Overexpression of miR-200a-3p promoted inflammation in sepsis-induced brain injury through ROS-induced NLRP3

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Abstract. Sepsis, a systemic inflammatory response syndrome induced by infection, is a common complication of trauma, burns, postoperative infection and critical disease, and is characterized by an acute onset and high fatality rate. The aim of the present study was to explore the possible molecular mechanisms of microRNA-200a-3p (miRNA-200a-3p) on inflammation during sepsis. Reverse transcription-quantitative PCR and gene microarray were used to measure the expression of miRNA-200a-3p. Tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-18 were searched by ELISA. The related proteins expression was measured using western blotting. The expression of miRNA-200a-3p was markedly higher in the sepsis model when compared with the normal control group. In addition, the expression of miRNA-200a-3p was upregulated by the miRNA-200a-3p plasmid in human brain microvascular endothelial cells treated with lipopolysaccharide, which further induced inflammation via the induction of NLR family pyrin domain containing 3 (NLRP3) and suppression of Kelch like ECH associated protein (Keap)-1/nuclear factor erythroid 2 like 2 (Nrf2)/heme oxygenase (HO)-1. The inhibition of Keap1/Nrf2/HO-1 attenuated the effects of anti-miRNA-200a-3p on inflammation. However, the inhibition of NLRP3 attenuated the effects of miRNA-200a-3p on inflammation. In conclusion, to the best of our knowledge, the results of the present study demonstrated for the first time that overexpression of miRNA-200a-3p promoted inflammation in sepsis-induced brain injury through reactive oxygen species-induced NLRP3.

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Introduction

As a type of systemic inflammatory response syndrome induced by the imbalanced pro- and anti-inflammatory mechanism, sepsis normally develops as a result of severe infections, burns, brain injury, trauma, shock and major surgery, causing serious pathophysiological changes in vital organs (1). The mortality of patients with sepsis-induced brain injury combined with sepsis shock and multiple organ dysfunction has been reported to be as high as 80-90% worldwide (2,3).

The NLR family pyrin domain containing 3 (NLRP3) pathway has been demonstrated to play an important role in the pathogenesis and development of sepsis lung injury, and the inhibition of the NLRP3 pathway could not only block the aggressive inflammatory response of sepsis, but also suppress its excessive anti-inflammatory response, thereby protecting important organ functions (4). Multiple cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and growth factors can induce signal transduction by activating the NLRP3 pathway (5). NLRP3 protein is downstream of Janus kinase 2, which is critically involved in the inflammatory mediator response and immunoregulation of sepsis (6). NLRP3 can be phosphorylated and activated through the NLRP3 pathway, subsequently transmitting the extracellular signal to the cell nucleus, binding with the target gene promotor in the nucleus, inducing the transcription of the target gene, and thereby initiating the release of a series of cell cytokines (4).

MicroRNAs (miRNAs/miRs), the complete or incomplete matching on the 3'-untranslated region (UTR) of the target gene mRNAs, can degrade mRNA or inhibit its translation and regulate protein expression at the post-transcriptional level (7). Bioinformatics prediction has discovered that miRNAs act on an extensive range of target genes and can regulate the expression of ~30% of human genes (7). It has also been shown that the host cell could produce miRNA immediately after being stimulated by the pathogenic microorganism, thereby participating in the innate immune response, promoting the release of inflammatory factors and inducing immune hyperfunction (7,8). In addition, miRNAs could induce cell apoptosis or degrade inflammatory factors, leading to immunosuppression (8). A previous study has verified that miRNAs are involved in the immunoregulation of sepsis, and exert important regulatory effects (8). miRNAs act on a wide range of target genes and participate in the regulation of multiple biological

processes, including immunity, metabolism, differentiation, proliferation and carcinogenesis (8). Zhang *et al* (9) revealed that miR-200a-3p promotes β-amyloid-induced neuronal apoptosis in Alzheimer's disease. The results of the present study indicated the possible molecular mechanisms underlying the effect of miRNA-200a-3p on inflammation during sepsis.

Materials and methods

Ethics statement and mouse model of cecal ligation and puncture (CLP). Male C57BL/6J mice (5-6 weeks, 18-20 g; n=12) were obtained from Animal Research Center of Xiamen University and housed under standard laboratory conditions at 21-23˚C with 55-60% humidity, a 12-h light/dark cycle and free access to food and water. Mice of sepsis model were anesthetized intraperitoneally with 35 mg/kg pentobarbital sodium. Once under anesthetization, the abdominal area of sepsis model mice was shaved and disinfected. The cecum was exposed for 15 min and ligatured at 2/3, and punctured with a 27-gauge needle. The wound was then sterilized and closed by applying a simple suture. Sham group mice were only anesthetized intraperitoneally with 35 mg/kg pentobarbital sodium. The present study was approved by The First Affiliated Hospital of Xiamen University on Animal Care.

Reverse transcription-quantitative PCR (RT-qPCR). After 24 h of CLP, mice were anesthetized intraperitoneally with 35 mg/kg pentobarbital sodium, and peritoneal fluid was extracted using a sterile syringe and cultured in RPMI-1640 supplemented with 2% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The single-cell suspensions were washed with PBS three times and peritoneal macrophages (PMs) were collected at 800 x g for 20 min at 4˚C. Total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into cDNA using the RealMasterMix First Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd.) at 42˚C for 60 min and 82˚C for 10 sec. RT-qPCR was performed in the Applied Biosystems 7500 Real-time PCR system using SYBR Premix ExTag™ (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. Data were presented as the relative expression level by comparing Cq values (10). The thermocycling conditions were as follows: Denaturation at 94˚C for 5 min, amplification for 40 cycles at 94˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 1 min. qPCR was performed using the following primers: miR-200a-3p forward 5'-AACACT GTCTGGTAACGATGTCGT-3' and reverse, 5'-CATCTT ACCGGACAGTGCTGGA-3'; U6 forward, 5'-GCTTCGGCA GCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACG AATTTGCGTGTCAT-3'.

Behavior assessment. The white open field consisted of an open square-shaped area with 30 cm high walls. Mice were placed in the center of the field, and were observed for 5 min to measure their locomotor activity. Testing videos were analyzed using Any-maze™ software 4.99 (Stoelting Co.). The field was filled with water to 150x50 cm in depth at 23˚C and mice where then placed in the water in different quadrants and allowed to search for the hidden platform (1 min); if mice failed to find the platform they were guided to it. Mice underwent 6 training trials a day with 25 min inter-trial intervals. After 5 days of training, the mice were given a probe test with the platform removed. Mice were released from the west quadrant, and allowed to swim for 1 min and their swimming paths recorded.

Gene microarray. Total RNA was extracted using the mirVana™ miRNA Isolation kit (cat. no. AM1561; Ambion; Thermo Fisher Scientific, Inc.). Total RNA was then amplified using the Low Input Quick Amp WT Labeling kit (cat. no. 5190-2943; Agilent Technologies, Inc.) according to the manufacturer's instructions. The slides were scanned using a Agilent Microarray Scanner (cat. no. G2565CA; Agilent Technologies, Inc.) and analyzed using Feature Extraction software 10.7 (Agilent Technologies, Inc.).

Cell culture and transfection. Human brain microvascular endothelial cells (HBMECs) were purchased from ScienCell Research Laboratories, Inc. and were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. HBMEC cells (70%) were plated in 6-well plates 24 h before transfection and were then transfected with 100 ng of miRNA-200a-3p plasmid (5'-TAA CACTGTCTGGTAACGATGT-3' and 5'-GCGGGTCACCTT TGAACATC-3'), 100 ng of small interfering RNA (si)-NLRP3 (cat. no. sc-45469; Santa Cruz Biotechnology, Inc.) plasmid and 100 ng of negative plasmid (5'-TTCTCCGAACGTGTC ACGT-3' and 5'-TTCTCTAGAACGTGTCAT-3'; Sangon Biotech Co., Ltd.) in Opti-MEM using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.). The medium was removed after 6 h. Then, at 24 h post-transfection HBMECs were treated with 50 ng/ml lipopolysaccharide (LPS) for 2 h at 37˚C, as described previously (11).

