Effects of microRNA-223 on morphine analgesic tolerance by targeting NLRP3 in a rat model of neuropathic pain

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Abstract
Objective: To investigate the effects of microRNA-223 on morphine analgesic tolerance by targeting NLRP3 in a rat model of neuropathic pain.

Methods: Our study selected 100 clean grade healthy Sprague-Dawley adult male rats weighing 200 to 250 g. After establishment of a rat model of chronic constriction injury, these rats were divided into 10 groups (10 rats in each group): the normal control, sham operation, chronic constriction injury, normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups. The real-time quantitative polymerase chain reaction assay, Western blotting, and enzyme-linked immunosorbent assay were used for detecting the mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, Interleukin (IL)-1β, and IL-18 in sections of lumbar spinal cord. Immunohistochemistry was applied for detecting the positive rates of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18.

Results: The paw withdrawal threshold and percentage maximum possible effect (%MPE) were higher in chronic constriction injury group when compared with the normal control and sham operation groups. Behavioral tests showed that compared with the chronic constriction injury and normal saline groups, the morphine and miR-223 + morphine groups showed obvious analgesic effects. Expressions of miR-223 in the miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine were significantly higher than those in the chronic constriction injury, normal saline, and morphine groups. Compared with chronic constriction injury, normal saline and morphine groups, the mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18 were significantly decreased in the miR-223 and miR-223 + morphine groups, while mRNA and protein expressions of NLRP3, apoptosis-associated speck-like protein, Caspase-1, IL-1β, and IL-18 were significantly increased in the NLRP3 and NLRP3 + morphine group.

Conclusion: Our study provides strong evidence that miR-223 could suppress the activities of NLRP3 inflammasomes (NLRP3, apoptosis-associated speck-like protein, and Caspase-1) to relieve morphine analgesic tolerance in rats by down-regulating NLRP3.

Keywords
MicroRNA-223, NLRP3, morphine analgesic tolerance, neuropathic pain

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Introduction

Neuropathic pain (NP) refers to the pain caused by damages in the somatosensory system or diseases, whose prevalence is estimated to possibly lie between 6.9% and 10% in the general population with millions of patients all over the world given no exact cause and no treatment methods. The most common causes include toxicity, surgery, radiation, trauma, and congenital disorders. Currently, medication remains the mainstay for NP treatment with most commonly used opioids treating chronic and intractable pain. Morphine, which can be used for intrathecal infusion therapy for NP, prevents pain signals from being transmitted to the center pivot so that pain signals cannot be delivered to the brain cortex, and the pain is controlled. Morphine is widely used in the clinical application for NP treatment with good analgesic effects and can be considered for first-line use in select clinical circumstances. However, the long-term application of morphine can result in morphine analgesic tolerance, and produce a special kind of hyperalgesia, decreasing effectiveness over time and increasing psychological burden of patients. Currently, there are many drugs treating morphine analgesic tolerance, but side effects, safety issues, and other problems brought by those drugs are outstanding. Molecular targeted therapy, which has been well applied in a variety of diseases in recent years, attracts more and more attention in the field of medicine, and a relevant study has confirmed changes in miR expression in NP. MicroRNA (miR) is a class of short non-coding RNAs with a length of about 22 nucleotides. Currently, the role of miRs in NP has received more attention in the field of medicine, and a relevant study has confirmed changes in miR expression in NP. MiR-223, expressed in myeloid cells, shows a negative expression in many diseases, such as leukemia, hepatitis B, influenza, lymphoma, and inflammation. NLRP3 is a type of inflammasome (a multi-protein complex produced by tissue cells after receiving endogenous and exogenous stimulation) which can be activated by cathepsins. In recent years, studies have confirmed that miR-223 can inhibit inflammation, thereby avoiding collateral damage. Recently, it is found that NLRP3 is one of the target genes of miR-223. A study has showed that miR-223 negatively targeting NLRP3, thereby promoting the secretion of certain macrophages. Currently, it is considered that miR-223 involved in the development of inflammatory response as a regulatory molecule. However, no relevant studies have reported whether miR-223 targeting NLRP3 participates in NP and morphine analgesic tolerance. Therefore, the aim of our study is to investigate the effects of miR-223 on morphine analgesic tolerance by targeting NLRP3 in a rat model of NP.

Materials and methods

Ethical statement

The experimental procedures in present study were carried out with the approval of the First Affiliated Hospital of Zhengzhou University. Breeding and use of laboratory animals in this experiment strictly adhered to the International Association for the Study of pain guidelines for the care and protection of laboratory animals.

Experimental animals and groups

In this study, clean grade healthy pure breed Sprague-Dawley male rats (n = 100) (200 ~ 250 g) were obtained from Hayes Lake Animal experiments Ltd. (Shanghai, China). The rats were group-housed (five rats per cage) in a relatively constant feeding environment (a normal circadian rhythm) at 20 ~ 25°C, with free access to food and water. After one week of feeding, the rats were randomly assigned to 10 groups with 10 rats per group, including the normal control, sham operation, chronic constriction injury (CCI), normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups. Different drug interventions were performed on different groups according to following steps (Table 1). Seven days after CCI, the intrathecal administration was conducted in the normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups, and the doses were as follows: the volumes of 10 mg/kg morphine (Shenyang First Pharmaceutical Factory, Shenyang, Liaoning, China), miR-223 lentivirus vectors, and NLRP3 lentivirus vectors were all 5μL (intravitreal injection of titer of miR-223 lentivirus vectors was 2 × 10^6 TU/mL; intravitreal injection of titer of NLRP3 lentivirus vectors was 2 × 10^8 TU/mL; lentivirus vectors were constructed by Shanghai GeneChem Co., Ltd., Shanghai, China). Drugs were first injected for intervention, and then catheters were flushed with normal saline, with a total volume of 20μL per rat. Rats were administered at 8:00 every morning, which lasted for 10 days. Then rats were scarified, and spinal cord tissues were preserved for further studies.

