Dexmedetomidine Attenuates Blood-Spinal Cord Barrier Disruption Induced by Spinal Cord Ischemia Reperfusion Injury in Rats

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Abstract

\textbf{Background/Aims:} Dexmedetomidine has beneficial effects on ischemia reperfusion (I/R) injury to the spinal cord, but the underlying mechanisms are not fully understood. This study investigated the effects and possible mechanisms of dexmedetomidine on blood-spinal cord barrier (BSCB) disruption induced by spinal cord I/R injury. \textbf{Methods:} Rats were intrathecally pretreated with dexmedetomidine or PBS control 30 minutes before undergoing 14-minute occlusion of aortic arch. Hind-limb motor function was assessed using Tarlov criteria, and motor neurons in the ventral gray matter were counted by histological examination. The permeability of the BSCB was examined using Evans blue (EB) as a vascular tracer. The spinal cord edema was evaluated using the wet-dry method. The expression and localization of matrix metalloproteinase-9 (MMP-9), Angiopoietin-1 (Ang1) and Tie2 were assessed by western blot, real-time polymerase chain reaction, and immunofluorescence. \textbf{Results:} Intrathecal preconditioning with dexmedetomidine minimized the neuromotor dysfunction and histopathological deficits, and attenuated EB extravasation after spinal cord I/R injury. In addition, dexmedetomidine preconditioning suppressed I/R-induced increase in MMP-9. Finally, Dexmedetomidine preconditioning enhanced the Ang1-Tie2 system activity after spinal cord I/R injury. \textbf{Conclusions:} Dexmedetomidine preconditioning stabilized the BSCB integrity against spinal cord I/R injury by inhibition of MMP-9, and enhancing the Ang1-Tie2 system.
Introduction

Spinal cord ischemia reperfusion (I/R) injury is one of the most serious complications of thoracoabdominal aortic aneurysm operation, which even causes paraplegia [1]. When the spinal cord is subjected to an ischemic injury, the local vasculature is disrupted and the blood-spinal cord barrier (BSCB) is compromised. Similar to the blood-brain barrier (BBB), BSCB is the physiological and metabolic substance diffusion barrier between the microvascular and surrounding tissues, which plays a very important role in maintaining the internal environment stability of the central nervous system. As shown in our previous studies, BSCB disruption plays an important role in spinal cord I/R injury [2-6].

Dexmedetomidine, a α2 adrenergic receptor agonist, is widely used in clinical anesthesia and intensive care unit, whose organ protective effect has been a major research topic in the recent years. Dexmedetomidine exerts its protective effect by regulating the immune system, reducing inflammation, and activating anti-apoptosis signal pathway. Recent studies suggest that dexmedetomidine can protect the spinal cord from I/R and improve ischemic tolerance [7, 8]. However, little is known about the effects of dexmedetomidine on BSCB disruption after spinal cord I/R injury.

Matrix metalloproteinase-9 (MMP-9) has been implicated in the disruption of BSCB [2, 3, 6]. Angiopoietin-1 (Ang1) reduces endothelial permeability and enhances vascular stabilization through binding to Tie2 receptor in endothelial cells [9]. Ang1 reduces VEGF-induced BBB permeability, which is associated with a decrease of MMP-9 activity [10].

The primary objective of this study was to investigate the potential protective effects of dexmedetomidine on the BSCB in a rat I/R model. Secondary objectives were to evaluate the role of MMP-9 and Ang1/Tie2 system in I/R-induced BSCB breakdown, with and without pretreatment with dexmedetomidine.

Materials and Methods

Animals

All experimental procedures were conducted with the approval of the Ethics Committee of China Medical University and in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health publication No. 85-23, National Academy Press, Washington DC, revised 1996). Male Sprague-Dawley rats, weighing 200-250g were used in this study. All rats were neurologically intact before anesthesia and housed in standard cages with free access to food and water and separately housed after surgery.

Experimental protocol

One hundred twenty rats were randomly assigned to three groups by means of the random number table. The DEX group (n=40) received 1μg/kg intrathecal dexmedetomidine at L_{5-6} segments of the spinal cord, and underwent 14 minutes occlusion of aortic arch 30 minutes after the intrathecal injection. The sham group (n=40) and I/R group (n=40) received intrathecal equivalent volume of phosphate buffered saline (PBS) as control, and aortic arch exposure or cross-clamping for 14 minutes was performed 30 minutes after the intrathecal injection. Rats were anesthetized with an overdose of pentobarbital and the L_{4-6} segments of spinal cords were rapidly collected for histological study, measurement of BSCB integrity and spinal cord edema, immunofluorescence staining, western blotting and real-time PCR at 48 h after surgery. In each group, eight rats were used to for the measurement of blood gas analysis and glucose; eight rats were used to evaluate neurological function, histological study and immunofluorescence staining; eight rats were injected EB dye intravenously for measurement of blood-spinal cord barrier (BSCB) leakage; eight rats were used for western blotting and real-time PCR and the other eight rats were used for the measurement of spinal cord edema.

