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ORIGINAL ARTICLE

miR-30a-5p Augments the Anti-inflammatory Effects of Dexmedetomidine in LPS-induced BV2 Cells

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LPS로 유도된 BV2 세포에서 Dexmetomidine이 갖는 항염증효과에 대한 miR-30a-5p의 시너지 효과

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ABSTRACT

Neuroinflammation is defined as a neurological inflammation within the brain and the spinal cord. In neuroinflammation, microglia are the tissue-resident macrophages of the central nervous system, which act as the first line of defense against harmful pathogens. Dexmedetomidine (Dex) has an anti-inflammatory effect in many neurological conditions. Additionally, the microRNA-30a-5p (miR-30a-5p) mimic has been proven to be effective in macrophages in inflammatory conditions. This study aimed to investigate the synergistic anti-inflammatory effects of both miR-30a-5p and Dex in lipopolysaccharide (LPS)-induced BV2 cells. This study showed that miR-30a-5p and Dex decreased nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation in LPS-induced BV2 cells. MiR-30a-5p and Dex alleviated tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), LPS-induced phosphorylation c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinase (ERK) and p38. Also, the expression of the NOD-like receptor pyrin domain containing 3 inflammasome (NLRP3), cleaved caspase-1, and ASC was inhibited. Furthermore, LPS-stimulated nitric oxide (NO) production, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) expression were attenuated by Dex and miR-30a-5p. Our results indicate that a combination of Dex and miR-30a-5p, attenuates NF-κB activation, the mitogen-activated protein kinase (MAPK) signaling pathway, and inflammatory mediators involved in LPS-induced inflammation and inhibits the activation of the NLRP3 inflammasome in LPS-activated BV2 cells.

INTRODUCTION

Microglia is the brain's endogenous immune cells, which arise from yolk-sac fetal macrophages. Microglia respond to central nervous system diseases with complex reactions, often called 'activation' [1, 2]. In brain inflammation, microglia is activated and release inflammatory mediators, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interlukin-6 (IL-6). Also, the expression of inducible NO synthesis (iNOS) and cyclooxygenase-2 (COX-2) seems to be increased [3]. Lipopolysaccharide-induced microglia evoked higher pro-inflammatory gene expression and increased several anti-inflammatory genes [4].

MicroRNAs (miRNAs) are endogenous non-coding RNAs

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with an average length of 18~22 nucleotides. miRNAs regulate target genes expression by interacting with the complementary sites of the 3' untranslated region (3'-UTR) of mRNAs to induce mRNA degradation [5]. The interaction of miRNAs serve as an important regulator in diverse biological processes, including cell survival [6]. A number of studies have reported that various types of miRNAs are actively used in experiments. Those miRNAs that can alleviate inflammation states are miRNA-20b [7], miRNA-30a-5p [8], miRNA-30c-5p [9], and miRNA-544a [10]. Recently, miR-30a-5p has been reported to suppress spinal cord injury-induced inflammatory response [8]. However, the mechanism that miR-30a-5p regulates neuro-inflammation in microglia cells remains unknown.

Dexmedetomidine, which is known as a highly selective α_2-adrenoceptor agonist, has shown sedative, analgesic and anxiolytic effects after administration to ill patients [11]. In a recent study, effects of Dex have been shown to attenuate pro-inflammatory response and exhibit neuroprotection in spinal cord ischemia. Moreover, Dex can increase microglia-associated anti-inflammatory cytokines levels [12]. However, the effect of using miR-30a-5p in combination with Dex on microglia with inflammatory response was not confirmed. Therefore, we hypothesized that miR-30a-5p and Dex work together, they create more synergistic effects than their respective effects and will be excellent in reducing neuro-inflammation in BV2 cells. The aim of this study is to investigate how miR-30a-5p and Dex alleviated neuroinflammation under that LPS-induced stimulus and to demonstrate its associated signaling pathways in microglia activity.

