 18-G cannula, and a 0.9-mm-diameter polyurethane catheter was inserted into the intrathecal space. The catheter tip was set 2 cm beyond the cannulation site. The rabbits were then placed in the supine position, and the right femoral artery was exposed. Heparin sodium (100 U/kg) was administered as an intravenous bolus for approximately 10 min before occlusion of the infrarenal abdominal aorta. The right femoral artery was incised, and a 4-Fr Fogarty occlusion balloon catheter (Edwards Lifesciences, Irvine, Calif) was inserted up to the infrarenal abdominal aorta. The occlusion catheter was advanced 15 cm from the inguinal ligament and inflated for 5 seconds in the sham group and for 15 minutes in the ischemia, nonoxygenated, and oxygenated groups. Previous reports indicate that occlusion of the infrarenal abdominal aorta for 15 minutes led to paraparesis in rabbits.[21,22] At 15 minutes after aortic occlusion release, the artificial CSF was injected into the rabbits in the nonoxygenated and oxygenated groups.

Once the procedures were completed, all catheters were removed, and the rabbits were returned to their cages after regaining consciousness. The timeline of the experimental maneuver is shown in Figure 1. Forty-eight hours later, all rabbits were neurologically evaluated for hind limb
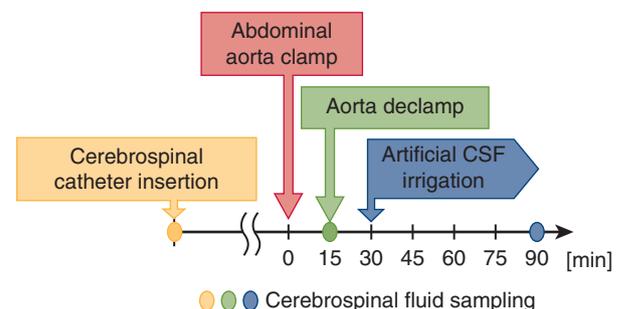
FIGURE 1. The timeline of the experiment. Artificial cerebrospinal fluid was irrigated into the intrathecal cavity in the nonoxygenated and oxygenated groups, with no irrigation in the sham and ischemia groups. *CSF*, Cerebrospinal fluid.

function using the modified Tarlov score[23] and then euthanized via the acute administration of efficient potassium chloride under deep anesthesia. The twelfth thoracic vertebra and sixth lumbar vertebra were immediately incised, and the spinal cords were removed.

Six rabbits exhibited macroscopic laceration on the surface of the spinal cord and were excluded from further evaluation. These lacerations most likely were attributable to a suboptimal technique for intrathecal catheter insertion, which could have compromised our entire comparative evaluations. Therefore, we replaced those animals with 6 other rabbits for the experiment. Blood pressure, rectal temperature, PO_2 of arterial blood, hemoglobin concentration, and CSF pressure—all of which are known to influence neurologic function—were monitored. All the animals had free access to water and food before and after the experiment. This study was conducted in accordance with the Regulation for Animal Experiments and Related Activities and approved by the Animal Ethical Committee at Tohoku University (2017MdA-079).

Measurement of CSF-PO$_2$

CSF-PO$_2$ was measured on intrathecal catheter insertion in the sham group; on intrathecal catheter insertion and 15 and 75 minutes after spinal cord ischemia in the ischemia group; and on intrathecal catheter insertion, 15 min after spinal cord ischemia, and 75 minutes after the completion of continuous artificial CSF injection in the nonoxygenated and oxygenated groups.

Neurologic Evaluation

Neurologic function of the hind limbs was assessed at 48 hours after spinal cord ischemia using the modified Tarlov score[23] as follows: 1, slight movement; 2, sits with assistance; 3, sits alone; 4, weak hop; and 5, normal hop. We video recorded the evaluation of neurologic outcome, and 2 blinded investigators performed the assessment and assigned a Tarlov score.

Histopathological Evaluation

The removed spinal cord was immediately dipped in 10% neutral buffered formalin solution (Wako Pure Chemical Industries, Osaka, Japan). Subsequently, paraffin-embedded samples were created, and cross-sections of the spinal cord were stained with hematoxylin and eosin. The histopathological examination was performed by a pathologist who was blinded to the study groups. The gray matter at L2 and L3 was divided into 4 sections in the vertical and horizontal directions, with the central canal as the center. One section from the anterior horn was selected, and the anterior horn neurons were counted. Only neurons with a cell body with a minor diameter 4-fold larger than the nucleus of a glial cell were considered anterior horn neurons, to exclude neurons with pyknotic changes and small cross-sections. We defined normal anterior horn neurons as those with a polygonal body with round and basophilic nuclei and degenerated anterior horn neurons as being eosinophilic, without nuclei, or chromatolytic neurons. The histopathological evaluation was performed using the values of normal anterior horn neurons.

