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Original article

Dexmedetomidine preconditioning plays a neuroprotective role and suppresses TLR4/NF-κB pathways model of cerebral ischemia reperfusion

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ABSTRACT

Background: Dexmedetomidine has been reported to play an efficient role on multi-organ protection. Our study aims to investigate the neuroprotective of dexmedetomidine preconditioning on cerebral ischemic reperfusion (I/R) injury and investigate the underlining signaling mechanisms.
Methods: Cerebral I/R models were established with SD rats through middle cerebral artery occlusion (MCAO). After 2 h of ischemia followed by 7 days of reperfusion, the degree of cerebral tissue injury was detected by HE, Nissl and TUNEL staining. Glial fibrillary acidic protein (GFAP) positive and TNF-α positive cells were stained by immunohistochemistry and counted under microscope. TLR4, NF-κB and TIR-domain containing adapter-inducing interferon-β (TRIF) expression were detected by real time PCR and western blot.
Results: Dexmedetomidine preconditioning markedly prevented the ischemia-induced cellular damage observed from HE and Nissl staining in hippocampus and cortex. Dexmedetomidine observably decreased the number of apoptotic cells in TUNEL staining. Besides, yohimbine could specifically suppress the protective effect of dexmedetomidine. GFAP expression was distinctly inhibited by dexmedetomidine preconditioning (10 μg/kg, 20 μg/kg) in cerebral ischemia area. Dexmedetomidine preconditioning inhibited the expression of TLR4 and NF-κB and increased that of TRIF.
Conclusion: The results of this study suggest that dexmedetomidine preconditioning plays a neuroprotective role against I/R injury. Dexmedetomidine might suppress TLR4/NF-??B pathway and drive TLR4/TRIF signaling pathway to reduce the inflammatory injury.

1. Introduction

Because of the absence of effective neuroprotective therapeutic method, the cerebral I/R injury is a major medical problem desperately required to be further explored. Cerebral ischemic diseases have become one of major intraoperative and postoperative complications [1]. After cerebral ischemia, recanalization is the most effective approach for treatment of acute cerebral infarct, but ischemia/reperfusion (I/R) injury is frequently caused by recovery of blood [2]. Cerebrum is sensitive to ischemia/hypoxia and cerebral I/R injury usually causes irreversible cerebral injury. Until now, there are various clarified cerebral I/R injury mechanism, including energy exhaustion, intracellular calcium overload, inflammatory response, free radical damage, etc. [3,4].

Toll-like receptor 4 (TLR4), a member of the TLR family, plays a pivotal role in both innate and adaptive immune responses. In the inflammatory response, TLR4 pathway is motivated by precipitating factor and leads to activation of nuclear factor kappa B (NF-κB) [5]. Then, follow-up immune and inflammatory genes/factors, such as IL-1/6 and TNF-α, are induced in cascade reaction [6,7]. TLR4/NF-κB signaling pathway activated by I/R injury mediates the expression of various inflammatory factors, which aggravates the inflammatory response of retinal damage. TLR4 is the main receptor of endotoxin/LPS response [8], and studies have proved the vital effect of CD14 and LPS binding protein in LPS effective mechanism [9].

Dexmedetomidine, a new type of high selectivity α_2 adrenoreceptor agonist, is an effective sedative agent applied on short-term calm for mechanical ventilation patients and also

Abbreviation: I/R, ischemia reperfusion; TLR4, toll-like receptor 4; NF-κB, nuclear factor kappa B; TRIF, TIR-domain containing adapter-inducing interferon-β; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion.

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provides effective analgesia [10,11]. Studies have shown that α_2 receptor agonists has an effectively neuroprotection role intra-operative and postoperative of neurosurgery, which arouse the interests of researchers, but the mechanism is unknown. Except for hypnotic sedation and analgesia, further researches also find that dexmedetomidine has anti-inflammatory effect. Dexmedetomidine preconditioning before intestines ischemia could inhibit the intestinal mucosa epithelial apoptosis and inflammatory response, and promote intestinal trauma repair process [12]. Both in vivo and in vitro of animal experiments have showed that dexmedetomidine has a protective effect for the heart, kidney, brain I/R injury [13].

