Dexmedetomidine post-conditioning attenuates cerebral ischemia following asphyxia cardiac arrest through down-regulation of apoptosis and neuroinflammation in rats

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Abstract

**Background and Purpose** Neuroprotection strategies after cardiac arrest (CA)/cardiopulmonary resuscitation (CPR) remain key areas of basic and clinical research. This study was designed to investigate the neuroprotective effects of dexmedetomidine following resuscitation and potential mechanisms.

**Methods** Anesthetized rats underwent 6-minute asphyxia-based cardiac arrest and resuscitation, after which the experimental group received a single intravenous dose of dexmedetomidine (25μg/kg). Neurological outcomes and ataxia were assessed after the return of spontaneous circulation. The serum levels and brain expression of inflammation markers was examined, and apoptotic cells were quantified by TUNEL.
**Results:** Neuroprotection was enhanced by dexmedetomidine post-conditioning after the return of spontaneous circulation. This enhancement was characterized by the promotion of survival, neurological function scores and coordination. In addition, dexmedetomidine post-conditioning attenuated the serum levels of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α at 2h, as well as interleukin (IL)-6 at 2, 24, and 48h. TUNEL staining showed that the number of apoptotic cells in the dexmedetomidine post-conditioning group was significantly reduced compared with in the control group. Further western blot analysis indicated that dexmedetomidine markedly reduced the levels of caspase-3 and nuclear factor-kappa B (NF-κB) in the brain.

**Conclusions:** Dexmedetomidine post-conditioning had a neuroprotective effect against cerebral injury following asphyxia-induced cardiac arrest and improved the survival rate. The mechanism was associated with the down-regulation of apoptosis and neuroinflammation.

**Keywords:** Dexmedetomidine, post-conditioning, cerebral ischemia, asphyxia cardiac arrest, apoptosis, neuroinflammation

**Background**

Cardiac arrests (CA) is a leading cause of death worldwide\(^1\),\(^2\). Although recent developments in cardiopulmonary resuscitation (CPR) techniques and post-resuscitation care have improved the chances of survival, there are still high rates of death and disability following the restoration of spontaneous circulation (ROSC) \(^3\).
Survivors of CA suffer from painful sequelae, including anoxic brain injury, myocardial dysfunction and the systemic ischemia/reperfusion response, which are described as post-CA syndrome, whereby cerebral injury is the main reason of death and disability after ROSC. The brain consumes the largest amount of oxygen of all organs, and is highly susceptible to disruptions of blood flow. Sudden cardiac arrest induces complete cerebral ischemia, followed by a cascade of detrimental events that can lead to immediate and delayed brain damage, including excitotoxicity, oxidative stress and inflammation. What’s more, cardiopulmonary resuscitation can lead to reperfusion injury, which may exacerbate brain damage. Neurons in affected areas of brain undergo delayed cell death, which disrupts the shaping of neural circuits and ultimately leads to both motor and cognitive dysfunction. Because of the high incidence of CA and the complex etiology of cerebral ischemia-reperfusion injury, it is urgent to find a therapeutic strategy to attenuate post-CA brain injury.

Dexmedetomidine (Dex) is a specific agonist of α2-adrenergic receptors that has been used as a sedative in intensive care since 1999, and also as an adjuvant to reduce the dosage of other anesthetics. Recently, a growing body of research found neuroprotective effects of Dex in different experimental models of cerebral injury. While the uncontrolled, systemic inflammatory response is a critical cause of brain injury following ischemia/reperfusion (I/R), it was found that Dex can reduce the expression of inflammatory factors after brain I/R injury, which may be related with the inhibition of the toll-like receptor-4/NF-κB (TLR-4/NF-κB) pathway. On the other hand, overproduction of free radicals and destruction of natural antioxidant
function is another cause of I/R-induced brain injury. Additionally, studies have shown that Dex can attenuate oxidative stress\textsuperscript{14-16}. What’s more, the antiapoptotic effect of Dex is likely caused by an improvement of mitochondrial function and the inhibition of neuronal autophagy\textsuperscript{17}. However, whether these pharmacological effects of Dex could alleviate post-CA brain damage was not known, and the underlying mechanism have not been fully understood.