RNA Preparation and Microarray Hybridization

The removed spinal cords were immediately dissected and fresh-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

Subsequently, total RNA (80 ng) was amplified at 40°C for 2 hours using the Agilent Low RNA Input Quick Amplification Kit (Agilent Technologies, Santa Clara, Calif), following the manufacturer's protocol. Complementary RNA was labeled at 40°C for 2 hours with cyanine 3. The sample was incubated with a microarray slide (020908; Agilent Technologies) for 17 hours. Microarray signals were scanned in a microarray scanner (Agilent Technologies). The images were processed using Feature Extraction version 10.7.3.1 software (Agilent Technologies), which provided normalized cyanine 3 channel intensity values for each spot on an array. Data were normalized at the 75th percentile using GeneSpring version 14.5 (Agilent Technologies). Expression data were analyzed using the Functional Annotation Clustering tool in DAVID version 6.8 (https://david.ncifcrf.gov/), an online bioinformatics resource that provides functional and pathway enrichment analysis.[24] A P value <.05 was considered to indicate statistical significance on the basis of Bonferroni-adjusted P values. A gene count >2 served as the cutoff criterion, and the classification stringency was set to medium.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed on spinal cord samples. Total RNA (1 μg) from each sample was reverse-transcribed using the Applied Biosystems High-Capacity cDNA Reverse-Transcription Kit (Thermo Fisher Scientific, Waltham, Mass). The qRT-PCR assays were performed in 96-well plates in an ABI 7300 real-time PCR instrument (Thermo Fisher Scientific). PCR analyses were conducted with gene-specific primers and fluorescently labeled Taq for interleukin (IL)-1β (Oc03823250_s1), IL-6 (Oc04097051_m1), and tumor necrosis factor (TNF; Oc03397715_m1) mRNAs (designed by Applied Biosystems/Thermo Fisher Scientific). The reaction mixture (30 μL) containing 1.5 μL of cDNA template, 1.0 μL of each primer, 7.5 μL of distilled water, and 10 μL of TaqMan Universal PCR Master Mix (Applied Biosystems/Thermo Fisher Scientific) was amplified as follows: denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds, and 60°C for 1 minute. Each value was normalized to the housekeeping gene *GAPDH* (Applied Biosystems/Thermo Fisher Scientific; Oc04097051_m1). The fold change in expression was then obtained using the $2^{-\Delta\Delta CT}$ method. All qRT-PCR reactions were performed in triplicate.

Statistical Analysis

The determination of the sample size was based mainly on power analysis using the data obtained from the previous experiment[17] with a power of 0.80 and $\alpha = 0.05$, with the assumption of a similar degree of improvement by therapeutic intervention with oxygenated artificial CSF. Normal data distribution was tested using the Shapiro–Wilk test. The variance of continuous variables in the intraoperative data was analyzed with the Kruskal–Wallis test. The PO$_2$ values were analyzed by 1-way repeated-measures analysis of variance followed by the Tukey–Kramer multiple-comparisons test for post hoc testing. The number of anterior horn cells and extent of modified Tarlov scores were compared among groups using the Mann–Whitney U test followed by Bonferroni correction for post hoc testing. qRT-PCR results were analyzed using one-way analysis of variance, followed by the Tukey-Kramer multiple-comparisons test for post hoc testing. Statistical analyses were performed using JMP Pro 13.0.0 software (SAS Institute, Cary, NC).

RESULTS
Preserved CSF-PO$_2$ by Oxygenated Artificial CSF After Spinal Cord Ischemia-Reperfusion

The baseline characteristics were essentially the same across the 4 study groups (Table 1). In addition, CSF pressure after aortic occlusion was not significantly different among the ischemia, nonoxygenated, and oxygenated groups ($P = .27$). The baseline CSF-PO$_2$ values measured immediately after insertion of the intrathecal catheter were 130.3 $\pm$ 16.6 mm Hg in the ischemia group, 138.0 $\pm$ 13.3 mm Hg in the nonoxygenated group, 130.0 $\pm$ 26.7 mm Hg in the oxygenated group, and

TABLE 1. Intraoperative data

Variable	Sham group	Ischemia group	Nonoxygenated group	Oxygenated group	*P* value
Samples, n	5	5	5	5	
Body weight, kg	2.89 ± 0.03	2.92 ± 0.06	2.90 ± 0.03	2.87 ± 0.03	.62
Mean blood pressure, mm Hg	78.5 ± 9.8	83.3 ± 10.8	78.9 ± 10.9	79.1 ± 9.5	.84
Mean rectal temperature, °C	37.60 ± 0.61	37.98 ± 0.44	38.00 ± 0.58	38.48 ± 0.69	.16
Mean arterial blood PO_2, mm Hg	142.5 ± 42.7	125.2 ± 43.4	125.8 ± 52.1	138.1 ± 19.9	.87
Mean hemoglobin concentration, g/dL	11.10 ± 1.60	11.20 ± 0.76	11.40 ± 0.41	11.18 ± 0.64	.96
CSF pressure at baseline, mm Hg	10.4 ± 2.0	10.5 ± 4.0	10.2 ± 1.3	10.8 ± 3.3	.99
CSF pressure after aortic occlusion, mm Hg	—	14.0 ± 1.5	10.4 ± 1.6	11.2 ± 1.7	.27

Data are mean $\pm$ SD. *PO_2*, Partial pressure of oxygen; *CSF*, cerebrospinal fluid.

132.3 ± 31.3 mm Hg in the sham group (Figure 2). The CSF-PO_2 values after 15 minutes of spinal cord ischemia were 116.0 ± 42.2 mm Hg in the ischemia group, 124.0 ± 28.6 mm Hg in the nonoxygenated group, and 111.0 ± 30.6 mm Hg in the oxygenated group. After 1 hour of replacement with artificial CSF, the CSF-PO_2 values were 103.3 ± 15.0 mm Hg in the nonoxygenated group and 134.8 ± 19.8 mm Hg in the oxygenated group.

