miRNA-20b inhibits cerebral ischemia-induced inflammation through targeting NLRP3

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Abstract. The present study was designed to investigate the role of microRNA (miRNA)-20b in the inflammatory response during cerebral ischemia and the underlying mechanism following cerebral ischemia. A reverse transcription quantitative polymerase chain reaction assay was used to measure the expression of miRNA-20b, and tumor necrosis factor α, interleukin (IL)-6, IL-18 and IL-1β levels were measured using ELISA. In addition, the protein expression levels of NOD-like receptor pyrin domain containing 3 (NLRP3), caspase-1, IL-1β and IL-18 were determined by western blot analysis. It was determined that the expression of miRNA-20b during cerebral ischemia was increased compared with the control group. The overexpression of miRNA-20b increased the levels of IL-1β and IL-18 in the cerebral ischemia group through activation of the NLRP3 signaling pathway. Conversely, the downregulation of miRNA-20b suppressed IL-1β and IL-18 levels in cerebral ischemia via suppression of the NLRP3 signaling pathway. Additionally, the overexpression of miRNA-20b increased the levels of adenosine 5'-triphosphate (ATP) and reactive oxygen species (ROS) in the cerebral ischemia group, which were decreased following the downregulation of miRNA-20b. The inhibition of NLRP3 decreased the pro-inflammatory effects of miRNA-20b in cerebral ischemia. Suppression of ATP decreases the pro-inflammatory effects of miRNA-20b in cerebral ischemia. Suppression of ROS also decreases the pro-inflammatory effects of miRNA-20b in cerebral ischemia. Collectively, the present study provided novel insight into the role of miRNA-20b upregulation in the promotion of inflammation following cerebral infarction, suggesting that the miRNA-20b/NLRP3 axis may be a putative therapeutic target in cerebral ischemia.

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Introduction

Stroke is one of the primary diseases threatening human health. According to the most recent analysis of the global disease burden, stroke is second among all causes of mortality worldwide (1). Concurrently, brain tissue injury is also the leading cause of permanent disability (1). At present, with an increase in the size of the aging population in China, China ranks first in the world in terms of the number of patients suffering from strokes (2). The morbidity and mortality in China are increased compared with those in economically developed countries, including European and the USA. It remains a serious threat to human health in China. Concomitantly, the incidence of stroke indicates a younger trend in China (2): The ages of onset and mortality of patients suffering strokes in China is decreasing, compared with the United States of America (3). A previous study have indicated that the primary subtype of stroke classification is ischemic stroke, accounting for ~80% of the overall stroke incidence (3). The high morbidity, mortality and recurrence rates of ischemic stroke lead to heavy financial burdens on families and society (4). Therefore, it is an urgent global problem to be solved, of how to minimize the neurological dysfunction following stroke and to restore the injured neurological functions as soon as possible (3).

The pathological process of cerebral ischemia-reperfusion injury is complicated and affected by multiple factors (5). The mechanisms of the occurrence and action of cerebral ischemia-reperfusion injury involve the interaction of multiple factors (6). The inflammatory process serves a considerably important role during cerebral ischemia-reperfusion injury (5). With the increasing level of understanding of cerebral ischemia-reperfusion injury, multiple studies have provided novel ideas to prevent and treat this condition (7). Among them, blocking the inflammatory cascade following reperfusion is an ideal strategy to improve cerebral injury following ischemia-reperfusion events. However, the timing of the inhibition of the inflammatory reaction is an important problem in the success of this treatment (7).

Cerebral infarction is a serious vascular complication of diabetes. Diabetes combined with acute cerebral infarction accounts for 20-25% of the total number of cases of cerebral infarction (8). Larger infarction areas and more severe symptoms of diabetes are more likely to result in progressive strokes, with poorer prognoses (9). At present, no effective and

feasible therapeutic approaches have been made available for diabetic cerebral infarction. MicroRNAs (miRNAs) are a class of small non-coding RNAs, containing 20-22 nucleotides (10). Previous studies have suggested that miRNAs regulate gene expression by modulating the translational process of their targeted mRNAs. They are widely involved in various biological processes, including cell differentiation, proliferation and apoptosis (10,11).

In previous years, a study demonstrated that the activation of NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome serves an important role in cerebral ischemia-reperfusion injury (12). The NLRP3 inflammasome is a complicated group of multiprotein complexes (12). It belongs to the family of intracytoplasmic pattern recognition receptors (PRRs) that recognize exogenous pathogens and intrinsic endogenous danger signals, including during ischemia-reperfusion injury (13). In addition, the NLRP3 inflammasome regulates innate immunity and acquired immunity, which is a key molecular pathway for the inflammatory cascade. A variety of studies have suggested that the structure, function, expression and distribution of the NLRP3 inflammasome may affect the NLRP3-mediated inflammatory reaction (13,14). It is involved in multiple pathophysiological processes, including immunity, ischemia-reperfusion injury and cerebral degenerative disease (13). Ischemia-reperfusion injury is an inflammatory cascade involving the interaction of multiple factors. In previous years, a number studies have demonstrated that the NLRP3 inflammasome, an important PPR in innate immunity, is involved in the inflammation-associated injury of ischemia-reperfusion injury (14). Therefore, blocking or inhibiting the activation of NLRP3 may become a novel therapeutic target in ischemic cerebrovascular disease (14). Therefore, the present study aimed to investigate the role of miRNA-20b in inflammation of cerebral ischemia and the underlying mechanism following cerebral ischemia.

Materials and methods

Animal model. Sprague-Dawley rats (male, 5-6 week, n=12, 170-200 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed at 22-23°C, 55-60% humidity, 12-h dark cycle light, free access to food and water. Rats were anesthetized using 35 mg/kg pentobarbital sodium (intraperitoneally). A nylon filament with its cusp slightly rounded by heat was advanced into the lumen of the internal carotid artery to occlude the right middle cerebral artery (MCA) for 1 h, and the filament was subsequently withdrawn to allow reperfusion. All experiments were performed in compliance with guidelines for the ethical use of animals of Hebei General Hospital. The present study was approved by the Hebei Municipal Committee of Science and Technology.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA from hippocampus tissue samples and HUVECs cell samples was extracted using a mirVana miRNA Isolation kit (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was reverse transcribed using the PrimeScript RT reagent kit at 37°C for 30 min and 84°C for 10 sec. RT-qPCR was performed using SYBR Premix Ex Taq (Takara Bio., Inc., Otsu, Japan) on an ABI PRISM 7500

Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The primers of miR-20b were as follows: Forward, 5'-TGTCAACGA TACGCTACGA-3' and reverse, 5'-GCTCATAGTGCAGGT AGA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTA AAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGT CAT-3'. PCR amplification was performed at 95°C for 10 min prior to 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed at 72°C for 5 min. miRNA was measured using the $2^{-\Delta\Delta Cq}$ method (15).

miRNA microarray. Total RNA from HUVECs and rat tissues was labeled and hybridized with the miRCURY™ LNA Array (v.16.0; Exiqon; Qiagen, Inc., Valencia, CA, USA) and analyzed using Agilent Feature Extraction Software (version A.10.7.3.1; Agilent Technologies, Inc., Santa Clara, CA, USA). The statistical significance of the miRNAs was analyzed by Agilent Feature Extraction Software.

ELISA. Serum samples of rats and cell supernatants samples were collected at 1,000 x g for 10 min at 4°C. TNF-α (cat. no. H052), interleukin (IL)-6 (cat. no. H007), IL-18 (cat. no. H015) and IL-1β (cat. no. H002) levels were measured using ELISA kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China).

