European Review for Medical and Pharmacological Sciences 2018; 22: 6507-6516

Dexmedetomidine protects liver cell line L-02 from oxygen-glucose deprivation-induced injury by down-regulation of microRNA-711

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Abstract. – OBJECTIVE: In liver transplantation, long-time portal vein blocking causes the occurrence of ischemic liver injury. Dexmedetomidine, a widely admired anesthetic, has been reported as a protective agent on organs under ischemic condition. The objective of this study was to reveal the role and underlying mechanism of dexmedetomidine in ischemic liver injury.

MATERIALS AND METHODS: L-02 cells were treated with dexmedetomidine during 6 h of oxygen-glucose deprivation (OGD) exposure. The expression of microRNA-711 (miR-711) in cell was overexpressed by miRNA transfection. Then, the following parameters were observed: cell viability, apoptosis, the expression of apoptosis-related proteins, and the expression and the release of Interleukin 1 beta (IL-1β) and Tumor necrosis factor alpha (TNF-α).

RESULTS: Apoptosis and inflammation were induced following OGD exposure in L-02 cells, as cell viability was impaired, apoptotic cell rate was increased, caspase-3, and caspase-9 was cleaved, and the expression and release of TNF-α and IL-1β were increased. Dexmedetomidine attenuated OGD-induced apoptosis and inflammation, and dexmedetomidine down-regulated the expression of miR-711. Also, dexmedetomidine blocked the activation of p38 mitogen-activated protein kinase (p38MAPK) and Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) signaling upon OGD. Moreover, when miR-711 was overexpressed, dexmedetomidine did not protect L-02 cells against OGD, and did not block p38MAPK and JAK/STAT signaling pathways.

CONCLUSIONS: Dexmedetomidine ameliorated OGD-induced cell apoptosis and inflammation in L-02 cells, exerting protective activities in ischemic liver injury. The anti-OGD effects of dexmedetomidine might be realized by down-regulation of miR-711 and suppression of p38MAPK and JAK/STAT signaling pathways.

Key Words:
Dexmedetomidine, Oxygen-glucose deprivation (OGD), L-02 cell, miR-711, Ischemic injury.

Introduction

Ischemic injury is frequently occurs following hemorrhagic shock, surgical resection, and solid organ transplant[1]. A number of cells can be saved if the blood supply restored rapidly. But inappropriate reperfusion will further impair organ function, as the excessive free radicals and pro-inflammatory cytokines attack the cells post-reperfusion[2]. Ischemic condition has become a serious obstacle in liver transplantation[3]. Investigation of novel, safe and effective ways for liver cell protection will largely improve patient survival during hepatectomy.

Dexmedetomidine, a selective α2 adrenoceptor (α2-AR) agonist, can inhibit the neuronal discharge by stimulating α2-AR in brain and spinal cord. It has been introduced to anaesthesia practice[4] as it produces dose-dependent sedation, anxiolysis, and analgesia without respiratory depression[5,6]. Additionally, it appears to be protective in a broad range of organs, including lung, heart, kidney, spinal, brain, and liver. An *in vivo*

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study found that dexmedetomidine was associated with the reduced infarct size in ischemia/reperfusion injury in rat[7]. Dexmedetomidine has also been reported to protect patients with acute kidney injury from experiencing further tissue damage[8]. Regarding the liver, sporadic studies[9-11] have revealed the protective potentials of dexmedetomidine against hepatic lipid peroxidation and histological damage in sepsis and in ischemia-reperfusion animal model. But the mechanism of the protective action is still unclear.

An increasing number of investigations[12,13] have indicated that microRNAs (miRNAs) act as key modulators in the process of ischemic injury. miR-711 was highly expressed in response to ischemia-reperfusion injury in cardiomyocytes, and it promoted apoptosis of cardiomyocytes via transportation of the nuclear factor kappa B (NF-κB) into the nucleus[14]. Another research[15] has concerned miR-711 as an anti-fibrotic gene in myocardial infarction. Additionally, changes in miR-711 have been reported after liver injury and inflammation. miR-711 was elevated in liver tissues but exhibited lower level in plasma samples in acetaminophen-induced liver injury[16]. Thus, we are interested to investigate whether miR-711 was involved in the pathogenesis of ischemic liver injury.