In the ischemia group, the CSF-PO_2 after 75 min of reperfusion was 85.3 ± 10.0 mm Hg, significantly lower than the CSF-PO_2 at baseline ($P < .01$; Figure 2, A). In the nonoxygenated group, the CSF-PO_2 after ischemia-reperfusion followed by CSF irrigation was significantly lower than that at baseline despite irrigation with nonoxygenated artificial CSF ($P = .027$; Figure 2, A). In the oxygenated group, the CSF-PO_2 after ischemia-reperfusion followed by
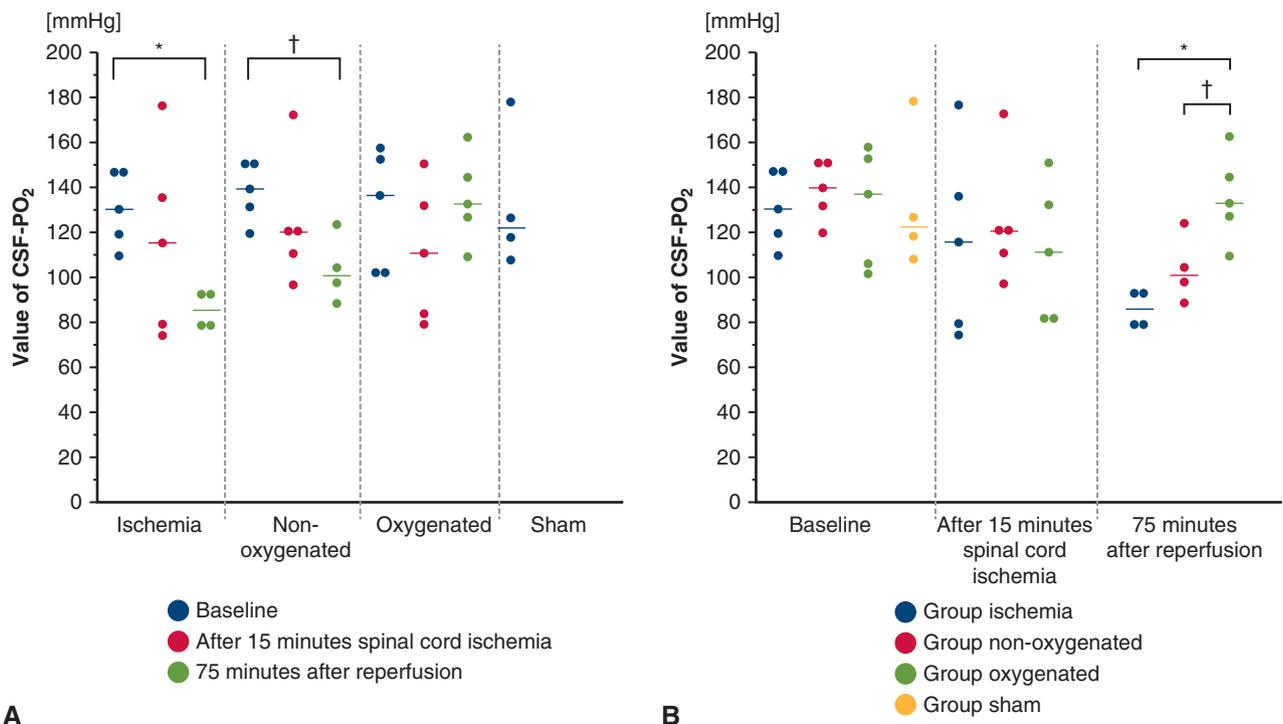
FIGURE 2. Cerebrospinal fluid (*CSF*) partial pressure of oxygen (*PO_2*) was decreased after ischemia-reperfusion injury and increased after oxygenated artificial CSF irrigation. A, Intragroup comparisons of differences between baseline, after 15 minutes of spinal cord ischemia, and after CSF replacement. CSF-PO_2 tended to decrease after spinal cord ischemia, with further decreases even after reperfusion in the ischemia and nonoxygenated groups but was restored to the baseline level after oxygenated artificial CSF irrigation in the oxygenated group. B, Intergroup comparisons of PO_2 at time points throughout the experiment. CSF-PO_2 was significantly higher in the oxygenated group compared with the ischemia and nonoxygenated groups at 75 minutes after reperfusion. Data are presented as *colored dots*; *horizontal lines* indicate medians.*$P < .01$; †$P < .05$.

oxygenated artificial CSF irrigation was significantly higher than that in the ischemia and nonoxygenated groups ($P < .01$ and $P = .031$, respectively; Figure 2, B). No significant difference was found between the ischemia and non-oxygenated groups ($P = .35$).

Improved Neurologic Function by Oxygenated Artificial CSF

The mean modified Tarlov score was significantly higher in the oxygenated group compared with the ischemia and nonoxygenated groups ($P < .01$ and $P = .019$, respectively), whereas no significant difference was found between the nonoxygenated and ischemia groups ($P = .279$; Figure 3).