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs) were purchased from the Type Culture Collection of the Chinese academy of sciences (Shanghai, China) and grown in M199 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; both HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 0.1% gelatin and 1% penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) with 5% CO_2 at 37°C. HUVECs were transfected by 100 ng of miRNA-20b (5'-GAAAAGCAG GAAGGACCCTCGCCCTTCAAACCC-3'), 100 ng of anti-miRNA-20b (5'-GGGTTTGAAGGGCGAGGGTCC TTCCTGCTTTTC-3) or 100 ng of negative mimics (5'-TTC TCCGAACGTGTCACGT-3') using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) for 4 h. Then, the cells were cultured in M199 medium without FBS in a hypoxia incubator (Sanyo, Osaka, Japan) at 37°C, 5% CO_2, 94% N_2 and 1% O_2 for 12 h. The luciferase assay was performed using TransMessenger Transfection Reagent (Tiangen Biotech Co., Ltd., Beijing, China).

Luciferase reporter assays. The fragment was designated as NLRP3 and miRNA-20b mimics. The recombinant reporter pGL3M vectors (Promega Corporation, Madison, WI, USA) with NLRP3 were co-transfected with miRNA-20b into HUVECs using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) for 4 h. The method of normalization was used as comparison with *Renilla* luciferase activity. After 48 h of transfection, luciferase activity levels normalized to *Renilla* activity was measured using an automatic micro-plate reader with the Dual-Luciferase Reporter Assay System (Promega Corporation).

ATP and ROS assay. Cell was collected at 1,000 x g for 10 min at 4°C and used to measured ATP level using ATP kits
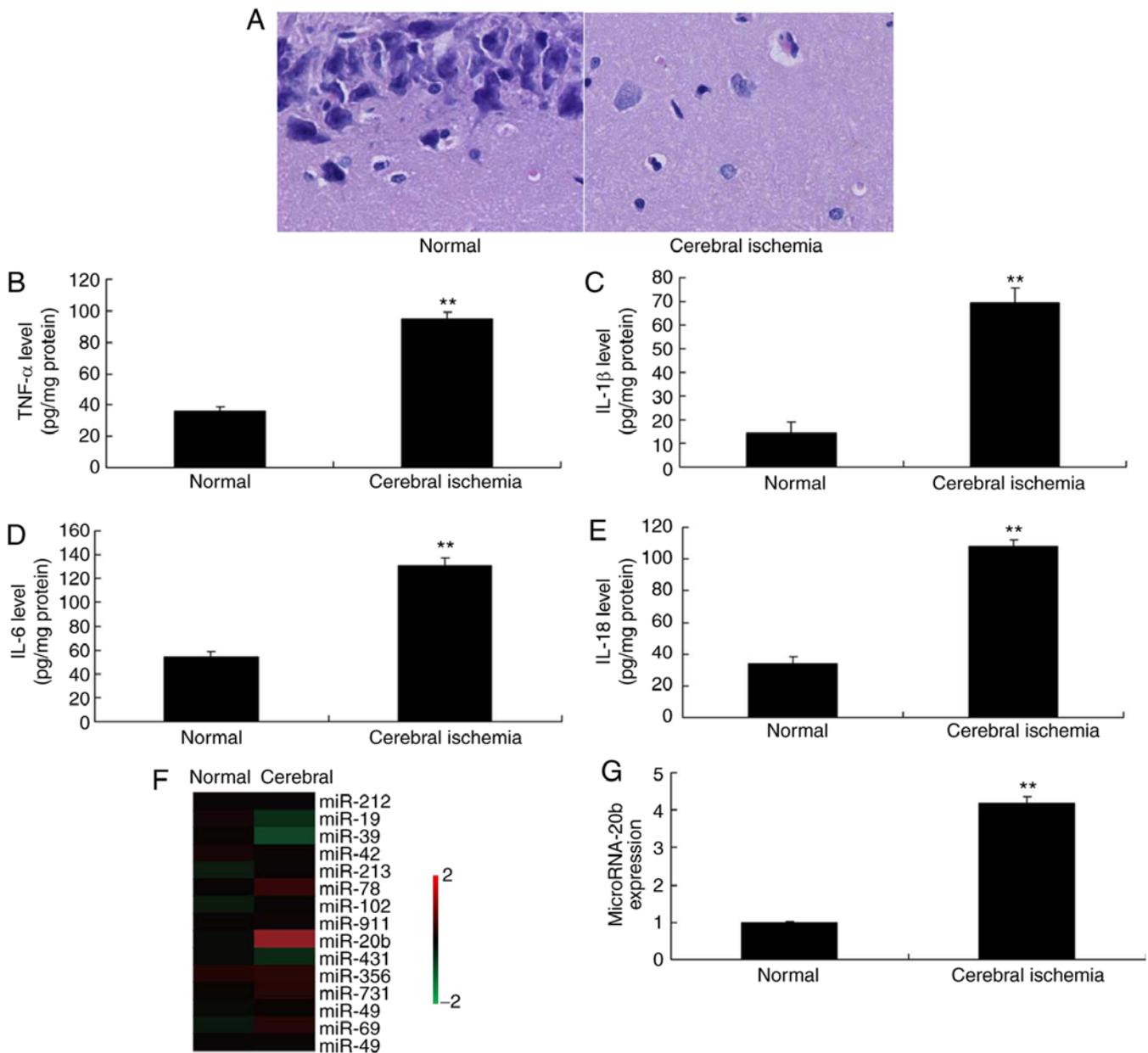
Figure 1. Expression of miRNA-20b in cerebral ischemia. (A) Hematoxylin and eosin staining of hippocampus tissues, magnification, x200. Serum levels of (B) TNF-α, (C) IL-1β, (D) IL-6 and (E) IL-18. (F) Microarray and (G) quantitative polymerase chain reaction analysis of miRNA-20b expression. **P<0.01 vs. normal control group. Normal, normal control group; Cerebral ischemia, cerebral ischemia group; miRNA, microRNA; TNF-α, tumor necrosis factor α, IL, interleukin.

(cat. no. S0026). ROS levels were measured using ROS kits (cat. no. S0033; both Beyotime Institute of Biotechnology).

Hematoxylin and eosin (H&E) staining. Sections were stained with H&E assay for 10 min at room temperature. H&E staining was performed on hippocampus tissues and sections were viewed under confocal microscopy (Leica SP5, Argon laser; Leica Microsystems, Inc., Buffalo Grove, IL, USA; magnification x200).