Oxygen-glucose deprivation (OGD) is one of the critical pathological processes, which is involved in a series of ischaemic diseases. In this study, human liver cell line L-02 was subjected with OGD to mimic an *in vitro* model of ischemic liver injury. Thereafter, the effects of dexmedetomidine on OGD-injured liver cells were monitored. The regulatory role of dexmedetomidine treatment in the expression of miR-711 was tested to explore the underlying mechanism of which dexmedetomidine protected liver cells against OGD. The findings of this study will highlight the potential of dexmedetomidine application in the protection of liver during hepatectomy.

Materials and Methods

Cell Culture

Human liver cell line L-02 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI)-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2.5 g/L glucose (Sigma-Aldrich, Basel, Switzerland), 0.11 g/L sodium pyruvate (Sigma-Aldrich, Basel, Switzerland), and 15% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA). The cells were maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Study Design

For testing the effect of dexmedetomidine on OGD-induced injury, L-02 cells were allocated into three groups. 1) OGD group, culture medium was replaced by a glucose-free RPMI-1640 medium, and the cells were cultured under anaerobic condition (5% CO_2 and 95% N_2) at 37°C for 6 h. 2) Control group, cells were cultured in complete medium under normoxia. 3) OGD + DEXM group, cells were treated with 5 μM dexmedetomidine (TargetMol, Boston, MA, USA) during OGD stimulation.

miRNAs Transfection

miR-711 mimic and the negative control (NC) were synthesized by GenePharma Co. (Shanghai, China) and were transfected into L-02 cells by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The sequences for miR-711 mimic were as follows: sense, 5'-GGGACCCAGGGAGAGACGUAAG-3', anti-sense, 5'-UACGUCUCUCCCUGGGUCCCUU-3'. The sequences for the scrambled NC were 5'-UCACAACCUCCUAGAAAGAGUAGA-3'. The final concentrations of miR-711 mimic and NC were 50 and 100 nM, respectively. At 48 h of transfection, cells were collected and the transfection efficiency was verified by the quantitative real-time polymerase chain reaction (qRT-PCR).

CCK-8 Assay

L-02 cells were seeded in 96-well plates with a density of 5×10^3 cells/well. After treatment, the cells were washed twice with phosphate buffer saline (PBS; Beyotime, Shanghai, China). 10 μL Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kyushu, Japan) solution was added into each well, and the plates were allowed for culturing at 37°C for 4 h. The absorbance was then measured by a Microplate Reader (Bio-Rad, Hercules, CA, USA) under 450 nm.

Apoptosis Assay

After treatment, cells (1×10^5 per sample) were collected and stained by Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) according to the manufacturer's protocol. The stained cells were then analyzed by a flow cytometer (Beckman Coulter,

Fullerton, CA, USA). The percentage of early apoptotic cells (FITC-positive and PI-negative) were calculated by FlowJo software (Tree Star, San Carlos, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA) Assay

After treatment, cell culture supernatant was collected for testing the concentrations of tumor necrosis factor-alpha (TNF-α) and Interleukin-1 beta (IL-1β) by using human ELISA kits (ab181421 and ab100562, Abcam, Cambridge, MA, USA).

qRT-PCR

Total RNAs were prepared using TRI Reagent (Sigma-Aldrich, Basel, Switzerland). The cDNAs were synthetized by PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). qRT-qPCR was conducted using SYBR Green detection system (iQ™ SYBR® Green Supermix, Bio-Rad, Hercules, CA, USA). The expressions of TNF-α and IL-1β were normalized to the β-actin. For testing the expression changes of miR-711, TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used for reverse transcription. qRT-PCR was performed by using TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA). U6 acted as an internal control in this system. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primary sequences were listed as follows. IL-1β: forward 5'-CAGCTACGAATCTCCGACCAC-3', reverse 5'-GGCAGGGAACCAGCATCTTC-3'; TNF-α: forward 5'-ATGTGCTCCTCACCCACACC-3', reverse 5'-GTCGGTCACCCTTCTCCAGCT-3; β-actin: forward 5'-AAGAGAGGCATCCTCACCCT-3', reverse 5'-TACATGGCTGGGGTGTTGAA-3'; miR-711: forward 5'-ACACTCCAGCTGGGGGGACCCAGGGAGAGA-3', reverse 5'-TGGTGTCGTGGAGTCG-3'; U6: forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Western Blot