The present study used a rat model of CA/CPR to investigate the protective effects of Dex against brain injury and investigate the potential mechanisms.

**Methods**

**Animals:** All the experiments were approved by the Animal Care and Use Committee of the Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital and the animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). A total of 30 male Sprague-Dawley rats, weighing 350-450g, were obtained from the Chengdu Dashuo Experimental Animal Centre of Sichuan, China. The animals were housed at a constant temperature (23±1°C) on a 12h light/dark cycle with free access to food and water, two rats were placed in per cage. These housing environments were maintained until the animals were sacrificed under deeply anesthesia with isoflurane for brain tissue harvest.

**Asphyxial cardiac arrest model:** The rat asphyxial CA model was established as reported previously\textsuperscript{18}, with minor modifications as follows. Each rat was anesthetized
using an intraperitoneal injection of pentobarbital sodium solution (45mg/kg) and mechanically ventilated (respiratory frequency 60 bpm, tidal volume 8 ml/kg) using a Harvard Ventilator (Model 683, Harvard Apparatus, Holliston, MA, USA). A rectal probe was inserted to monitor the body temperature of the rats, which was maintained at 36°C ± 1°C using a heating pad. The right femoral artery and vein were exposed. A venous indwelling catheter (24G) filled with heparin saline was placed in the femoral artery and connected to a pressure transducer (Powerlab 16/30, AD-Instruments, Australia) to monitor the arterial blood pressure. Another 24G venous indwelling catheter was placed into the femoral vein for fluid infusion. The rats were monitored for at least 10 minutes to record the baseline. After muscle relaxation with cisatracurium besilate (0.2mg/kg), asphyxial CA was induced by clamping the tube in the trachea and stopping the ventilator. CA was defined as a systolic blood pressure (SBP) < 25 mmHg. Six minutes after CA, CPR and mechanical ventilation were initiated. External anterior to posterior chest compressions at a depth of 1/3 diameter of the rat thorax were carried out at a frequency of 200/min. During resuscitation, epinephrine (0.01mg/kg), 5% sodium bicarbonate (0.36ml/kg) and 0.9% saline (0.5ml) were injected into femoral vein through the indwelling catheter. Restoration of spontaneous circulation (ROSC) was defined as the return of spontaneous sinus rhythm, with SBP > 60 mmHg, which was maintained for at least 10 minutes. Spontaneous respiration was carefully monitored every 5 minutes. The rats were weaned from the ventilator after spontaneous respiration totally recovered. Finally, the venous indwelling catheters were withdrawn from the right femoral artery and vein.
**Experimental protocol:** All rats were randomly assigned to three groups: 1) sham operation group (sham, n=6); 2) CA/CPR without any treatment (control, n=12); 3) CA/CPR plus post-treatment with dexmedetomidine (Dex, n=12). The tail of each rat was marked by different color markers according group design, and the cage was labeled the group name. The sham rats went through all the operational procedures except for cardiac arrest and CPR. After restoration of spontaneous circulation, rats in the Dex and control groups received a single intravenous injection of Dex (25mg/kg, Hengrui Medicine, Jiangsu, China) or the same volume of saline, respectively. Dex or saline was pumped into the vein using a micro-infusion pump for about 30min.

