의학박사 학위논문

백서의 일시적 전뇌허혈 모델에서 dexmedetomidine의 뇌보호효과: TLR-4/NF-κB pathway의 비활성화를 통한 항염증효과

Dexmedetomidine confers neuroprotection against transient global cerebral ischemia/reperfusion injury in rats: Anti-inflammatory effect through inactivation of the TLR-4/NF-κB pathway

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지도교수 박 희 평

이 논문을 김유진 박사학위논문으로 제출함.

2017년 4월

서울대학교 대학원

의학과 임상의과학 전공

김 유 진

김유진의 박사학위논문을 인준함

2017년 7월

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Dexmedetomidine confers neuroprotection against transient global cerebral ischemia/reperfusion injury in rats: Anti-inflammatory effect through inactivation of the TLR-4/NF-κB pathway

by

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A Thesis submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine (Clinical Medical Sciences) at Seoul National University College of Medicine

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Abstract

Dexmedetomidine confers neuroprotection against transient global cerebral ischemia/reperfusion injury in rats: Anti-inflammatory effect through inactivation of the TLR-4/NF-κB pathway

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Objective: Dexmedetomidine (DXM) has anti-inflammatory effects, which is considered an important mechanism of DXM-induced neuroprotectionfrom cerebral ischemia/reperfusion injury. We determinedwhethertheanti-inflammatory effects of DXM are associated with inhibition of the toll-like receptor (TLR)-4/nuclear factor kappa B (NF-κB) pathway in a rat model of transient global cerebral ischemia/reperfusion injury.

Methods: Fifty rats were randomly assigned to one of five groups (10 rats/group): Group S received no treatment; Group C underwent transient global ischemia(10 min); Group D received DXM 30 min before ischemia; Group R received resatorvid, a selective TLR-4 antagonist, 30 min before ischemia; and Group RD received resatorvid and DXM 30 min before ischemia. The numbers of necrotic and apoptotic cells and the levels of TLR-4, NF-κB, and caspase-3 were assessed 1 day after ischemia, and pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) were measured before ischemia and 2,6, and 24 h thereafter.

Results: The necrotic and apoptotic cell counts and levels of TLR-4, NF-κB, and caspase-3 were higher in Group C than in other groups. TNF-α were higher in Group C than in other groups 2 h

afterischemia,whereasIL-6 were higher in Group C 6 h after ischemia. IL-1βwas higher in Group C than in Group D 6 and 24 h after ischemia.

Conclusions: Our findings suggest that the anti-inflammatory action of DXM via inactivation of the TLR-4/NF-κB pathway, in part, may explain DXM-induced neuroprotection after cerebral ischemia.

Keywords: inflammation;dexmedetomidine; neuroprotection; cerebral ischemia; TLR-4; NF-κB

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Introduction

Inflammatory response after a stroke has been known to increase cerebral infarct size and worsens clinical outcomes[1-3]. After the cerebral ischemia, free radicals, so called reactive oxygen species (ROSs) are increased, which induce the expression of inflammatory cytokines and chemokines [4]. Subsequently, microglia activation and upregulation of cell adhesion molecules (CAMs) were induced, and they mediate the interaction between endothelial cells and leukocytes, which called leukocyte rolling and diapedesis. Simultaneously, nuclear factor kappa-B (NF-κB) and inducible nitric oxide synthase (iNOS) are also activated, which further exacerbates oxidative stress and cytokine production. This post-ischemic neuroinflammatory cascade leads to dysfuction of blood-brain barrier (BBB), cerebral edema, and neuronal cell death [3, 5]. Therefore, targeting the neuroinflammatory pathway has become one of the important neuroprotective strategies after acute cerebral ischemia.

Dexmedetomidine (DXM) is a selective α_2-adrenergic receptor agonist, which was approved for sedation of intensive care units (ICUs) by the US Food and Drug administration in 1999. Since then, a growing body of research reported other clinical implications such as adjuvant anesthetics, analgesics, and anxiolytics [6, 7]. Recently, DXM was shown to have additional neuroprotective effects in various *in vitro* and *in vivo* experimental models [8-10]. One possible mechanism of neuroprotection is anti-inflammation, and in fact, it was found that DXM reduces the expression levels of inflammatory cytokines after cerebral ischemia/reperfusion (I/R) injury [8, 11]. Although activation of the α2-adrenoreceptor pathway has been suggested as one mechanism by which DXM counters inflammation [12], the precise mechanism remains unclear.

Toll-like receptors (TLRs) play a fundamental role in pathogen recognition and activation of innateimmunity. They recognize pathogen-associated molecular patterns (PAMPs) which are expressed on infectious agents. TLRs are ubiquitous in nature, and their expression levels are

associated with activation of the innate immune system in response to pathogens, cytokines, and environmental stressors[13, 14]. Of the 13 known mammalian TLR subtypes, TLR-4 is activated in the ischemic brain and its inhibition attenuates cerebral I/R injury [14, 15]. Moreover, it activates nuclear factor kappa B (NF-κB) signaling, thereby triggering the transcription of many pro-inflammatory genes. DXM has been reported to attenuate TLR-4-related inflammatory reactions after I/R injury to various organs [16-18]. However, to the best of our knowledge, no study has evaluated the effects of DXM on TLR-4-related neuroinflammatory responses that develop after cerebral I/R injury.

Therefore, we determined whether the anti-inflammatory effects of DXM are associated with inhibition of the TLR-4/NF-κB pathway, thereby reducing the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, after transient global cerebral I/R injury in rats.

Materials and Methods

The animal protocol was approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (IACUC No. 14-0257-S1A1). All animal experiments and care followed the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health (Bethesda, MD, USA). Male Sprague-Dawley rats aged 10–16 weeks and weighing 350–380 g were used. The rats were housed under a 12 h day-night cycle at 20°C.The rats was fasted for 12–16 h before the experiments, but water was freely allowed.

The rats were anesthetized via intraperitoneal (i.p.) injection of zoletil (a 1:1 w/w combination of 125 mg tiletamine and 125 zolazepam [20 mg/kg]) and xylazine (5 mg/kg). After tracheal intubation, the rats were ventilated with 50% oxygen and 50% nitrogen (both v/v). The corneal and pedal reflexes and responses to tail pinching were intermittently checked to maintain an adequate level of anesthesia. Additional zoletil (10 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) were given when the reflexes or response to tail pinching was detected, or when the heart rate or systolic arterial pressure attained >20% of the baseline level. Femoral artery was catheterized to allow continuous arterial pressure monitoring and blood sampling. Core body temperature was checked using a rectal probe, and was maintained at 37°C using a heating/cooling board. A subcutaneous thermistor was implanted below the right temporalis muscle, adjacent to the skull, to monitor brain temperature (TCAT-2 Temperature Controller; Harvard Apparatus, Holliston, MA, USA) and maintain at about 37.5°Cusing an infrared lamp.

Fifty rats were randomly assigned to one of five groups (10 rats/group) using computer-generated random numbers (Fig. 1): 1) Group S received no treatment; 2) Group C underwent transient global cerebral ischemia (10 min); 3) Group D received DXM (100 μg/kgi.p.) 30 min before ischemia [19]; 4) Group R received resatorvid (a selective TLR-4 antagonist; 3 mg/kg i.p.) 30

min before ischemia [20]; and 5) Group RD received resatorvid 3 mg/kg and DXM 100 μg/kgi.p. 30 min before ischemia.

The transient global cerebral ischemia model used was as previously described [21]. Briefly, ischemia was induced via bilateral common carotid artery (CCA) ligation under conditions of arterial hypotension. A laser Doppler monitoring system (Moor Instruments VMS-LDF2, Axminster, UK) was used to monitor cerebral blood flow (CBF) during the procedure. The sensor of the monitor was placed (and fixed with bone cement) 1–2 mm posterior and 4–5 mm lateral to the bregma on the left or right skull hemisphere after a small midline skin incision had been made on the same side. For blood withdrawal and re-infusion, the right jugular vein was cannulated with a silicone catheter. After heparinization (50 units), blood was quickly drawn until the mean arterial pressure (MAP) attained 25–30 mm Hg and the reduction in regional CBF was 50% from baseline. Then both CCAs were clamped with vascular clips for 10 min, and the MAP was maintained at 25–30 mmHg during the ischemia. The clips were carefully removed from CCAs and the withdrawn blood was slowly re-infused. After the procedure, the rats were given 0.5% bupivacaine injections around the incision sites and were allowed to recover from anesthesia at room temperature. Arterial blood gas, hemoglobin, glucose, and MAP were measured 10 min before ischemia, during ischemia, and 30 min after reperfusion.