MATERIALS AND METHODS

1. Materials

Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were provided by Corning (New York, NY, USA). Heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) were purchased from Gibco (Life Technologies, Gaithersburg, MD, USA). LPS and Dex were obtained from Sigma-Aldrich (St. Louis, MO, USA). TNF-α and IL-6 were measured by an enzyme-linked immunosorbent assay (ELISA) using the mouse TNF-α and IL-6 DuoSet® ELISA kits (R&D systems, MN, USA) according to the manufacturer's instructions. The following antibodies used for western blot: anti-ERK1/2, anti-p-ERK1/2, anti-p38, anti-p-p38, anti-NF-κB, anti-NLRP3, anti-COX-2, anti-iNOS, anti-rabbit IgG HRP, and anti-mouse IgG HRP (Cell Signaling Technology, Danvers, MA, USA); and anti-JNK, anti-p-JNK, anti-caspase-1 (p20), anti-ASC, anti-β-actin, and anti-Lamin-B1 (Santa Cruz Biotechnology, Dallas, TX, USA).

2. Cell culture

BV2 cells were obtained from the Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine (Seoul, South Korea) and were maintained in DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% FBS at 37°C. The cells were incubated in humidified atmosphere containing 5% CO_2. To stimulate BV2 cells and mimic the inflammatory environment, we used to Lipopolysaccharide (1 μg/mL) for 24 hrs.

3. miRNA transfection

After seeding BV2 cells in a 6-well plate, it was confirmed that 70~80% confluency to transfection. MiR-30a-5p mimic was synthesized by Genolution (Seoul, Korea). Cells were transfected with miRNA using Lipofectamine 3000 (Invitrogen, Waltham, Massachusetts, USA) in a serum-free medium, according to the manufacturer's instructions. The cells were incubated for 24 h before performing other experiments.

4. Protein isolation and western blot analysis

For nuclear and cytoplasmic protein extraction, BV2 cells were washed twice with cold 1× PBS and centrifuged at 500 g for 3 min. First, the cell pellet was suspended in cytoplasmic extraction reagent Ⅰ of NE-PER® (Thermo

Fisher, Rockford, IL, USA) by vortexing. The suspension was incubated on ice for 10 min followed by the addition cytoplasmic extraction reagent II of NE-PER® (Thermo Fisher, Rockford, IL, USA), vortexed for 5 sec, incubated on ice for 1 min and centrifuged for 5 min at 16,000 g. The supernatant fraction was transferred to a pre-chilled tube. The insoluble pellet fraction, which contains nuclei, was resuspended in nuclear extraction reagent by vortexing during 15 sec and incubated on ice of 10 min, then centrifuged for 10 min at 16,000 g. The supernatant constituting the nuclear extract. BV2 cells were lysed using Radioimmunoprecipitation assay buffer (Thermo Scientific, Waltham, MA, USA) to prepare whole cell extracts. The protein concentrations were determined using the Lowry protein assay. Thereafter, samples with an equal amount of protein were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10~12% SDS gels. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories Inc, Hercules, CA, USA), and then the membrane was incubated overnight at 4°C with the primary antibodies, followed by incubation for 1 h at room temperature with either the anti-rabbit IgG HRP or the anti-mouse IgG HRP secondary antibody. The protein bands on the membrane were visualized by an enhanced chemiluminescence detection system (Vilber Lourmat, Collégien, France) and then captured using ChemiDoc imaging system.

5. ELISA

The levels of the cytokines, such as TNF-α and IL-6 (R&D systems, Minneapolis, MN, USA) were detected with ELISA kits, according to the manufacturer's instructions. In brief, cultured cell supernatants were collected following various treatments. The absorbance of each solution was measured at 450 nm using a microplate reader. All assays were performed as three independent experiments. The levels of cytokines were calculated using the standard value obtained from a linear regression equation.

6. Nitric oxide assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with NO plus detection kit (iNtRON Biotechnology, Seongnam, Gyeonggi-do, Korea). BV2 cells (2.4×105 cells/mL) were plated onto 6-well plates and transfected with miR-30a-5p for 24 h, prior to treat with LPS and Dex for 24 h. Cell supernatants were collected and NO production was measured. The absorbance was measured at 540 nm using a microplate reader. Nitrite concentration was determined from a nitrite standard curve.