Our study aims to explore the neuroprotection of dexmedetomidine preconditioning in cerebral I/R injury and investigate the potential mechanism of TLR4 pathway.

2. Materials and methods

2.1. Animals and cerebral ischemia reperfusion model

Total of 80 male Sprague-Dawley rats (260∼300 g) were randomized into 4 groups (n=16). Groups include sham-operated group (Sham), I/R group (I/R), I/R and dexmedetomidine group (I/R+Dex), I/R and dexmedetomidine and Yohimbine group (I/R +Dex+Yoh). The study was approved by the Institutional Animal Care and Use Committee of Shandong Provincial Hospital and Shandong University. The animal experiments were followed the NIH guidelines for the Care and Use of Laboratory Animals (1996). I/R injury was induced by 2 h of middle cerebral artery occlusion (MCAO) and followed by 24 h of reperfusion [14]. 7 days later, rats were anesthetized with intraperitoneal injection of 10% chloral hydrate. 30 min before the ischemia, Dex (10 μg/kg, 20 μg/kg) or Yoh (0.1 mg/kg) were synchronously or respectively administered through intraperitoneal injection in various group.

2.2. Nissl staining

Rats were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Chest was incised and heart-lung were exposed. Then, abdominal aorta was cliped and perfusion was merely applied on upper body and brain. 100 ml saline and paraformaldehyde were respectively perfused into heart. Afterwards, rats were executed and brain tissue were stripped carefully. The brain were fixed in paraformaldehyde for 4–6 h, and then were dehydrated, paraffin embedded and sliced. Sections were stained with Nissl staining solution for 5–10 min at 35–50 °C. The next steps were 95% ethanol dehydration for 2 min and xylene transparency for 5 min. Sections were observed under light microscope (Leica DM IRB, Heidelberg, Germany).

2.3. TUNEL staining

Paraffin sections were preparated as above mentioned and described methods in our previous section. Apoptosis of the retinal tissue was detected by the In-Situ Cell Death Detection Kit-POD (Roche, Mannheim, Germany) according to the manufacturer's instructions. Sections were rinsed with TBS buffer. Endogenous peroxidase was inactivated by 200 μl 3% H_2O_2 and incubated for 5 min at room temperature. Samples were incubated in 200 μl equilibration buffer at room temperature for 30 min and placed in TdT labelling reactions mixture at 37 °C for 1.5 h. Then, samples were incubated in 60 μl block buffers, 100 μl DAB for 10 min, and then were stained by methyl green. Number of the TUNEL-positive cells were counted by light microscopy (Leica, Heidelberg, Germany) under 400 magnification.

2.4. Flow cytometry

Cells were washed with PBS and resuspended in 110 μl binding buffer (pH 7.3). Staining was applied with 5 μl FITC annexin-V and propidiumiodide (Becton Dickinson, Heidelberg, Germany). After 400 μl of binding buffer was added and flow cytometric analyzes were performed (Attune, Darmstadt, Germany), cells were incubated at room temperature for 15 min. Finally, the apoptosis was detected by flow cytometer.

2.5. Immunohistochemical staining

As above mentioned, frozen sections were preparated into 4 μm thickness, and fixate in 4% paraformaldehyde phosphate buffer. Then, sections were blocked in goat serum liquid and added GFAP monoclonal and TNF-α polyclonal antibody at 4 °C overnight. Sections were incubated with mice second antibody for 20 min at 37 °C and horseradish peroxidase marked streptomycin avidin for 20 min at 37 °C. The rest steps were DAB developing, hematoxylin redyeing and sealing.

2.6. ELISA of serum TNF-a

Venous blood (3 ml) was taken. Serum was separated and stored under −80 °C. The concentration of TNF-a was detected by Enzyme Linked Immunosorbent Assay (ELISA) Kit (Meixuan Co. Ltd, Shanghai, China) according to the manufacturer's instructions.