Intrathecal Injection

As previously described [11], a stainless needle attached to a microsyringe was inserted between the L5 and L6 vertebrae of conscious rat. A sudden slight flick of the tail indicated the needle entered into the
subarachnoid space. Ten microlitres of dexmedetomidine or PBS was injected over a period of more than 30 seconds. The needle was removed after a 15-second waiting time. The rats were included in the study only if they had a normal hind-limb motor function just before I/R was induced.

Surgical procedure

The spinal cord I/R model was induced by occluding the aortic arch for 14 minutes through a left lateral thoracotomy approach, as previously reported [12]. All rats were anesthetized with intraperitoneal injection of 4% sodium pentobarbital at an initial dose of 50 mg/kg. Lung ventilation was achieved using a small animal ventilator (Harvard Apparatus, Holliston, USA) with endotracheal intubation. Body temperature was continuously monitored with a rectal probe and was maintained at 37.5 ± 0.5 °C with the aid of a heated operating table. Catheters were inserted into the left carotid artery and the tail artery to measure proximal and distal blood pressure, respectively (Spacelabs Medical Inc, Redmond, USA). Rats were placed on the lateral position and a small transverse incision between the 2nd and 3rd rib was made below the left forelimb and shoulder. After entering the thoracic cavity, aortic arch was separated and exposed. Under direct visualization, the aortic arch was cross-clamped between the left common carotid artery and the left subclavian artery. Occlusion was confirmed using a laser Doppler blood flow monitor (Moor Instruments, Devon, UK). Ischemia was confirmed as a 90% decrease in flow measured at the femoral artery. The ischemia lasted for 14 minutes, after which the clamp was removed, followed by 48 h of reperfusion. Sham-operated rats underwent the same procedure, but no occlusion of the aortic arch was performed. Arterial blood gas analysis and blood glucose were measured before ischemia (baseline), 14 minutes after ischemia, and 20 min after reperfusion. All rats were allowed to recover in a plastic box at 28 °C for 4 h, and subsequently placed in their cages with free access to food and water.

Neurological assessment

The motor functions of the rats were assessed at 48 h after the procedure using the following Tarlov scale [13]: 0 = no lower limb function; 1 = perceptible lower limb function with weak antigravity movement only; 2 = some movement of lower limb joints with good antigravity strength, but inability to stand; 3 = ability to stand and walk, but not normally; and 4 = normal motor function.

Histological study

Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin. In cases in which the cytoplasm was diffusely eosinophilic, the large motor neuron cells were considered to be necrotic or dead. When basophilic stippling (containing Nissl substance) was seen, the motor neuron cells were considered to be viable or alive. The intact motor neurons in the ventral gray matter were counted in three sections for each rat by a blinded investigator, and the results were averaged.

Measurement of EB extravasation

In animal studies, BSCB disruption is demonstrated by the extravasation of vascular tracers into the spinal cord parenchyma. After survival time of 48 h, Evans blue (EB) content and fluorescence were used for quantitative and qualitative examination of extravasations induced by BSCB disruption after I/R, as previously reported in our studies [2, 3]. The 2% EB dye (10 mL/kg; Sigma) was slowly intravenously administered. After the EB circulated for 1 h, the rats were anesthetized and perfused through the left ventricle with 500 mL/kg saline, and the spinal cords were removed. First, the spinal cord tissue was weighed and soaked in methanamide for 24 h (60°C), and then centrifuged. The absorption of the supernatant was measured at 632 nm with a microplate reader (BioTek, Winooski, USA). The content of EB was measured as µg/g of spinal cord tissue using a standardized curve. Second, the spinal cord tissue was fixed in 4% paraformaldehyde and sectioned (10 mm), kept frozen and sealed in a dark container before measurement of EB fluorescence. EB staining was visualized using a BX-60 (Olympus, Melville, USA) fluorescence microscope (green zone).