Western blot analysis. Total protein was extracted from HUVEC samples using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) from cell samples and protein concentration was determined by a bicinchoninic acid Protein Assay kit. A total of 30 μg protein was subjected

to 10% SDS-PAGE lysis and transferred to polyvinylidene fluoride membranes. Membranes were blocked in 5% skimmed milk (Beyotime Institute of Biotechnology) for 2 h at room temperature and incubated with antibodies against NLRP3 (cat. no. 13158; 1:1,000), caspase-1 (cat. no. 3866; 1:1,000; both Cell Signaling Technology, Inc., Danvers, MA, USA), IL-18 (ab191860; 1:1,000; Abcam), IL-1β (cat. no. 12242; 1:1,000) and GAPDH (5174; 1:5,000; both Cell Signaling Technology, Inc.) at 4˚C overnight. The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) at 37˚C for 1 h. Bands were visualized using the electrochemical luminescence (ECL) western detection reagents and analyzed using Image-ProPlus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).
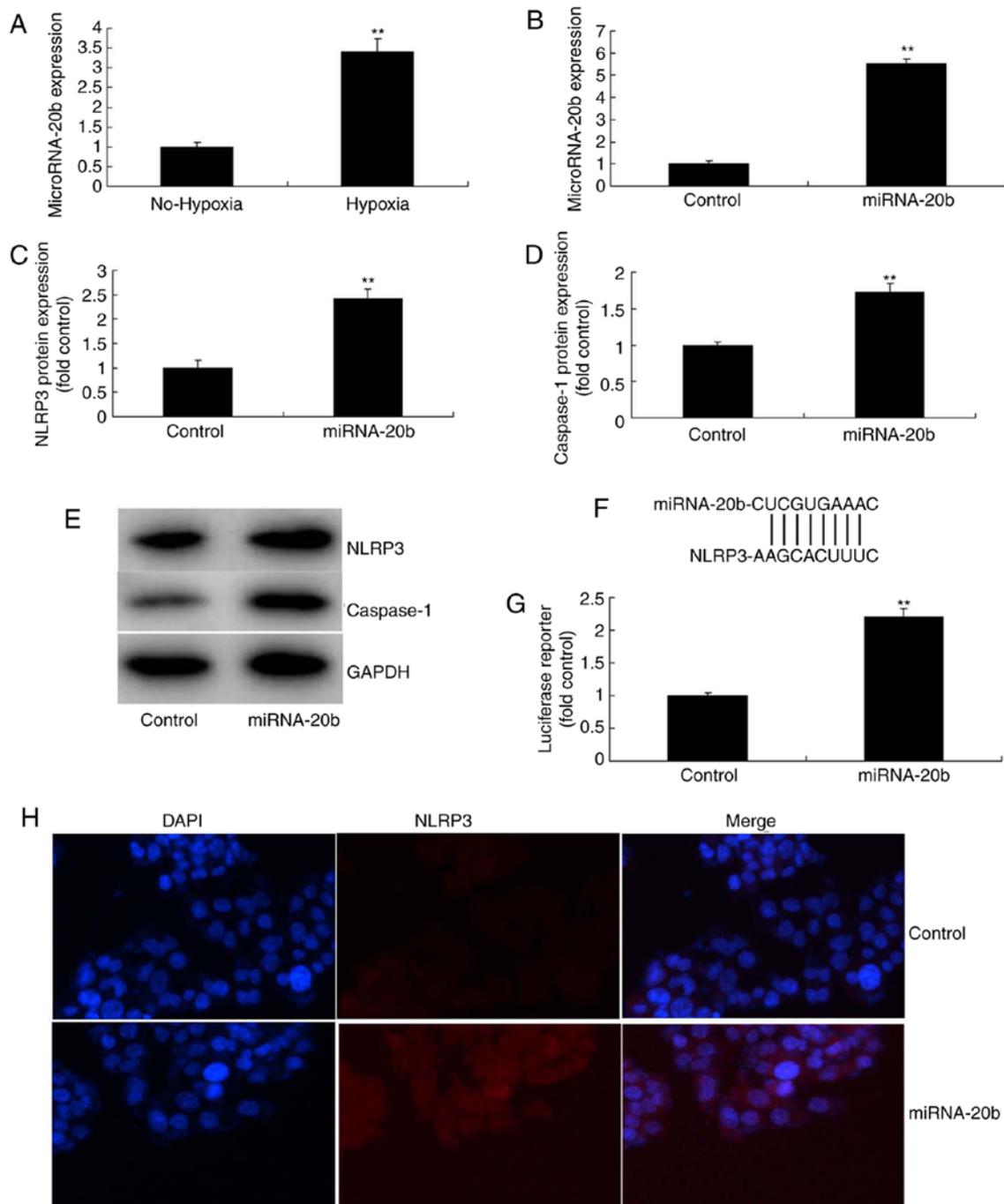
Figure 2. miRNA-20b overexpression regulates the NLRP3 signaling pathway in an *in vitro* hypoxia model. (A) miRNA-20b expression. (B) Quantitative polymerase chain reaction analysis of miRNA-20b expression. Densitometric data of (C) NLRP3 and (D) caspase-1 protein expression and (E) western blot analysis of NLRP3 and caspase-1 protein expression. (F) miRNA-20b directly targets NLPR3. (G) Luciferase reporter activity following miRNA-20B overexpression. (H) Immunohistochemistry results. **P<0.01 vs. control group. miRNA, microRNA; NLRP3, NOD-like receptor pyrin domain containing 3; control, negative control group; miRNA-20b, miRNA-20b over-expression group.

Immunohistochemistry. HUVECs were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology) containing 0.25% Triton-X100 for 1 h at room temperature and incubated with NLPR3 (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. Then, cells were washed with PBST for 20 min and incubated with goat anti-rabbit IgG-CruzFluor™ 555 (cat. no. sc-362262; 1:100; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h. Then, cells were washed with PBST for 20 min and stained with DAPI for 20 min in the dark at room temperature. Cell was obtained by confocal microscopy (Leica SP5, Argon laser; Leica Microsystems, Inc.; magnification x100).

Statistical analysis. Data are presented as the means ± standard deviation. Differences among two groups were assessed by Student's t-test for comparison of 2 groups or one-way analysis of variance followed by Tukey's post hoc test for three groups using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.
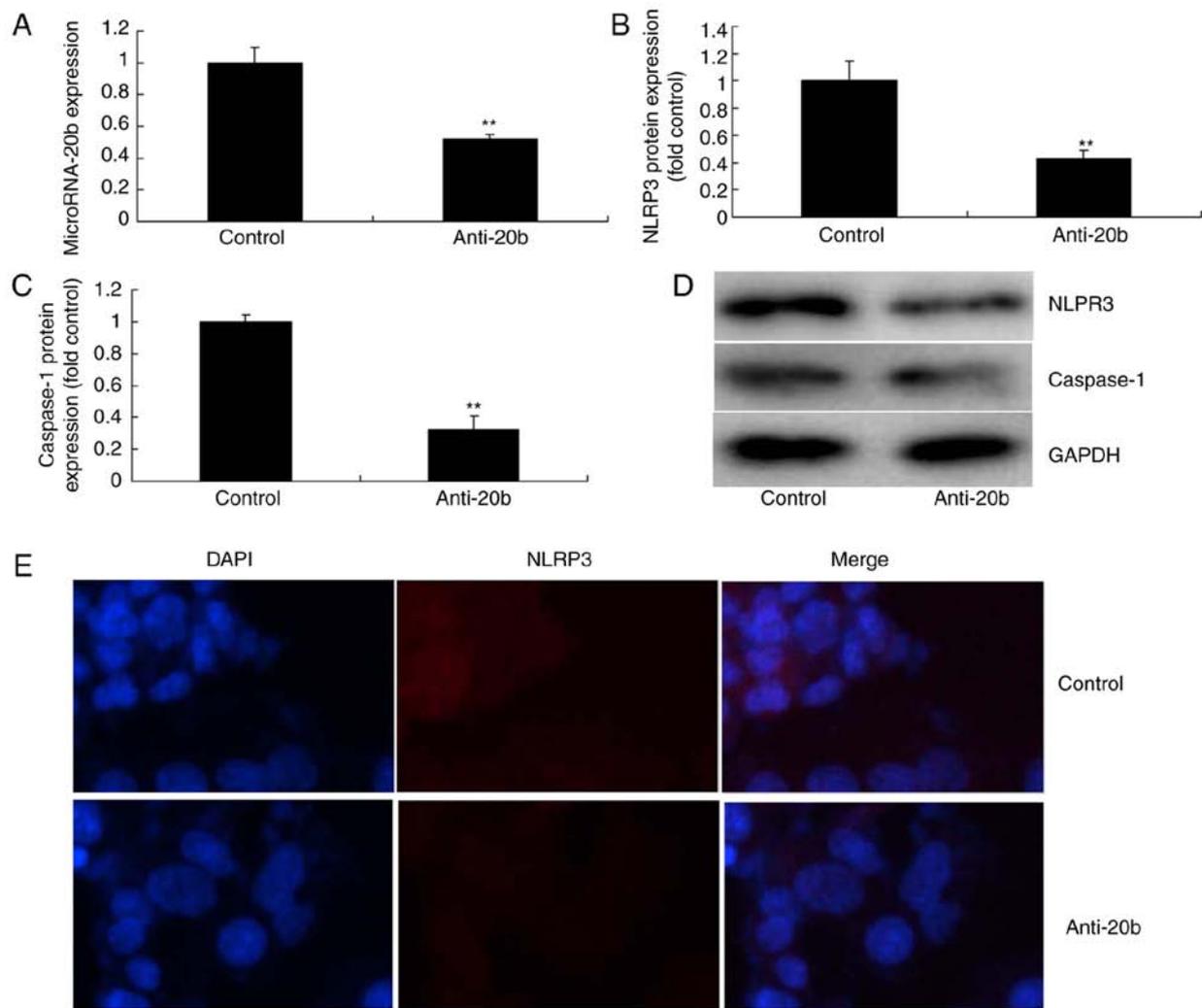
Figure 3. miRNA-20b downregulation regulates NLRP3 signaling pathway *in vitro*. (A) Quantitative polymerase chain reaction analysis of miRNA-20b expression. Densitometric data of (B) NLRP3 and (C) caspase-1 protein expression and (D) western blot analysis of NLRP3 and caspase-1 protein expression. (E) Immunohistochemistry results. $^{**}P<0.01$ vs. negative control group. miRNA, microRNA; NLRP3, NOD-like receptor pyrin domain containing 3; IL, interleukin; control, negative control group; Anti-20b, miRNA-20b downregulation group.