2.7. Real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, Calif, USA) according to the manufacturer's instructions. The RNA concentration was detected by ultraviolet spectrophotometer at 260–280 nm and the appropriate OD value was 1.8-2.0. The total reverse transcription reaction system was 20 μl to synthesize cDNA with SuperScript First-Stand Synthesis system (Invitrogen, Carlsbad, Calif, USA). The PCR primer were as following: TLR4, forward: 5′-CAG CAAAGTCCCTGA TGACA-3′, reverse: 5′-GTC TCCACAGCCACCAG ATT-3′; NF-κB, forward: 5′-TGT AGGAGTGGGTAG GGC-3′, reverse: 5′-CTGAG CAAC CAG-GAATA GAC-3′; TRIF, forward: 5′- AGACCCCTACAGCCAGGTCT-3′, reverse: 5′-GGC ATGGAGAAGCTTTG ACT-3′; β-actin, forward: 5′-TTC CTTCTTGGGTATGG AAT-3′, reverse: 5′-GAG CAATGATCTTGA TCTTC-3′. PCR amplification was carried out by ABI PRISM 7900 thermocycler using SYBR Premix Taq (Applied Biosystems, Foster, Calif, USA). The reaction conditions contains: initial denaturation at 94 °C for 50s, 96 °C for 14s for 40 cycles, 60 °C for 25 s, 72 °C for 3 min, final elongation step at 72 °C for 10 min. The results was calculated compare with β-actin with the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot

After I/R injury, tissue were dissected and splited by 200 μl RIPA Lysis Buffer (Santa Cruz, Dallas, TX, USA). The solution was put in EP tubes and incubated for 40 min on ice. Then, EP tubes were centrifuged 10000 rpm for 20 min. Protein concentrations were measured comparison to BCA. Protein and sample buffer were subjected to SDS-PAGE, and then was transferred onto PVDF membrane. PVDF membrane were blocked in blocking buffer, composed with TBST and 5% fat-free milk, for 1 h on shaking table at room temperature. Then, it was incubated overnight at 4 °C with the primary antibody. The following primary antibodies were used for immunoblotting: TLR4 (1:1000, Cell Signaling), NF-κB (1:1000, Abcam), TRIF (1:1000, Santa Cruz), caspase 3 (1:1000, Santa Cruz) β-actin (1:2000, Cell Signaling). Then, the PVDF membrane was diluted in secondary antibody (1:500, Santa Cruz,) 4 °C overnight.

Finally, the PVDF membrane was washed again with TBST for 10 min. Quantification of the protein bands were performed using the software Quantity One (Bio-Rad Laboratories, Hercules, CA). Data were normalized against β-actin.

2.9. Statistical analysis

Data was analyzed and expressed as mean±standard deviation. The inter-group differences were analyzed by Student's *t*-test or one-way ANOVA. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Degree of cerebral tissue injury

After 2 h of ischemia followed by 7 days of reperfusion, the degree of cerebral tissue injury was detected by HE and Nissl staining (Fig. 1). Cortex and hippocampus were obviously damaged. There were obvious nuclear pyknosis, gliocyte swelling and interstitial edema in cortex and hippocampus. Dissolution and necrosis of central Nissl body existed in neurons. Demyelination and gliosis occured in hippocampal pyramidal cell. This results revealed that dexmedetomidine preconditioning (10 μg/kg) decreased the I/R injury, which was suppressed by yohimbine (0.1 mg/kg).

3.2. Apoptosis of neurons

Apoptosis of neurons were detected by TUNEL staining, flow cytometry and western blot (Fig. 2). In TUNEL assay, apoptotic cells were dyed brown There was a large number of apoptotic neurons induced by I/R injury in cortex. Afterwards, flow cytometry and western blot showed that dexmedetomidine observably decreased the number of apoptotic cells compared with I/R group. Results showed that dexmedetomidine preconditioning reduced the apoptosis of neurons, which was specifically suppressed by yohimbine.