Oxygenated Artificial CSF Preserved Normal Anterior Horn Cells

The mean normal anterior horn neuron count was 2.7 ± 2.7, 10.2 ± 2.2, 10.4 ± 2.2, and 9.2 ± 1.9 at L2 and 1.4 ± 1.0, 6.8 ± 1.4, 8.8 ± 0.7, and 12.0 ± 2.8 at L3 in the ischemia, nonoxygenated, oxygenated, and sham groups, respectively (Figure 4, A and B). There were significantly fewer normal anterior horn neurons in the ischemia group than in the nonoxygenated and oxygenated groups at L2 and at L3 ($P < .01$; Figure 4, A and B). Representative histopathological images of the anterior horn in the 4 study groups are shown in Figure 4, C.

CSF Oxygenation Suppressed Inflammatory Response

Compared with the sham group, the ischemia group had 644 gene signals that were up-regulated by more than

twofold. A DAVID functional annotation clustering analysis of these genes produced 3 enriched functional clusters under high-stringency conditions (Table 2). Gene Ontology terms "inflammatory response" (GO:0006954), "positive regulation of I-$\kappa\beta$ kinase/NF-$\kappa\beta$ signaling" (GO:0043123), and "cellular response to mechanical stimulus" (GO:0071260) showed high enrichment scores with strong confidence levels. Thirty-three genes related to inflammatory response were enriched among the differentially expressed genes, and of the 33 genes in the "inflammatory response" category, 10 were related with "cytokine." The heat map of each group in these 10 genes (Figure 5) shows that gene expression involved in the inflammatory response was suppressed marginally in the nonoxygenated group and remarkably in the oxygenated group compared with the ischemia group. The processed signal values in these 10 genes are presented in Table 3. Expressions of IL-6, TNF, and IL-1β associated with neuronal inflammation from the glial cells were suppressed in the oxygenated group (Figure 6, A).

We confirmed the selected microarray results by comparing them with relative mRNA levels obtained by qRT-PCR (Figure 6, B). IL-6 expression was significantly decreased in the oxygenated group compared with the ischemia group ($P = .048$) and likely down-regulated in the nonoxygenated group, but the difference was not statistically significant ($P = .053$). Moreover, TNF expression was significantly lower in the oxygenated group compared with the ischemia group ($P = .049$). No significant differences in IL-1β expression were found between the ischemia group and with the other groups, whereas the oxygenated showed a tendency toward lower IL-1β expression compared with the ischemia group.

DISCUSSION

We have demonstrated that CSF oxygenation with nano-bubbles after ischemia-reperfusion can mitigate spinal cord injury, and that its therapeutic effect is associated with the suppression of inflammatory responses in the spinal cord. This treatment modality can provide a paramount alternative pathway to supply oxygen to the spinal cord with imminent irreversible damage. In case of brain infarction, oxygen delivery can be directly resumed in multiple ways, such as tissue plasminogen activator administration and catheter intervention. Contrarily, spinal cord infarction after thoracoabdominal aortic aneurysm repair cannot be managed with similar treatments. Given that spinal cord infarction typically develops from occlusion of the small-caliber critical segmental artery connected to the artery of Adamkiewicz or from profound reduction of miniscule collateral flow into the spinal cord, recanalization of the main stem into the cord is not readily achieved. Thus, oxygenated artificial CSF irrigation may supply oxygen via the CSF for postoperative spinal cord ischemic injury.