Results

Expression of miRNA-20b in vivo of cerebral ischemia. In order to investigate the potential implication of miRNA-20b on cerebral ischemia, brain tissue samples were collected. H&E staining was performed on the hippocampus, which revealed increased neurocyte cell death in the cerebral ischemia group, compared with the control group (Fig. 1A). Serum TNF-α, IL-6, IL-18 and IL-1β levels were increased in rats with cerebral ischemia compared with the control group (Fig. 1B-E). Microarray and qPCR was used to analyze the expression of miRNAs in rats with cerebral ischemia, which demonstrated that miRNA-20b expression was increased in rats in the cerebral ischemia group compared with the control group (Fig. 1F-1G). These alterations in cases of cerebral ischemia suggested that miRNA-20b may be involved in the molecular pathogenesis of cerebral ischemia.

miRNA-20b regulates the NLRP3 signaling pathway in vitro. To additionally investigate the potential role of miRNA-20b in regulating the inflammatory response of cerebral

ischemia, microarray analysis was also utilized to analyze the expression of miRNA-20b in an *in vitro* model. It was demonstrated that miRNA-20b expression was increased in the cerebral ischemia group compared with the control group (Fig. 2A). Then, miRNA-20b mimics were used to increase the expression of miRNA-20b in the *in vitro* model (Fig. 2B), followed by investigation of the mechanism of action of miRNA-20b on the inflammatory response during cerebral ischemia. The results of microarray analysis indicated that the overexpression of miRNA-20b induced the protein expression of NLPR3 and caspase-1 in cerebral ischemia compared with the control group (Fig. 2C-E). The luciferase reporter assay demonstrated that miRNA-20b directly targeted NLPR3, and that the activity of the luciferase reporter was increased by the overexpression of miRNA-20b, compared with the control group (Fig. 2F-G). The immunofluorescence assay indicated that the overexpression of miRNA-20b induced NLPR3 protein expression in the cerebral ischemia group compared with the control group (Fig. 2H).

Next, whether miRNA-20b downregulation inhibited the NLRP3 signaling pathway in cerebral ischemia was
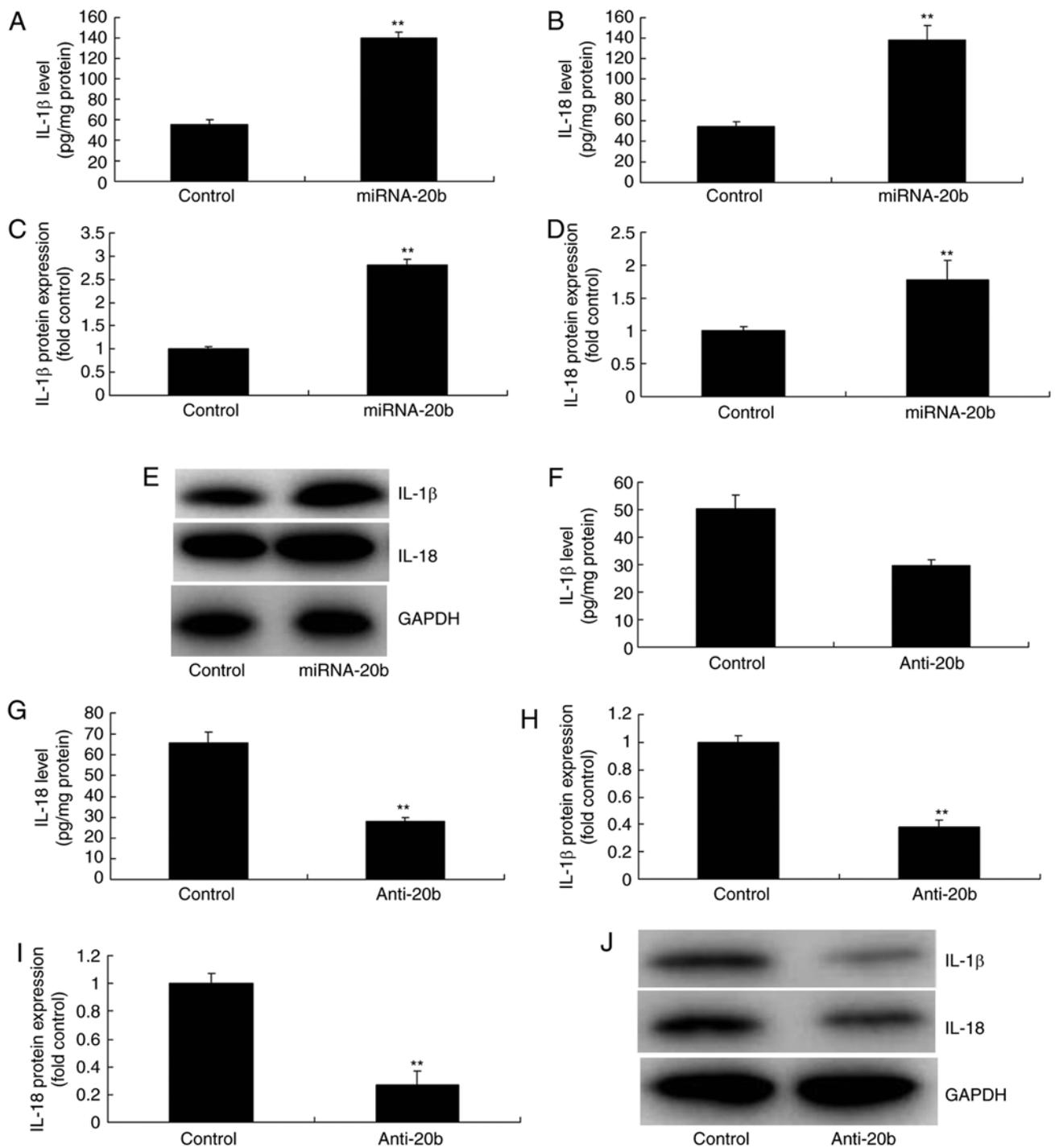
Figure 4. miRNA-20b regulates IL-18 and IL-1β levels *in vitro*. (A) IL-1β and (B) IL-18 levels, densitometric analysis of (C) IL-18 and (D) IL-1β protein expression and (E) western blot analysis of IL-18 and IL-1β protein expression following miRNA-20b overexpression. (F) IL-1β and (G) IL-18 levels, densitometric analysis of (H) IL-18 and (I) IL-1β protein expression and (J) western blot analysis of IL-18 and IL-1β protein expression following miRNA-20b downregulation. **P<0.01 vs. negative control group. miRNA, microRNA; IL, interleukin; control, negative control group; miRNA-20b, miRNA-20b over-expression group; Anti-20b, miRNA-20b downregulation group.