7. Statistical analysis

All data are presented as mean±standard error and are representative of three independent experiments. The SPSS statistical software package (Version 18.0, USA) was used for the analysis of variance (ANOVA), as appropriate. Additionally, individual differences among each group were compared through one-way ANOVA, followed by Scheffe and Dunnett T3 methods. Results with $P<0.05$ were considered statistically significant.

RESULTS

1. miR-30a-5p and Dex decrease NF-κB nuclear translocation in LPS-induced BV2 cells

According to recent studies, Dex treatment inhibited neuro-inflammation and decreased NF-κB activation in BV2 cells. Also, miR-30a-5p has been reported to ameliorates phosphorylation of NF-κB in the inflammatory state. In LPS-induced microglia, activated NF-κB translocates to the nucleus and binds to the target genes, including cytokine genes. Eventually, pro-inflammatory cytokines mRNA and protein expression is induced. Here, we wanted to determine the synergistic inhibition effect of the NF-κB pathway by combining Dex with miR-30a-5p. In Figure 1, we confirmed the translocation of NF-κB in LPS-induced BV2 cells. Each of Dex administration and miR-30a-5p transfection in LPS-

induced BV2 cells showed reduced NF-κB activation compared with the LPS-only treated group. It can be seen that NF-κB activation was decreased meaningfully in co-treatment of the Dex administration and miR-30a-5p transfection group.

2. miR-30a-5p promotes downregulation of pro-inflammatory cytokines in LPS-induced BV2 cells with Dex

The types of pro-inflammatory cytokines induced by LPS are diverse, and they regulate cell activation, differentiation and returning of the immune cells to infection sites aim to control and eliminate intracellular pathogens. As illustrated in Figure 2, NF-κB pathway-associated pro-inflammatory cytokines, TNF-α and IL-6 were significantly decreased in Dex administration and miR-30a-5p transfection respectively in BV2 cells. Furthermore, group in which co-treatment with miR-30a-5p and Dex were performed had much lower levels of cytokine. These data suggest that the application of miR-30a-5p enhanced the inhibitory effect of Dex on pro-inflammatory cytokines in LPS-induced BV2 cells.

3. MAPK signaling pathway was downregulated by miR-30a-5p and Dex in LPS-induced BV2 cells

In order to elucidate the molecular mechanisms associated with the anti-inflammatory effects of Dex and miR-30a-5p, we investigated the activation of MAPK signaling pathway which is known as the representative pathway involved in the regulation of the synthesis of inflammation mediators. c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, are the three major mitogen-activated protein kinases (MAPKs). The effects of Dex and miR-30a-5p are shown in Figure 3, which confirmed that phospho-form of JNK, ERK, and p38 decreased in LPS-induced BV2 cells. In the group treated with Dex and miR-30a-5p transfection, the expression of p-JNK, p-ERK and p-38 were significantly decreased compared with LPS treated group.