At 1 day after ischemia, rats were anesthetized with 20 mg/kg zoletili.p. and decapitated. The brains were quickly removed and divided transversely into two parts using a rat brain slice matrix. The anterior part of each brain was placed in liquid nitrogen and stored at -80°C for later Western blot analysis, while the posterior part was fixed in buffered 10% (v/v) formalin for histopathological examination. Paraffin wax-embedded brain regions were sliced into serial coronal sections 5 μm in thickness and stained with hematoxylin and eosin (H&E) and terminal deoxynucleotidyltransferasedUTP nick end-labelling (TUNEL) using an Apoptag Peroxidase *In*

Situ Apoptosis Detection Kit S7100 (Millipore Corp., Billerica, MA, USA). An investigator unaware of group assignment evaluated the total number of cells and the number of necrotic or apoptotic cells in the hippocampal CA1 region, using a light microscope method [21]. Necrotic neurons were identified by karyolytic or pyknotic nuclei and cytoplasmic shrinkage. TUNEL-positive cells with blue-stained apoptotic bodies were considered as apoptotic cells. In total, six optical fields (left: three, right: three) of the hippocampal CA1 sector were examined under high-power magnification (400×); the percentage of necrotic or apoptotic cells was calculated as the ratio of the number of necrotic or apoptotic cells to the total number of cells in each field.

Western blotting for TLR-4, NF-κB, and caspase-3 was performed with the aid of anti-NF-κB p65, anti-TLR-4, and anti-cleaved caspase-3 antibodies (Cell Signaling Technology, Beverly, MA, USA). Total proteins were extracted from transversely excised brain tissue (including the anterior hippocampus) and proteins were detected using an Amersham enhanced chemiluminescence kit (Amersham, Bucks, UK). Nuclear proteins were extracted using the NE-PER® kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Densitometry afforded quantification. The β-actin and histone H3 served as internal controls for the cytoplasmic proteins (TLR-4, caspase-3), and the nuclear protein, NF-κB, respectively.

Blood was sampled aseptically from the retro-orbital plexus before ischemia and at 2, 6, and 24 h following ischemia. Blood-containing tubes were allowed to stand at room temperature for 2 h, and sera were prepared by centrifugation at 3000 × g for 20 min at 4°C. The serum levels of TNF-α, IL-1β, and IL-6 were assayed using enzyme-linked immunoassay (ELISA) kits (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions.

The primary endpoint was the expression level of TLR-4. The secondary endpoints were the expression levels of NF-κB and caspase-3, serum levels of TNF-α, IL-1β, and IL-6, and percentages of necrotic and apoptotic cells in the hippocampal CA1 region. In a previous study, the expression

ratio of TLR-4/β-actin was approximately 0.58 ± 0.20 following cerebral I/R injury in rats [22]. We assumed that a 50% decrease of the expression ratio of TLR-4/β-actin in the DXM group would be considered significant. Considering type I error of 0.05 and a power of 80%, 10 rats in each group were needed.

The expression levels of TLR-4, NF-κB, and caspase-3, and the percentages of necrotic and apoptotic cells in the hippocampal CA1 region were compared using the Kruskal-Wallis test followed by the Mann-Whitney *U*-test. The serum levels of TNF-α, IL-1β, and IL-6, and physiological variables were compared using repeated-measures ANOVA, and the levels at each timepoint were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney *U* test. To adjust for multiple comparisons, a P value < 0.01 rather than a P value < 0.05 was considered statistically significant. All values were expressed as medians with interquartile ranges (Q1–Q3). SPSS software (version 22.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Both the MBP and regional CBF were higher in the sham group than in the other groups during the ischemic period ($P < 0.01$, respectively, Table 1). During the reperfusion, MBP was higher in Group D than in Groups C, R, and S ($P < 0.01$, respectively).

On post-ischemic day 1, many necrotic cells were observed in the hippocampal CA1 regions of Group C, whereas few were detected in Group S (Figure 2A). The percentage of necrotic cells was significantly higher in Group C than in Groups D, R, RD, and S ($P < 0.001$, 0.001, 0.001, and 0.003, respectively; Figure 2B). In addition, the percentage of necrotic cells was higher in Group D than in Groups RD and S ($P = 0.002$ and 0.002). Many apoptotic cells were observed in Group C, whereas few were evident in Group S (Figure 3A). The percentage of apoptotic cells was higher in Group C than in Groups D, R, RD, and S ($P = 0.007$, 0.004, 0.001, and 0.003, respectively) 1 day after ischemia (Figure 3B). In addition, the percentage of apoptotic cells was higher in Group D than in Group RD ($P = 0.003$).

The relative expression levels of TLR-4, NF-κB, and caspase-3 1 day after ischemia are shown in Figure 4. Cytoplasmic TLR-4 expression was higher in Group C than in Groups D, R, RD, and S ($P = 0.001$, 0.001, 0.008, and 0.002, respectively). The NF-κB expression in the nucleus was higher in Group C than in Groups D, R, RD, and S ($P < 0.001$, < 0.001, 0.001, and 0.002, respectively). The cytoplasmic expression of caspase-3 was higher in Group C than in Groups D, R, RD, and S ($P < 0.001$, < 0.001, 0.001, and 0.002, respectively).

The serum level of TNF-α peaked at approximately 2 h after ischemia in Group C (Figure 5A). At this time, the TNF-α level was significantly higher in Group C than in Groups D, R, RD, and S ($P = 0.003$, 0.002, 0.006, and 0.006, respectively). At 6 h after ischemia, the TNF-α level was significantly higher in Group C than in Group S ($P = 0.003$). The serum level of IL-6 peaked 6 h after ischemia in Group C (Figure 5B). At 2 h after ischemia, the serum level of IL-6 was significantly

higher in Group C than in Group RD (P = 0.004). At 6 h after ischemia, the serum IL-6 level was significantly higher in Group C than in Groups D, R, RD, and S (P = 0.001, < 0.001, 0.001, and 0.002, respectively). At 24 h after ischemia, the IL-6 level was significantly higher in Group C than in Groups R and RD (P = 0.005 and 0.007, respectively).The serum IL-1β level peaked 24 h after ischemia (Figure 5C). At 6 h after ischemia, the level of IL-1β was significantly higher in Group C than in Groups D, R, RD and S (P = 0.002, 0.003, 0.003, and 0.003, respectively). At 24 h after ischemia, the IL-1β level was significantly higher in Group C than in Groups D, RD, and S (P = 0.006, 0.001, and 0.002, respectively).

Table 1. Physiological variables during ischemia/reperfusion period.

		Group C (n =10)	Group D (n = 10)	Group R (n = 10)	Group RD (n = 10)	Group S (n = 10)
pH	Baseline	7.38 (7.36-7.39)	7.36 (7.34-7.37)	7.37 (7.35-7.39)	7.35 (7.32-7.37)	7.37 (7.36-7.38)
	Ischemia	7.46 (7.38-7.49)	7.45 (7.42-7.48)	7.43(7.35-7.46)	7.40 (7.39-7.43)	7.37 (7.35-7.40)
	Reperfusion	7.33 (7.28-7.35)	7.34 (7.28-7.35)	7.31 (7.30-7.33)	7.35 (7.25-7.39)	7.37 (7.35-7.38)
$PaCO_2$(mmHg)	Baseline	38.0 (37.1-41.8)	41.3 (33.9-42.7)	41.0 (37.3-44.1)	42.3 (40.6-45.1)	46.6 (34.2-48.4)
	Ischemia	29.2 (26.5-34.9)	31.1 (27.2-32.8)	32.6 (29.3-37.5)	29.7 (25.1-33.2)	41.2 (40.7-47.6)
	Reperfusion	44.6 (40.0-50.0)	46.3 (40.9-49.7)	48.3 (45.4-49.8)	45.8 (39.0-52.1)	45.6 (45.5-46.3)
PaO_2 (mmHg)	Baseline	285.1 (271.3-315.7)	277.7 (260.3-289.5)	295.9 (288.7-302.2)	299.7 (287.6-315.9)	278.8 (264.2-291.3)
	Ischemia	255.4 (241.4-287.6)	272.3 (255.8-286.1)	302.4 (278.2-313.1)	295.5 (273.6-314.7)	255.7 (242.7-318.2)
	Reperfusion	244.1 (219.1-270.6)	245.7 (235.9-262.3)	283.5 (238.5-308.1)	251.4 (219.4-282.6)	255.9 (238.9-284.3)
Hb (g/dl)	Baseline	10.0 (9.5-11.1)	10.3 (8.9-12.4)	11.6 (11.1-12.0)	12.0 (10.8-12.3)	11.4 (9.8-11.9)
	Ischemia	9.3 (6.3-10.5)	9.4 (8.1-10.6)	10.0 (8.8-10.2)	9.9 (9.6-10.1)	11.3 (10.7-11.7)
	Reperfusion	11.0 (10.6-11.5)	10.6 (9.6-11.3)	11.1 (10.7-11.3)	11.4 (10.7-11.9)	11.5 (11.4-11.7)
Blood	Baseline	93.5 (68.8-107.8)	96.5 (71.5-122.5)	79.0 (66.5-94.5)	91.0 (73.3-110.8)	122.0 (77.0-144.0)
glucose	Ischemia	89.0 (64.0-123.8)	91.0 (73.3-123.8)	82.0 (74.5-112.0)	83.0 (69.3-89.8)	126.0 (94.5-137.0)
(mg/dl)	Reperfusion	114.5 (95.0-131.3)	122.0 (98.8-129.5)	90.0 (73.5-123.0)	99.0 (81.5-114.0)	124.0 (110.0-139.0)
MAP (mmHg)	Baseline	81.5 (79.8-86.5)	81.5 (72.0-88.0)	80.0 (78.0-97.5)	69.5 (63.0-120.8)	84.0 (81.5-89.0)
	Ischemia	29.5 (28.0-31.0)§	30.0 (29.0-30.0)§	28.0 (28.0-43.0)§	29.0 (28.0-29.0)§	84.0 (82.0-89.5)
	Reperfusion	94.0 (91.8-107.3)*, ‡	121.0(113.0-131.3)†,§	98.0 (89.0-110.5)	108.5 (105.8-122.0)	86.0 (83.0-94.0)
Laser	Baseline	81.9 (66.2-96.3)	77.8 (62.4-90.6)	77.0 (70.2-93.6)	95.0 (64.5-109.2)	77.1 (68.8-91.3)
Doppler	Ischemia	17.5 (13.3-26.0)	12.0 (10.7-16.1)	15.1 (12.4-25.3)	27.5 (12.0-34.2)	82.9 (76.7-97.5)
(PU)	Reperfusion	120.8 (104.7-134.8)§	111.6 (94.5-133.1 §	105.0 (93.9-116.4)§	123.4 (95.3-130.8)§	84.1 (75.2-99.1)