**Evaluation of neurological deficits:** Neurological examination was performed by an investigator who was blinded to the experimental design using the neurological deficit scores (NDS), which ranges from 80 (best) to 0 (brain dead) and includes a subscore of general behavioral deficit: consciousness as normal, stuporous or unresponsive and arousal with eye opening and respiration as normal, abnormal (hypo or hyperventilation) or absent. The NDS of the surviving rats was assessed at 24, 48 and 72h after CA/CPR. The brainstem function sub-scores were assessed as follows: (1) olfaction, as response to the smell of food; (2) vision, as head movement toward light; presence of (3) pupillary light reflex; (4) corneal reflex; (5) startle reflex; (6) response to whisker stimulation and (7) swallowing of liquids or solids. The sub-score for motor assessment included strength testing as normal, abnormal (either stiff or weak) and absence of movement. The sensory assessment sub-score included response to limb pinch as brisk withdrawal, weak or abnormal response (extension or flexion
posture) and no response. The motor behavior sub-score was assessed based on gait coordination as normal, abnormal or none. Balance on a beam was judged as normal if the rat could cross a 2 cm wide and 1m long beam suspended 0.5 m above the floor; abnormal if the rat attempts and does not continue or stays momentarily and falls. The assessment was scored as absent if the rat falls off immediately upon placing on the beam. Other evaluated behavioral reflex sub-scores include: (1) righting reflex (animal placed on its back and is able to correct to upright position); (2) turning alley (the animal is made to walk and turn back at the end of a 15 cm by 0.5 m alley); (3) visual placing (the animal is lifted and is able to visually orient itself to objects and depth); and (4) negative geotaxis (animal is placed on its back on a plane angled at 45° and the animal corrects itself and moves upward on the incline). The last subscore assesses the occurrence of seizures (convulsive or non-convulsive).

**Rotarod test:** The rotarod test is designed to evaluate the motor coordination and balance ability of rats. It includes adaptation training and a test process. Before CA surgery, the rats in each group were trained continuously for 3 days. The rotating bar fatigue meter was set to 4rpm. The animals were trained 3 times a day for at least 15min each time, and the interval between the two training sessions was at least 15min. The final testing was performed 5 days after ROSC. All surviving rats were individually placed on the rotating rod, the rotation speed was increased from 4rpm to 40rpm within 260s and the time from the beginning to the fall of the rat recorded. The test was repeated three times, and the average amount of time until falling was taken as the final result.
Serum levels of inflammatory factors: Retro-orbital blood samples (0.8-1.2 mL) were collected at 2, 24, and 48h after ROSC, the serum was separated by centrifugation at 12000 g for 10 min and immediately analyzed or stored at -80°C. The levels of IL-6 and TNF-a were analyzed using commercial ELISA kits (R&D systems) according to the manufacturer’s instructions. All measurements were carried out in duplicate.

Western blot analysis: Brain tissue was collected at 5 days after ROSC, and total protein lysates were prepared using lysis buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors cocktail (Sigma-Aldrich) and phosSTOP phosphatase Inhibitor Cocktail (Roche, Nutley, NJ, USA). The BCA assay kit (Thermo Fisher Scientific, USA) was used to measure the protein concentration. Samples comprising 20 μg total protein per lane was separated by SDS-PAGE and then transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk for about 1 h at room temperature and incubated with the following primary antibodies overnight at 4°C: rabbit polyclonal anti-caspase-3 antibody (1:1000; Cell Signaling Technology, USA); rabbit polyclonal anti-NF-κB antibody (1:1000 Protein-tech, China); α-tubulin (1:5000; Protein-tech, China,). After incubation with secondary antibodies, the immunoreactive bands were developed using enhanced chemiluminescence reagents (Pierce, IL, USA) and was visualized using GeneSnap software version 7.08. The protein amounts were quantified densitometrically using Image J software and normalized to the density of α-tubulin in the same sample. The results of rats from the different experimental groups were then normalized to the
mean values of the corresponding control animals.

**TUNEL staining:** The TUNEL assay was performed using the Apoptosis & Cell Death Assay kit (Merck Millipore, USA) according to the manufacturer’s instructions. Briefly, brain sections were incubated with proteinase K at room temperature for 30 min, and then incubated with the TUNEL reagent at 37°C for 1 h. The sections were then washed with PBS and counter-stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using a fluorescence microscope at 40× magnification. The results were quantified as apoptotic index (AI%), which was defined as the ratio of positive apoptotic cells to all cells in the same field of view.