Cellular protein was extracted by using RIPA lysis buffer (Beyotime, Shanghai, China). The BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used for testing the concentration of protein in whole-cell extract. An equal amount of protein was resolved over sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After blocking in 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C for detection of caspase-3 (ab32351), caspase-9 (ab32539), TNF-α (ab1793), IL-1β (ab200478), p38 mitogen-activated protein kinase (p38MAPK; ab170099), phospho (p)-p38MAPK (ab47363), Janus kinase 1 (JAK1; ab133666), p-JAK1 (ab215338), Signal transducer and activator of transcription protein 3 (STAT3; ab119352), p-STAT3 (ab76315), and β-actin (ab8227). All antibodies used in Western blotting were purchased from Abcam (Cambridge, MA, USA). The membranes were then incubated with the secondary antibody horseradish peroxidase conjugate for 1 h at room temperature. The positive signals were visualized by enhanced chemiluminescence (ECL) method, and the intensity of the signals was tested by Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Data were expressed as mean ± Standard Deviation (SD) from three independent experiments. Statistical analyses were performed in SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) using a one-way analysis of variance (ANOVA) followed by Duncan post-hoc test. A result with p-value < 0.05 was considered as statistical significant.

Results

OGD Induces Apoptosis and Release of Pro-Inflammatory Cytokines in L-02 Cells

To start with, L-02 cells were exposed to OGD for 0-12 h, and cell viability was monitored at each time period. As shown in Figure 1A, 2 h of OGD exposure induced a slight increase in cell viability, and the viability begun to decrease significantly at 4 h ($p < 0.05$). Since the viability was significantly reduced by 47.49% at 6 h of OGD, 6 h was selected as an OGD-stimulating condition for use in the next steps.

The impacts of 6 h of OGD on the apoptosis of L-02 cells were evaluated by detection of apoptotic cell rate and the expression changes of apoptosis-related factors. Figure 1B showed that the apoptotic cell rate in OGD group was significantly higher than that in the control group ($p < 0.05$). Figure 1C showed that both caspase-3 and caspase-9 were remarkably cleaved in OGD group compared to control group.

The impacts of OGD on the release of pro-inflammatory cytokines were tested. As data given in Figure 1D-1F, the mRNA ($p < 0.05$) and