analyzed. As indicated in Fig. 3A, anti-miRNA-20b mimics inhibited the expression of miRNA-20b in the cerebral ischemia group compared with the control group. In addition, the downregulation of miRNA-20b suppressed the protein expression of NLPR3 and caspase-1 in the cerebral ischemia group compared with the control group (Fig. 3B-D). Immunofluorescence assays visually demonstrated that the downregulation of miRNA-20b also suppressed NLPR3

protein expression in the cerebral ischemia group compared with the negative group (Fig. 3E). Together, the aforementioned data indicated that miRNA-20b is a potential target gene of NLRP3 in cerebral ischemia.

miRNA-20b regulates IL-18 and IL-1β levels in vitro. NLRP3 has been demonstrated to induce inflammation by regulating IL-18 and IL-1β to in cerebral ischemia (16). Therefore,
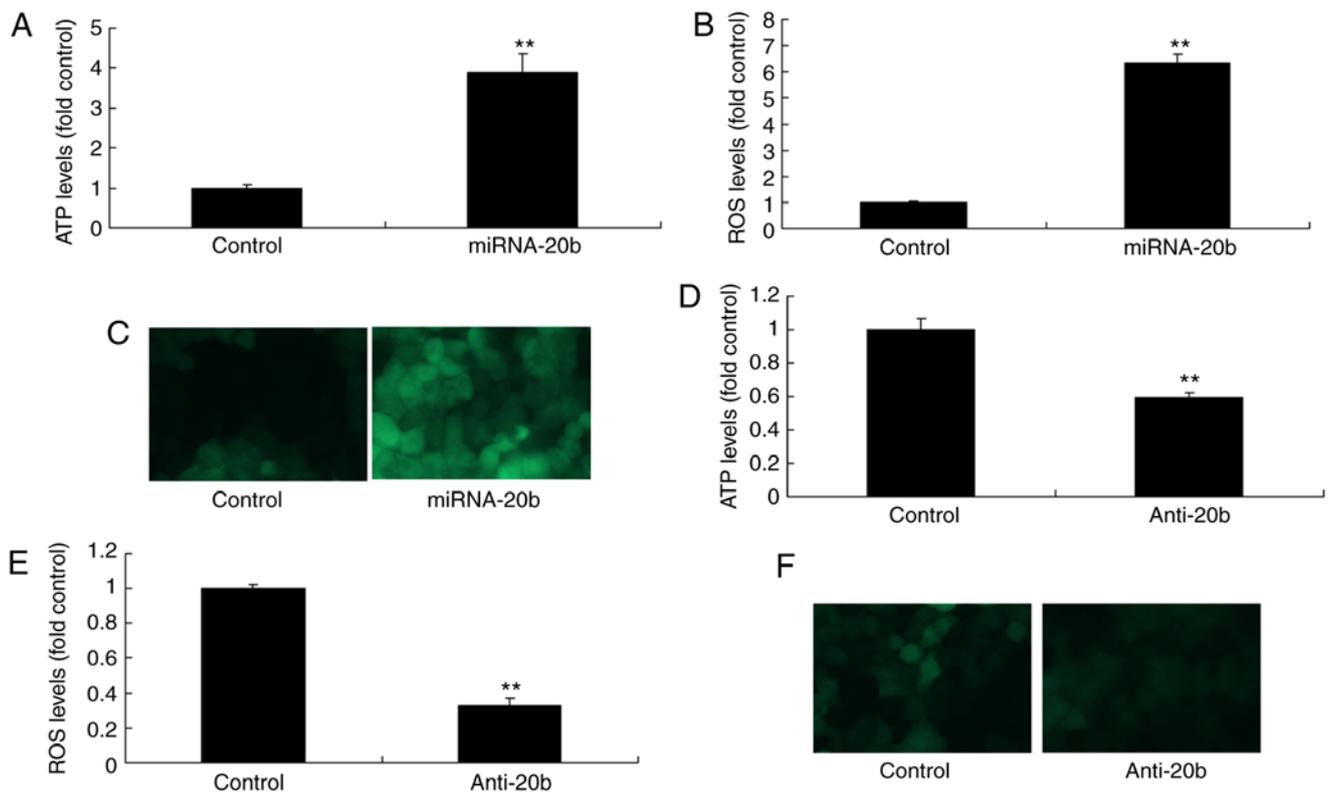
Figure 5. miRNA-20b regulates ATP and ROS levels during cerebral ischemia. (A) ATP and (B) ROS levels determined using ELISA, and (C) ROS levels measured by fluorescence microscope following miRNA-20b overexpression. (D) ATP and (E) ROS levels determined using ELISA, and (F) ROS levels measured by fluorescence microscopy following miRNA-20b downregulation. **P<0.01 vs. negative control group. miRNA, microRNA; ATP, adenosine 5'-triphosphate; ROS, reactive oxygen species; control, negative control group; miRNA-20b, miRNA-20b overexpression group; Anti-20b, miRNA-20b downregulation group.

whether miRNA-20b served a role in the expression of IL-18 and IL-1β *in vitro* was analyzed. As indicated in Fig. 4A-B, IL-18 and IL-1β levels in the supernatant were increased in the *in vitro* model by the overexpression of miRNA-20b compared with the control group. Western blot analysis data indicated that the overexpression of miRNA-20b induced the protein expression of IL-18 and IL-1β in the *in vitro* model compared with the control group (Fig. 4C-E). Then, the downregulation of miRNA-20b also decreased IL-18 and IL-1β levels in the supernatant in the *in vitro* model compared with the control group (Fig. 4F-G). In addition, the downregulation of miRNA-20b also suppressed the protein expression of IL-18 and IL-1β *in vitro* compared with the control group (Fig. 4H-J).

miRNA-20b modulates the levels of ATP and ROS in cerebral ischemia. In order to verify the association between miRNA-20b and NLRP3 in cerebral ischemia, ATP and ROS levels were moderating to NLRP3 signaling pathway (17). Therefore, whether miRNA-20b affected the levels of ATP and ROS during cerebral ischemia was assessed. As indicated in Fig. 5A, the overexpression of miRNA-20b increased the ATP and ROS levels in the cerebral ischemia group compared with the control group (Fig. 5A-C). On the contrary, the downregulation of miRNA-20b decreased ATP and ROS levels in the cerebral ischemia group compared with the control group (Fig. 5D-F). These results suggested that miRNA-20b regulates the NLRP3 signaling pathway via modulation of ATP and ROS levels during cerebral ischemia.