Measurement of spinal cord edema

The water content of the spinal cord was measured by a wet-dry method to provide a quantitative measure of edema. Animals were anesthetized, the L4-6 segment was immediately removed and weighed, and then dried at 110 °C for 24 h and reweighed. Percent water content was calculated as: (wet weight-dry weight)/wet weight × 100.
Immunofluorescence staining

Double immunofluorescence analysis was performed to measure the expression of MMP-9, Ang1 and Tie2 after I/R injury. The spinal cord was fixed and sectioned into 10 μm slices using a Leica CM3050 S cryostat. The sections were blocked with 10% bovine serum albumin for 1 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: mouse anti-MMP-9 (1:100, Abcam), rabbit anti-GFAP (1:800, Abcam), rabbit anti-Iba-1 (1:800, Wako, Germany), rabbit anti-NeuN (1:500, CST), goat anti-Ang1 (1:200, R&D), mouse anti-GFAP (1:800, CST), mouse anti-α-SMA (1:400, Sigma), goat anti-Tie2 (1:100, R&D), and mouse anti-CD31 (1:400, Abcam, Cambridge, UK). After incubation with Alexa 594-conjugated donkey anti-mouse IgG (1:500, Molecular Probes), Alexa 488-conjugated donkey anti-rabbit IgG (1:500, Molecular Probes) and Alexa 488-conjugated donkey anti-goat IgG (1:500, Molecular Probes, Eugene, USA) for 2 h at room temperature, the images were captured using a Leica TCS SP2 (Leica Microsystems, Buffalo Grove, USA) laser scanning microscope.

Western Blotting

The expressions levels of MMP-9, Ang1 and Tie2 in spinal cord tissue were determined by western blotting. Rat spinal cords were homogenized, and total proteins were purified using cell and tissue protein extraction reagents according to the manufacturer’s instructions (KC-415; Kang Chen, Shanghai, China). The antibodies used were as follows: mouse monoclonal anti-MMP-9 (1:500, Abcam), goat polyclonal anti-Ang1 (1:500, R&D), goat polyclonal anti-Tie2 (1:500, R&D) and horseradish peroxidase (HRP)-conjugated secondary antibodies (Bioss, Beijing, China). Semi-quantitation of scanned films was performed using Quantity One software (Bio-Rad, Italy).

Real-time PCR

Total RNA was extracted using TRIzol kit and converted to first-strand cDNA according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green SuperMix-UDG in Prism 7000 Q real-time PCR detection system (Applied Biosystems, Foster City, USA). The primer sequences were as follows. MMP-9: forward 5’-GCA ACG GAG ACG GCA AAC C-3’; reverse 5’-GAC GAA GGG GAA GAC GCA-3’. Ang1: forward 5’- GAA AAT TAT ACT CAG TGG GAA AAA-3’; reverse 5’- TTC TAG GAT TTT ATG CTC TAA TAA ACT-3’. Tie2: forward 5’-ATT GAT GGT ATT AAT ATT CAG GAC ACC-3’; reverse 5’-ATC GCC ATG GCA GGT TTT CCT GGT CCT CCT GTT. β-actin: forward 5’-CCT CTA TGC CAC AGT GC-3’; reverse 5’-CAT CGT ACT CCT GCT TGC TG-3’. Amplification was performed with using the following cycles: 50°C for 2 minutes (UDG incubation), 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for 30 seconds. All reactions were performed in triplicate. Melting curve analysis was performed to ensure the specificity of quantitative PCR. Data analysis was performed using the 2-ΔΔCT method described by Livak [14], with β-actin as the reference gene.

Statistical analysis

All data were expressed as means ± standard error of the mean (means ± SEM) and analyzed by SPSS software (version 17.0, SPSS Inc, Chicago, USA). All variables were normally distributed. Groups were compared with one-way analysis of variance (ANOVA), followed by Newman-Keuls post-hoc analysis. A P value of <0.05 was considered to be statistically significant.

Results

Physiologic Parameters

Table 1 indicates the physiologic parameters from all the groups. The distal mean blood pressure was decreased significantly during the aortic occlusion in the rats of I/R and DEX groups (P < 0.01). There was no significant hemodynamic difference between I/R group and DEX group during the ischemia and reperfusion. PH and BEecf values were decreased induced by ischemia and reperfusion in the rats of I/R and DEX groups (P < 0.05). Meanwhile, glucose was increased compared with baseline in I/R and DEX groups (P < 0.01). Also, there was no difference in PH, BEecf and glucose between I/R group and DEX group during the surgery.
Dexmedetomidine preconditioning improved neurological assessment scores after I/R injury

All rats showed normal hind limb motor function after intrathecal injection before the induction of ischemia. The individual neurological scores of the three groups after reperfusion are shown in Fig. 1A. The sham animals showed normal motor function of lower limbs throughout the observation period. Aortic occlusion for 14 minutes resulted in severe lower extremity neurological deficits in the I/R rats. Prophylactic dexmedetomidine enhanced the recovery of motor function (P < 0.01).