Values are presented as median with interquartile (Q1-Q3).

All parameters are measured at 30 minutes of stabilization (baseline), after 10 minutes of ischemia (ischemia), and after 30 minutes of reperfusion (reperfusion).

*: $P< 0.01$ vs. group D, †: $P< 0.01$ vs. group R, ‡: $P<0.01$ vs. group RD, §: $P< 0.01$ vs. group S.

Hb, hemoglobin; MAP, mean arterial pressure; PU, perfusion units.

	Surgical preparation		Ischemia	Reperfusion
Group S	Surgical preparation			
Group C	Surgical preparation		CCA occlusion	
Group D	Surgical preparation	DXM	CCA occlusion	
Group R	Surgical preparation	Resatorvid	CCA occlusion	
Group RD	Surgical preparation	Resatorvid + DXM	CCA occlusion	
Time	30 min	30 min	10 min	30 min

Figure 1.Experimental protocol.

In group D, dexmedetomidine 100 μg/kg is intraperitoneally administered 30 min before cerebral ischemia. In group R, resatorvid 3 mg/kg is intraperitoneally administered 30 min before cerebral ischemia. In group RD, resatorvid 3 mg/kg and dexmedetomidine 100 μg/kg are sequentially administered 30 min before cerebral ischemia. group S, sham; group C, control; group D, dexmedetomidine; group R, resatorvid; group RD, resatorvid + dexmedetomidine. DXM: dexmedetomidine; CCA: common carotid artery.

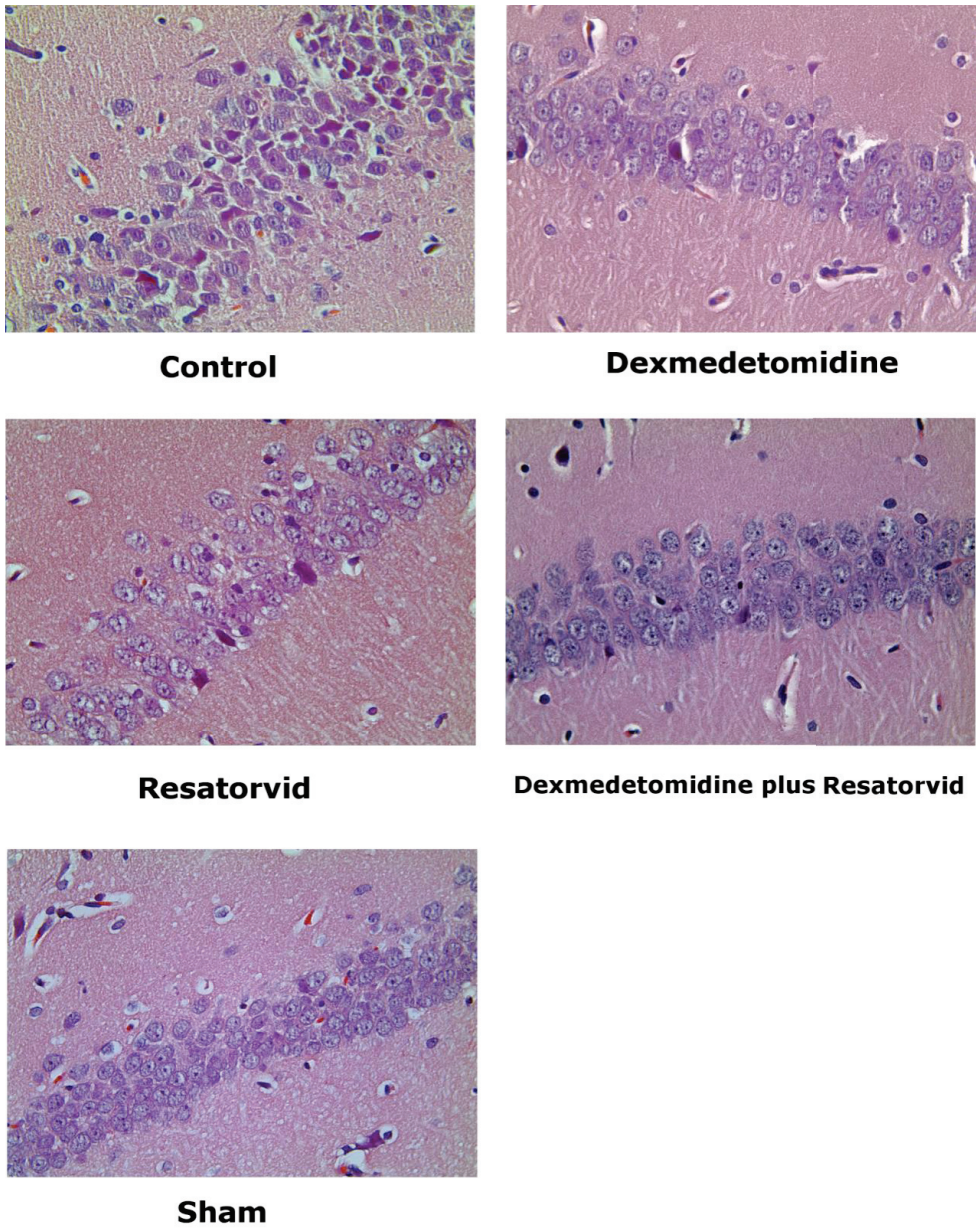


Figure 2(A) Representative photomicrographs from a single rat with H&E staining in the hippocampal CA1 regions one day after transient global cerebral ischemia.

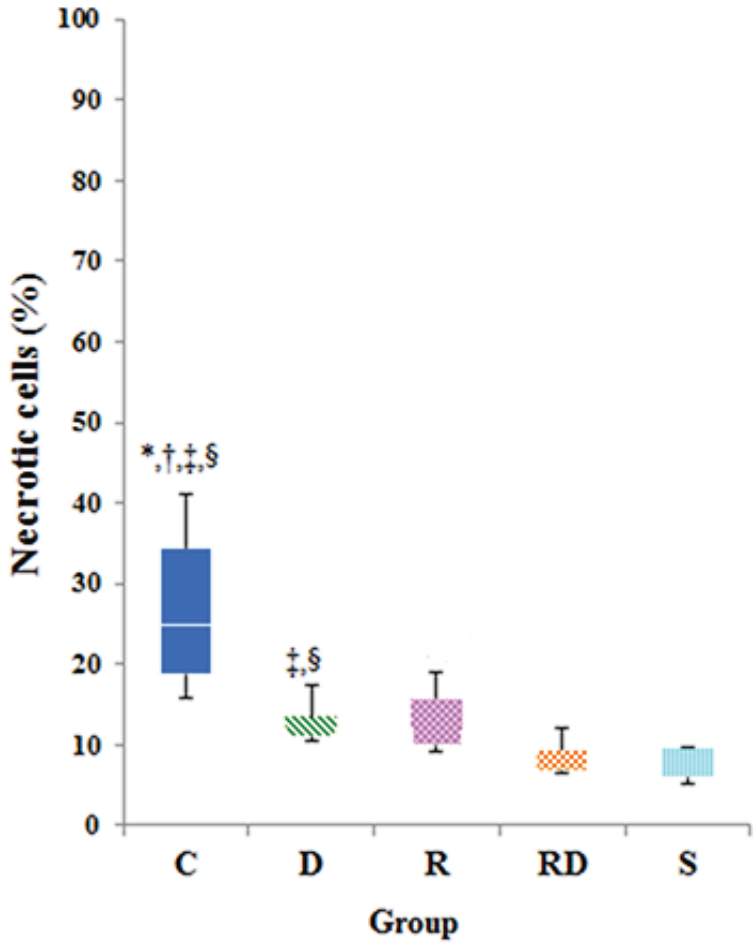


Figure 2(B) Percentage of necrotic cells in the hippocampal CA1 regions one day after ischemia. Box plot indicates median, inter-quartile, and full ranges. C, control; D, Dexmedetomidine; R, Resatorvid; RD, Resatorvid plus Dexmdetomidine; S, Sham.