Then, 100 ng of si-miRNA-200a-3p plasmids (5'-ATTGTG ACAGACCATTGCTACA-3') and 100 ng of si-Keap1 (5'-TGG GGTCGTCGGTCTAGGG-3'; cat. no. sc-43878; Santa Cruz Biotechnology, Inc.), 100 ng of si-Nrf2 (5'-AGTAGTACTACC TGAACCTC-3'; cat. no. sc-37030; Santa Cruz Biotechnology, Inc.) or 100 ng of si-HO-1 (5'-CCTACCGCAGTAGTGATG GTAA-3'; cat. no. sc-35554; Santa Cruz Biotechnology, Inc.) were co-transfected into cells using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h of transfection, HBMECs were treated with 50 ng/ml LPS for 2 h at 37˚C.

miRNA-200a-3p plasmids were transfected into cells using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.). After 6 h of transfection, 3 mmol/l of tempol (ROS inhibitor; MedChemExpress) was added to cells for 18 h at 37˚C and then HBMECs were treated with 50 ng/ml LPS for 2 h at 37˚C.

Measurements of intracellular reactive oxygen species (ROS). HBMECs were washed twice with PBS and then incubated in PBS containing 10 μM of dichlorodihydrofluorescein diacetate for 30 min at 37˚C. ROS levels were measured using a microplate reader (Tecan Group, Ltd.) at 450 nm and were visualized with an Olympus IX2-SL epifluorescence microscope (magnification, x200; Olympus Corporation).

Luciferase reporter assay. TargetScanHuman 7.2 (www.targetscan.org) was employed to evaluate the miR-200a-3p network signaling pathway, which indicated that NLRP3 and Keap1 expression may be regulated by miR-200a-3p. NLRP3-3'UTR and Keap1-3'UTR were constructed and purchased from GeneCopoeia, Inc. Cells ($1x10^6$ cell) were co-transfected with 100 ng of the reporter constructs, NLRP3-3'UTR or Keap1-3'UTR, with 100 ng of the aforementioned miR-200a-3p mimic or control mimic using Lipofectamine 2000™ (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Luciferase activity was measured using a dual luciferase reporter assay kit (GeneCopoeia, Inc.) after 48 h of transfection. Normalization was performed via comparisons with *Renilla* luciferase activity.

Western blotting. HBMECs, after transfection, were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology) and phosphatase inhibitors (Beyotime Institute of Biotechnology). The supernatants were collected after centrifugation at 10,000 x g for 15 min at 4˚C and used to quantitate protein concentration using BCA (Beyotime Institute of Biotechnology). An equal amount (50 μg) of protein was applied to 8-12% SDS-PAGE and transferred onto nitrocellulose filter membranes (Thermo Fisher Scientific, Inc.). Membranes were blocked with 0.1% TBST with 5% skim milk powder for 1 h at room temperature and incubated with primary antibodies against NLRP3 (cat. no. 13158; 1:1,000; Cell Signaling Technology, Inc.), caspase-1 (cat. no. 24232; 1:1,000; Cell Signaling Technology, Inc.), Keap1 (cat. no. 8047; 1:1,000; Cell Signaling Technology, Inc.), Nrf2 (cat. no. 12721, 1:1,000; Cell Signaling Technology, Inc.), HO-1 (cat. no. 86806; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174; 1:2,000; Cell Signaling Technology, Inc.) at 4˚C overnight. Membranes were washed with TBST and then incubated with anti-rabbit immunoglobulin G horseradish peroxidase-linked secondary antibody (cat. no. D110058; 1:5,000; Sangon Biotech Co., Ltd.) for 1 h at 37˚C. Protein bands were visualized using enhanced chemiluminescence (cat. no. C500044; Sangon Biotech Co., Ltd.) and analyzed using the Odyssey™ Infrared Imaging System (version 3.0; Gene Company, Ltd.). GAPDH protein expression was used as an internal control to show equal loading of the protein bands.

Enzyme-linked immunosorbent assay (ELISA). HBMECs after transfection were lysed with RIPA buffer (Beyotime Institute of Biotechnology) and phosphatase inhibitors (Beyotime Institute of Biotechnology). The supernatants were collected after centrifugation at 10,000 x g for 15 min at 4˚C and protein concentration was measured by BCA (Beyotime Institute of Biotechnology). An equal amount (10 μg) was then used to measure the cytokine concentrations of TNF-α (cat. no. H052), IL-1β (cat. no. H002), IL-6 (cat. no. H007) and IL-18 (cat. no. H015) by ELISA according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis. Data are presented as the mean ± standard error of the mean for each group of 3 experimental repeats using SPSS 17.0 (SPSS, Inc.). Differences between groups were compared by Student's t-test or one-way analysis of variance with Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miRNA-200a-3p in the sepsis model. The present study first explored whether the expression of miRNA-200a-3p was different between the sepsis model and the normal group. The results revealed that the expression of TNF-α, IL-1β, IL-6 and IL-18 were significantly increased in the sepsis model compared with sham group (Fig. 1A-D). The escape latency was markedly longer in the sepsis model group when compared with that of the sham group (Fig. 1E). However, the number of crossings and the time in the target quadrant was lower in the sepsis model group than in the sham group (Fig. 1F-H). In addition, the number of neuronal cells was lower in the hippocampal tissue of the sepsis model compared with the sham group (Fig. 1I). As shown in Fig. 1J and K, the expression of miRNA-200a-3p was significantly higher in the sepsis model group than in the sham group.

miRNA-200a-3p regulates NLRP3 and Keap1 expression in vitro. The present study examined the mechanism of miRNA-200a-3p *in vitro*. miRNA-200a-3p mimics were used to increase the expression of miRNA-200a-3p, compared with the negative group (Fig. 2A). The results of the heat map revealed that the overexpression of miRNA-200a-3p increased the expression of p65 and NLRP3, but reduced that of Keap1, Nrf2 and HO-1 *in vitro*, when in comparison with the negative group (Fig. 2B). The 3'-UTR of NLRP3 mRNA was the binding site of miRNA-200a-3p, and the luciferase assay activity levels were increased in the miRNA-200a-3p group, compared with the negative group (Fig. 2C and D). Moreover, the 3'-UTR of Keap1 mRNA was the binding site of miRNA-200a-3p, and the luciferase assay activity levels were reduced in the miRNA-200a-3p group when compared with the negative group (Fig. 2E and F). The network signaling pathway suggested that miRNA-200a-3p may regulate the expression of NLRP3 and Keap1 *in vitro* (Fig. 2G). Overexpression of miRNA-200a-3p significantly suppressed the protein expression of Keap1, Nrf2 and HO-1, and significantly induced that of NLRP3 and caspase-1 *in vitro*, when in comparison with negative group (Fig. 3A-F). Overexpression of miRNA-200a-3p significantly increased the levels of ROS, IL-1β and IL-18 *in vitro*, compared with the negative group (Fig. 3G-J).

Downregulation of miRNA-200a-3p expression on inflammation in vitro. To further explore the effects and mechanism of anti-miRNA-200a-3p on inflammation *in vitro*, anti-miRNA-200a-3p mimics was utilized to reduce the expression of miRNA-200a-3p *in vitro*, when compared with negative group (Fig. 4A). In addition, the protein expression of Keap1, Nrf2 and HO-1 was significantly increased, while that of NLRP3 and caspase-1 were significantly decreased *in vitro* following the downregulation of miRNA-200a-3p, compared with negative group (Fig. 4B-G). Downregulation of miRNA-200a-3p also reduced significantly ROS levels and inhibited the levels of IL-1β and IL-18 *in vitro*, when compared with the negative group (Fig. 4H-K).