3.3. Expression of GFAP and TNF-α in cerebral I/R area

Gliocyte were excessive activated in cerebral ischemia area. Expression level of glial fibrillary acidic protein (GFAP) was an important symbol of gliocyte activation. GFAP expression in cerebral ischemia area was detected by immunohistochemistry and protein positive cells were counted (Fig. 3). Serum TNF-α level was detected by ELISA. GFAP-positive,TNF-α- positive astrocyte and serum TNF-αincreased obviously in I/R group compared with Sham group. Besides, counting of positive cell numbers was increased accordingly. Dexmedetomidine preconditioning (10 μg/kg, 20 μg/kg) could distinctly inhibit the positive cell and decrease the cell number. Furthermore, higher concentration of dexmedetomidine was predicted more obvious inhibition.

3.4. Expression of TLR4, NF-κB and TRIF

To investigate the signaling pathway of TLR4 in cerebral I/R injury, we examined the expression of TLR4, NF-κB and TRIF with western blot (Fig. 4). Expression level of TLR4 and NF-κB were significantly elevated in I/R injury. Afterwards, dexmedetomidine (10 μg/kg, 20 μg/kg) could observably down-regulate the TLR4 and NF-κB expression and up-regulate the TRIF expression. Results

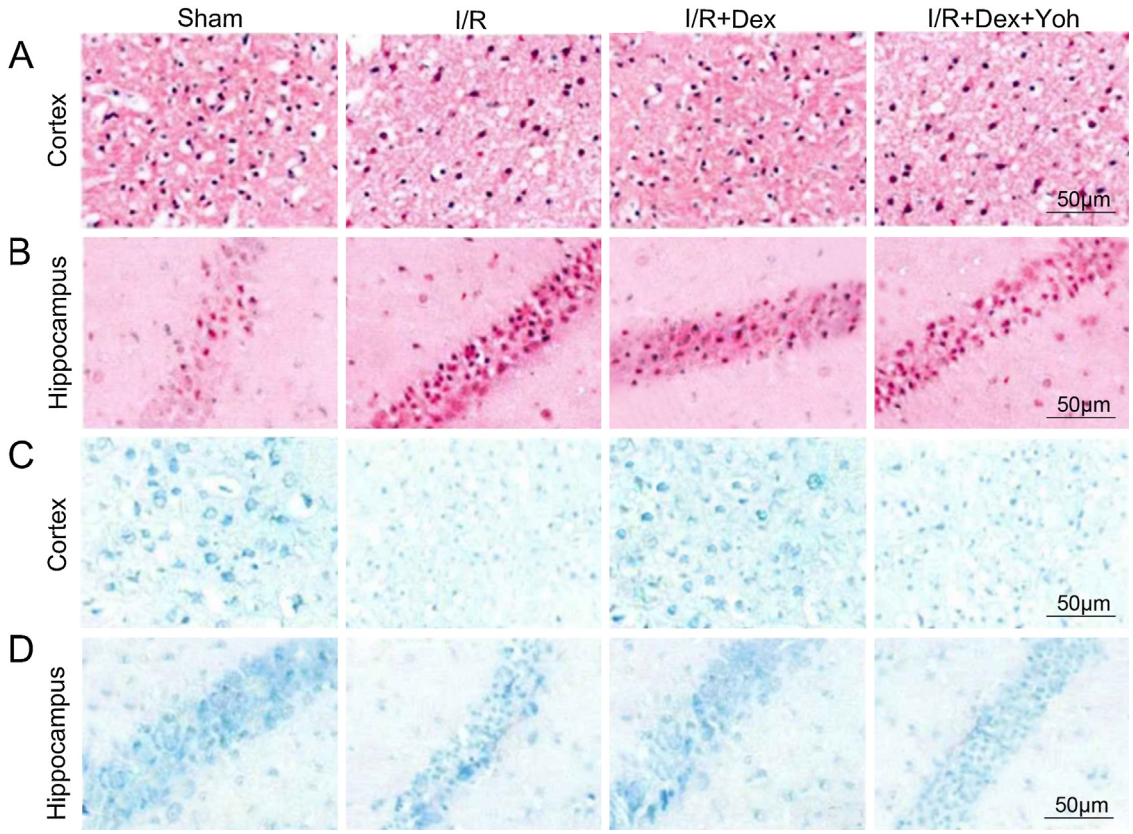

Fig. 1. Degree of cerebral tissue injury was detected by HE and Nissl staining. (A) HE staining of cortex. (B) HE staining of hippocampus. (C) Nissl staining of cortex. (D) Nissl staining of hippocampus. Scale was 50 μm at 400 magnification wih light microscope. Sham represents Shame, Dex represents dexmedetomidine preconditioning, Yoh represents yohimbine.