*: P < 0.01 vs. group D, †: P < 0.01 vs. group R, ‡: P <0.01 vs. group RD, §: P < 0.01 vs. group S.

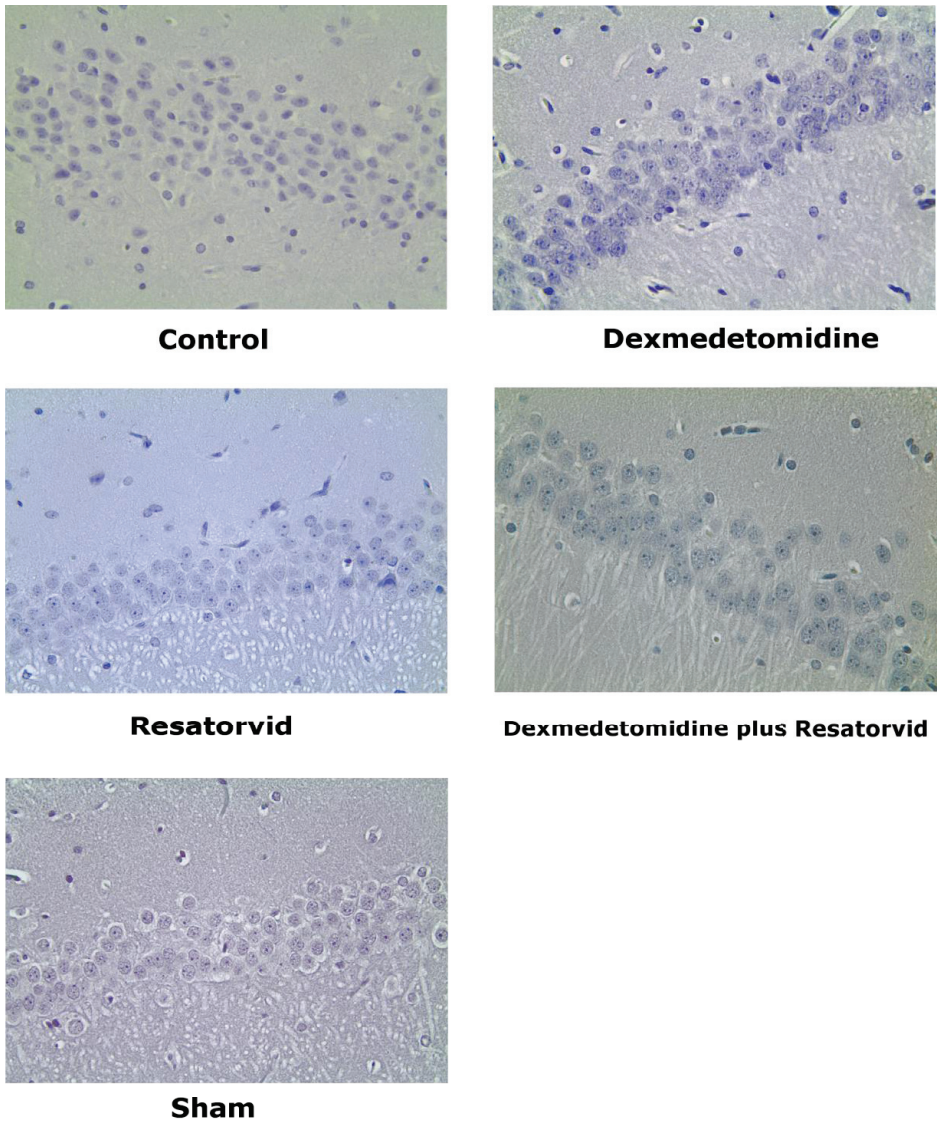


Figure 3(A) Representative photomicrographs from a single rat with TUNEL staining in the hippocampal CA1 regions one day after transient global cerebral ischemia.

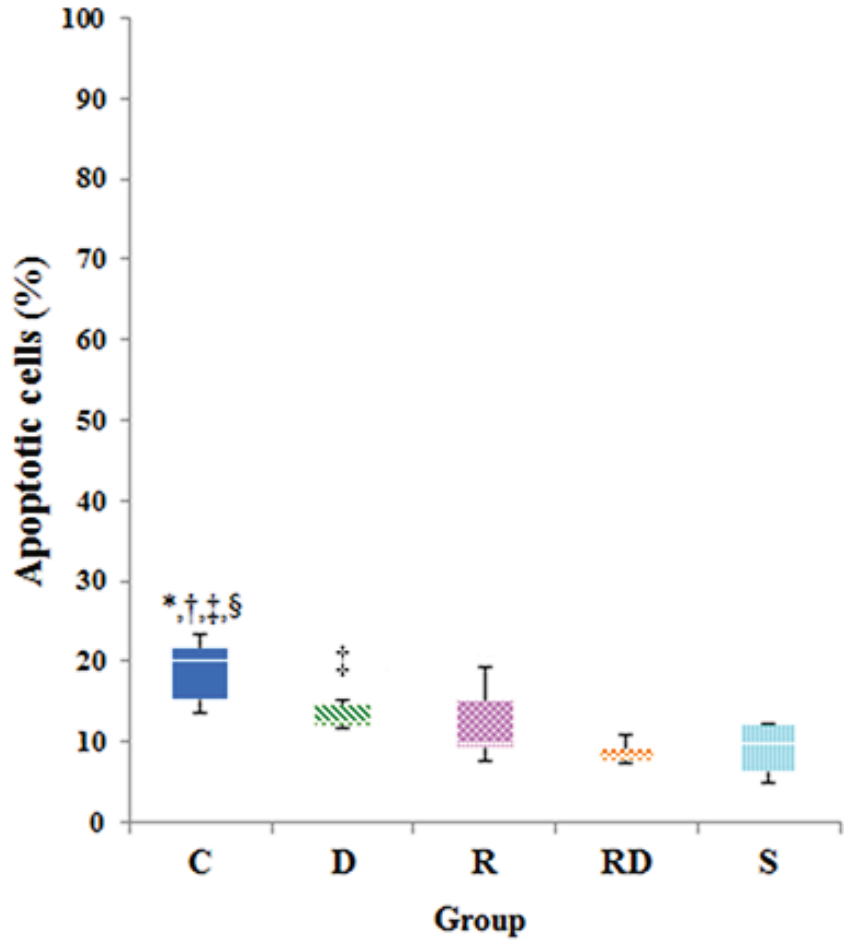


Figure 3(B) Percentage of apoptotic cells in the hippocampal CA1 regions one day after ischemia. Box plot indicates median, inter-quartile, and full ranges. C, control; D, Dexmedetomidine; R, Resatorvid; RD, Resatorvid plus Dexmdetomidine; S, Sham.

*: P < 0.01 vs. group D, †: P < 0.01 vs. group R, ‡: P <0.01 vs. group RD, §: P < 0.01 vs. group S

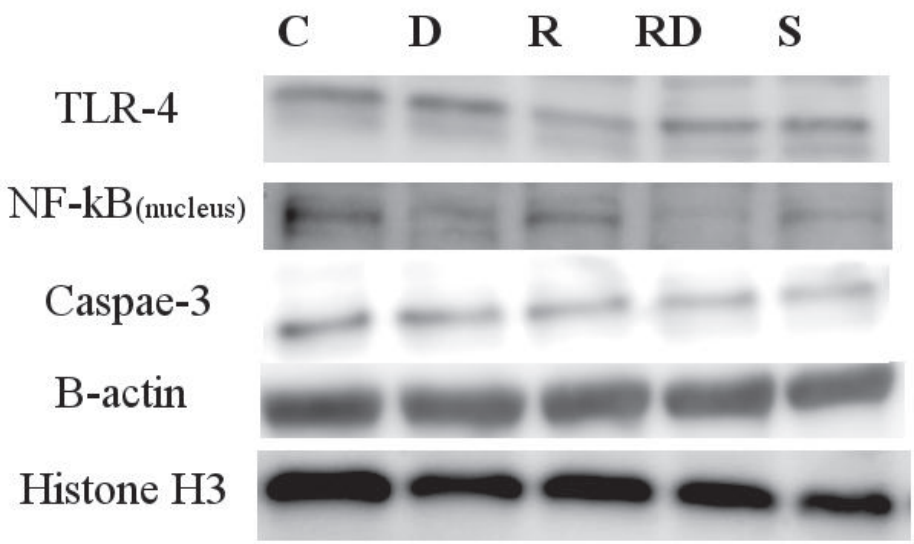


Figure 4 (A) Representative western blot analysis of Toll like receptor 4 (TLR-4), nuclear factor kappa B (NF- κB), and caspase-3 one day after transient global cerebral ischemia. C, control; D, Dexmedetomidine; R, Resatorvid; RD, Resatorvid plus Dexmdetomidine; S, Sham.