Dexmedetomidine preconditioning improved histological assessment scores after I/R injury

Histopathological changes after I/R injury are shown in Figs 1B-C. As compared to the sham controls, I/R resulted in significant loss of motor neurons (P < 0.01), whereas more intact motor neurons were observed in the operated rats with dexmedetomidine preconditioning (P < 0.01).

Dexmedetomidine preconditioning decreased BSCB leakage and spinal cord edema after I/R injury

The permeability of the BSCB was measured by extravasation, which was visualized as red fluorescence under the fluorescent microscope. As shown in Fig. 2A, I/R induced a significant increase in EB dye extravasation as compared to the sham group (P < 0.01); while dexmedetomidine preconditioning rescued the levels of extravasation following I/R injury (P < 0.01), indicating that dexmedetomidine preserved BSCB integrity. Meanwhile, EB content in spinal cord was less in the DEX group (8.31±0.83 μg/g) than in the I/R group (18.92±1.45 μg/g) (P < 0.01; Fig. 2B). In addition, I/R caused spinal cord edema, closely related to BSCB breakdown. In contrast, dexmedetomidine preconditioning decreased the spinal cord edema (P < 0.01; Fig. 2C).

Dexmedetomidine preconditioning inhibited up-regulation of MMP-9 after I/R injury

Western blot analysis indicated that MMP-9 level increased after injury, and dexmedetomidine preconditioning depressed the upregulation of MMP-9 (P < 0.01; Fig. 3A). Similarly, real-time PCR showed that dexmedetomidine preconditioning decreased messenger RNA (mRNA) expression of MMP-9, which was increased by I/R injury (P < 0.01;
Fig. 1. Effects of dexmedetomidine on neurological motor function and histological assessment of the spinal cord after spinal cord ischemia reperfusion (I/R) injury. A) Tarlov score at 48 h after I/R injury. Each symbol represents data for one rat (bar = median). B) Number of intact motor neurons in the ventral gray matter at 48 h after I/R injury. C) Representative sections of lumbar spinal cords stained with hematoxylin-eosin. Arrowheads indicate the normal intact neurons and arrows indicate the necrotic or dead neurons. Upper panel magnification 100×, scale bar = 200 μm; lower panel magnification 200×, scale bar = 100 μm. All data are represented as means ± SEM (n = 8 per group). *P < 0.01 versus sham; **P < 0.01 versus I/R.

Fig. 2. Effects of dexmedetomidine on blood-spinal cord barrier (BSCB) leakage and spinal cord edema after spinal cord I/R injury. A) Evans Blue (EB) extravasation fluorescence at 48-h after I/R injury. In the sham group, almost no red fluorescence was seen in the spinal cord parenchyma. After I/R injury, significant red fluorescence could be seen, especially in the gray matter. In contrast, red fluorescence was significantly weakened in the DEX group. B) Quantification data of EB content of spinal cord (μg/g). C) Quantification data of water content of spinal cord. All data are represented as means ± SEM (n = 8 per group). *P < 0.01 versus sham; **P < 0.01 versus I/R.
Further, immunofluorescence double labeling suggested that MMP-9 was mostly expressed in NeuN positive neurons. Iba-1 positive microglia and GFAP positive astrocyte also expressed a certain amount of MMP-9. Dexmedetomidine preconditioning inhibited MMP-9 expression in all these cells, which was increased by spinal cord I/R injury (Fig. 3C).

**Dexmedetomidine preconditioning enhanced the Ang1-Tie2 system after I/R injury**

As protective factors for angiogenesis and vascular stabilization, Ang1 and Tie2 increased slightly, but not significantly after spinal cord I/R injury. However, their protein and mRNA expression levels were significantly increased by dexmedetomidine preconditioning (P < 0.01; Figs 4A-B, Figs 5A-B). Double immunofluorescence labeling showed that Ang1 in GFAP positive astrocytes and α-SMA positive pericytes, and Tie2 in CD31 positive vascular endothelial cells were activated by dexmedetomidine (Fig. 4C, Fig. 5C).