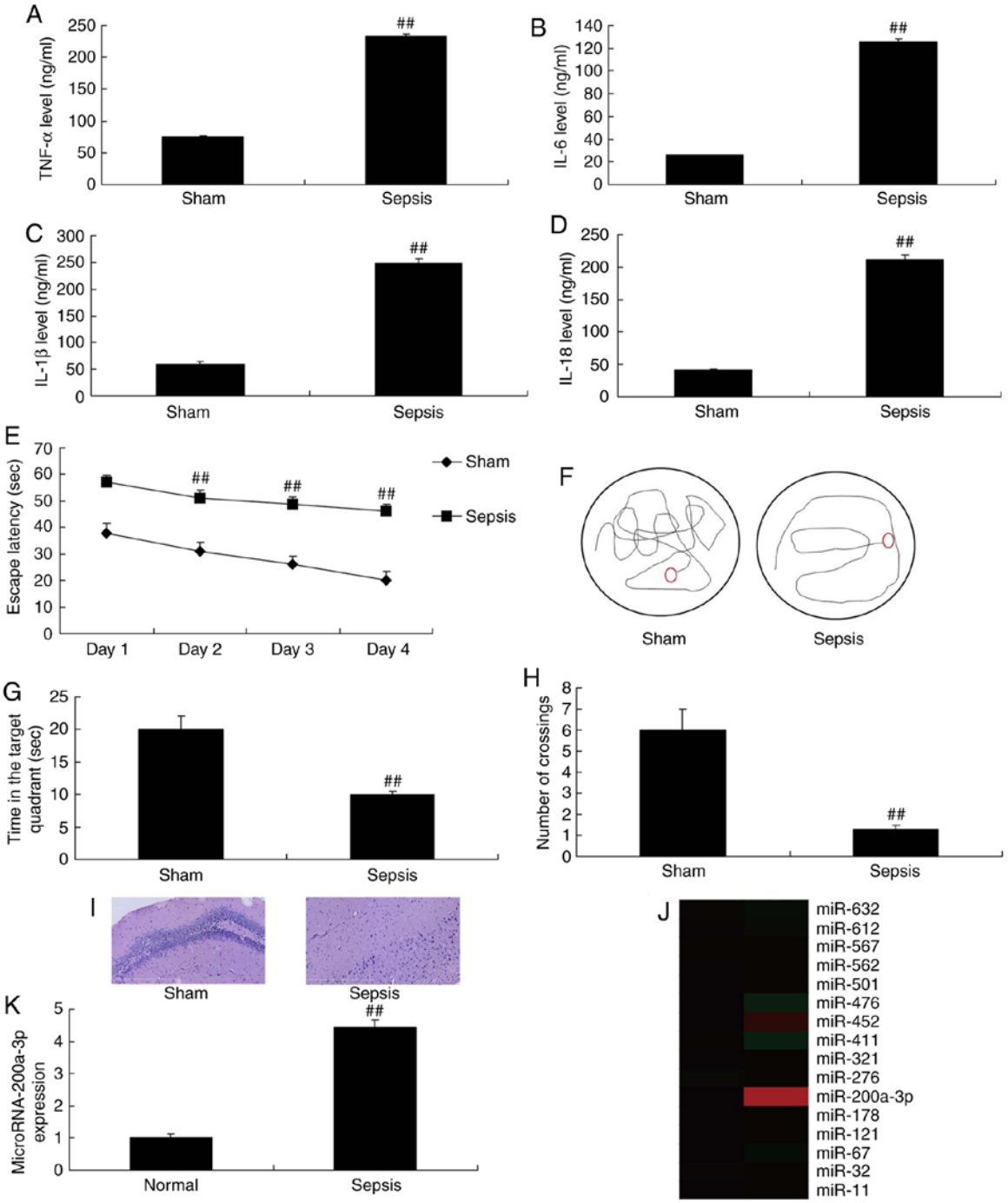

Figure 1. Expression of miRNA-200a-3p in sepsis model. (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-18 levels were determined by ELISA. The (E) escape latency, (F) tracking maps, (G) the time in the target quadrant and (H) the number of crossings were analyzed via behavior assessments. (I) Evaluation of the number of neuronal cells in hippocampal tissues (magnification, x100), (J) gene chip analysis and (K) reverse transcription-quantitative PCR for miRNA-200a-3p expression were also conducted. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. sham group. Sham, sham group; Sepsis, sepsis model group; TNF-α, tumor necrosis factor-α; IL, interleukin; miRNA/miR, microRNA.

Inhibition of Keap1 attenuates the effects of anti-miRNA-200a-3p on inflammation in vitro. In addition, the present study investigated whether Keap1 was a key signaling mediator and if it played important roles in the effect of miRNA-200a-3p on sepsis. si-Keap1 significantly suppressed the protein expression of Keap1, Nrf2 and HO-1, and significantly induced that of NLRP3 and caspase-1 *in vitro* following the downregulation of miRNA-200a-3p when compared with the miRNA-200a-3p downregulation group (Fig. 5A-F). Moreover, si-Keap1 significantly increased ROS levels and significantly promoted the levels of IL-1β and IL-18 *in vitro* following the downregulation of miRNA-200a-3p, compared with the miRNA-200a-3p downregulation group (Fig. 5G-J).

Inhibition of Nrf2 attenuates the effects of anti-miRNA-200a-3p on inflammation in vitro. To elucidate the role of Nrf2 in the effects of anti-miRNA-200a-3p on inflammation *in vitro*,

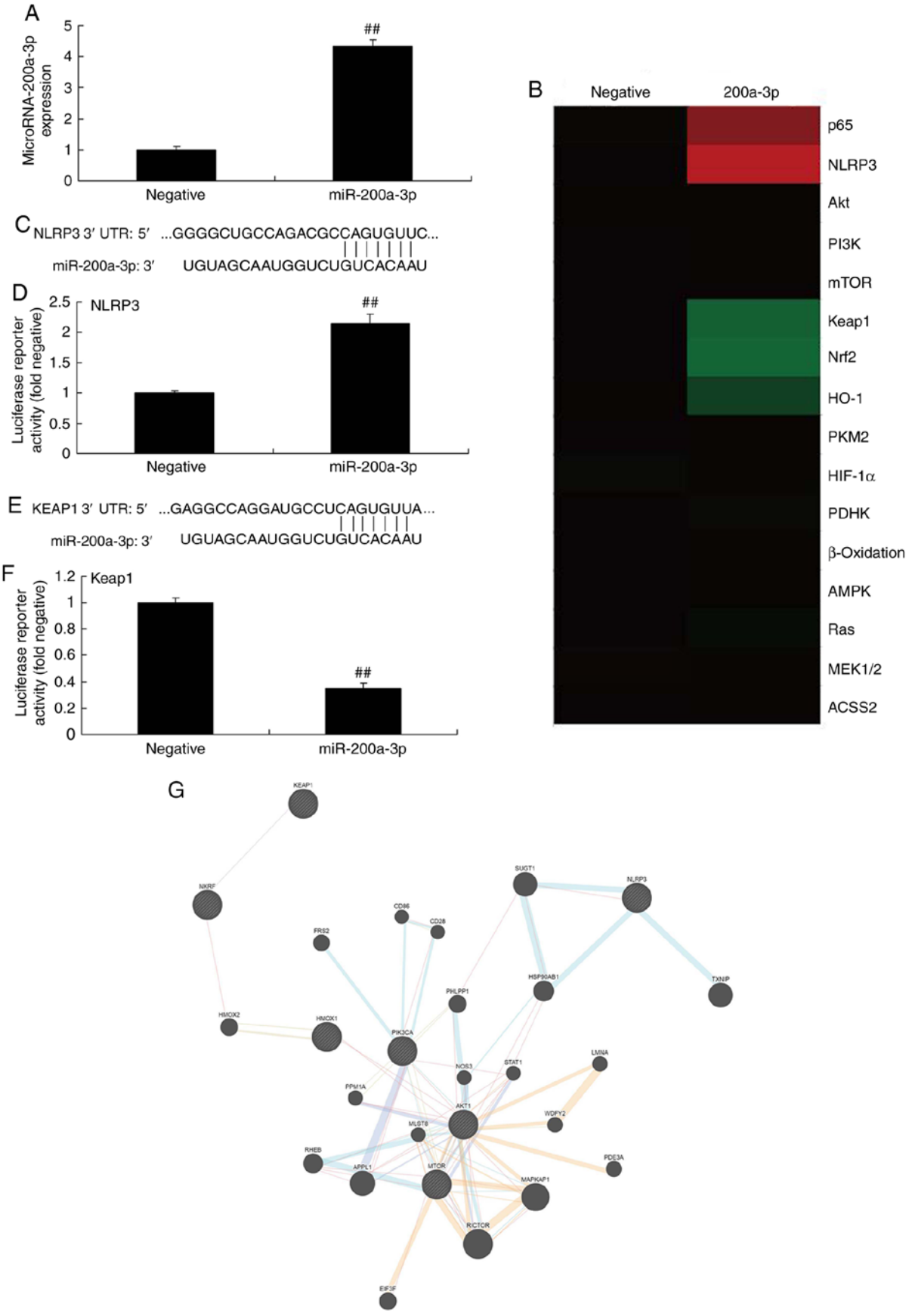

Figure 2. miRNA-200a-3p regulates NLRP3 and keap1 expression *in vitro* model. (A) miRNA-200a-3p expression in cells transfected with negative and miR-200a-3p plasmids. (B) Gene chip analysis for NLRP3. (C) The miRNA-200a-3p binding site in the 3'-UTR of NLRP3 mRNA, and (D) luciferase assay activity levels. (E) The miRNA-200a-3p binding site in the 3'-UTR of Keap1 mRNA, and (F) the luciferase assay activity levels. (G) The network signaling path. Data are presented as the mean ± standard error of the mean. ##P<0.01 vs. negative group. Negative, negative plasmid group; miR-200a-3p, miR-200a-3p overexpression group; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; UTR, untranslated region.