Suppression of ATP decreases the pro-inflammatory effects of miRNA-20b in cerebral ischemia. In order to confirm the role of ATP in the pro-inflammatory effects of miRNA-20b during cerebral ischemia, an ATP scavenger (10 µM INF39) was utilized to inhibit ATP levels, which suppressed the protein expression of NLRP3, caspase-1, IL-18 and IL-1β in the cerebral ischemia group with miRNA-20b overexpression, compared with the miRNA-20b overexpression alone group (Fig. 6A-F). The levels of IL-18 and IL-1β levels in the supernatant were also decreased in the cerebral ischemia group following overexpression of miRNA-20b and combined treatment with the ATP scavenger compared with the miRNA-20b overexpression alone group (Fig. 6G-H). Taken together, these results indicated that miRNA-20b regulates the NLRP3 signaling pathway by altering ATP levels during cerebral ischemia.

Suppression of ROS decreases the pro-inflammatory effects of miRNA-20b in cerebral ischemia. To validate the role of ROS in the pro-inflammation of miRNA-20b in cerebral ischemia, a ROS scavenger (1 mM NAC) was used to decrease ROS levels, which subsequently suppressed the protein expression of NLRP3, caspase-1, IL-18 and IL-1β in cerebral ischemia with miRNA-20b overexpression compared with the miRNA-20b overexpression group (Fig. 7A-F). The IL-18 and IL-1β levels in the supernatants were also decreased in the cerebral ischemia group following the overexpression of miRNA-20b and treatment with the ROS scavenger, compared with the miRNA-20b overexpression alone group (Fig. 7G-H). These results suggested that
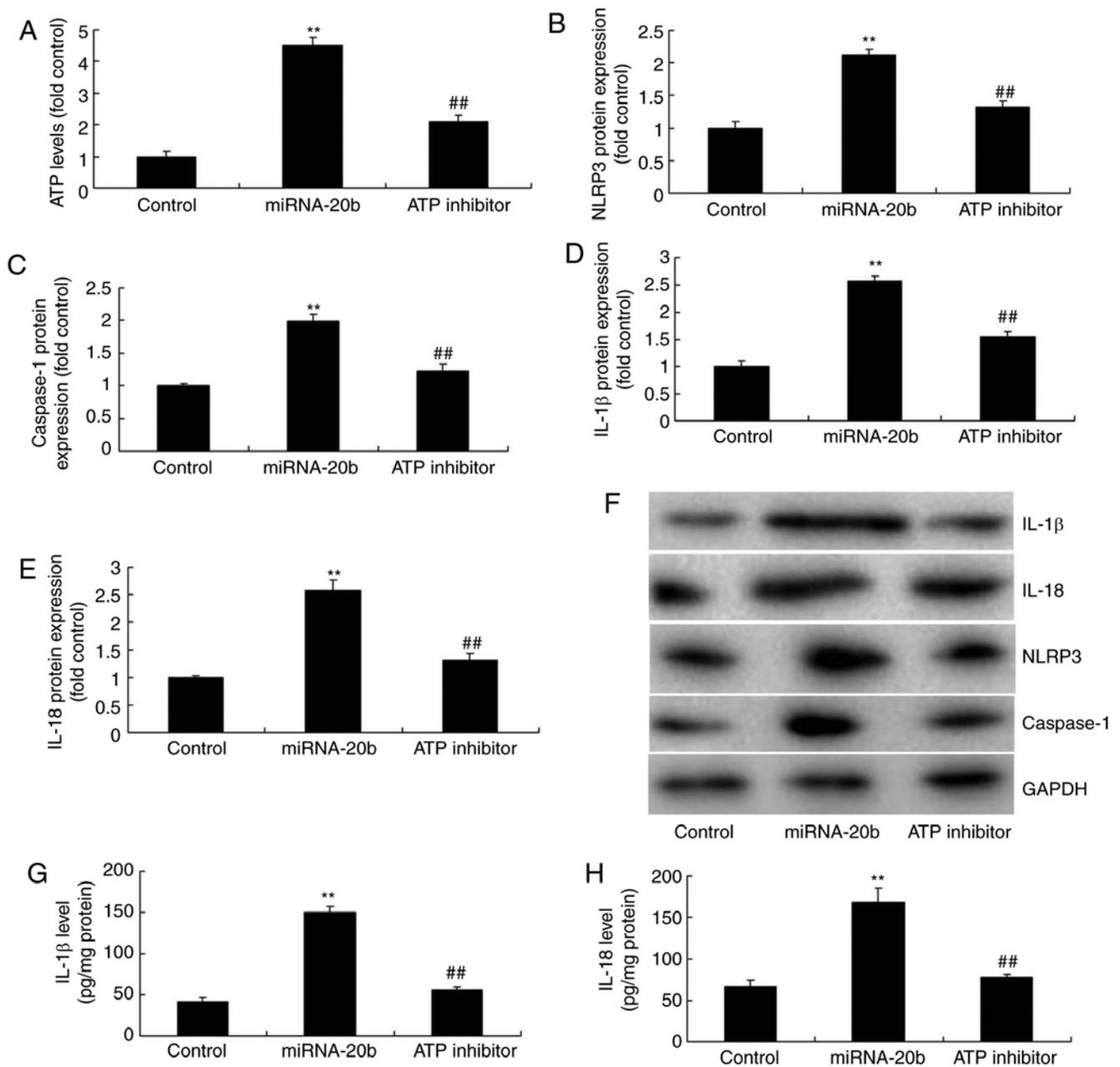
Figure 6. Suppression of ATP decreases the pro-inflammatory effect of miRNA-20b in cerebral ischemia. (A) ATP levels were determined using ELISA; densitometric analysis of (B) NLRP3, (C) caspase-1, (D) IL-18 and (E) IL-1β protein and (F) western blot analysis of NLRP3, caspase-1, IL-18 and IL-1 protein expression following miRNA-20b overexpression and ATP inhibition. Serum levels of (G) IL-1β and (H) IL-18 following miRNA-20b overexpression and ATP inhibition. **$P<0.01$ vs. negative control group. ##$P<0.01$ vs. miRNA-20b overexpression group. miRNA, microRNA; ATP, adenosine 5'-triphosphate; NLRP3, NOD-like receptor pyrin domain containing 3; IL, interleukin; control, negative control group; miRNA-20b, miRNA-20b overexpression group; ATP inhibitor, 10 μM INF39 and miRNA-20b overexpression group.

miRNA-20b regulates the NLRP3 signaling pathway by affecting the levels of ROS during cerebral ischemia.

Suppression of NLRP3 decreases the pro-inflammatory effects of miRNA-20b in cerebral ischemia. To additionally investigate the function of NLRP3 in the pro-inflammatory effects of miRNA-20b during cerebral ischemia, an NLRP3 inhibitor (5 nM MCC950) was used. It was demonstrated that the inhibition of NLRP3 suppressed the protein expression of NLRP3, caspase-1, IL-18 and IL-1β in cerebral ischemia compared with the miRNA-20b overexpression alone group (Fig. 8A-E). The NLRP3 inhibitor decreased IL-18 and IL-1β levels in the

supernatants of the cerebral ischemia in comparison with the miRNA-20b overexpression group (Fig. 8F-G). The schematic for how miRNA-20b inhibits cerebral ischemia-induced inflammation through targeting NLRP3 is demonstrated in Fig. 9.