**Discussion**

This is the first investigation to demonstrate the effectiveness of dexmedetomidine in reducing inflammation of the BSCB after I/R injury. Findings from this study show that dexmedetomidine preconditioning reduces BSCB permeability, preserves the number of intact motor neurons and extremity motor function after I/R injury to the spinal cord, which is mediated by the inhibition of MMP-9 and enhancement of the Ang1/Tie2 system.

Neuroprotective effects of dexmedetomidine have been reported in a cerebral ischemia model, and are related to excitation of alpha 2 receptors [15]. Dexmedetomidine can
promote focal adhesion kinase (FAK) phosphorylation, decrease pro-apoptotic factor caspase-3 expression, and enhance nerve cell survival through the excitement of alpha 2 receptors [16]. Moreover, mounting evidence indicates that dexmedetomidine might reduce inflammation [17–19]. Inflammatory factors play a critical role in disrupting the BSCB, while the barrier damage aggravates the nervous deficits [2–5]. MMP-9 is a potent regulator of acute inflammation [20]. Overexpressing MMP-9 destroys barrier integrity by digesting basement membranes of endothelial cells and tight junction proteins [21]. MMP-9 was shown to be upregulated and associated with BBB or BSCB dysfunction after stroke [22] and spinal cord I/R injury [2, 3]. Our recent study demonstrated that upregulated MMP-9 secreted by activated microglia was involved in invasion and migration of microglial cells, production of proinflammatory chemokine and neuronal apoptosis after spinal cord I/R injury [6]. In the present study, we found that MMP-9 upregulation was not confined to microglia; neurons and astrocytes also expressed amounts of MMP-9. In fact, most of the MMP-9 was expressed in NeuN positive neurons. Fortunately, dexmedetomidine preconditioning inhibited the expression of MMP-9 in all these cells.

Angiopoietins (Angs) are important regulators of vascular remodeling and maturation, which act on Tie2 receptors [23]. Ang1 was the first discovered vascular anti-permeability factor, which is involved in maintaining the integrity of blood vessels and preventing

![Fig. 4. Dexmedetomidine up-regulated Angiopoietin-1 (Ang1) after spinal cord I/R injury. A) Representative western blot and quantitative protein analysis of Ang1 in the spinal cord at 48 h after I/R injury. B) Real-time PCR of Ang1 mRNA expression. C) Immunofluorescence photomicrographs of Ang1 co-localized with astrocytes (GFAP) and pericytes (α-SMA), respectively at 48 h after I/R injury. All data are represented as means ± SEM (n = 8 per group). *P < 0.01 versus sham; **P < 0.01 versus I/R.](image-url)
microvascular leakage [24]. Ang1 reduces vascular leakage by strengthening PECAM-1 and VE-cadherin regulated interendothelial adhesion [25]. It is also implicated in the pathogenesis of spinal cord injury [26, 27]. Ang1 can combat VEGF-induced BSCB breakdown. Sustained delivery of VEGF165 and Ang1 using viral vectors has potential angiogenesis and vascular stabilization effects against spinal cord injury [26]. Alex Zacharek et al reported that transplantation of bone marrow stromal cells reduced the BBB permeability by increasing the expression of Ang1/Tie2 [9]. Similarly, the current study found a slight, but unremarkable increase in the expression of Ang1 and Tie2 after spinal cord I/R injury. In contrast, Ang1 and Tie2 expression were markedly increased and BSCB damage was reduced by dexmedetomidine preconditioning. This suggests that dexmedetomidine may alleviate disruption of the BSCB by enhancing the Ang1/Tie2 system, and plays a role in protecting the spinal cord.

The limitation in the present study arises from the time window to observe BSCB changes. Based on our previous study [4], aggravated BSCB disruption peaked at 48 h after spinal cord I/R injury. Hence, we focused on BSCB integrity at 48 h after reperfusion. A longer observation time would be important in future studies assessing the protective effects of dexmedetomidine on BSCB after I/R injury. Though dexmedetomidine has not been used in clinical at present, the protective effects of dexmedetomidine against spinal cord I/R injury have been clarified in either in vivo or in vitro animal models [8, 28]. Furthermore, dexmedetomidine have widely used in clinical medicine as an anesthesia adjuvant drug. It is thus clear that dexmedetomidine preconditioning will be a promising strategy to protect spinal cord.

In conclusion, the current study provides the first direct evidence that dexmedetomidine preconditioning stabilized the BSCB integrity against spinal cord I/R injury. This beneficial effect is mediated by inhibition of MMP-9 and enhancement of the Ang1-Tie2 system.
Disclosure Statement

The authors declare no conflict of interest.

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