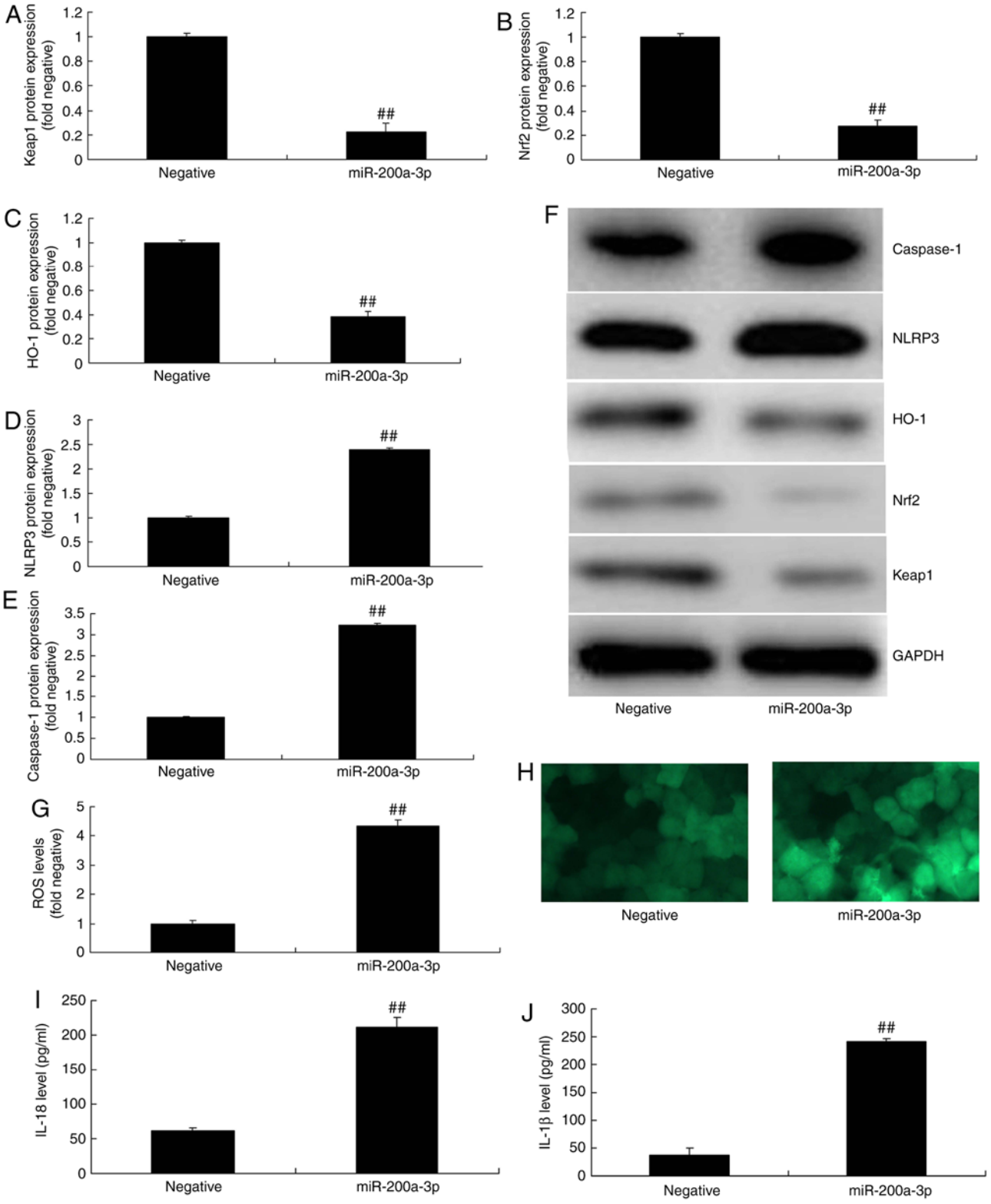

Figure 3. Effect of miRNA-200a-3p overexpression on inflammation *in vitro*. (A) Keap1, (B) Nrf2, (C) HO-1, (D) NLRP3 and (E) caspase-1 protein expressions were determined by (F) western blotting analysis. (G and H) ROS levels (magnification, x200), and (I) IL-18 and (J) IL-1β levels were also assessed. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group. Negative, negative group; miR-200a-3p, miR-200a-3p overexpression group; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

si-Nrf2 was administered, which suppressed the protein expression of Nrf2 and HO-1, and induced that of NLRP3 and caspase-1 *in vitro* following the downregulation of miRNA-200a-3p, in comparison with the miRNA-200a-3p downregulation group (Fig. 6A-E). si-Nrf2 also significantly promoted ROS levels and significantly increased the levels IL-1β and IL-18 *in vitro* following the downregulation of miRNA-200a-3p, compared with the miRNA-200a-3p downregulation group (Fig. 6F-I).