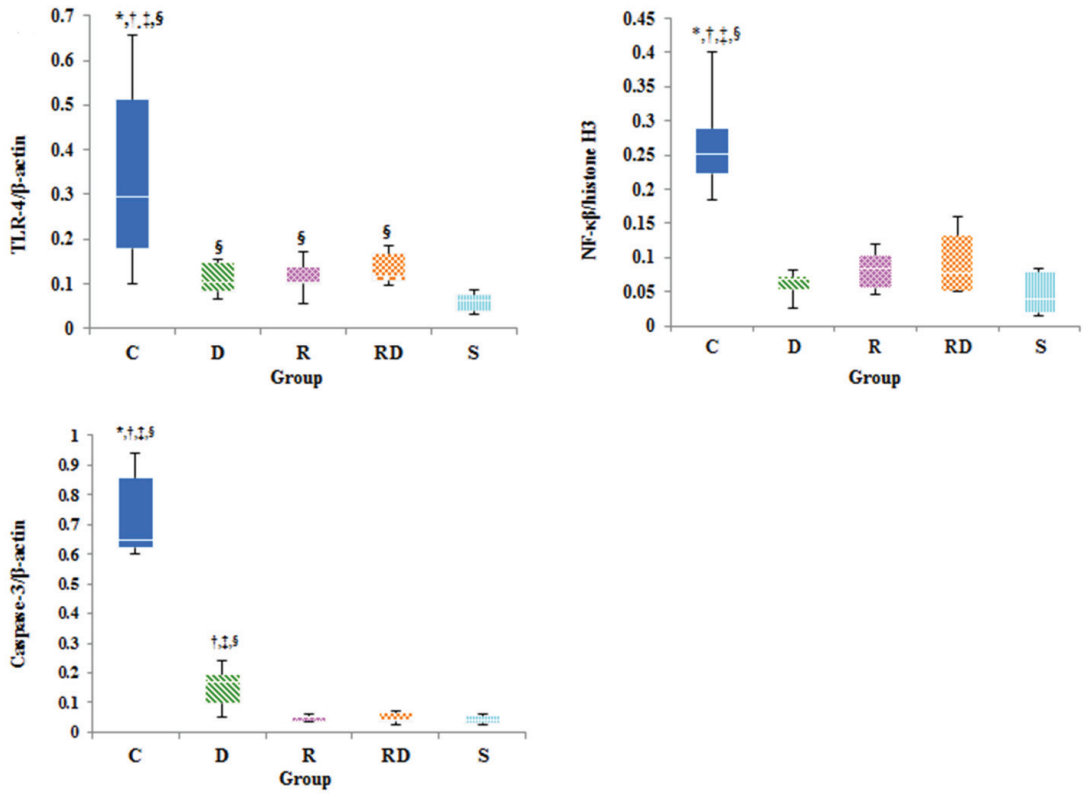
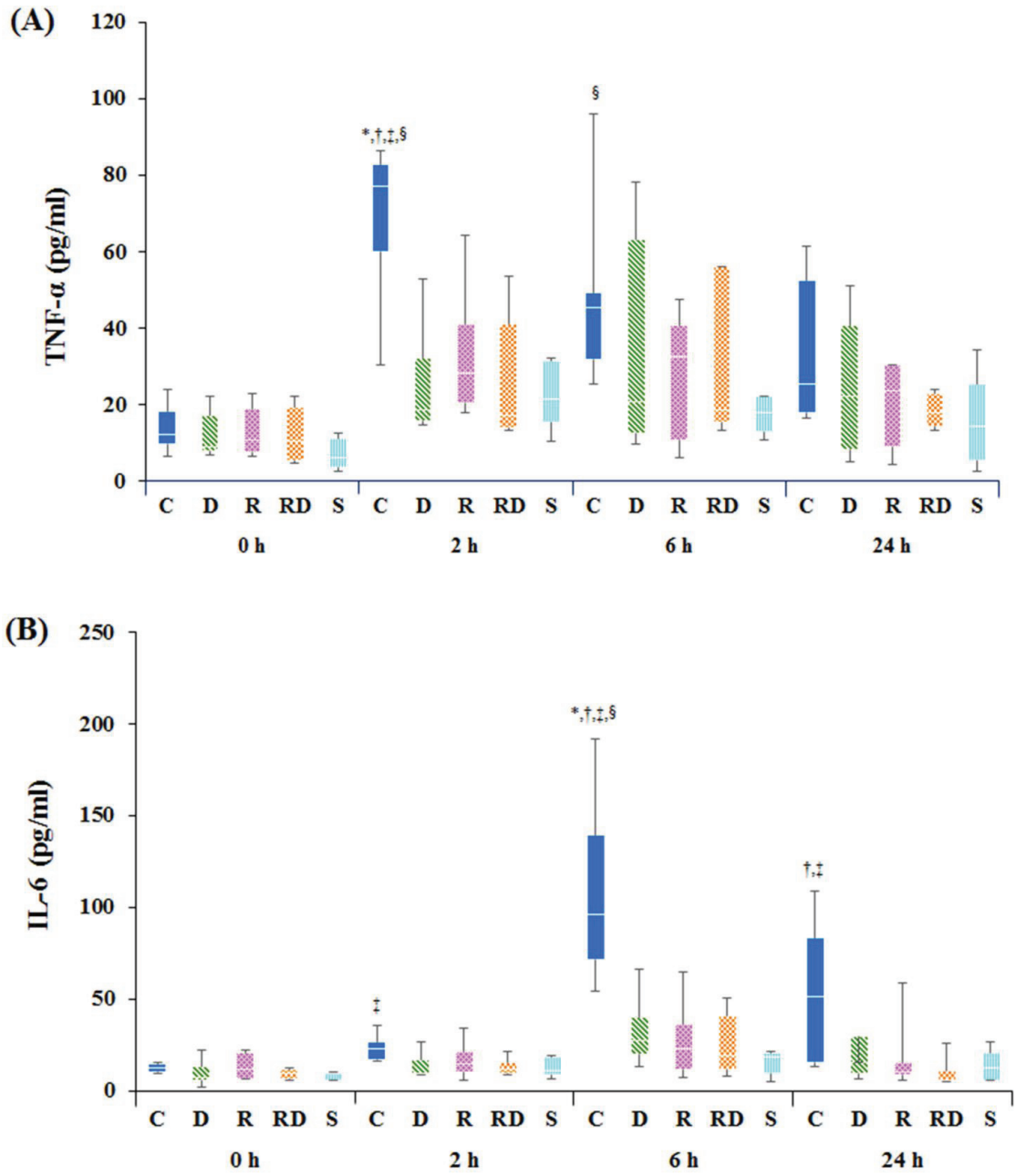


Figure 4(B) Densitometricevaluation of TLR-4, NF-κB, and caspase-3. Box plot indicates median, inter-quartile, and full ranges. ß-actin is used as a control for cytoplasmic proteins (TLR-4 and caspase-3) while Histone H3 for nuclear protein (NF-κB). KDa, Kilodalton. C, control; D, Dexmedetomidine; R, Resatorvid; RD, Resatorvid plus Dexmdetomidine; S, Sham.

*: P < 0.01 vs. group D, †: P < 0.01 vs. group R, ‡: P <0.01 vs. group RD, §: P < 0.01 vs. group S.