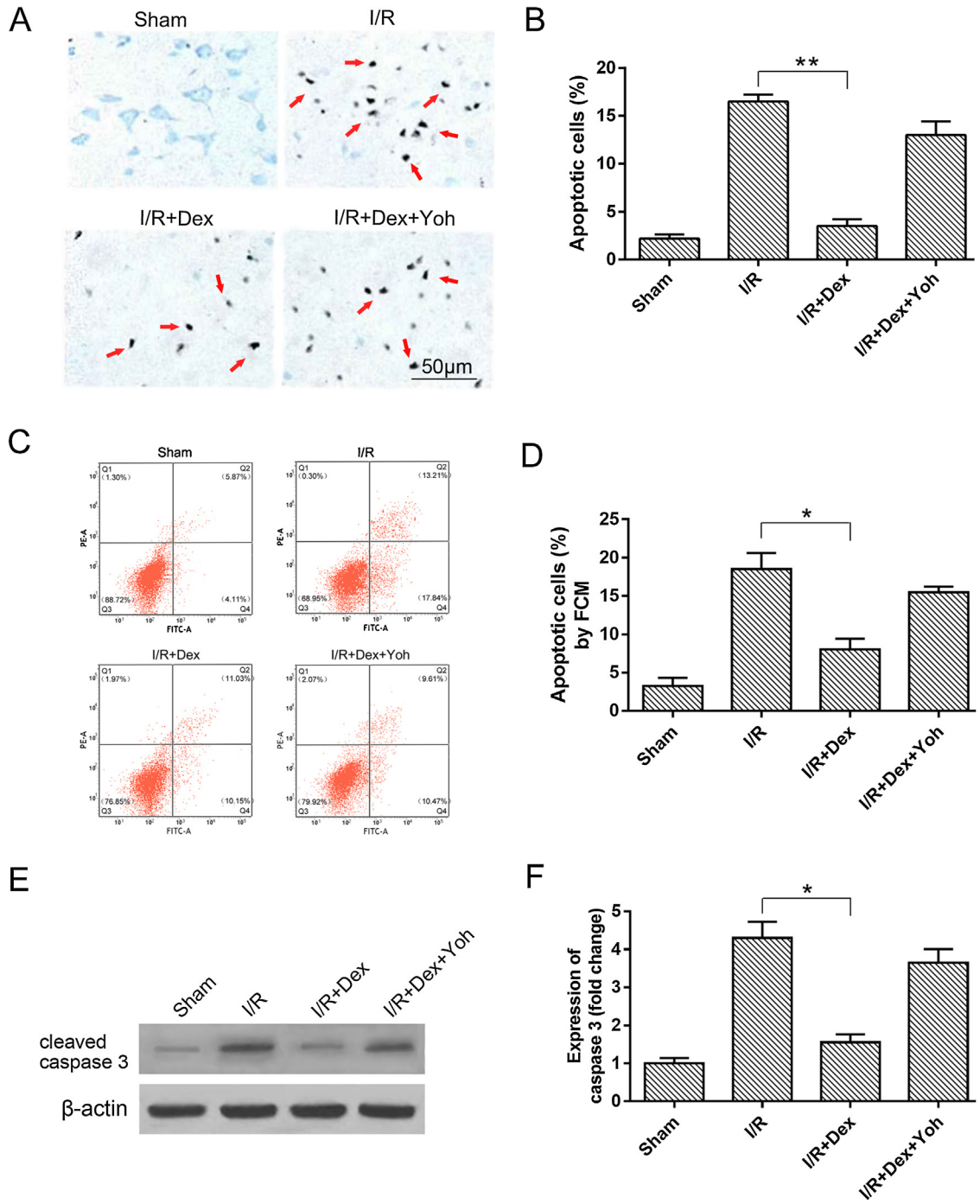

Fig. 2. TUNEL staining, flow cytometry and caspase-3 showed the apoptotic neurons. (A) TUNEL staining of cortex in each group. (B) The total apoptosis rates counted under light microscope. (C, D) Flow cytometry and apoptosis rate. (E, F) Western blot of caspase 3 and quantitative analysis. Data represent means ± standard deviation. Scale was 50 μm at 400 magnification wih light microscope. $^{**}P < 0.01$ represents statistical difference compared with I/R group.

showed that dexmedetomidine preconditioning inhibited the activation of TLR4 and NF-κB induced by I/R injury and increased expression of TRIF.

4. Discussion

In this study, we found that dexmedetomidine preconditioning plays a significant role in neuroprotective, which was verified by multifarious experiments. Besides, dexmedetomidine mediates inflammation-related TLR4 signaling pathway and regulates cerebral I/R injury.

In cerebral ischemia area, HE and Nissl staining showed that cortex and hippocampus were obviously damaged. There were obvious nuclear pyknosis, gliocyte swelling and interstitial edema (Fig. 1). Dissolution and necrosis of central Nissl body existed in neurons, and demyelination and gliosis occured in hippocampal pyramidal cell. TUNEL staining showed that increasing apoptotic neurons induced by I/R injury appear in cortex and dexmedetomidine (10 μg/kg) could observably reduce the apoptosis of neurons (Fig. 2). In order to confirm the inhibition of dexmedetomidine, we performed yohimbine (0.1 mg/kg), a selectively α_2 receptor blocker, as a control group. Results revealed that the