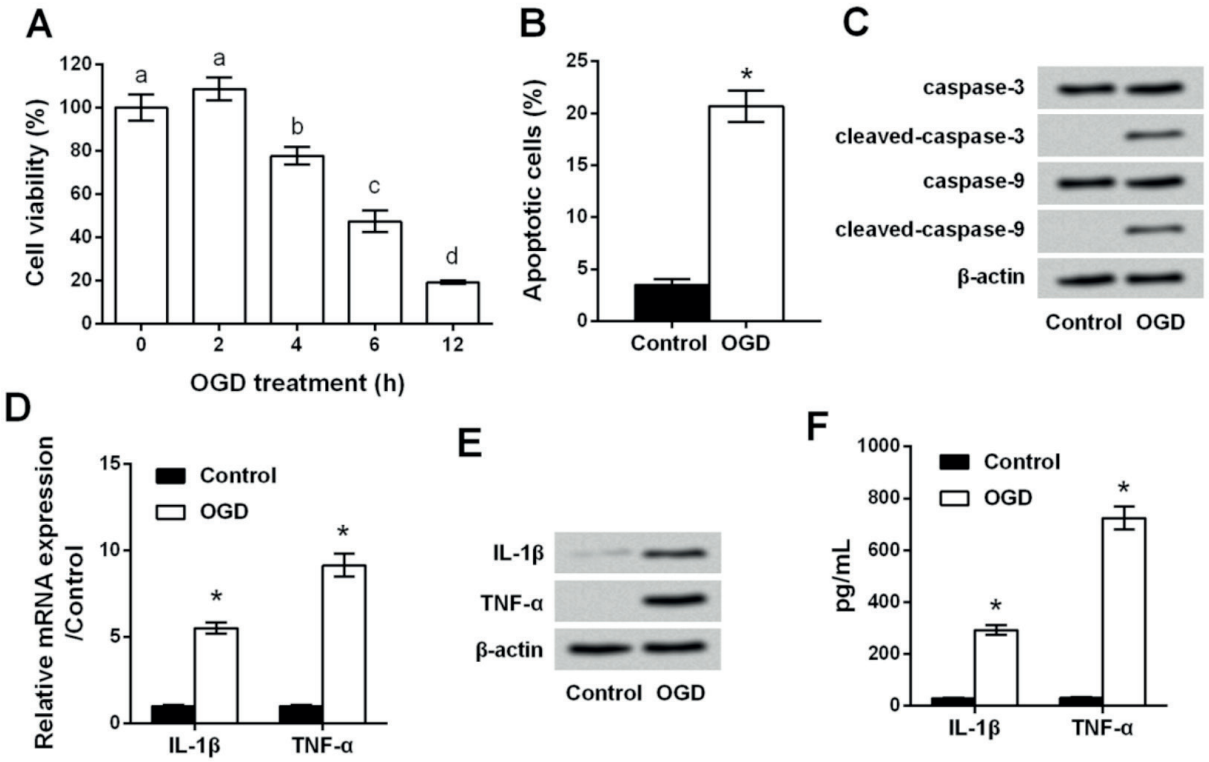

Figure 1. Effect of oxygen-glucose deprivation (OGD) on L-02 cells. ***A,*** Viability of L-02 cells after 0-12 h of OGD exposure. Different letters above the columns in the same phase indicate that the means of different groups were significantly different (ANOVA with Duncan procedure). ***B,*** Apoptotic cell rate. ***C,*** Protein expressions of apoptosis-related factors. ***D,*** mRNA levels of TNF-α and IL-1β. ***E,*** Protein levels of TNF-α and IL-1β. ***F,*** Concentrations of TNF-α and IL-1β in culture supernatant were tested, after 6 h of OGD exposure. *$p < 0.05$ *vs.* control group (ANOVA). Data represented as mean ± SD (n = 3).

protein levels of IL-1β and TNF-α, as well as the concentrations of IL-1β and TNF-α in the culture supernatant ($p < 0.05$) were all increased by OGD exposure.

Dexmedetomidine Ameliorates OGD-Induced Injury in L-02 Cells

L-02 cells were treated with the gradient of concentration of dexmedetomidine to test the cytotoxicity of dexmedetomidine on cell. As a result, 10 μM dexmedetomidine resulted in a significant decrease of cell viability ($p < 0.05$, Figure 2A). No significant change of viability was observed in cell treated with doses less than or equal to 5 μM. Then, L-02 cells were treated with 5 μM dexmedetomidine throughout the procedure of OGD stimulation. We found that viability was increased ($p < 0.05$, Figure 2B), apoptotic cell rate was decreased ($p < 0.05$, Figure 2C), and protein expressions of cleaved caspase-3 and caspase-9 were down-regulated (Figure 2D) in OGD + DEXM group than those in OGD group. Besides, the mRNA ($p < 0.05$, Figure 2E) and protein (Figure 2F) levels of IL-1β and TNF-α, as well as the concentrations of IL-1β and TNF-α in the culture supernatant ($p < 0.05$, Figure 2G) were remarkably decreased in OGD + DEXM group than those in OGD group.

Dexmedetomidine Down-Regulated the Expression of miR-711

L-02 cells were treated with various doses of dexmedetomidine for 6 h, after which the RNA level expressions of miR-711 was tested by qRT-PCR. Figure 3 indicated that the expressions of miR-711 were not significantly changed by dexmedetomidine with doses less than 1 μM. But, 5 μM of dexmedetomidine significantly reduced the expression of miR-711 ($p < 0.05$).

Dexmedetomidine Ameliorates OGD-Injured L-02 Cells Through Down-Regulation of miR-711

The expression of miR-711 in L-02 cells was then overexpressed by mimic transfection to see whether miR-711 was involved in the protective

actions of dexmedetomidine. As shown in Figure 4A, the expression of miR-711 was significantly overexpressed by mimic transfection ($p < 0.05$). Figure 4B-4G showed that the protection of dexmedetomidine on OGD-injured L-02 cells was ameliorated by miR-711 overexpression. That cell viability was reduced ($p < 0.05$), apoptotic cell rate was increased ($p < 0.05$), cleavage of caspase-3 and caspase-9 was promoted, as well as the expressions and release of IL-1β and TNF-α ($p < 0.05$) were elevated in miR-711-overexpressing cell.

Dexmedetomidine Blocks p38MAPK and JAK1/STAT3 Signaling Pathways

Furthermore, we detected the expression changes of core factors in p38MAPK and JAK1/STAT3 signaling pathways. We found that OGD significantly up-regulated the protein expressions of p-p38MAPK, p-JAK1, and p-STAT3 ($p < 0.05$, Figure 5A-B), and dexmedetomidine significantly down-regulated the expressions of them ($p < 0.05$). Interestingly, dexmedetomidine-induced down-regulations were attenuated by miR-711 overexpression ($p < 0.05$).