(A)
TNF-α (pg/ml)
120
100
80
60
40
20
0
*,†,‡,§
§
C D R RD S C D R RD S C D R RD S C D R RD S
0 h
2 h
6 h
24 h
(B)
IL-6 (pg/ml)
250
200
150
100
50
0
*,†,‡,§
‡
†,‡
C D R RD S C D R RD S C D R RD S C D R RD S
0 h
2 h
6 h
24 h

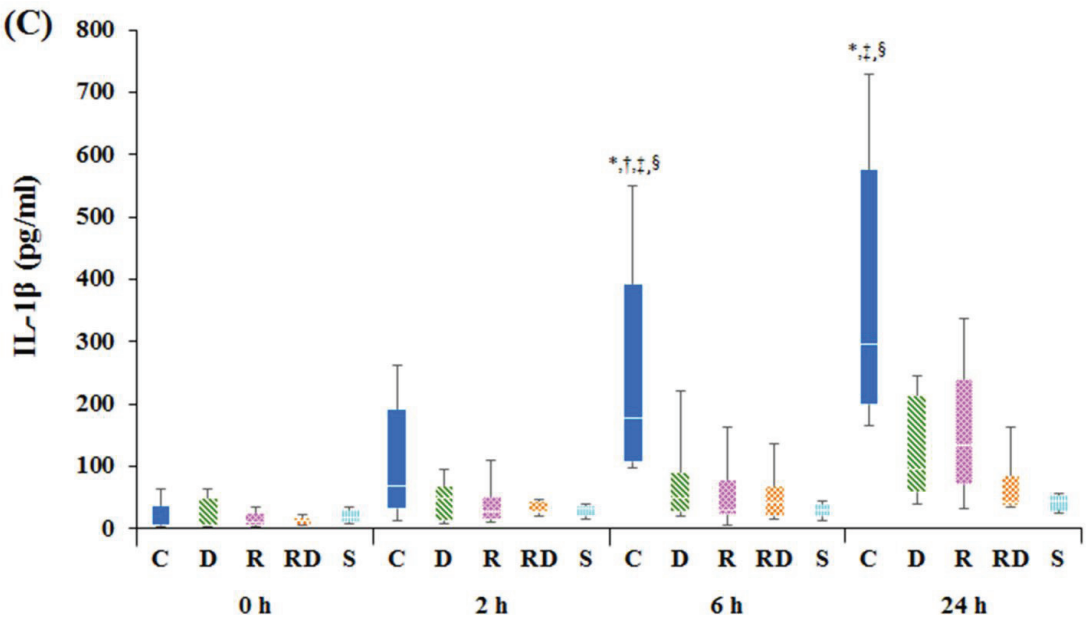


Figure 5 Serum levels of (A) Tumor necrosis factor-alpha (TNF-α), (B) Interleukin-6 (IL-6), and (C) Interleukin1 beta (IL-1β) are measured before and at 2, 6, and 24 h following transient global cerebral ischemia and assayed by ELISA. Box plot indicates median, inter-quartile, and full ranges. C, control; D, Dexmedetomidine; R, Resatorvid; RD, Resatorvid plus Dexmdetomidine; S, Sham.

*: $P < 0.01$ vs. group D, †: $P < 0.01$ vs. group R, ‡: $P < 0.01$ vs. group RD, §: $P < 0.01$ vs. group S.

Discussion

In this study, pre-ischemic DXM administration inhibited inflammation by decreasing TLR-4/NF-κB expression and production of pro-inflammatory cytokines, and reduced apoptosis by decreasing caspase-3 expression after transient global cerebral I/R injury in rats.

Inflammation is considered an important contributor to the pathophysiology of cerebral I/R injury and is known to exacerbate neuronal damage after such injury [4, 23]. TLRs are known as main agents of the innate immune response and their expression is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses [13, 14]. Specifically, TLR-4 regulates the brain response to stress and TLR-4 activation is known to be involved in brain damage and inflammation after cerebral I/R injury [24]. Previous studies demonstrated that the expression levels of TLR-4 on microglia, glial cells, neurons, and astrocytes were significantly increased after cerebral ischemic injury [15, 22, 24]. TLR-4 activates NF-κB signaling which encodes cytokines, chemokines, proteins of the complement system, various enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and matrix metalloproteinase-9 (MMP-9) [25]. In accordance with previous reports [15, 22, 24, 26],we found that the expression levels of TLR-4 and NF-κB were significantly higher in the control group than in the sham group, indicating that activation of the TLR-4/NF-κB pathway plays an important role in pathophysiology of global cerebral I/R injury.

In our study, TLR-4 expression was reduced in both resatorvid and DXM groups. Resatorvid, an exogenous synthetic antagonist for TLR-4, has been shown to disrupt the interaction of TLR-4 with its adaptor molecules, and thereby inhibit TLR-4 signal transduction and its downstream signaling events [27]. Various experimental investigations showed that resatorvid exerted neuroprotection by inhibiting TLR-4 activation and its downstream inflammatory signal transduction after cerebral injury, but not quantitatively suppressing the extent of TLR-4 expression [27, 28]. In this study, we did not take into account the exact molecular mechanisms by which resatorvid and

DXM decrease the extent of TLR-4 expression. Possible explanation for this phenomenon is that anti-inflammatory effects induced by both agents can affect the overall TLR-4 expression. More specifically, TLR-4 might be regulated by various mediators also activated by cerebral injury, such a heat shock proteins, lipopolysaccharides (LPS), and other damage-associated molecular patterns (DAMPs) [29]. The excitatory amino acid glutamate, which is rapidly released after cerebral ischemia, can regulate TLR-4 via and N-methyl-D-aspartic acid (NMDA)-dependent mechanism [30]. Widely known stress mediators such as epinephrine, norepinephrine, β_2 adrenergic receptor and corticotrophin releasing factor also can regulate TLR-4 expression [31, 32]. Such molecules may re-activate TLR-4 after cerebral ischemia like a vicious cycle. Indeed, previous studies have demonstrated the protective effects of DXM against cerebral ischemic injury via modulation of abovementioned mediators [33-36].

Besides resatorvid, various agents blocking TLR-4 have been known to reduce cerebral I/R injury. Ginkgolide B and progesterone also showed neuroprotection through inhibition of the TLR-4 signaling and reduced inflammation, although they are not specific TLR-4 blockers [37, 38]. This study also showed that the levels of TLR4/NF-κB in the brain was significantly lower in rats pre-treated with DXM after cerebral I/R injury compared to rats with no treatment. DXM has neuroprotective effects by inhibiting inflammation [8, 11].Previous studies demonstrated that suppression of pro-inflammatory cytokines production in the brain played a crucial role in DXM-induced neuroprotection after cerebral I/R injury [8, 11]. In this study, we investigated the role of TLR-4/NF-κB, not pro-inflammatory cytokines, in DXM-induced neuroprotection because pro-inflammatory cytokines resulting in brain damage are their downstream products. This study showed that DXM significantly reduced TLR-4/NF-κB expression and the extent of such decrease was similar in resatorvid treatment. Similar with this study, DXM was reported to exhibit renal and

pulmonary protection via anti-inflammatory effects mediated by inactivation of the TLR-4/NF-κB pathway [18, 39, 40].

In this study, the levels of serum pro-inflammatory cytokines were markedly increased after cerebral I/R injury and DXM significantly attenuated such an increase in the levels of serum pro-inflammatory cytokines. We speculated that the levels of serum pro-inflammatory cytokines might reflect the severity of inflammation in the brain. Cerebral I/R injury damages the cell membranes of neurons and glial cells, leading to the release of TNF-α, IL-6, and IL-1β (downstream products of the TLR-4/NF-B pathway) to the extracellular spaces. These cytokines enter the blood through the damaged blood-brain barrier (BBB). Therefore, the levels of serum pro-inflammatory cytokines can increase after cerebral I/R injury. Indeed, a previous study showed that the levels of pro-inflammatory cytokines were markedly increased in the brain tissue as well as in serum after cerebral ischemic and hypoxic injury, and that the levels of pro-inflammatory cytokines in serum were correlated with the severity of brain damage [41]. On the other hand, pro-inflammatory cytokines in serum may influence on TLR-4/NF-κB activity in the brain indirectly. Cerebral I/R injury results in an increase in the BBB permeability, allowing serum cytokines to reach the injured brain regions through the blood. At that sites, serum cytokines act as a powerful ligand of TLR-4 and reinforce TLR-4 and subsequent NF-κB activation. In addition, peripheral inflammatory cytokines upregulates cell adhesion molecules, which mediate the interaction between leukocytes and cerebral vascular endothelial cells, leading to infilatration of leukocytes into the brain parenchyme. Leukocytes promote the release of various inflammatory mediators and free radicals, which aggravate neuronal damage [4, 42, 43]. Taken together, our results indicate that anti-inflammation of DXM via inactivation of the TLR-4/NF-κB pathway is involved in DXM-induced neuroprotection although it is difficult to explain the exact mechanism of DXM-induced TLR-4 suppression.