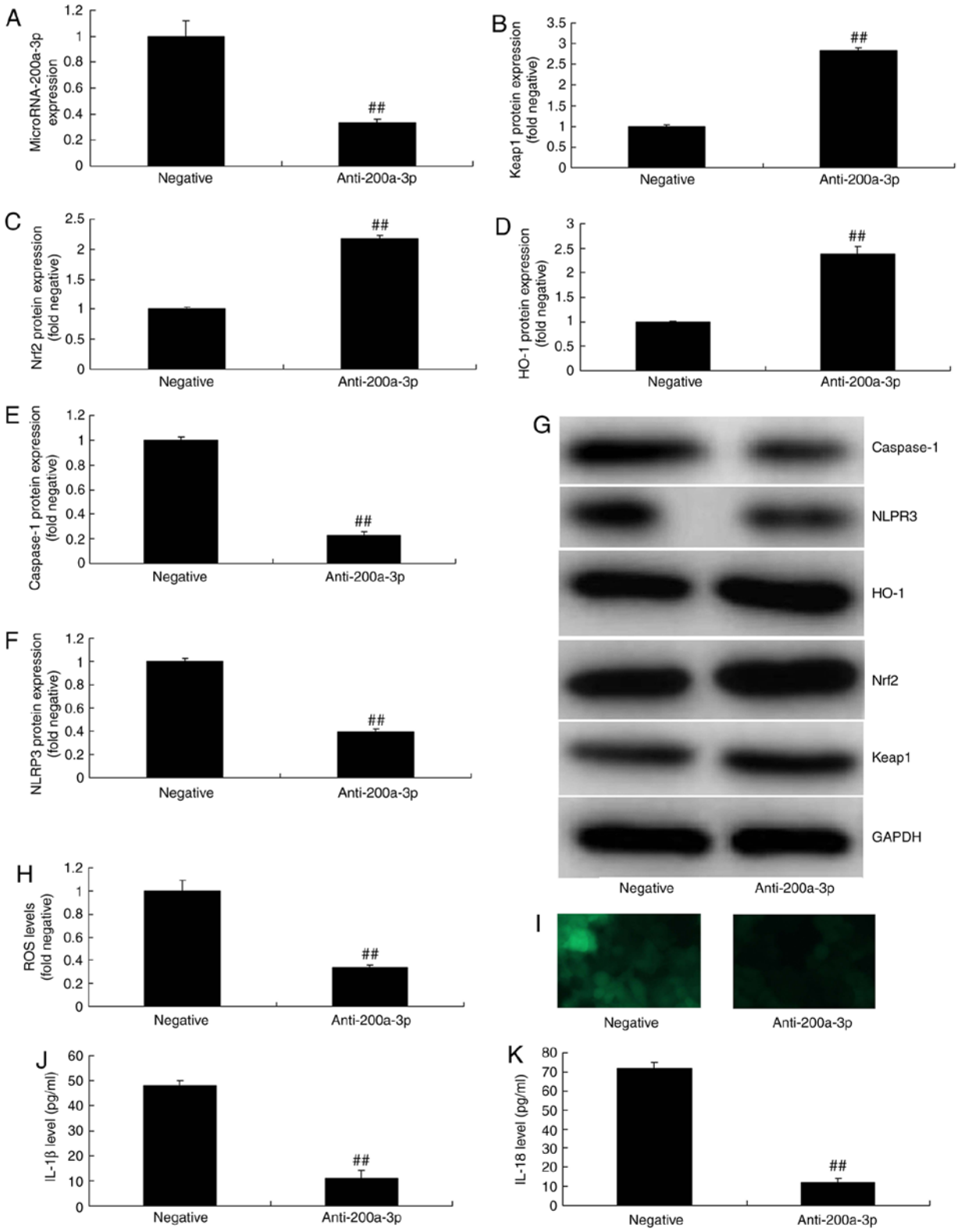

Figure 4. Effect of miRNA-200a-3p downregulation on inflammation *in vitro*. (A) miRNA-200a-3p expression following anti-miRNA-200a-3p plasmid transfection. (B) Keap1, (C) Nrf2, (D) HO-1, (E) caspase-1 and (F) NLRP3 protein expressions were determined by (G) western blotting analysis. (H and I) ROS levels (magnification, x200), and (J) IL-1β and (K) IL-18 levels were also assessed. Data are presented as the mean ± standard error of the mean. $^{##}$P<0.01 vs. negative group. Negative, negative group; anti-200a-3p, miR-200a-3p downregulation group; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

Inhibition of HO-1 attenuates the effects of anti-miRNA-200a-3p on inflammation in vitro. To further elucidate the roles of HO-1 in the effects of anti-miRNA-200a-3p on inflammation *in vitro*, si-HO-1 was applied to reduce the protein expression of HO-1. si-HO-1 administration induced the expression of NLRP3 and caspase-1 *in vitro* following

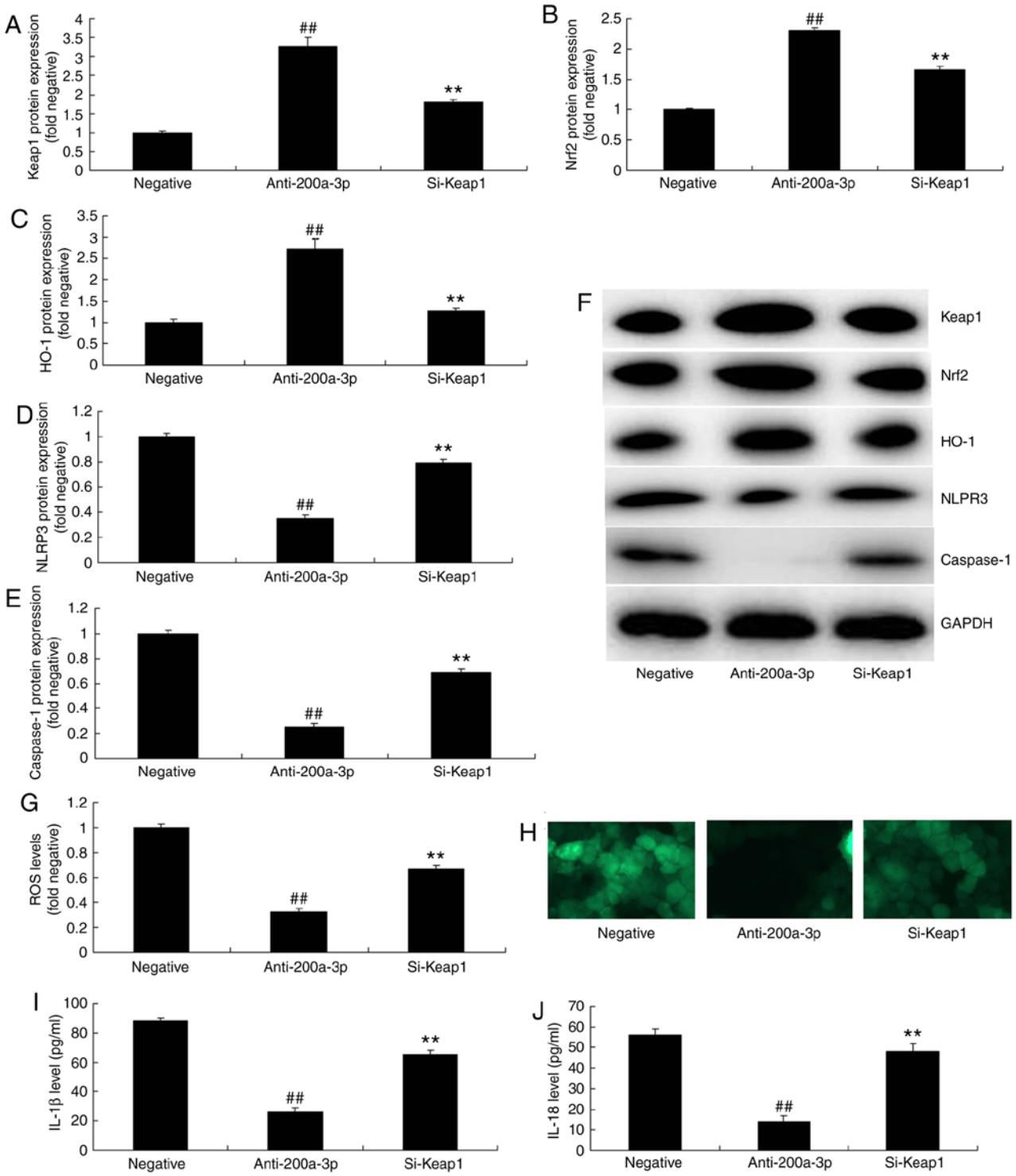

Figure 5. Inhibition of Keap1 reduces the effects of anti-miRNA-200a-3p on inflammation *in vitro*. (A) Keap1, (B) Nrf2, (C) HO-1, (D) NLRP3 and (E) caspase-1 protein expressions were determined by (F) western blotting analysis. The levels of (G and H) ROS (magnification, x200), (I) IL-1β and (J) IL-18 were also assessed. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group; **P<0.01 vs. miR-200a-3p downregulation group. Negative, negative group; anti-200a-3p, miR-200a-3p downregulation group; si-Keap1, downregulation of miR-200a-3p and si-Keap1 group; si-, small interfering RNA; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

the downregulation of miRNA-200a-3p, in comparison with the miRNA-200a-3p downregulation group (Fig. 7A-F). The application of si-HO-1 significantly promoted ROS levels, and increased IL-1β and IL-18 levels *in vitro* following the downregulation of miRNA-200a-3p, compared with the miRNA-200a-3p downregulation group (Fig. 7E-H).

ROS is involved in the effect of miRNA-200a-3p on inflammation in vitro. The present study further investigated the mechanisms of miRNA-200a-3p in ROS-regulated inflammation *in vitro*. As shown in Fig. 8A-E, the administration of ROS inhibitor (3 mmol/l tempol) reduced ROS levels, and significantly suppressed the protein expression of