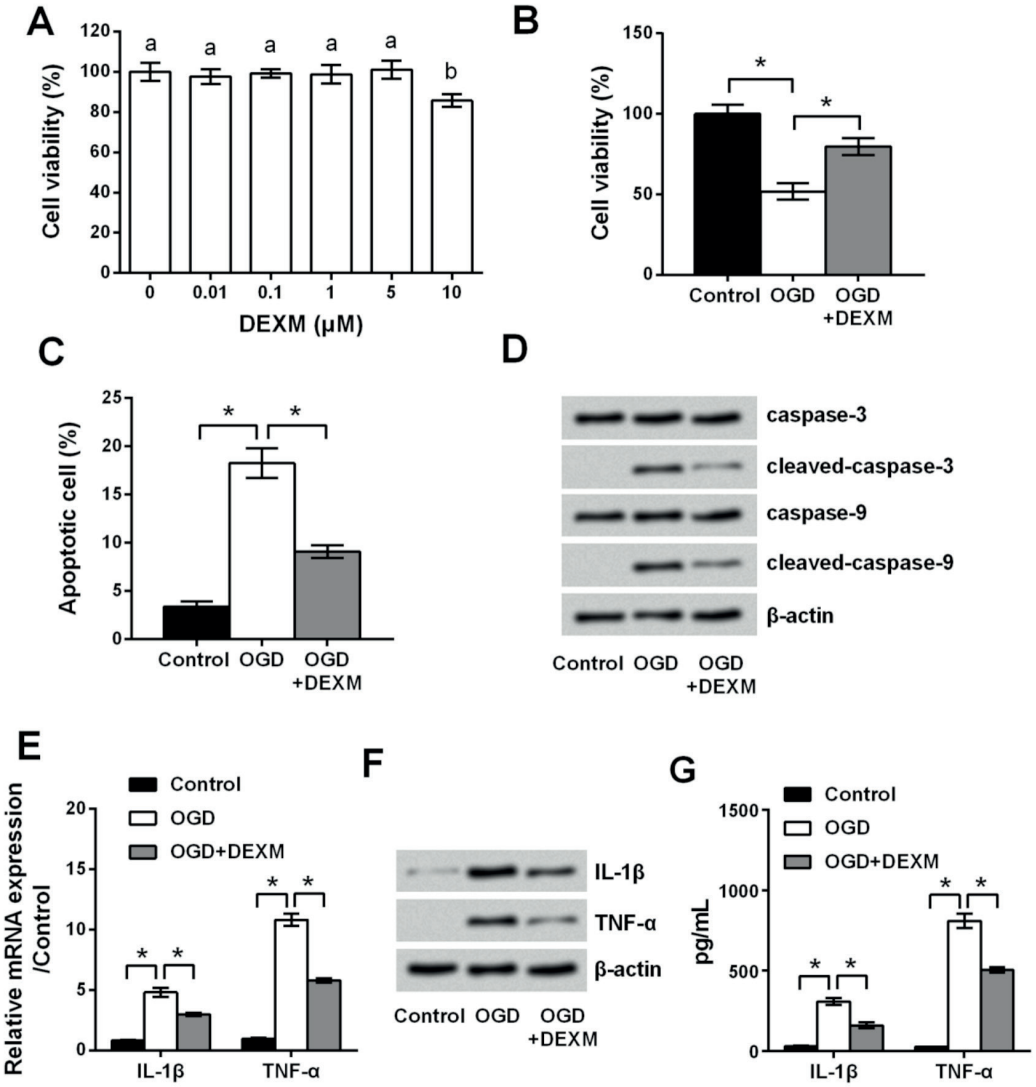

Figure 2. Effect of dexmedetomidine (DEXM) on oxygen-glucose deprivation (OGD)-injured L-02 cells. ***A,*** Viability of L-02 cells after 0~10 μM of DEXM treatment. Different letters above the columns in the same phase indicate that the means of different groups were significantly different (ANOVA with Duncan procedure). ***B,*** Viability. ***C,*** Apoptotic cell rate. ***D,*** Protein expressions of apoptosis-related factors. ***E,*** mRNA levels of TNF-α and IL-1β. ***F,*** Protein levels of TNF-α and IL-1β. ***G,*** Concentrations of TNF-α and IL-1β in culture supernatant were tested, after 6 h of OGD exposure with or without DEXM treatment. * $p < 0.05$ *vs.* the indicated group (ANOVA). Data represented as mean ± SD (n = 3).