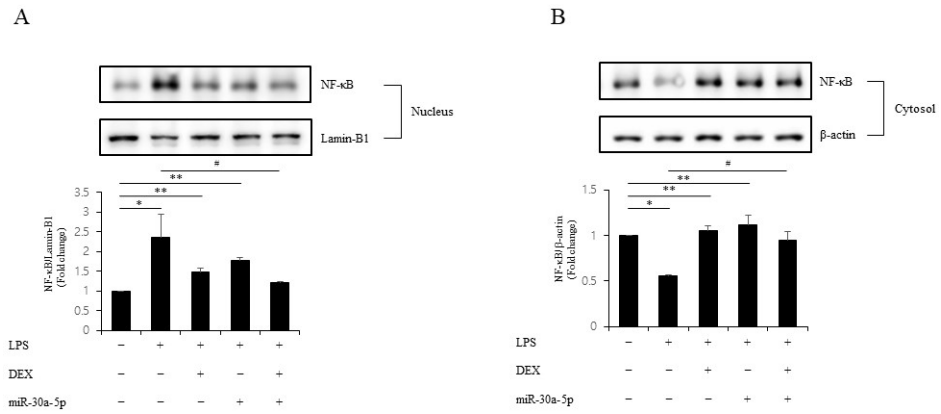

Figure 1. miR-30a-5p and Dex decrease nuclear NF-κB translocation in LPS-induced BV2 cells. BV2 cells were transfected with miRNA-30a-5p (50 nM) for 24 h, then treated Dex (20 μg/mL) and LPS (1 μg/mL) for 24 h. (A, B) The translocation of NF-κB was detected by western blot. Quantification of NF-κB ratio normalized onto β-actin in cytosolic protein and Lamin-B1 in nucleus protein were represented in bar graph. Data are presented as the means±S.D. Values of $^{*}P<0.05$, $^{**}P<0.01$ versus control; $^{\#}P<0.05$ compared with LPS group.

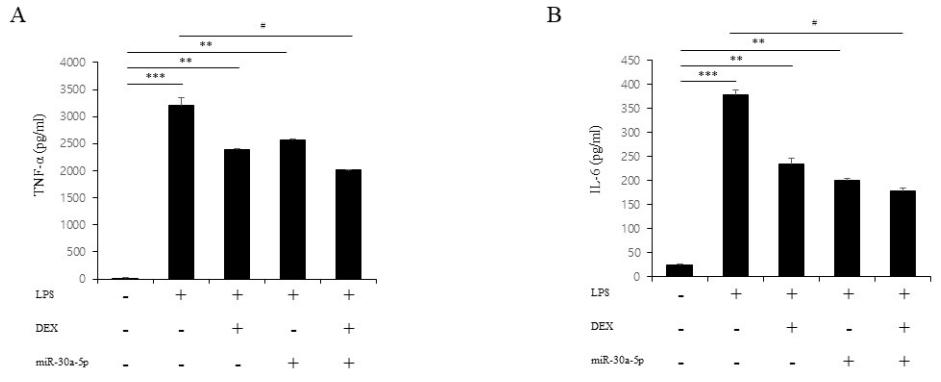

Figure 2. miR-30a-5p and Dex promote downregulation of pro-inflammatory cytokines in LPS-induced BV2 cells. BV2 cells were transfected with miRNA-30a-5p (50 nM) for 24 h, then treated Dex (20 μg/mL) and LPS (1 μg/mL) for 24 h. The level of TNF-α (A) and IL-6 (B) was measured by ELISA. Data are presented as the means±S.D. Values of $^{**}P<0.01$, $^{***}P<0.001$ versus control; $^{\#}P<0.05$ compared with LPS group.

4. miR-30a-5p and Dex alleviated NLRP3-inflmmasome activation through NF-κB signaling in LPS-induced BV2 cells

The NLR family pyrin domain containing3 inflammasome has been proposed to be upstream when stimulated by priming stimulus by a pathogen (PAMP), or damage-associated molecular pattern (DAMP) respectively. Upon activation, the NLRP3 protein interacts with ASC via pyrin domain (PYD), and the caspase recruitment domain (CARD) domains of ASC recruit the CARD domain of pro-caspase-1 to form the NRLP3 inflammasome. So, we investigated the change in expression when the components constituting the NLRP3 inflammasome were activated through western blot analysis. In Figure 4, NLRP3, ASC, cleaved caspase-1 were significantly upregulated in LPS-induced BV2 cells. Conversely, NLRP3 and cleaved caspase-1 protein expression are downregulated in those treated with Dex and transfected miR-30a-5p group. Although single treatment of Dex or miR-30a-5p did not inhibit ASC expression by LPS, co-treatment with miR-30a-5p and Dex hindered the increase of ASC. Comprehensively, it can be demonstrated that the action of Dex and miR-30a-5P together suppressed the activity of NLRP3 inflammasome, a mediator of inflammation.