FIGURE 3. Neurologic function 48 hours after ischemia-reperfusion injury assessed using the modified Tarlov score. The lower the score, the more severe the neurologic impairment. A score of 5 reflects intact neurologic function. Data are presented as *colored dots*; *horizontal line* represents the median. *$P < .01$; †$P < .05$; ‡$P = .279$.
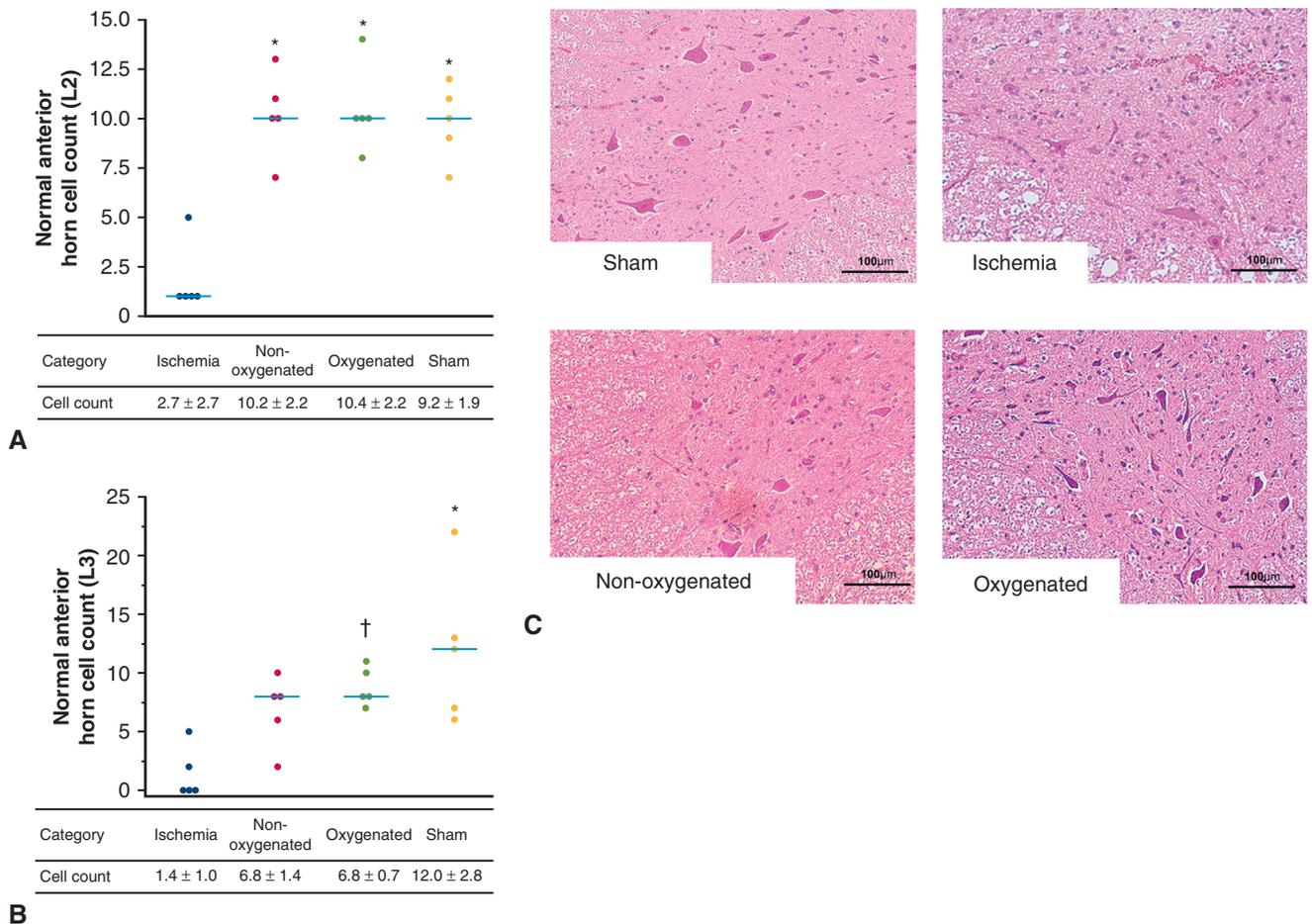
FIGURE 4. Normal large motor neurons preserved in nonoxygenated, oxygenated, and sham groups. A, Assessment of large motor neuron cell count at L2, in which the normal anterior horn neurons were significantly preserved in the nonoxygenated, oxygenated, and sham groups compared with the ischemia group. B, Assessment of large motor neuron cell count at L3, where the normal anterior horn neurons were significantly preserved in the nonoxygenated, oxygenated, and sham groups versus the ischemia group. C, Representative histopathological images of the spinal cord in the sham, ischemia, nonoxygenated, and oxygenated groups (hematoxylin and eosin staining; original magnification 200×). In the sham group, no ischemic injury was detected. In the ischemia group, many motor neurons were found to be degenerated with eosinophilic or chromatolytic changes, and normal neurons were diminished. In the nonoxygenated and oxygenated groups, normal neurons with basophilic nuclei were relatively well preserved. Data in A and B are presented as *colored dots*; *horizontal lines* represent medians. In A, *$P < .01$ vs the ischemia group. In B, †$P < .05$ versus the ischemia group.

The important difference between previous studies[17] and the present study is in the performance of CSF oxygenation. Here infrarenal abdominal aortic occlusion to cause spinal cord ischemia was performed before CSF oxygenation. In short, ischemia-reperfusion injury definitely occurred, unlike in the previous study. Some results of the present study demonstrate the reaction of ischemia-reperfusion injury; for example, decreased CSF-PO$_2$ was sustained even at 75 minutes after reperfusion, whereas irrigation with oxygenated artificial CSF partially restored a higher CSF-PO$_2$, but not to a remarkable extent. We achieved >250 mm Hg CSF-PO$_2$ before the ischemic insult after irrigation with oxygenated artificial CSF in our previous experiment. Thus, it is reasonable to speculate that the spinal cord, into which the ischemia-reperfusion injury was induced, consumes a

significant amount of oxygen from the CSF. Previous studies also demonstrated a correlation between spinal cord ischemia and decreased CSF-PO$_2$ in the intrathecal cavity in canine and swine models.[25,26] These findings are compatible, at least in part, with our observations. The CSF condition may not reflect only the condition in the spinal cord; significant cross-talk may occur between the CSF and spinal cord.