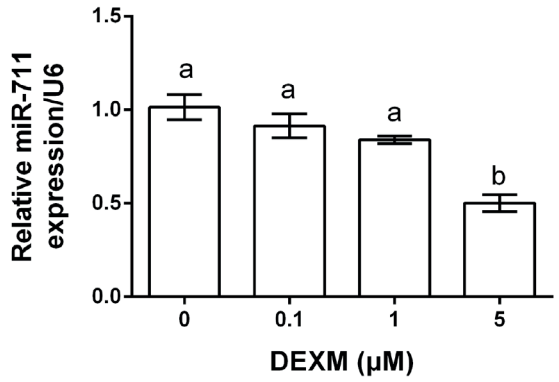

Figure 3. Effect of dexmedetomidine (DEXM) on the expression of miR-711. RNA level expressions of miR-711 were tested after 0-5 μM of DEXM treatment. Different letters above the columns in the same phase indicate that the means of different groups were significantly different (ANOVA with Duncan procedure). Data represented as mean ± SD (n = 3).

Discussion

In liver transplantation, long-time portal vein blocking can cause the occurrence of ischemic liver injury. Recently, multiple investigations have evidenced dexmedetomidine, a widely admired anesthetic, has protective effect on organs under ischemic condition. In the current study, *in vitro* data suggested the protective role of dexmedetomidine in OGD-injured liver cells. Dexmedetomidine attenuated OGD-induced apoptosis and inflammation in L-02 cells. miR-711 was low expressed in response to dexmedetomidine treatment. Besides, miR-711, and p38MAPK and JAK1/STAT3 signaling pathways might be implicated in the protective actions of dexmedetomidine.

Dexmedetomidine is used for anaesthesia and sedation in intensive care units (ICU) and in operating rooms prior to or during surgical procedures[6]. Accumulating evidence[7,17,18] has shown that dexmedetomidine plays protective roles in organs during many pathophysiological processes, especially during ischemia/reperfusion injury. Apoptosis is the major pathway of cell death following ischemic injury[19]. Preconditioning and postconditioning application of dexmedetomidine has been reported to reduce OGD-induced neuronal death via deactivation of caspase-3 (an executioner of apoptosis)[20,21], suggesting dexmedetomidine as a therapeutic agent to prevent brain damage induced by ischemia/reperfusion[22]. In addition to apoptosis, inflammation is another critical event during ischemic injury[23]. Dexmedetomidine protected organs against ischemia/reperfusion injury partially by suppressing inflammatory reaction. After ischemia, dexmedetomidine significantly reduced pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6[24,25], and thus conferred protection in neuron and liver. In the present study, apoptosis and inflammation were induced following OGD exposure in L-02 cells, as cell viability was impaired, apoptotic cell rate was increased, caspase-3 and caspase-9 was cleaved, and the expression and release of TNF-α and IL-1β were increased. Dexmedetomidine attenuated OGD-induced apoptosis and inflammation, indicating a protective role of dexmedetomidine in ischemic liver injury. This observation was consistent with previous studies[11,26,27], in which the liver protective effects of dexmedetomidine *in vivo* and *in vitro* have been reported.

miRNAs regulate approximately 60% of genes on the post-transcriptional level and play important roles in ischemia/reperfusion injury[12,13]. The role of miRNAs in response to ischemic stimuli is coming to light themselves as targets for liver protection and restoration following ischemia[28,29]. Huang et al[30] *in vitro* revealed that dexmedetomidine protected OGD-induced neuronal injury via down-regulation of miR-29b. Dexmedetomidine was also reported[31] to protect PC12 cells from lidocaine-induced cytotoxicity through up-regulation of miR-let-7b. In the present study, we for the first time pointed out that dexmedetomidine protected liver cells against OGD-induced injury via down-regulation of miR-711. This finding indicated that dexmedetomidine administration in combination with miRNA changes, and these miRNAs might be pivotal executors of dexmedetomidine's protective effects.

The liver protective mechanism of dexmedetomidine is still not clear. But recent studies have demonstrated the protection might be attributable to the enhancement of Nuclear factor, erythroid 2 like 2 (Nrf2) pathway and the suppression of MAPK, Caspase-3/Poly (ADP-Ribose) polymerase (PARP)[24], and Toll-like receptor 4 (TLR4)/NF-κB[26] pathways. Herein, we explored the effects of dexmedetomidine on the activation of p38MAPK and JAK/STAT signaling pathways to reveal whether the liver protective effects of dexmedetomidine were realized by modulation of these two signaling.