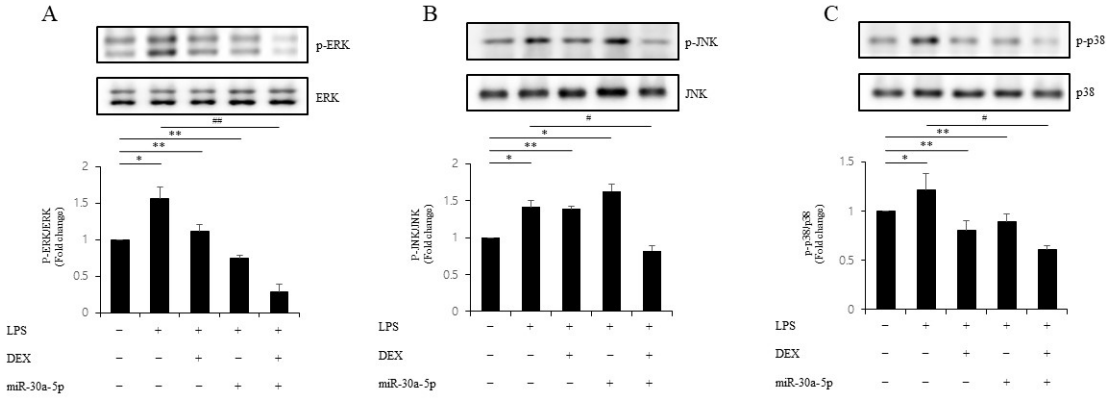

Figure 3. MAPK signaling pathway was downregulated by miR-30a-5p and Dex in LPS-induced BV2 cells. BV2 cells were transfected with miRNA-30a-5p (50 nM) for 24 h and then incubated with Dex (20 μg/mL) and LPS (1 μg/mL) for 24 h, The phosphorylation level of ERK (A), JNK (B) and p-38 (C) were detected by western blot. Data are presented as the means±S.D. Values of $^{*}P<0.05$, $^{**}P<0.01$ versus control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with LPS group.

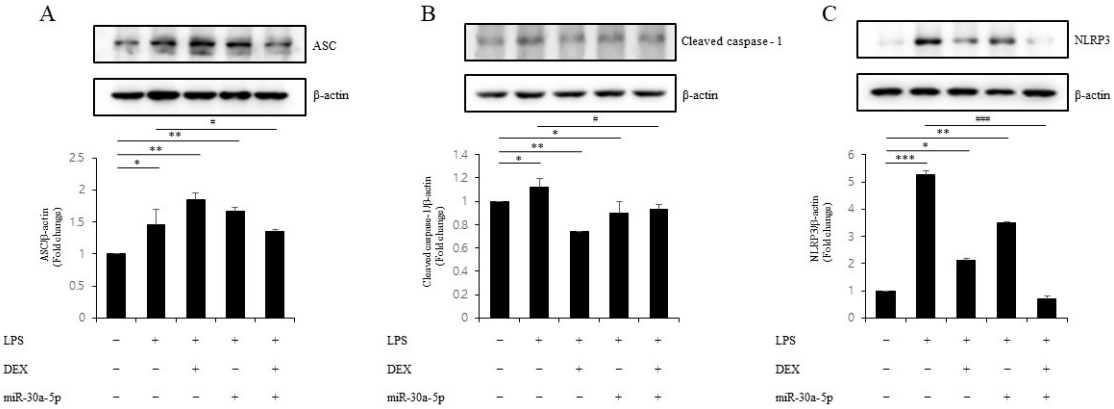

Figure 4. miR-30a-5p and Dex alleviated NLRP3-inflmmasome activation in LPS-induced BV2 cells. The component of NLRP3 inflammasome were detected by western blot. miRNA-30a-5p (50 nM) transfected for 24 h and then incubated with Dex (20 μg/mL) on LPS-induced BV2 cells. Analysis of NLRP3 (A), ASC (B) and cleaved caspase-1 (C) expression in BV2 cells. Quantification bar plots of NLRP3, ASC and Cleaved caspase-1 normalized with respect to β-actin. Data are presented as the means±S.D. Values of $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ compared with LPS group.