**Statistical analysis** SPSS 20.0 software (IBM Corp., USA) was used for statistical analysis. The survival curves were determined using the Kaplan-Meier method and compared with the log-rank test. Repeated test results are presented as means ± SD and differences with P-values < 0.05 were considered statistically significant. Charts were rendered using GraphPad Prism 6.0 software.

**Results**

**Dex improves survival and hemodynamics after CA/CPR**

Application of Dex significantly improved the survival at 5 days after CA/CPR. The survival rates of the rats in the Control and Dex groups were 50 and 66.7%, respectively. All five rats in the Sham group survived. The log-rank test showed that the differences were statistically significant (p < 0.05) (Fig. 1A). Compared with the basal level, the mean arterial pressure (MAPs) of the control and Dex groups decreased significantly after resuscitation, and notable decreases were observed at
15-25 min after ROSC ($P < 0.05$) (Fig. 1B). After resuscitation, the heart rate (HR) decreased in both groups, with no marked difference between the two groups ($P > 0.05$), and both returned to the baseline 1 h after ROSC (Fig. 1C).

**Dex attenuated the impairment of nerve and motor function following CA/CPR**

The NDS was evaluated at 24, 48 and 72 h after CA/CPR. In the sham group, the NDS score was approximately 80 at all the time points. After CA, the NDS of the control group was obviously decreased compared with the sham group. Treatment with Dex markedly attenuated the neurological deficit score (Fig. 2A).

In addition to neurological function disorders, CA/CPR can also damage motor function. In this study, the rotarod test was used to assess the ataxia of rats at 5 days after CA. After CA/CPR, the surviving rats in the Control group showed significantly poorer scores in all indicators, including the average rotarod speed (Fig. 2B), the total time on the rotarod (Fig. 2C), rotation speed at fall (Fig. 2D) and the total walking distance (Fig. 2D). As can be seen in the corresponding figures, the application of Dex effectively attenuated the neurological impairment.

**Dex reduced the expression of pro-inflammatory factors following CA/CPR**

To evaluate the anti-inflammatory effect of Dex following CA/CPR operation, the serum levels of IL-1β and TNF-α, as well as the expression of NF-κB in brain tissues were evaluated at 2, 24 and 48 hours after CA/CPR. Compared with the sham group, the serum levels of IL-1β and TNF-α (Figs. 3A and B) and the brain tissue expression of NF-κB (Fig. 3C) were significantly increased following CA/CPR. However, application of Dex after resuscitation decreased the production of IL-1β and
TNF-α, while also blocking the increase of NF-κB in the brain (Fig. 3).

Dex inhibited the expression of proteins related to neuronal apoptosis following CA/CPR.

To understand whether the protective effect of Dex is related to neuronal apoptosis, TUNEL staining and western blot analyses were performed to assess the percentage of apoptotic neurons and expression of the proapoptotic factor caspase-3. According to TUNEL staining, there were few positive cells in the sham group, but their percentage increased after CA/CPR (Fig. 4A). Furthermore, Dex significantly decreased the number of TUNEL-positive neurons (Fig. 4A). The proportion of TUNEL-positive neurons was done by cell counting in a single field of view, which showed that the apoptosis index of CA/CPR rats was significantly increased, while Dex effectively blocked this increase (Fig. 4B). In addition, treatment with Dex significantly reduced the expression of caspase-3 (Figs. 4C and D).

Discussion

In this study, we explored the potential effects of Dex on the survival and neurological function of rats after CA/CPR. The main findings of this study include: 1) treatment with Dex after resuscitation improved the survival rate of rats after CA/CPR; 2) Dex ameliorated the CA/CPR-induced neurological deficits; and 3) Dex may exert its protective effect by reducing inflammation and inhibiting apoptosis.