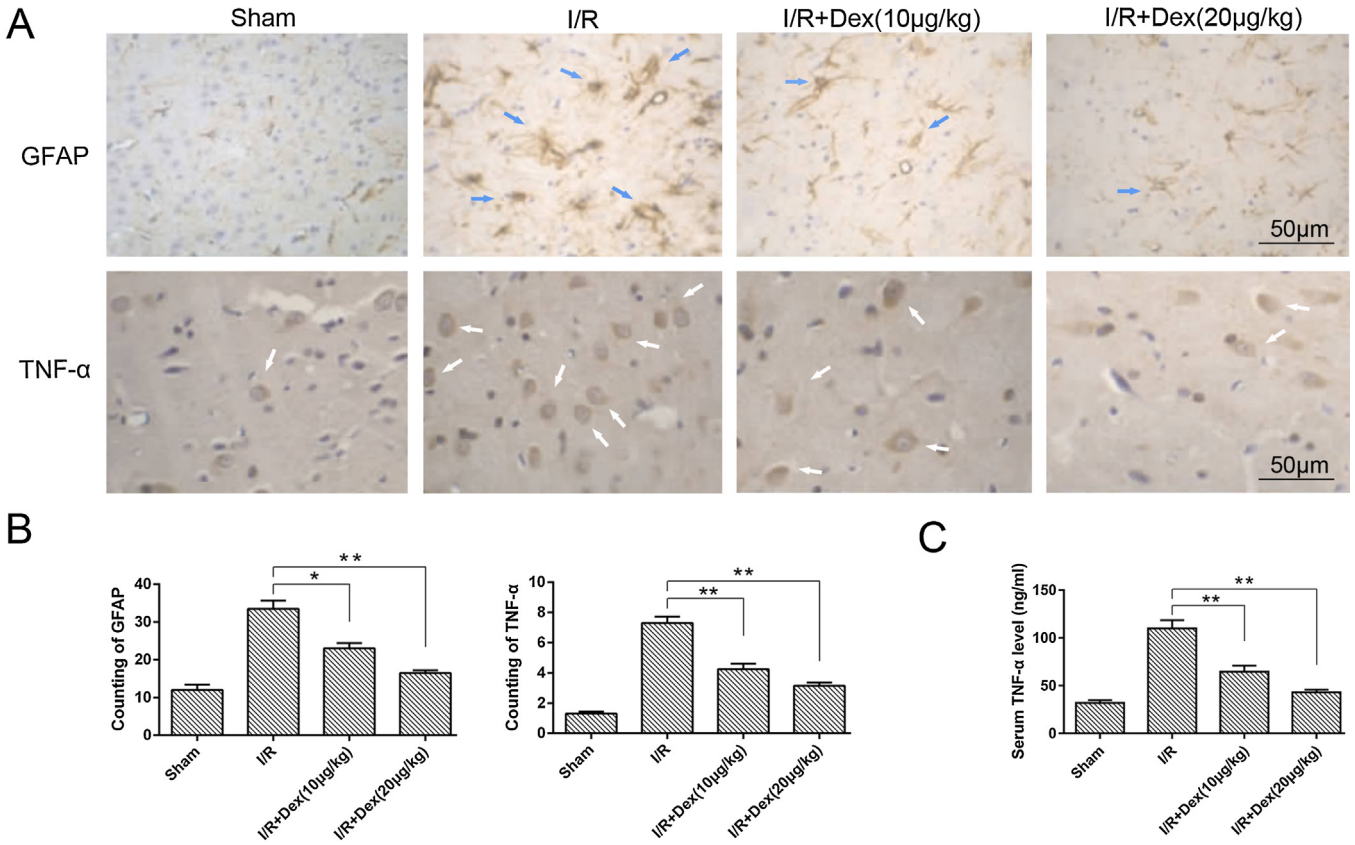

Fig. 3. GFAP and TNF-α expression were detected by immunohistochemistry and ELISA. (A) GFAP and TNF-α labeled cells in ischemic area of brain after 2 h of MCAO followed by 24 h of reperfusion. (B) Counting of positive cell numbers. Scale was 50 μm at 400 magnification under light microscope. (C) Serum TNF-α level. *P < 0.05, **P < 0.01 represents statistical difference compared with I/R group.

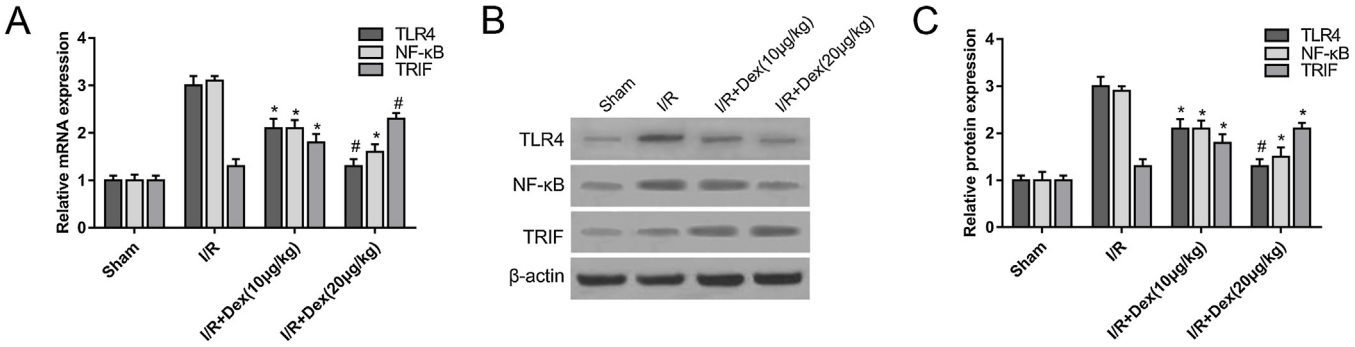

Fig. 4. Expression of TLR4, NF-κB and TRIF detected by real time PCR and western blot. (A) Relative mRNA expression of TLR4, NF-κB and TRIF. (B) Representative band images of TLR4, NF-κB and TRIF. (C) Quantitative analysis of the band densities for TLR4, NF-κB and TRIF. *P < 0.05, #P < 0.01 represents statistical difference compared with I/R group.

neuroprotective function of dexmedetomidine was almost counteracted by yohimbine, which indicated that dexmedetomidine veritably alleviate the cerebral I/R injury.