Sequential activation of caspases, a family of proteases, plays a pivotal role in cellular apoptosis in the central nervous system. Apoptotic stimuli such as ischemic injury trigger activation of initiator caspases, and subsequently the caspase cascade, finally leading to apoptotic cell death [44, 45].Of the various subtypes of caspases, caspases-3 and -9 are the two principal caspases involved in neuronal cell death [44].Specifically, activated caspase-9 cleaves and activates caspase-3, triggering neuronal apoptosis. Many reports have shown that caspase-3 activity increases after cerebral I/R injury [21, 46].In this study, pretreatment with DXM markedly reduced neuronal apoptosis by inhibiting caspase-3 expression. Such reduction in apoptosis is another important mechanism by which DXM induces neuroprotection. DXM attenuates apoptosis by inhibiting activation of the intrinsic apoptotic cascade [46].

We found that DXM reduced TLR-4/NF-κB expression to an extent similar to resatorvid and DXM plus resatorvid. However, histopathologically, the numbers of necrotic and apoptotic cells were significantly fewer in the DXM plus resatorvid group than in either single-treatment group. One possible reason is that the dose of DXM or resatorvid given may have been insufficient to trigger maximal neuroprotection against cerebral I/R injury. Although the dosages of DXM and resatorvid used in this study were based on those of previous experimental studies [20, 27],their therapeutic ranges have not been fully investigated. Another possibility is that in addition to its anti-inflammatory action, DXM may confer neuroprotective effects via various other mechanisms including reducing calcium entry into cells, acting as an antioxidant, suppressing excitatory neurotransmitters, activating an extracellular signal-regulated kinase, activating the mitochondrial potassium ATP-dependent channel, and exerting anti-apoptotic effects [33, 47, 48]. Therefore, when DXM and resatorvid are given together, a synergistic neuroprotective effect would be expected.

In clinical field, 1.0 μg/kg of DXM intravenous infusion is given over 10 minutes and titrated to achieve the desired clinical effect (sedation) with a range of 0.5-1.0 μg/kg/h. A high dose (100

μg/kg) was administered in this study. However, common perception of estimation of dose based on the body weight alone is not the right approach between species. Larger animals required smaller drug dose on weight basis compared to smaller animals, because they usually have lower metabolic rates and slower physiological process [49]. Among the various methods of converting dose from animal to human, the US Food and Drug Administration (FDA)'s current guidance [50] recommends considering the sizes of individual species based on body surface area which is related to metabolic rate of an animal established through evolutionary adaption of animals to their size. In this guidance, human equivalent dose (HED), which means "a dose in human anticipated to provide the same degree of effects as that observed in animals at a given dose", can be determined by the following equation:

$$HED = \text{Animal dose} \times (\text{Animal } K_m / \text{Human } K_m)$$

K_m: correction factor estimated by dividing the average body weight (kg) of species to its body surface area (m^2)

Because the standard K_m value in rats is 6 and K_m for human is 37 according to the FDA's guidance, HED is approximately 16.2 μg/kg. Although it remains un-solving that how to convert the intraperitoneal dose to intravenous dose, it seems that much more dose may be needed for human to show neuroprotection of DXM compared to common clinical dose (1.0μg/kg) .

There were several limitations to this study. First, we did not measure neuroprotective effects afforded by different doses of DXM because we used a single dose of DXM. Second, neuroprotective effects of post-ischemic DXM were not explored, as it was given prior to cerebral ischemia. Because interventions are usually delivered after cerebral I/R injury, post-treatment dosing might be more clinically relevant. Third, we did not explore the long-term beneficial effects of DXM on TLR-4 mediated inflammation. Clinically, the inflammatory cascade can contribute to brain damage for several days after such injury [4]. Fourth, we did not perform neurological behavioral

tests. Although we found among-group histopathological differences, neurological data may have strengthened our results. Fifth, we did not measure the levels of pro-inflammatory cytokines in the brain tissue directly. However, a previous study reported that the changes the levels of pro-inflammatory cytokines in serum were similar to that in the brain after cerebral ischemic and hypoxic injury [41]. Finally, only TLR-4 of various TLRs was investigated. However, two studies found that TLR-2 plays an important role in the development of brain ischemic damage [51, 52]. Thus, further studies are needed to explore the effects of DXM on other types of TLRs.

In conclusion, DXM pretreatment afforded neuroprotection against transient global cerebral I/R injury in rats and inhibited the TLR-4/NF-κB pathway and production of pro-inflammatory cytokines. Our findings suggest that anti-inflammatory action of DXM mediated via inactivation of the TLR-4/NF-κB pathway, at least in part, may explain the DXM-induced neuroprotection evident after cerebral ischemia.

References

1. Montaner, J., A. Rovira, C.A. Molina, et al., Plasmatic level of neuroinflammatory markerspredict the extent of diffusion-weighted image lesions in hyperacute stroke. J Cereb Blood Flow Metab, 2003;23:1403-1407.

2. Rallidis, L.S., M. Vikelis, D.B. Panagiotakos, et al., Inflammatory markers and in-hospital mortality in acute ischaemic stroke. Atherosclerosis, 2006;189:193-197.

3. Smith, C.J., H.C. Emsley, C.M. Gavin, et al., Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. BMC Neurol, 2004;4:2.

4. Shah, I.M., I.M. Macrae, and M. Di Napoli, Neuroinflammation and neuroprotective strategies in acute ischaemic stroke - from bench to bedside. Curr Mol Med, 2009;9:336-354.

5. Simard, J.M., T.A. Kent, M. Chen, et al., Brain oedema in focal ischaemia: molecular pathophysiology and theoretical implications. Lancet Neurol, 2007;6:258-268.

6. Bhana, N., K.L. Goa, and K.J. McClellan, Dexmedetomidine. Drugs, 2000;59:263-268; discussion 269-270.

7. Ramsay, M.A. and D.L. Luterman, Dexmedetomidine as a total intravenous anesthetic agent. Anesthesiology, 2004;101:787-790.

8. Eser, O., H. Fidan, O. Sahin, et al., The influence of dexmedetomidine on ischemic rat hippocampus. Brain Res, 2008;1218:250-256.

9. Peng, M., Y.L. Wang, C.Y. Wang, and C. Chen, Dexmedetomidine attenuates lipopolysaccharide-induced proinflammatory response in primary microglia. J Surg Res, 2013;179:e219-225.

10. Kuhmonen, J., J. Pokorny, R. Miettinen, et al., Neuroprotective effects of dexmedetomidine in the gerbil hippocampus after transient global ischemia. Anesthesiology, 1997;87:371-377.

11. Tanabe, K., R. Matsushima-Nishiwaki, O. Kozawa, and H. Iida, Dexmedetomidine suppresses interleukin-1beta-induced interleukin-6 synthesis in rat glial cells. Int J Mol Med, 2014;34:1032-1038.

12. Ma, D., M. Hossain, N. Rajakumaraswamy, et al., Dexmedetomidine produces its neuroprotective effect via the alpha 2A-adrenoceptor subtype. Eur J Pharmacol, 2004;502:87-97.

13. O'Neill, L.A., How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol, 2006;18:3-9.

14. Vartanian, K.B. and M.P. Stenzel-Poore, Toll-Like Receptor Tolerance as a Mechanism for Neuroprotection. Translational Stroke Research, 2010;1:252-260.

15. Gao, Y., X. Fang, H. Sun, et al., Toll-like receptor 4-mediated myeloid differentiation factor 88-dependent signaling pathway is activated by cerebral ischemia-reperfusion in hippocampal CA1 region in mice. Biol Pharm Bull, 2009;32:1665-1671.

16. Jiang, L., L. Li, J. Shen, et al., Effect of dexmedetomidine on lung ischemiareperfusion injury. Mol Med Rep, 2014;9:419-426.

17. Wu, Y., Y. Liu, H. Huang, et al., Dexmedetomidine inhibits inflammatory reaction in lung tissues of septic rats by suppressing TLR4/NF-kappaB pathway. Mediators Inflamm, 2013;2013:562154.

18. Yao, H., X. Chi, Y. Jin, et al., Dexmedetomidine Inhibits TLR4/NF-kappaB Activation and Reduces Acute Kidney Injury after Orthotopic Autologous Liver Transplantation in Rats. Sci Rep, 2015;5:16849.

19. Jeon, Y.T., J.W. Hwang, Y.J. Lim, et al., Postischemic sevoflurane offers no additional neuroprotective benefit to preischemic dexmedetomidine. J Neurosurg Anesthesiol, 2013;25:184-190.

20. Wang, Y.C., P.F. Wang, H. Fang, et al., Toll-like receptor 4 antagonist attenuates intracerebral hemorrhage-induced brain injury. Stroke, 2013;44:2545-2552.

21. Lee, H., Y.H. Park, Y.T. Jeon, et al., Sevoflurane post-conditioning increases nuclear factor erythroid 2-related factor and haemoxygenase-1 expression via protein kinase C pathway in a rat model of transient global cerebral ischaemia. Br J Anaesth, 2015;114:307-318.