Several potential mechanisms can explain how oxygen in the CSF is delivered to the spinal cord tissue if the tissue consumes oxygen within the CSF. With regard to the traditionally considered CSF circulation, CSF is known to be absorbed across the arachnoid villi around the brain and spinal cord.[27] However, Greitz and colleagues[28] reported that the CSF circulation occurs not only through this pathway, but

TABLE 2. Functional annotation clustering (DAVID) for overexpressed signals in the ischemia group

Up-regulation in the ischemia group compared with the sham group	n	*P* value
Inflammatory response	18	2.2E-02
Positive regulation of I-κB kinase/NF-κB signaling	16	2.9E-02
Cellular response to mechanical stimulus	9	3.0E-02

The left column defines the Gene Ontology category, and n indicates the number of significant genes. The probability that a greater or equal number would be found by chance (*P* < .05) with Bonferroni-adjusted *P* value is presented.

also through the brain capillaries to the brain tissue. Following this striking report, several researchers began to reconsider the CSF circulation.[29-31] Nedergaard and colleagues[32] and Iliff and colleagues[33] proposed a "glymphatic system" consisting of glial cells and the lymphatic system. The concept of the glymphatic system is that CSF passes specifically via the space surrounded by the abluminal surface of the blood vessel and apical processes of astrocytes. The authors demonstrated that the CSF enters the brain parenchyma through the paravascular spaces surrounded by penetrating arteries using fluorescent tracers of certain molecular weights in mice. Although they did not specifically mention oxygen delivery in association with CSF circulation, it can be naturally inferred that oxygen is carried with the CSF. Thus, if the CSF is distributed deeply inside the parenchyma via the glymphatic system, oxygen should be delivered into the spinal cord parenchyma. Moreover, the intrathecal route has been clinically used for drug delivery to treat various diseases, including stroke, neurodegenerative diseases, and brain tumors.[34]

The efficacy of intrathecal drug delivery even with protein has been demonstrated in animal and human studies. Thus, the intrathecal administration of various agents seems to be a validated treatment. This concept may support the observations of the present experiment.

A possible concern may be related to the practical limit for sustained replacement of CSF with oxygenated artificial CSF. Placement of an indwelling catheter in the spinal cavity generally carries a risk of infection, which limits the duration of this therapy. However, we postulate that CSF oxygenation can fulfill its expected role as an alternative oxygen supply during critical days. Etz and colleagues[3] demonstrated the important 5-day window between segmental artery occlusion and the development of a robust collateral pathway around the intraspinal and paraspinous arterial networks. The nanobubble technology can be applied in this critical time window, which could be a bridging method before sufficient collateral flow compensates for the oxygen demand in the spinal cord.

Nonetheless, whether the delivered oxygen exerts its protective function against ischemia-reperfusion injury even after insult to the spinal cord remains controversial. At the ischemic region, 3 parenchymal vascular states exist in various proportions: the ischemic core, penumbra, and benign oligemia. In these states, the penumbra is the tissue region at risk of recruitment into the ischemic core; thus, it is the principal treatment target for reperfusion. The earlier the penumbra is reperfused, the less the region is involved in the ischemic core. The recruitment of the penumbra into the ischemic core is thought to be related to excitotoxicity, the spread of depression, oxidative stress, and the inflammatory response.[18] Iadecola and colleagues[35] identified immunity
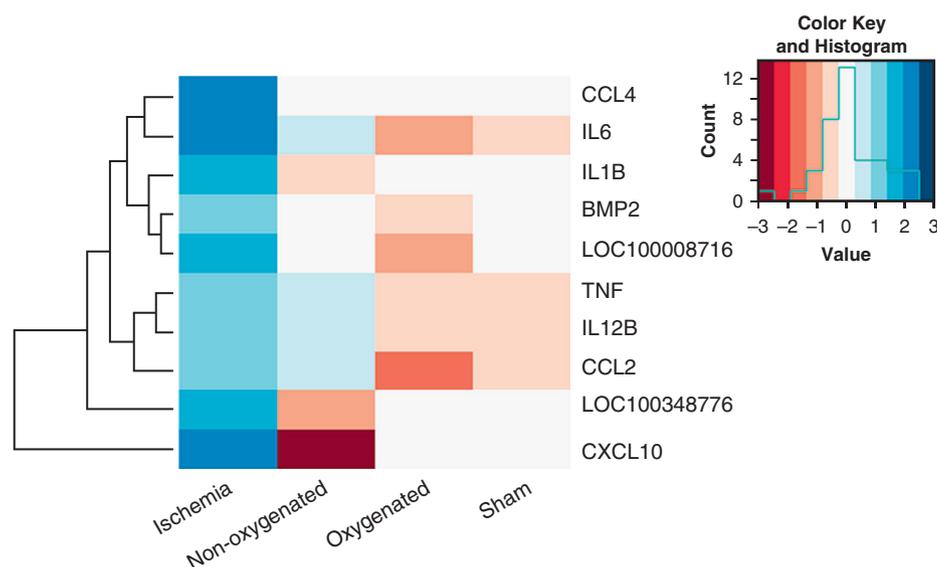
FIGURE 5. Gene expression involved in inflammatory responses was suppressed in the oxygenated group compared with the ischemia group. The gene expression values targeted by the ischemia group in the "inflammatory response" and "cytokine" categories are depicted by hierarchical clustering in the ischemia, nonoxygenated, oxygenated, and sham groups.