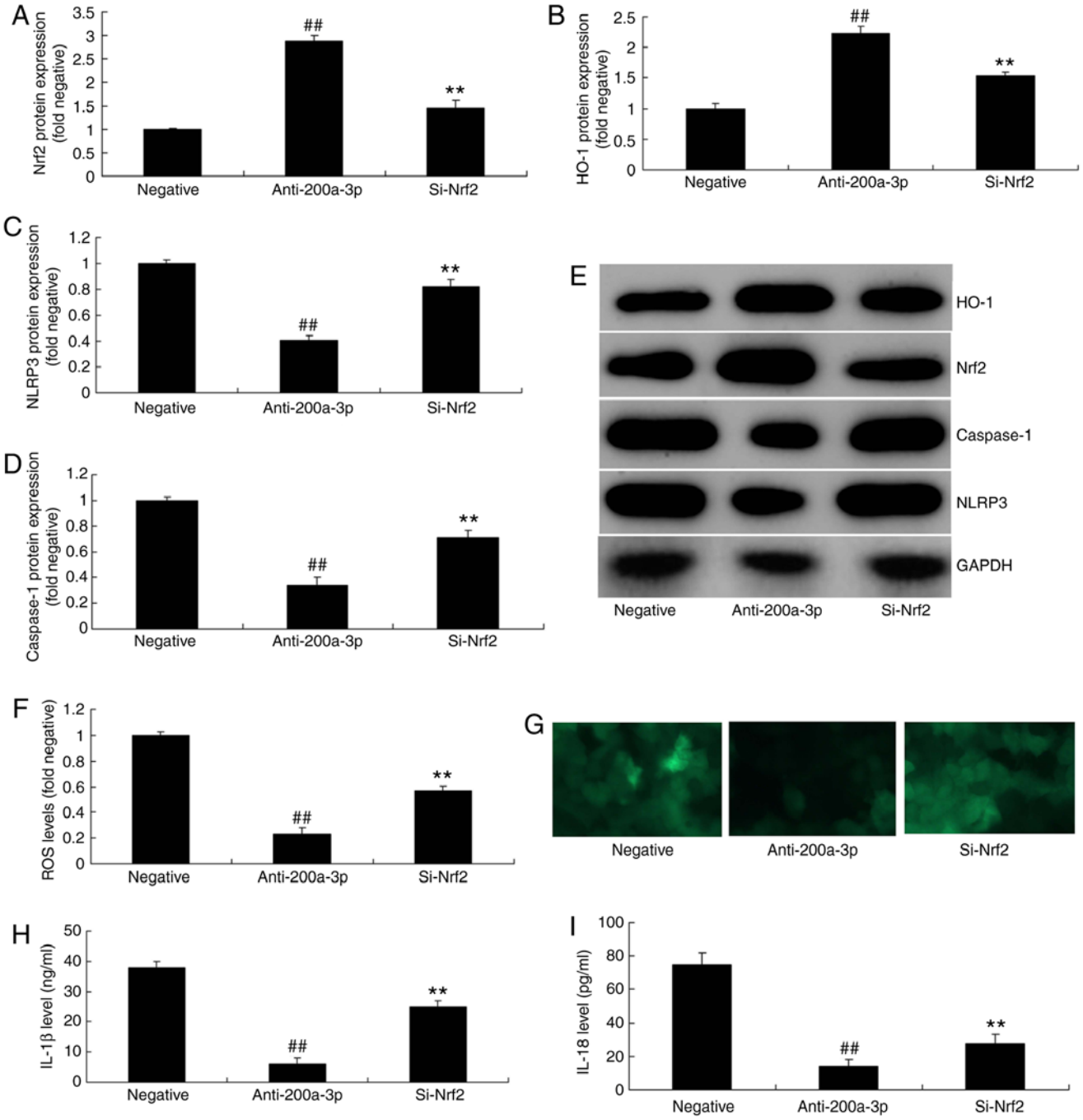

Figure 6. Inhibition of Nrf2 reduces the effects of anti-miRNA-200a-3p on inflammation *in vitro*. (A) Nrf2, (B) HO-1, (C) NLRP3 and (D) caspase-1 protein expressions were determined by (E) western blot analysis. The levels of (F and G) ROS (magnification, x200), (H) IL-1β and (I) IL-18 were also assessed. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group; **P<0.01 vs. miR-200a-3p downregulation group. Negative, negative group; anti-200a-3p, miR-200a-3p downregulation group; si-Nrf2, downregulation of miR-200a-3p and si-Nrf2 group; si-, small interfering RNA; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

caspase-1 and NLRP3 *in vitro* following the overexpression of miRNA-200a-3p, compared with the miRNA-200a-3p overexpression group. The inhibition of ROS also attenuated the effect of miRNA-200a-3p on IL-1β and IL-18 levels *in vitro*, when compared with the miRNA-200a-3p overexpression group (Fig. 8F-G).

Inhibition of NLRP3 attenuates the effects of miRNA-200a-3p on inflammation in vitro. To further elucidate the roles of NLRP3 in the effects of miRNA-200a-3p on inflammation *in vitro*, si-NLRP3 was employed to reduce the protein expression of NLRP3 and caspase-1 *in vitro* following the overexpression of miRNA-200a-3p, when in comparison with the miRNA-200a-3p overexpression group (Fig. 9A-C). si-NLRP3 also reduced IL-1β and IL-18 levels *in vitro* following the overexpression of miRNA-200a-3p, compared with the miRNA-200a-3p overexpression group (Fig. 9D and E).

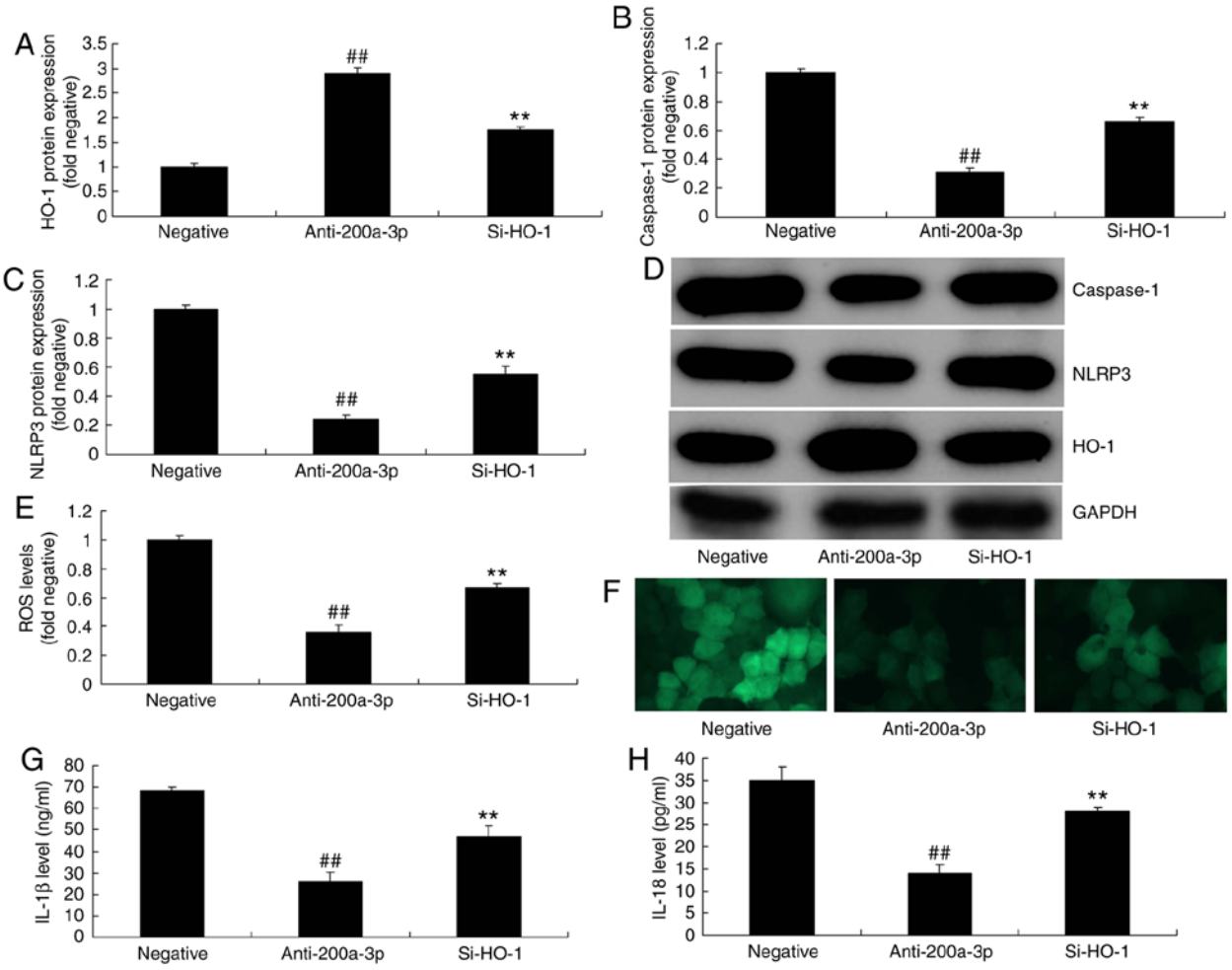

Figure 7. Inhibition of HO-1 reduces the effects of anti-miRNA-200a-3p on inflammation *in vitro*. (A) HO-1, (B) caspase-1 and (C) NLRP3 protein expressions were determined by (D) western blot analysis. The levels of (E and F) ROS (magnification, x200), (G) IL-1β and (H) IL-18 were also assessed. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group; **P<0.01 vs. miR-200a-3p downregulation group. Negative, negative group; anti-200a-3p, miR-200a-3p downregulation group; si-HO-1, downregulation of miR-200a-3p and si-HO-1 group; si-, small interfering RNA; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