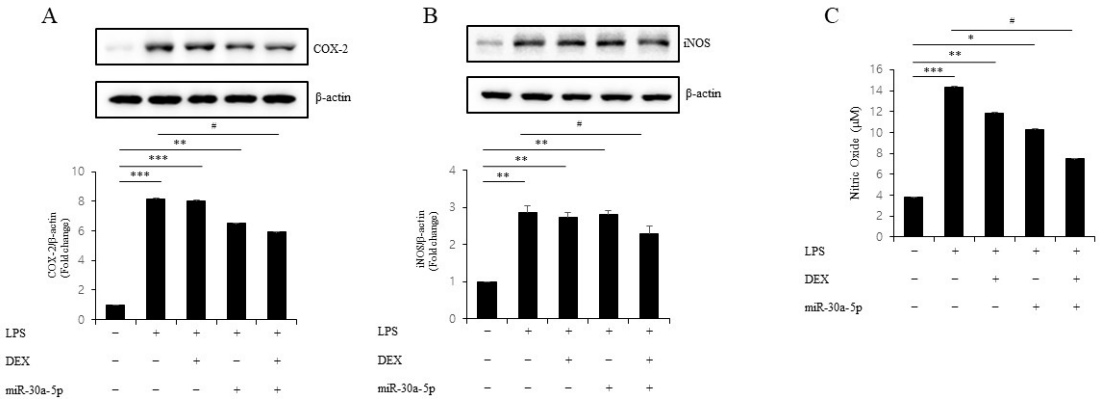

Figure 5. Inhibition of COX-2, iNOS and NO expression by miR-30a-5p and Dex in LPS-induced BV2 cells. BV2 cells were transfected with miRNA-30a-5p (50 nM) for 24 h, then pretreated with Dex (20 μg/mL) for 24 h, followed by treatment with LPS (1 μg/mL) for 24 h. (A, B) COX-2 and iNOS were detected by western blot. (C) The NO concentration in supernatant was using Griess reagent. Data are presented as the means±S.D. Values of $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control; $^{\#}P<0.05$ compared with LPS group.

5. Inhibition of COX-2, iNOS expression and NO by miR-30a-5p and Dex in LPS-induced BV2 cells

The transcription of specific target genes such as iNOS, COX-2 and NO syntheses were related to IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) which is the NF-κB subunit protein. iNOS, COX-2 and NO syntheses are controlled by signaling pathways, including MAPK. In Figure 5, The protein levels of iNOS and COX-2 and NO production were higher in LPS-induced BV2 cells than in the group that was treated with Dex or miR-30a-5p transfection, with the largest decrease in processing Dex and miR-30a-5p group.

DISCUSSION

Dex improves neurological recovery and reduces tissue damage after spinal cord injury (SCI), which is associated with modulation of microglial responses [13]. The anti-inflammatory effects of Dex on LPS-induced BV2 microglia is mediated through pathways involving TLR4 and NF-kB [14] or might be achieved by the inhibition of glycolysis [15]. In rats with lung injury, Dex's protective effect is possibly due to the activation of the phosphoinositide 3 kinase (PI3K)/serine/threonine kinase (Akt) pathway following upregulation of the CB2 receptor and hindering the assembly of the NLRP3 inflammasome through the ubiquitin-autophagy pathway or decreasing c-Fos nuclear protein levels [16, 17]. Moreover, Dex increases microglial M2 polarization by inhibiting phosphorylation of ERK1/2, by which it exerts anti-inflammatory effects in BV2 cells [18].

Various miRNA-based therapies could be considered as an attractive strategy to recover microglial inflammation. Let-7a was shown to inhibit the production of pro-inflammatory mediators, including iNOS, IL-6, and nitrate [19]. MiR-340 mimics enhanced anti-inflammatory effects of Dex in LPS-stimulated BV2 cells via inhibiting NF-κB and proinflammatory cytokines [20]. Previous studies have clarified that miR-30a-5p is endogenous and has been reported to suppress the progression of various types of cancer. According to Li et al, miR-30a-5p inhibits phosphorylation levels of STAT3, JNK, p56 and IκBα. Also, miR-30a-5p ameliorates LPS-induced inflammatory injury in human A549 cells and mice via targeting Runt-related transcription factor 2 (RUNX2) [21].