TABLE 3. Genes targeted by the ischemia group in the "inflammatory response" and "cytokine" categories

ID	Accession no.	Ischemia group	Nonoxygenated group	Oxygenated group
BMP2	NM_001082650	2.32	0.91	0.59
CCL2	NM_001082294	2.98	1.96	0.54
CCL4	NM_001082196	4.36	1.24	0.98
CXCL10	XM_002717106	5.98	0.15	1.43
IL12B	XM_002710347	2.64	1.55	0.72
IL1B	NM_001082201	2.35	0.69	0.7
IL6	NM_001082064	5.27	1.5	0.66
LOC100008716	XM_002717113	2.3	0.74	0.47
LOC100348776	XM_002719246	3.26	0.6	1.34
TNF	NM_001082263	3.07	1.65	0.97

Each value was normalized by the sham group.

and inflammation as key elements of stroke pathobiology. They reported that activated microglia led to the release of proinflammatory mediators such as IL-6, TNF, and IL-1β. These signal pathways promote inflammatory responses and cause further damage to nerve tissue. Several reports also have revealed the involvement of inflammatory responses in the ischemic brain and the spinal cord by demonstrating up-regulated expression of inflammatory cytokines such as IL-6, TNF, and IL-1β.[19,36] In our present study, IL-6 and TNF expression levels were increased in the ischemia
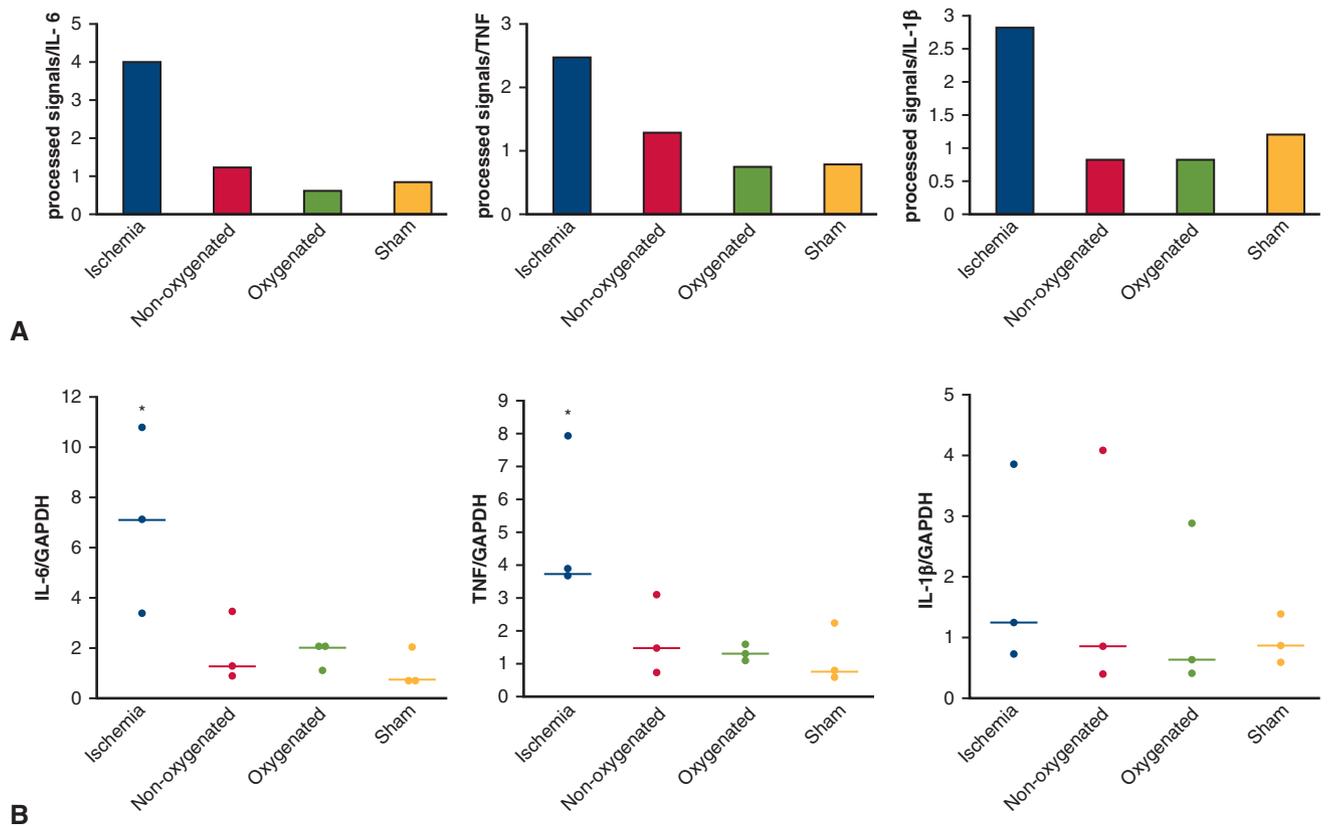
FIGURE 6. Gene expression levels of interleukin (IL)-6 and tumor necrosis factor (TNF) in were significantly suppressed in the oxygenated group compared with the ischemia group. A, Gene expression of IL-6, TNF, and IL-1β in the microarray. The y-axis represents the expression of the processed signals. B, The selected inflammatory genes, IL-6, TNF, and IL-1β, obtained from the DNA microarray experiment were validated using quantitative reverse-transcription polymerase chain reaction. The y-axis represents the $2^{-\Delta\Delta CT}$ data. Data are presented as *colored dots*, and *horizontal lines* represent medians. *$P < .05$ vs the oxygenated group.
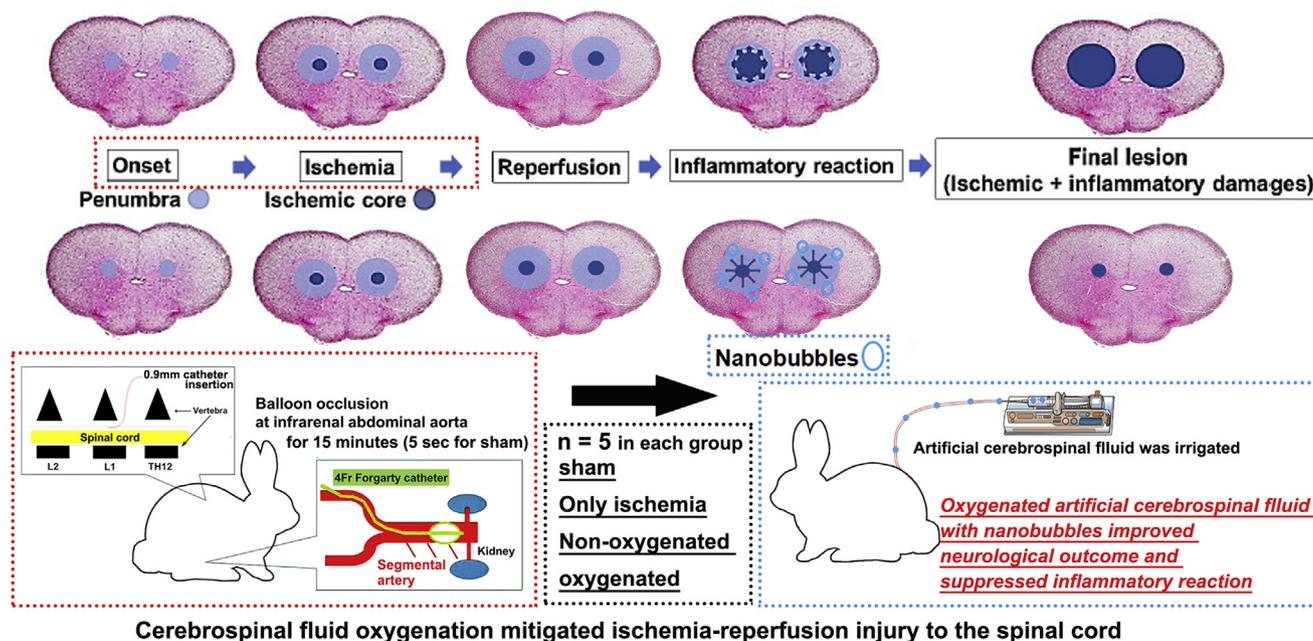
FIGURE 7. Proposed mechanism of treatment of ischemia-reperfusion injury to the spinal cord by cerebrospinal oxygenation. Oxygenated artificial cerebrospinal fluid created with nanobubble technology provides oxygen to the penumbra area and minimizes expansion of the ischemic core caused by the inflammatory reaction.