Discussion

As a type of systemic inflammatory response syndrome induced by the imbalance between the pro- and anti-inflammatory mechanism, sepsis is generally caused by severe infection, burns, trauma, shock and major surgery (12). The major pathophysiological process of sepsis is the intense self-destructive systemic inflammatory response caused by the excessive expression of the pro-inflammatory factors and other inflammatory mediators (13). Sepsis often leads to serious pathophysiological alterations in the vital organs (14). The mortality of sepsis patients with sepsis shock and multiple organ dysfunction has been suggested to be as high as 80-90% (2). To the best of our knowledge, the present study demonstrated for the first time that the expression of miRNA-200a-3p was higher in the sepsis model group than in the sham group.

miRNAs can enhance the immunocompetence of the body mainly by regulating the synthesis and release of inflammatory factors (15). In the case of sepsis, miRNAs can upregulate the expression of miRNA-155, enhance the release of inflammatory factors and inhibit the synthesis of anti-inflammatory factors (15). In the pathophysiological process of sepsis, miRNAs can promote the synthesis of inflammatory factors, enhance immune function, induce the apoptosis of immune cells and induce immunosuppression (16). miRNAs are involved in the precise regulation of the genesis, development and outcome of sepsis at various levels, such as the inflammatory response, immune cell differentiation and apoptosis. It is speculated that these miRNAs may possess regulatory effects on the differentiation of the effector T-cell (16). Thus, the present study next determined that overexpression of miRNA-200a-3p promoted the levels of IL-1β and IL-18 *in vitro*.

ROS, key cytokines in the inflammatory response, are produced early in the inflammatory response, reaching a peak rapidly, and in turn inducing the production of downstream cytokines, such as NLRP1, NLRP3, IL-1β, IL-6 and IL-8, thereby mediating a series of inflammatory cascade reactions (17). ROS, which are considered to be advanced stage inflammatory factors, play a key role in the lethal process of severe sepsis, which is also characterized by delayed secretion and long release time in relation to NLRP3 (18). In addition, the present study demonstrated that overexpression of

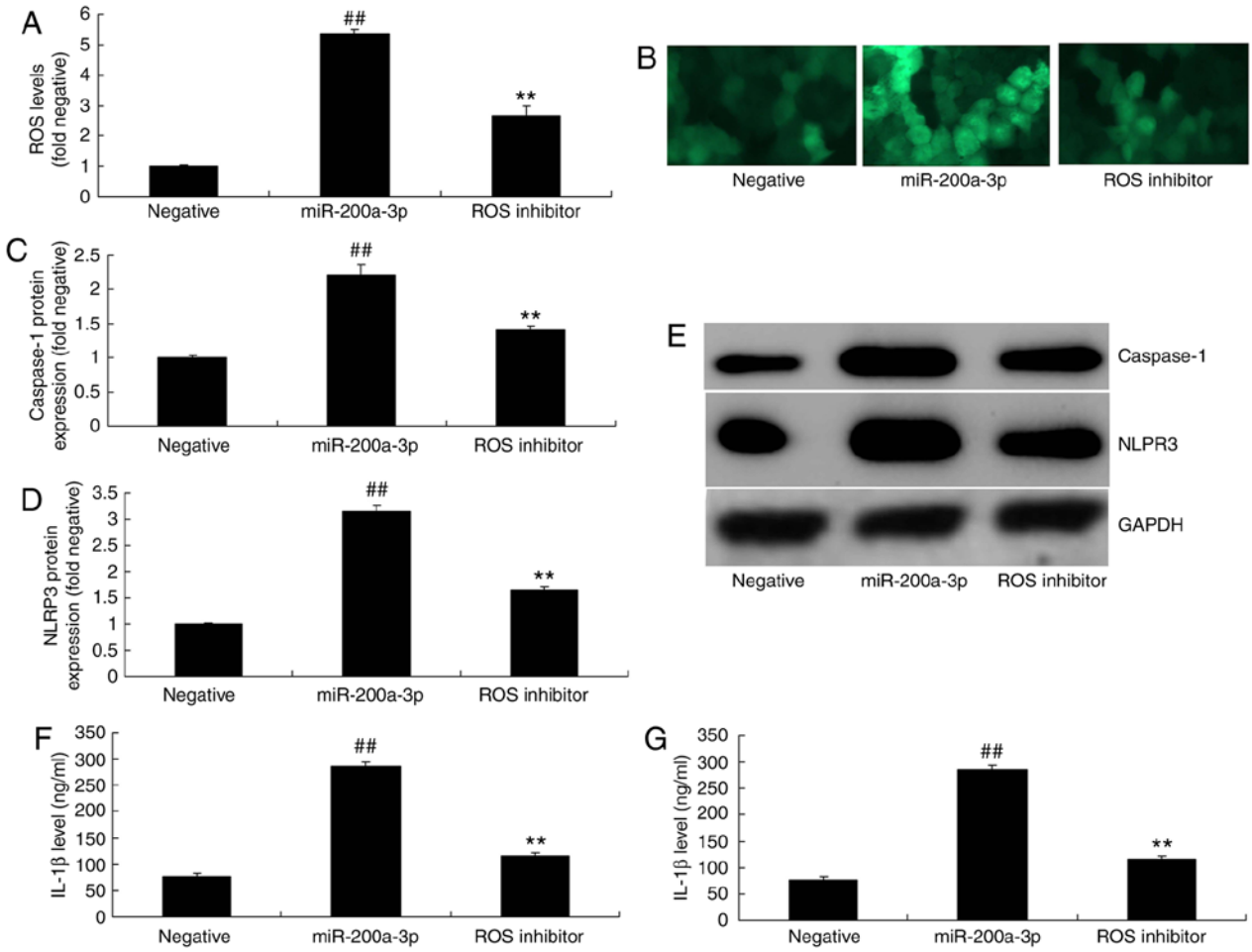

Figure 8. ROS participates in the effect of miRNA-200a-3p on inflammation *in vitro*. (A and B) ROS levels were assessed in cells treated with miR-200a-3p plasmid and ROS inhibitor (magnification, x200). (C) Caspase-1 and (D) NLRP3 protein expressions were determined by (E) western blotting analysis. (F) IL-1β and (G) IL-18 levels were also determined. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group; **P<0.01 vs. miR-200a-3p overexpression group. Negative, negative group; miR-200a-3p, miR-200a-3p overexpression group; ROS inhibitor, overexpression of miR-200a-3p and ROS inhibitor group; miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

miRNA-200a-3p increased ROS levels and promoted IL-1β and IL-18 levels *in vitro*. Xiao *et al* (19) indicated that the p38/p53/miR-200a-3p feedback loop promotes oxidative stress-mediated liver cell death. The present study revealed only the effects of miRNA-200a-3p on inflammation, and as ROS also regulates oxidative stress, this is experiment alone is insufficient; we will analyze the effects of miRNA-200a-3p on oxidative stress in a future study.

NLRP3 is an important factor for amplifying and continuing inflammation as it can promote the activation, differentiation and infiltration of macrophages, upregulate the expression of adhesion molecules, enhance the inflammatory reaction, promote the activation and aggregation of neutrophils, release a large amount of elastase and oxygen free radicals (which result in lung capillary endothelial cell and alveolar epithelial cells injury), vessel extracellular matrix destruction, increased pulmonary vascular permeability, and they can also induce severe alveoli and pulmonary interstitial edema (4,20). NLRP3 is an important factor that results in acute respiratory distress syndrome, which can be used to evaluate the degree of inflammatory response in patients with systemic infection and can serve as a monitoring index for inflammation treatment (4). The present data revealed that the overexpression of miRNA-200a-3p suppressed the protein expression of Keap1, Nrf2 and HO-1 and induced that of NLRP3 and caspase-1 *in vitro*. The inhibition of NLRP3 attenuated the effects of miRNA-200a-3p on inflammation *in vitro*. Furthermore, Ding *et al* (21) reported that curcumin and allopurinol ameliorated fructose-induced hepatic inflammation via miR-200a-mediated thioredoxin interacting protein/NLRP3 inflammasome inhibition in rats.