Discussion

Much improvement has been made in the diagnostic and therapeutic approaches for ischemic stroke in the previous 2 decades. Revascularization or reperfusion therapy remains a key treatment for patients suffering from ischemic stroke (3). Thrombolysis or endovascular treatment, delivered within
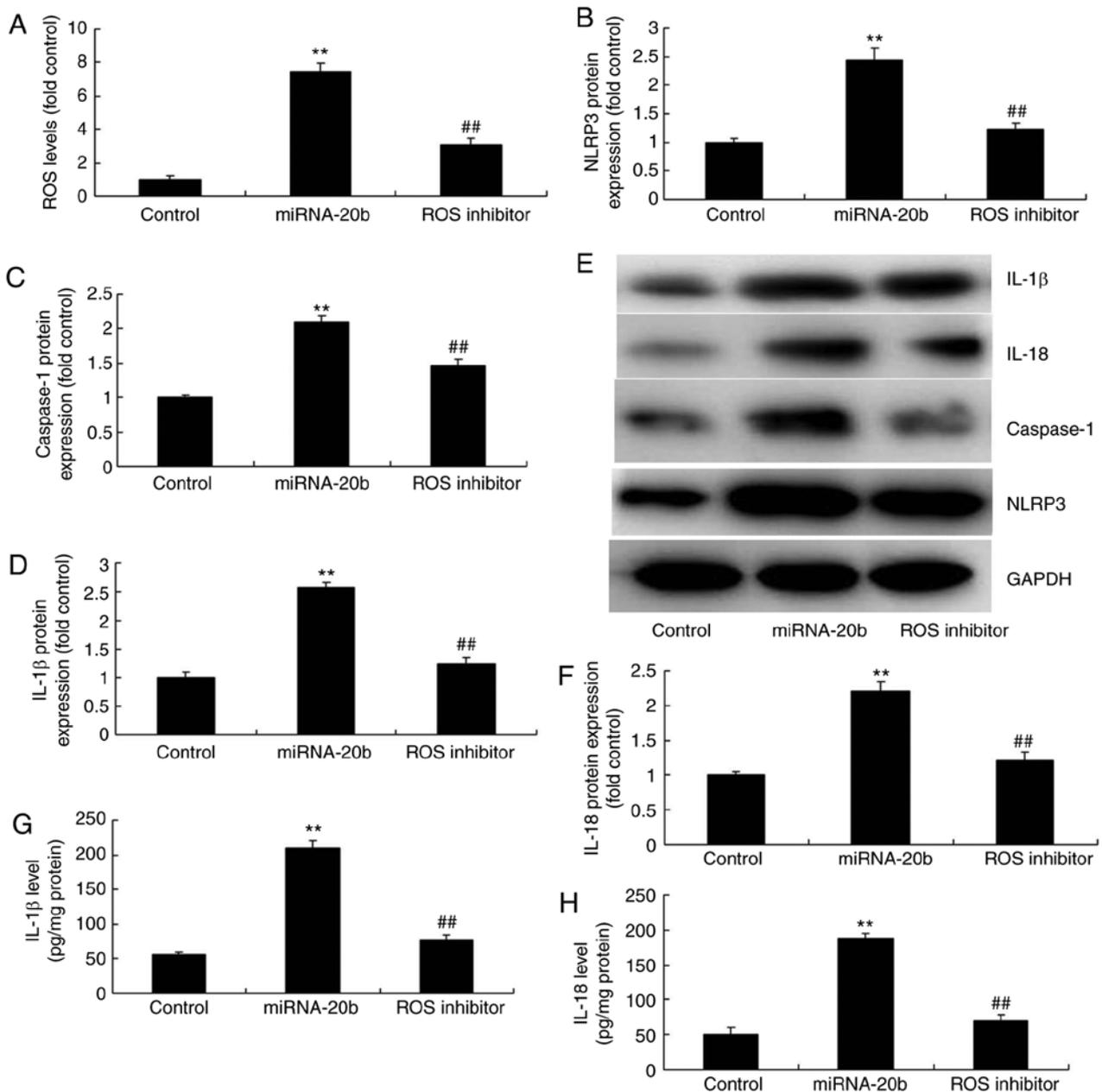
Figure 7. Suppression of ROS decreases the pro-inflammatory effect of miRNA-20b during cerebral ischemia. (A) ROS levels were determined using ELISA. Densitometric analysis of (B) NLRP3, (C) caspase-1, (D) IL-18 and (E) IL-1β protein expression and (F) western blot analysis of NLRP3, caspase-1, IL-18 and IL-1 protein expression following miRNA-20b overexpression and ROS inhibition. Serum levels of (G) IL-1β and (H) IL-18 following miRNA-20b overexpression and ROS inhibition. **P<0.01 vs. negative control group. ##P<0.01 vs. miRNA-20b overexpression group. miRNA, microRNA; ROS, reactive oxygen species; NLRP3, NOD-like receptor pyrin domain containing 3; control, negative control group; miRNA-20b, miRNA-20b overexpression group; ROS inhibitor, 1 mM NAC and miRNA-20b overexpression group.

an effective time window, may rescue cells in the ischemic penumbra (18). Thereby, it may decrease the risk of neurological deficits, decrease morbidity and mortality and increase survival. Ultimately, it may improve the outcomes and future quality of life for patients. However, revascularization may lead to ischemia-reperfusion injury (4). In the present study, it was identified that miRNA-20b expression was increased in rats with cerebral ischemia compared with the control group. Ahmad *et al* (19) demonstrated that miRNA-20b is upregulated in brain metastases from primary breast cancer.

During cerebral ischemia-reperfusion, there are numerous inflammatory factors in the ischemic area (20). In addition, the activation and infiltration of inflammatory cells, and the synthesis and secretion of adhesion molecules, are considered to be cascade reactions that reciprocally promote each other (21). Therefore, the inflammatory response serves an important role in the mechanism of cerebral ischemia-reperfusion injury. In the present study, it was identified that the overexpression of miRNA-20b increased IL-18 and IL-1β levels in an *in vitro* model of cerebral ischemia. Ma *et al* (22) suggested that miRNA-20b decreased the incidence in asthmatic mice.