22. Zhu, L., T. Ye, Q. Tang, et al., Exercise Preconditioning Regulates the Toll-Like Receptor 4/Nuclear Factor-kappaB Signaling Pathway and Reduces Cerebral Ischemia/Reperfusion Inflammatory Injury: A Study in Rats. J Stroke Cerebrovasc Dis, 2016;25:2770-2779.

23. Doll, D.N., T.L. Barr, and J.W. Simpkins, Cytokines: their role in stroke and potential use as biomarkers and therapeutic targets. Aging Dis, 2014;5:294-306.

24. Caso, J.R., J.M. Pradillo, O. Hurtado, et al., Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation, 2007;115:1599-1608.

25. del Zoppo, G., I. Ginis, J.M. Hallenbeck, et al., Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. Brain Pathol, 2000;10:95-112.

26. Ridder, D.A. and M. Schwaninger, NF-kappaB signaling in cerebral ischemia. Neuroscience, 2009;158:995-1006.

27. Hua, F., H. Tang, J. Wang, et al., TAK-242, an antagonist for Toll-like receptor 4, protects against acute cerebral ischemia/reperfusion injury in mice. J Cereb Blood Flow Metab, 2015;35:536-542.

28. Garate, I., B. Garcia-Bueno, J.L. Madrigal, et al., Toll-like 4 receptor inhibitor TAK-242 decreases neuroinflammation in rat brain frontal cortex after stress. J Neuroinflammation, 2014;11:8.

29. Tsan, M.F. and B. Gao, Endogenous ligands of Toll-like receptors. J Leukoc Biol, 2004;76:514-519.

30. Glezer, I., H. Zekki, C. Scavone, and S. Rivest, Modulation of the innate immune response by NMDA receptors has neuropathological consequences. J Neurosci, 2003;23:11094-11103.

31. Kizaki, T., T. Izawa, T. Sakurai, et al., Beta2-adrenergic receptor regulates Toll-like receptor-4-induced nuclear factor-kappaB activation through beta-arrestin 2. Immunology, 2008;124:348-356.

32. Chaniotou, Z., P. Giannogonas, S. Theoharis, et al., Corticotropin-releasing factor regulates TLR4 expression in the colon and protects mice from colitis. Gastroenterology, 2010;139:2083-2092.

33. Engelhard, K., C. Werner, E. Eberspacher, et al., The effect of the alpha 2-agonist dexmedetomidine and the N-methyl-D-aspartate antagonist S(+)-ketamine on the expression of apoptosis-regulating proteins after incomplete cerebral ischemia and reperfusion in rats. Anesth Analg, 2003;96:524-531, table of contents.

34. Cai, Y., H. Xu, J. Yan, et al., Molecular targets and mechanism of action of dexmedetomidine in treatment of ischemia/reperfusion injury. Mol Med Rep, 2014;9:1542-1550.

35. Matsumoto, M., M.H. Zornow, B.C. Rabin, and M. Maze, The alpha 2 adrenergic agonist, dexmedetomidine, selectively attenuates ischemia-induced increases in striatal norepinephrine concentrations. Brain Res, 1993;627:325-329.

36. Chiu, K.M., T.Y. Lin, C.W. Lu, and S.J. Wang, Inhibitory effect of glutamate release from rat cerebrocortical nerve terminals by alpha2 adrenoceptor agonist dexmedetomidine. Eur J Pharmacol, 2011;670:137-147.

37. Yu, W.H., X.Q. Dong, Y.Y. Hu, et al., Ginkgolide B reduces neuronal cell apoptosis in the traumatic rat brain: possible involvement of toll-like receptor 4 and nuclear factor kappa B pathway. Phytother Res, 2012;26:1838-1844.

38. Wang, Z., G. Zuo, X.Y. Shi, et al., Progesterone administration modulates cortical TLR4/NF-kappaB signaling pathway after subarachnoid hemorrhage in male rats. Mediators Inflamm, 2011;2011:848309.

39. Chi, X., X. Wei, W. Gao, et al., Dexmedetomidine ameliorates acute lung injury following orthotopic autologous liver transplantation in rats probably by inhibiting Toll-like receptor 4-nuclear factor kappa B signaling. J Transl Med, 2015;13:190.

40. Gu, J., P. Sun, H. Zhao, et al., Dexmedetomidine provides renoprotection against ischemia-reperfusion injury in mice. Crit Care, 2011;15:R153.

41. Li, S.J., W. Liu, J.L. Wang, et al., The role of TNF-alpha, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy. Eur Rev Med Pharmacol Sci, 2014;18:905-909.

42. Amantea, D., M. Certo, R. Russo, et al., Early reperfusion injury is associated to MMP2 and IL-1beta elevation in cortical neurons of rats subjected to middle cerebral artery occlusion. Neuroscience, 2014;277:755-763.

43. Tuttolomondo, A., D. Di Raimondo, R. di Sciacca, et al., Inflammatory cytokines in acute ischemic stroke. Curr Pharm Des, 2008;14:3574-3589.

44. Yuan, J. and B.A. Yankner, Apoptosis in the nervous system. Nature, 2000;407:802-809.

45. Namura, S., J. Zhu, K. Fink, et al., Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. J Neurosci, 1998;18:3659-3668.

46. Tanaka, H., H. Yokota, T. Jover, et al., Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. J Neurosci, 2004;24:2750-2759.

47. Wang, Y., R. Han, and Z. Zuo, Dexmedetomidine post-treatment induces neuroprotection via activation of extracellular signal-regulated kinase in rats with subarachnoid haemorrhage. Br J Anaesth, 2016;116:384-392.

48. Huang, R., Y. Chen, A.C. Yu, and L. Hertz, Dexmedetomidine-induced stimulation of glutamine oxidation in astrocytes: a possible mechanism for its neuroprotective activity. J Cereb Blood Flow Metab, 2000;20:895-898.

49. Nair, A.B. and S. Jacob, A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm, 2016;7:27-31.

50. Rockville, M. Guidance for Industry: Estimating the Maximum Safe Starting Dose in Adult Healthy Volunteer. 2005; Available from:https://www.fda.gov/downloads/drugs/guidances/ucm078932.pdf.

51. Ziegler, G., D. Harhausen, C. Schepers, et al., TLR2 has a detrimental role in mouse transient focal cerebral ischemia. Biochem Biophys Res Commun, 2007;359:574-579.

52. Lehnardt, S., S. Lehmann, D. Kaul, et al., Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. J Neuroimmunol, 2007;190:28-33.

국문초록

목적: Dexmedetomidine의 항염증 효과는 뇌의 허혈/재관류 손상에 대한 신경보호에서 중요한 역할을 하고 있다. 본 연구는 쥐의 일시적 전뇌허혈/재관류 손상모델에서 Dexmedetmidine의 항염증을 통한 뇌보호 효과와 Toll like receptor (TLR) -4/ Nuclear factor kappa B (NF-κB) 경로와의 연관성에 대하여 알아보고자 하였다.

방법: 50 마리의 쥐를 무작위로 5 군으로 나누었다: 겉보기수술군 (group S, n=10), 전뇌허혈군 (group C, 10분간 전뇌허혈, n=10), Dexmedetomidine 전처치군 (group D, 허혈 30분전 복강내 100 μg/kg의 Dexmedetomidine 투여, n=10), Resatorvid 전처치군 (group R, 허혈 30분전 복강내 3mg/kg의 선택적 TLR-4 억제제 Resatorvid 투여, n =10), Resatorvid 및 Dexmedetomidine 전처치군 (group RD, n=10). 허혈 하루 후 세포자멸수와 괴사수, TLR-4, NF-κB, caspase-3의 단백질 발현 정도를 평가하였다. 허혈 전과 허혈 후 2시간, 6시간, 24시간째에 혈장내의 tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6)의 수치를 측정하였다.

결과: 괴사세포수와 자멸세포수는 전뇌허혈군에서 다른 군들에 비해 유의하게 낮게 관찰되었다. TLR-4, NF-κB, caspase의 발현 역시 전뇌허혈군에서 다른 군들에 비해 높게 계측되었다. TNF-α는 허혈 후 2시간째에 전뇌허혈군에서 다른 군들에 비해 높았고, IL-6는 허혈 후 6시간, IL-1는 허혈후 6시간째와 24시간째 다른 군들에 비해 높게 측정되었다.

결론: 쥐모델을 이용한 일시적 전뇌허혈/관류손상에서 dexmedetomidine 전처치는 TLR-4/NK-κB 경로를 불활성화시켜 항염증 효과를 보였다. 상기 결과는 뇌허혈로 인한 손상과정에서 dexmdetomidine이 나타내는 뇌보호효과의 기전과 관련될 수 있을 것이다.

주요어: 뇌보호 효과; 염증; Dexmdedetomidine; TLR-4; NF-κB; 뇌허혈

학번: 2014-30907