In recent years, an increasing amount of attention has been paid to the signal transduction mechanism during the pathogenesis and development of sepsis (22). A recent discovery has indicated that the Keap1/Nrf2/HO-1 signaling pathway played an important role in ROS transduction (22). The Keap1/Nrf2/HO-1 pathway can mediate multiple-cytokine-regulated cell growth, differentiation, proliferation and apoptosis, thereby playing a key role in the process of sepsis, driving ROS production; Keap1/Nrf2/HO-1 also plays a critical role in the ROS mediator response (22). In addition, the Keap1/Nrf2/HO-1 signaling pathway plays an important role in the development of the multiple-organ dysfunction of sepsis (23). In the present study, the

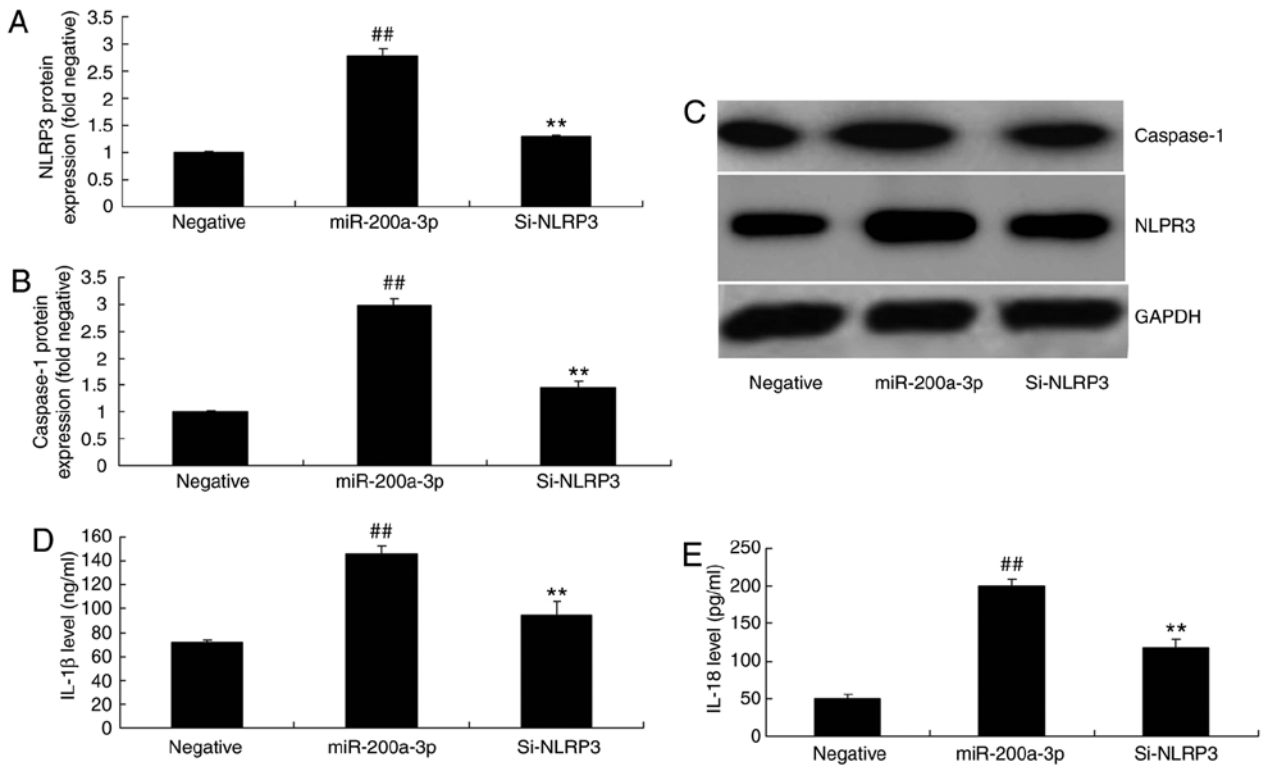

Figure 9. Inhibition of NLRP3 reduces the effects of miRNA-200a-3p on inflammation *in vitro*. (A) NLRP3 and (B) caspase-1 protein expressions were determined by (C) western blotting analysis. (D) IL-1β and (E) IL-18 expression levels were also evaluated. Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. negative group; **P<0.01 vs. miR-200a-3p overexpression group. Negative, negative group; miR-200a-3p, miR-200a-3p overexpression group; si-NLRP3, overexpression of miR-200a-3p and si-NLRP3 group; si-, small interfering RNA; miRNA/miR, microRNA; NLRP3, NLR family pyrin domain containing 3; IL, interleukin.

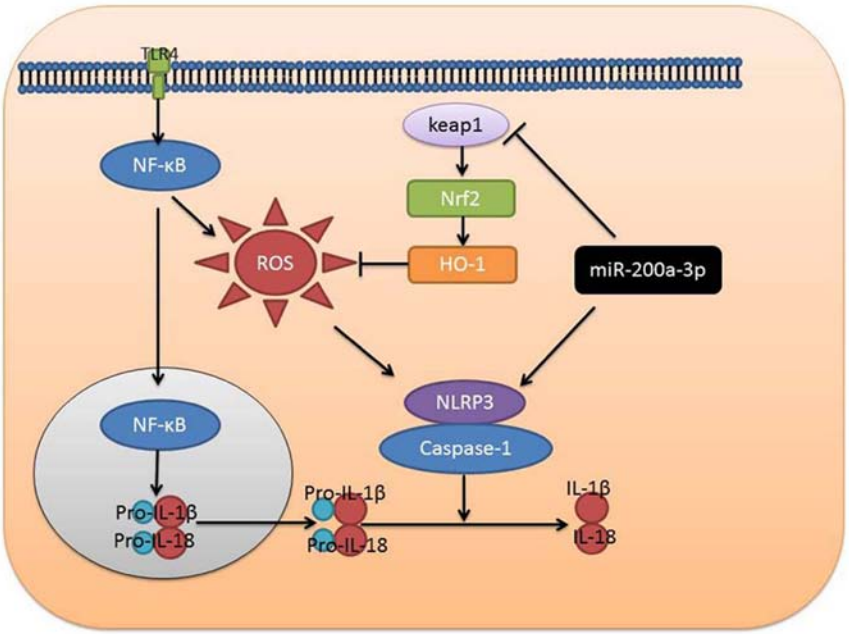

Figure 10. Overexpression of miR-200a-3p promotes inflammation in sepsis-induced brain injury through Keap1/Nrf2/HO-1/ROS-induced NLRP3. miR, microRNA; NLRP3, NLR family pyrin domain containing 3; Keap1, Kelch like ECH associated protein-1; Nrf2, nuclear factor erythroid 2 like 2; HO-1, heme oxygenase; ROS, reactive oxygen species; IL, interleukin.

inhibition of Keap1, Nrf2 or HO-1 attenuated the effects of anti-miRNA-200a-3p on inflammation *in vitro*. Wei *et al* (24) suggested that miR-200a-3p/141-3p coordinated Keap1-Nrf2 signaling in renal mesangial cells and the renal cortex of diabetic mice. Therefore, these results indicated that Keap1/Nrf2/HO-1-regulated miRNA-200a-3p in sepsis.

In conclusion, the present study examined the roles of miRNA-200a-3p in sepsis-induced brain injury. The results

demonstrated that miRNA-200a-3p promoted sepsis through Keap1/Nrf2/HO-1/ROS-induced NLRP3 in sepsis-induced brain injury (Fig. 10). Therefore, the present findings indicate that miRNA-200a-3p may provide a better understanding of sepsis-induced brain injury and help to identify potential therapeutic targets for sepsis-induced brain injury.

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Availability of data and materials

The data sets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZW designed the experiment, analyzed the data and wrote the manuscript. JY, JC, SC and HY performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The First Affiliated Hospital of Xiamen University on Animal Care.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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