CA/CPR induces systemic ischemia-reperfusion (I/R) injury, which activates the immune system and causes a systemic inflammatory response. During CA/CPR, leukocytes, macrophages and tissue-resident immune cells recognize the injury and
release primary cytokines, which in turn induce the recruitment and activation of leukocytes, largely amplifying the inflammatory response\textsuperscript{19, 20}. The brain uses 20 percent of the body’s oxygen and calories\textsuperscript{21}, and can therefore suffer severe damage due to CA. As the crucial resident immune cells of the central nervous system (CNS), microglia express various cytokine receptors, recognizing IL-1 and TNF-\(\alpha\), among many others. Consequently, microglia will be over-activated after I/R injury and release excess pro-inflammatory cytokines, impairing neural function\textsuperscript{22, 23}. A growing body of evidence suggests that inflammation is crucial for the pathogenesis of neurological deficits after CA/CPR\textsuperscript{24-27}. In this study, we found that the serum levels of TNF-\(\alpha\) and IL-6 were significantly increased after ROSC in the CPR group. The levels of pro-inflammatory factors peaked at 2h and returned to baseline levels within 48h after ROSC. Treatment with Dex attenuated the increase of TNF-\(\alpha\) and IL-6, improving the neurological outcomes. CA/CPR can cause sympathetic nerve over-excitation, which may exacerbate further inflammation and cause significant neurotoxicity\textsuperscript{28, 29}. Dex is a highly specific agonist of the \(\alpha_2\)-adrenergic receptor, and is commonly used as an adjuvant anesthetic. Furthermore, several studies demonstrated the anti-inflammatory effect of Dex in different models. For instance, Dex was found to significantly improve cognitive function after carotid endarterectomy by inhibiting CNS inflammation\textsuperscript{30}. Moreover, Zheng and colleagues showed that Dex inhibited CNS neuroinflammation after traumatic brain injury (TBI), and reduced the expression of the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome\textsuperscript{31}. 
Additionally, previous research suggests that post-treatment with Dex could attenuate early brain injury (EBI) induced by subarachnoid hemorrhage (SAH), and that it exerts its protective effect by inhibiting the activation of the TLR4/NF-κB pathway, the release of pro-inflammatory cytokines and the expression of the NLRP3 inflammasome²³. NF-κB is a transcription factor that regulates many genes, especially inflammation-related cytokines. Dex was shown to reduce the expression of Toll-like receptor 4 and suppress the activation of NF-κB by interacting with the α-2 receptor³³. The results of this study are in agreement with this theory, and we found that treatment with Dex can effectively suppress the phosphorylation of NF-κB following CA/CPR.

Recently, there has been increasing evidence that neuronal apoptosis is also a key reason for CNS dysfunction after I/R⁵,³⁴. Apoptotic programmed cell death is mainly induced by specific proteins such as Apaf-1, as well as proteins in the Bcl-2 and caspase families³⁵. In mammalian cells, apoptosis is triggered by two main pathways, called the intrinsic pathway and the extrinsic pathway, which both converge in the activation of caspase-³⁶,³⁷. Neuronal apoptosis is initiated by the cleavage of caspase-3 and results in DNA breakdown, degradation of cytoskeletal components, and the production of apoptotic particles, which are finally engulfed by phagocytic cells³⁸. CA/CPR compromises the integrity of the blood-brain barrier and activates microglial cells, resulting in the release of inflammatory mediators and reactive oxygen species. These toxic chemicals inhibit the production of neurotrophic factors and disturb the effective communication between brain cells³⁹. In this study, TUNNEL
staining showed a significant increase in the number of apoptotic neurons following CA/CPR, which could be effectively alleviated by Dex. The level of cleaved caspase-3 is universally recognized as a specific marker of apoptosis\textsuperscript{17}. Our findings indicated that Dex decreased the concentration of cleaved caspase-3 in brain tissues, which was in accordance with the results of TUNEL-staining. Previous research demonstrated that Dex exerts its antiapoptotic effect by reducing the levels of pro-inflammatory factors and ROS\textsuperscript{40, 41}. In addition, Dex improves the survival of neurons by activating the brain-derived neurotrophic factor/ tropomyosin-related kinase B(BDNF/TrkB )pathway\textsuperscript{42, 43}.