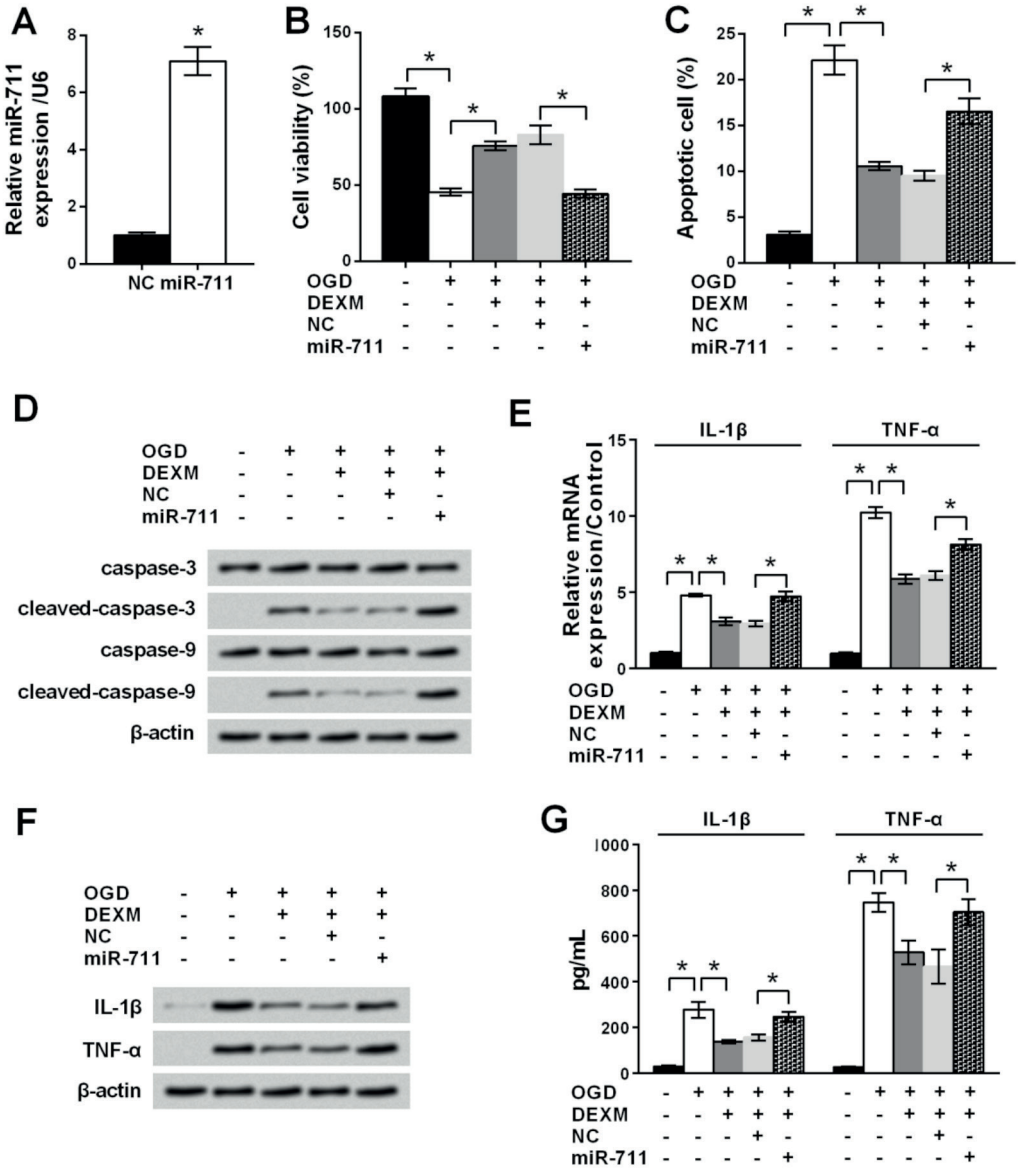

Figure 4. Effect of miR-711 overexpression on dexmedetomidine (DEXM)-mediated apoptosis and inflammation upon oxygen-glucose deprivation (OGD). ***A,*** RNA level expressions of miR-711 in L-02 cells, after transfection with miR-711 mimic (miR-711 group) or the negative control (NC group). ***B,*** Viability. ***C,*** Apoptotic cell rate. ***D,*** Protein expressions of apoptosis-related factors. ***E,*** mRNA levels of TNF-α and IL-1β. ***F,*** Protein levels of TNF-α and IL-1β. ***G,*** Concentrations of TNF-α and IL-1β in culture supernatant were tested in miR-711-transfected or NC-transfected cells. After transfection, the cells were treated with or without DEXM upon OGD exposure for 6 h. *$p < 0.05$ *vs.* the indicated group (ANOVA). Data represented as mean ± SD (n = 3).