Researches have shown that many inflammatory mediators and cytokines play an important role in I/R injury. TNF-α is a major proinflammatory cytokines and important initiator, which is produced in macrophage, astrocytes, microglia and neurons of brain tissue. After cerebral ischemia, astrocytes are very active, including gliocyte hyperplasia and GFAP overexpression. Moreover, a variety of inflammatory factor are synthesized and mediate inflammatory response to case brain tissue damage [15]. Our results showed that GFAP-positive and TNF-α positive astrocyte increased obviously in I/R group compared with Sham group (Fig. 3). Dexmedetomidine preconditioning could observably reduce the GFAP and TNF-α expression. Furthermore, higher concentration (20 μg/kg) of dexmedetomidine exerts more obvious inhibition than lower concentration (10 μg/kg). Recently, researchers pay more attentions to the relationship of astrocyte activation and I/R injury. It has previously been considered that the excessive activation of astrocytes hinder the repair and functional recovery of neurons [16]. At the same time, astrocytes release glutamic acid to cause toxicity of amino acids and inflammation factors to aggravat neuronal damage [17]. Dexmedetomidine pretreatment has been tested to significantly suppress the inflammatory responses, as evidenced by lower TNF-α level and NF-κB activity, and alleviated overexpression of microglia and astrocytes in the hippocampus [18]. Dexmedetomidine preconditioning and postconditioning could reduce renal dysfunction

induced by I/R, inhibit inflammatory response, up-regulate the expression of protein ZO-1 and occludin [13].

Numerous TLRs express in the brain of mammal. It's mainly TLR2 and TLR4 to mediate organ injury. Therefore, blockage of inflammatory response induced by TLR receptor activation is the main approach to reduce tissues and organs damage [19]. Relevant researches have confirmed that TLR4 mediate the central nervous system damage and the expression of TLR4 mainly caused by microglial cells damage [20]. As an inflammatory signal receptor, the activation of TLR could start the release of a series of inflammatory factors, such as TNF-α and IL-6, and subsequent a cascade of inflammatory response. After cerebral I/R, abundant TLR4 express and mediate subsequent inflammatory reaction to cause brain damage. In lung tissues of septic rats, dexmedetomidine (10 and 20??g/kg) significantly decreased mortality and pulmonary inflammation, and inhibited TLR4/MyD88 expression and NF-??B activation [21,22]. Evidence indicates that the inflammatory response, associated with pro-inflammatory cytokines IL-1 and TNF-α, plays a vital role in cerebtal dysfunction following I/R [23].

To elucidate the TLR4 mediated mechanisms underlying the preconditioning effects of dexmedetomidine, we examined the mRNA and protein expression changes of TLR4, NF-κB and TRIF induced by ischemic stress in the cerebrum. Our results showed that expression level of TLR4 and NF-κB were significantly elevated in cerebral ischemia area (Fig. 4). Our study confirm that dexmedetomidine exerts neuroprotection against inflammation following I/R injury. The protective effect is on the basis of the suppression of TLR4/NF-κB pathway and pro-inflammatory factors TNF-α. Besides, dexmedetomidine preconditioning selectively drives TLR4/TRIF signaling and microglia in the prevention of cerebral ischemic damage [24]. The preventive effect of dexmedetomidine is mediated by TLR4/TRIF signaling and microglia. TLR4/TRIF signaling and microglia have been proved to play a key role in the prevention of retinal ischemic damage [25]. Therefore, the potential mechanisms of dexmedetomidine need to be investigated to develop a future therapy for ischemic precaution.

Conflicts of interest

The study and authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.06.051.

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