group but decreased in the oxygenated group. The anterior horn cells were better preserved histopathologically in the oxygenated group compared with the ischemia group. These findings imply that the penumbra could survive with the oxygen provided by the oxygenated artificial CSF, and that subsequent inflammatory responses were significantly suppressed, minimizing expansion of the ischemic core (Figure 7).

Intriguingly, the inflammatory reaction was more likely to be suppressed in the nonoxygenated group than in the ischemia group, although there was no significant intergroup difference. Furthermore, a significantly greater number of anterior horn cells were preserved in the nonoxygenated group compared with the ischemia group. Based on this result, we speculated that the inflammatory response was suppressed and the anterior horn cells were preserved because the spinal cord tissue consumed normobaric oxygen dissolved in normal artificial CSF. Supporting this idea, in the nonoxygenated group, the CSF-PO_2 value was significantly decreased after the irrigation of normal artificial CSF compared with baseline. Based on these results, we believe that the functional outcome improved in an oxygen concentration–dependent manner.

The present study has some limitations. First, the potentially harmful impact related to CSF irrigation was not studied. Furthermore, increased intrathecal pressure compromises spinal cord perfusion. In this experiment, we drained the CSF intermittently when the intrathecal pressure exceeded 30 mm Hg, because we could not use a

double-lumen catheter for the rabbits owing to their miniature size. To translate this nanobubble technology into clinical practice, a double-lumen catheter must be applied for simultaneous CSF irrigation and drainage. The effect of continuous CSF irrigation is a subject for a future study. Second, we did not evaluate all inflammation-related genes because of the limited amount of RNA derived from the spinal cord tissue. We selected IL-6, TNF, and IL-1β among the inflammation-related genes because many studies on stroke previously indicated strong associations of these cytokines with neurologic inflammation. A future study is needed to elucidate inflammatory genes specific to spinal cord ischemic injury.

Nanobubble technology to treat for spinal cord Ischemic Injury

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VIDEO 1. The main findings and implications of the study are summarized by Dr Saiki. Video available at: https://www.jtcvs.org/article/S2666-2736(20)30067-X/fulltext.

CONCLUSIONS

We have demonstrated that CSF oxygenation with nanobubbles after spinal cord ischemic injury can ameliorate ischemic insult and suppress inflammatory responses in the rabbit spinal cord (Video 1). This new therapy may enable a symptom-driven postoperative intervention and serve as a bridging method to supply oxygen to the spinal cord tissue before a collateral network develops. This information should be adopted in further studies aimed at improving spinal cord protection in thoracoabdominal aortic repair.

Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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