p38MAPK and JAK/STAT signaling pathways were chosen to be investigated because they were previously documented to be associated with ischemic liver injury[32,33]. Herein, we demonstrated that p38MAPK and JAK/STAT signaling pathways were significantly activated by OGD, which were in line with previous studies[34,35]. OGD-induced activation of p38MAPK and JAK/STAT signaling pathways were eliminated by dexmedetomidine. This phenomenon was in line with a previous study[36] performed in rat kidney which was injured by ischemia/reperfusion. Moreover, dexmedetomidine did not eliminate the activation of these two signaling when miR-711 was overexpressed. Altogether, these data suggested that dexmede-

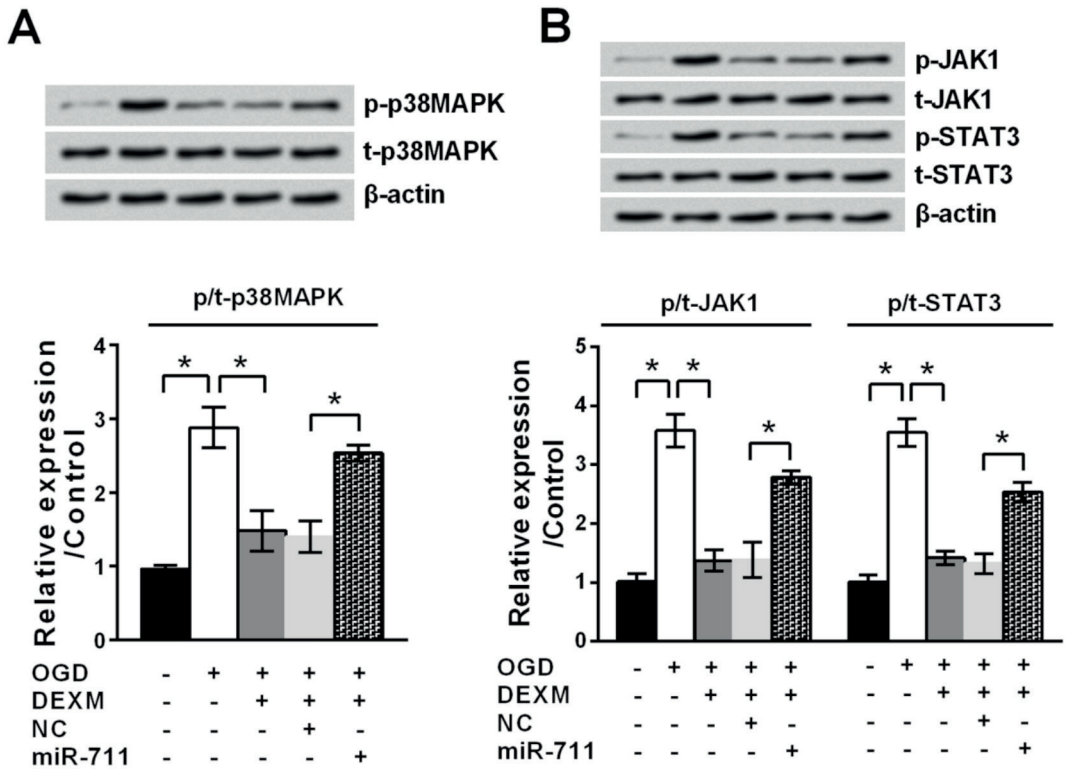

Figure 5. Effect of dexmedetomidine (DEXM) on the activation of p38MAPK and JAK/STAT signaling pathways. ***A,*** Phosphorylation of p38MAPK. ***B,*** JAK1 and STAT3 in miR-711-transfected or NC-transfected L-02 cells. After transfection, the cells were treated with or without DEXM upon OGD exposure for 6 h. * $p < 0.05$ *vs.* the indicated group (ANOVA). Data represented as mean ± SD (n = 3).

tomidine blocked p38MAPK and JAK/STAT signaling pathways upon OGD possibly via down-regulation of miR-711. However, further investigations are required to reveal whether p38MAPK and JAK/STAT signaling pathways are implicated in the protective effects of dexmedetomidine against OGD in liver cells.

Conclusions

We observed that dexmedetomidine ameliorated OGD-induced cell apoptosis and inflammation in L-02 cells, exerting protective activities in ischemic liver injury. The anti-OGD effects of dexmedetomidine might be realized by down-regulation of miR-711 and suppression of p38MAPK and JAK/STAT signaling pathways.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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