The current study also has some inadequacies and limitations. For example, we were not able to use a concentration gradient to confirm the best therapeutic dose due to limitations in the number of experimental animals that can be handled. What’s more, exploration of the possibility of combination therapy, such as dexmedetomidine combined with hypothermia therapy, is still awaiting further research.

**Conclusions**

Our findings indicate that post-resuscitation treatment with dexmedetomidine has a significant neuroprotective effect and attenuates neurological disorders following CA/CPR. The potential mechanism through which dexmedetomidine exerts its protective effects is likely related to the suppression of neuroinflammation and promotion of neuron survival by inhibiting apoptosis.

**Abbreviations**

AI: apoptotic index; BDNF: brain-derived neurotrophic factor; CA: cardiac arrest;
CNS: central nervous system; CPR: cardiopulmonary resuscitation;

DAPI: 4',6-diamidino-2-phenylindole; Dex: dexmedetomidine; EBI: early brain injury; HR: heart rate; IL: interleukin; I/R: ischemia reperfusion; MAPs: mean arterial pressure; NDS: neurological deficit scores; NF-κB: nuclear factor-kappa B; NIH: National Institutes of Health; NOD: nucleotide-binding oligomerization domain;

NRLP3: (NOD)-like receptor family pyrin domain containing 3; ROSC: restoration of spontaneous circulation; SBP: systolic blood pressure; SAH: subarachnoid hemorrhage; TBI: traumatic brain injury; TNF-α: tumor necrosis factor alpha; TrkB: tropomyosin-related kinase B; TLR-4: toll-like receptor 4.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Animal Care and Use Committee of the Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. And the animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Consent to publish**

Not Applicable.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article and supporting data can be obtained from the corresponding author upon reasonable request.
Competing interests

The authors declare they have no competing interests.

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Authors’ contributions

LQ and DF contributed to the concept and design of the study. GQL and LQ and PG collected and analyzed the data. GQL and LQ wrote the original draft. DF critically reviewed and revised the manuscript. All authors have read and approved the final manuscript.

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**Appendices**

**Figure 1** Dex improved the survival and hemodynamics of rats following cardiac arrest and cardiopulmonary resuscitation (CA/CPR). (A) Survival of the Sham, Control and Dex groups. (B and C) Changes in the mean arterial pressures (MAP) and heart rate (HR) in the Control and Dex groups after CA/CPR. Data are expressed as
the means ± SD.(n=6-12). *P < 0.05

**Figure 2** Dex attenuated the impairment of neurological and motor function after CA/CPR. (A) The neurological deficit score evaluated at 24, 48 and 72 hours after ROSC. (B–D) Rotarod performance tests were conducted at 5 days after ROSC. The results are shown as the average speed of the rotarod (B), the total time of walking on the rotarod (C), the rotation speed at falling (D) and the total distance of rat the walking on the rotarod (E). The data are presented as the means ± SD.(n=6).

***p<0.001; **p<0.01; *p<0.05

**Figure 3** Dex reduced the expression of pro-inflammatory cytokines after CA/CPR. The serum levels of IL-1β(A) and TNF-α (B) at 2, 24 and 48 hours after resuscitation. (C–D) The protein levels of NF-κB in brain tissues. The data are presented as the means ± SD.(n=6) *p<0.05

**Figure 4** Dex inhibits the apoptosis of neurons after CA/CPR. CA/CPR-induced apoptosis, as assessed by TUNEL staining. (A) The quantitative analysis results of TUNEL staining. (B) Treatment with Dex decreased the levels of cleaved caspase-3. (D) Western blot analysis results, normalized to α-tubulin. The data are expressed as the means ± SD. (n=4-6).*P< 0.05