Through inhibition of lactate dehydrogenase A, miR-30a-5p dampens glycolysis in breast cancer cells. Moreover, the anti-tumor functions were also confirmed in lung cancer, pancreatic cancer and liver cancer [22]. Additionally, recent studies have shown that the over-expression of miR-30a-5p prevented the MAPK/ERK

signaling and inhibits inflammatory responses in microglia of SCI [8]. The mature miRNA duplex is classified into two strands, 3p and 5p. The target gene is determined according to the completion of a mature miRNA duplex having a certain strand [5]. In our previous experiment, miR-30a-5p was designed to target LPS-induced inflammatory response-related gene.

Dex and miR-30a-5p act on various inflammatory pathways, respectively, and consequently show anti-inflammatory effects. Therefore, it was possible to hypothesize that the combined treatment of Dex and miR-30a-5p would show greater inflammation relief. In this study, our results demonstrate that Dex and miR-30a-5p can down-regulates LPS-induced neuroinflammatory responses in microglial cell. Currently, simultaneous administration of small molecular chemotherapeutic drug with gene medicine are common and effective ways to treat various diseases. Zhang et al demonstrated that combining doxorubicin and miR-21 inhibitor significantly reduced tumor cell proliferation, invasion and migration in glioblastoma [23]. Upregulated miR-381-5p strengthens the effect of Dex preconditioning to protect to protect against myocardial ischemia-reperfusion injury [24]. As shown in our study, we have confirmed that miR-30a-5p in combination with Dex inhibits LPS-mediated NF-kB nuclear translocation and upregulation of pro-inflammatory cytokines such as TNF-α and IL-6 in BV2 cells. Although miR-30a-5p transfection alone did not inhibit JNK phosphorylation and NLRP3 inflammasome activation by LPS remarkably, co-treatment with miR-30a-5p and Dex conclusively decreased MAPK phosphorylation and suppress NLRP3 inflammasome activation in LPS-activated BV2 cells. In addition, treatment with Dex and miR-30a-5p downregulates inflammatory mediators secreted by LPS-induced BV2 cells, such as NO, iNOS and COX-2.

The inflammatory response is described in four stages. Inflammatory inducers are detected by inflammatory sensors and inflammatory mediators are released, eventually affecting cells and tissue. In this study, it was confirmed that miR-30a-5p and Dex alleviated the effects of LPS, an inflammatory inducer, and lowered all the inflammatory mediator levels in BV2 cells, an inflammatory sensor. Collectively, our findings suggest that co-treatment with Dex and miR-30a-5p can develop anti-inflammatory effects in LPS-induced BV2 cells shown in Figure 6. Furthermore, inflammation-related factors and signaling pathways, which are activated in LPS-induced inflammation, decreased, so it can be explained that Dex and miR-30a-5p have a synergistic effect against LPS-induced microglial inflammation.

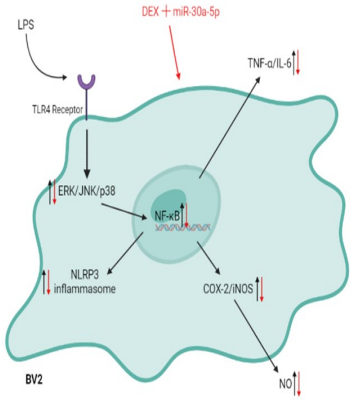

Figure 6. A schematic diagram showing the effect of miR-30a-5p and Dex in LPS-induced BV2 cell. Co-treatment with miR-30a-5p and Dex inhibited LPS-induced BV2 cells inflammatory response. The results showed that decreased NF-κB translocation and pro-inflammatory cytokines. In addition, miR-30a-5p and Dex alleviated MAPK signaling pathway and NLRP3 inflammasome activation. The inflammatory mediators iNOS, COX-2 and NO also tend to decrease.

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Conflict of interest: None

Author's information (Position): Kim JE, Graduate Student; Yang SJ, Professor.

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