NLRP3 is primarily expressed in immune organs and peripheral immune cells (13). It has been recently identified
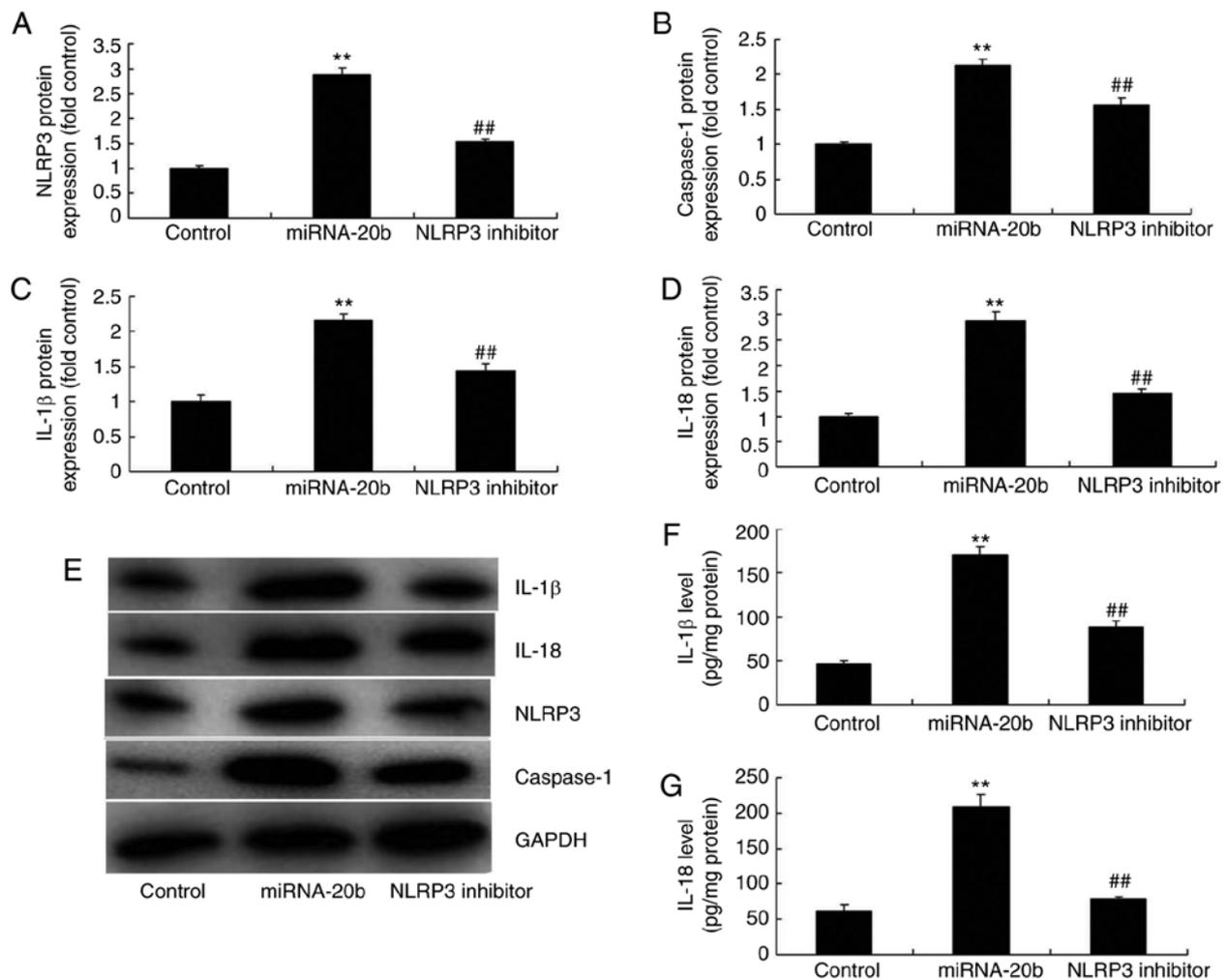
Figure 8. Suppression of NLRP3 decreases the pro-inflammatory effect of miRNA-20b in cerebral ischemia. Densitometric analysis of (A) NLRP3, (B) caspase-1, (C) IL-18 and (D) IL-1β protein and (E) western blot analysis of NLRP3, caspase-1, IL-18 and IL-1 protein expression. Serum levels of (F) IL-1β and (G) IL-18 levels. **P<0.01 vs. negative control group. $^{##}$P<0.01 vs. miRNA-20b overexpression group. miRNA, microRNA; NLRP3, NOD-like receptor pyrin domain containing 3; IL, interleukin; control, control negative group; miRNA-20b, miRNA-20b overexpression group; NLRP3 inhibitor, 5 nM MCC950 and miRNA-20b overexpression group.

to be expressed in the central nervous and cardiovascular systems, with abundant expression in vascular endothelial cells (13). A previous study demonstrated that the expression of NLRP3 was also detected on the wall of intracranial aneurysms (13). Besides, the expression of NLRP3 on the wall of ruptured aneurysms was significantly increased compared with that on the walls of non-ruptured aneurysm (23). These data suggest that NLRP3 may be involved in the formation and progression of intracranial aneurysms. By constructing mouse models of MCA occlusion, NLRP3 has been demonstrated to be primarily expressed in microglia and vascular endothelial cells, and expressed in neurons and astrocytes to a lesser degree (23). The data of the present study demonstrated that the overexpression of miRNA-20b induced NLPR3 and caspase-1 protein expression in cerebral ischemia. Coskun *et al* (24) suggested that miR-20b, miR-98, miR-125b-1*, and let-7e* are novel potential diagnostic biomarkers in ulcerative colitis. So, these results demonstrated that miRNA-20b could be a biomarker for cerebral ischemia.

Numerous studies have demonstrated that NLRP3 expression is upregulated and that the NLRP3 inflammasome is activated following cerebral ischemia (25). Additionally, the neuronal function may be protected by regulating the activity of NLRP3 inflammasome, leading to improved prognoses (25,26). It has been demonstrated that the protein expression levels of NLRP3, apoptosis-associated speck-like protein containing a CARD and caspase-1 are increased, the NLRP3 inflammasome is activated and increased levels of IL-1β and IL-18 are secreted following cerebral ischemia and hypoxia by *in vitro* and *in vivo* studies (26). Caspase-1 inhibitors can suppress neuronal apoptosis and decrease ischemia-reperfusion injury (26). In addition, immunoglobulin treatment may result in reduced activities of NLRP1 and NLRP3 inflammasome, and decrease the infarction size and mortality rate (26). These results additionally demonstrated that the NLRP3 inflammasome is involved in the immune inflammatory reaction following cerebral ischemia-reperfusion injury (26). The present study identified that the suppression of NLRP3 decreased the pro-inflammatory effect of miRNA-20b in cerebral ischemia. In agreement with this, Lou *et al* (27) indicated that miRNA-20b may alleviate the inflammatory response in mice with tuberculosis via targeting the NLRP3/caspase-1/IL-1β pathway.
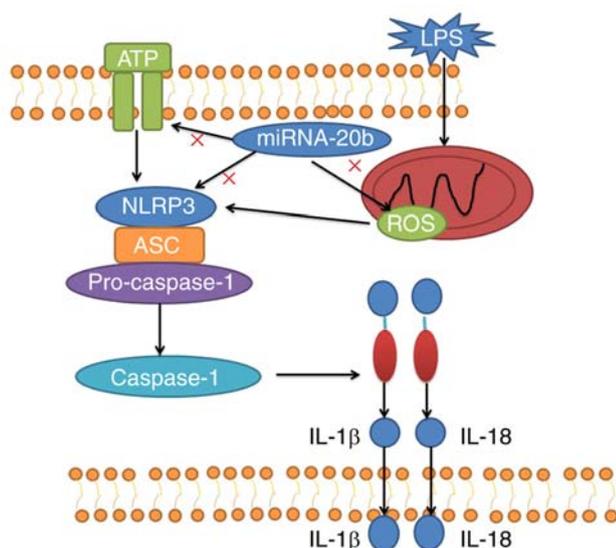
Figure 9. miRNA-20b inhibits cerebral ischemia-induced inflammation through targeting NLRP3. miRNA-20b suppressed ATP, ROS and NLRP3 signaling pathway to reduce IL-1β and IL-18 levels in cerebral ischemia. IL, interleukin; ROS, reactive oxygen species; NLRP3, NOD-like receptor pyrin domain containing 3; ATP, adenosine triphosphate; miRNA, microRNA-20b; LPS, lipopolysaccharide.

In conclusion, the present study provided novel insight into the roles of miRNA-20b upregulation in the promotion of inflammation following cerebral infarction via the NLRP3 signaling pathway. The identification of the miRNA-20b/NLRP3 axis may provide novel insight into the potential molecular mechanisms of cerebral infarction.

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Availability of data and materials

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

Authors' contributions

LL designed the experiment. JZ, HW, LD and SS performed the experiments. LL and JZ analyzed the data. LL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed in compliance with guidelines for the ethical use of animals of Hebei General Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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