Establishment of a rat model of CCI

Surgical procedures were performed under sterile conditions. After anesthetized by the intraperitoneal injection of sodium pentobarbital (40 mg/kg, Sigma, St. Louis, MO, USA), the rats were fixed in a prone position. Hair on the left hind leg was removed, and the skin was disinfected. Then the sciatic nerve was exposed by skin incision and blunt separation of the thigh muscles.
and was suture ligated (n = 4, interval 1 mm, appropriate compactness) using non-absorbable sutures (number 4–0 chromic catgut, Johnson & Johnson, Arlington, TX, USA). After surgery, the rats were injected with penicillin (30,000 units, Sigma, St. Louis, MO, USA) to prevent infection. Only sciatic nerve trunk in the sham operation group rats was exposed without ligation. Other surgical procedures were the same with injury groups. After surgery, rats were fed in a quiet and warm environment without strong light and noise stimulation. If hind limb paralysis or death occurred in the model rats after surgery, those rats were excluded. Behavioral tests were performed on rats after surgery to identify whether CCI models were established successfully.

**Intrathecal catheterization in rats**

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg), and fixed in a prone position. After routine disinfection, atlantooccipital membranes were exposed by skin and fascia incision and blunt separation of muscle. Atlantooccipital membranes were gently picked, and then a gush of cerebrospinal fluid could be seen. Polyurethane microspinal catheters (0.12 mm inner diameter, 0.35 mm outer diameter, Lewiston, NY, USA) were slowly implanted into the lumbar interspace through the laceration. Rats with hind limb paralysis, tail paralysis, or impaired motor function after intrathecal catheterization were excluded. Rats without impaired motor function were selected for identification of catheterization success, in which rats received an injection of 2% lidocaine (15 μL, Sigma, St. Louis, MO, USA) in the lumbar catheter. If double-leg paralysis occurred about 20 s later, and rats returned to the normal within 30 min, intrathecal catheterization was considered to be successful.

**Behavioral tests**

The morphine-produced analgesia was measured with the paw withdrawal threshold (PWT) and percentage maximum possible effect (%MPE) in the tail flick test. After rats were familiar with the testing environment and became quiet, behavioral tests began. The PWT was measured using von Frey filaments (DanMic Global, San Jose, CA, USA). The filament was applied directly to the plantar hind paw, and the force of each application was limited within 8 s. Withdrawal of paw during the application was considered as a positive response, otherwise it was considered as a negative response. If no withdrawal was observed, another filament was used. The test was continuously performed four times with an interval of 2 min between two stimuli. The mean value was the PWT. Rats were placed on the tail-flick apparatus, and the light radiation was focused on one-third of the distance from the tail distal end to the base. A cut off time of 15 s was used to avoid tissue damage in the tail. The test was continuously performed four times with an interval of 5 min between two light radiations. The threshold of pain was calculated as the average of four consecutive tail flick latencies (the period from the start of light radiation to the withdrawal of the tail). %MPE = (post-drug threshold of pain – pre-drug threshold of pain)/(cut off time – pre-drug threshold of pain) × 100%.

**Luciferase reporter gene assay**

TargetScan (http://www.targetscan.org/vert_61/), online bioinformatics software, concluded that NLRP3 is a downstream target gene of miR-223. The luciferase-3’-UTR reporter constructs were generated by introducing the 3’-UTRs of NLRP3 carrying the putative miR-223 binding site into a luciferase reporter vector.
A plasmid vector containing the wild-type loci of miR-223 binding NLRP3 (NLRP3 wild) and a plasmid vector containing the mutant loci of miR-223 binding NLRP3 (NLRP3 mut) were designed and constructed. NLRP3-3'UTR primer sequences synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) are as follows (Table 2). After the sequence was verified by sequencing, HEK293 cells (ATCC) were co-transfected with 80 ng NLRP3 wild plasmid or NLRP3 mut plasmid, along with 40 ng dual fluorescence plasmid carrying firefly and Renilla luciferase reporters, and the miR-223 mimic, miR-223 inhibitor, or the control (Shanghai GenePharma Co., Ltd., Shanghai, China). After HEK293 cells were incubated at 37°C for 24 h, proteins were extracted and analyzed by the dual-Luciferase reporter assay (Promega, Madison, WI, USA).

**Tissue sampling**

On day 11 after drug intervention, when rats in each group completed behavioral tests, they were sacrificed after receiving an intraperitoneal injection of pentobarbital (40 mg/kg). Rats were fixed with pre-cooled normal saline (250 mL) and 4% paraformaldehyde (500 mL) through cardiac perfusion. Complete spinal cord of rats (the CCI, normal saline, morphine, miR-223, NLRP3, miR-223 + morphine, NLRP3 + morphine, and miR-223 + NLRP3 + morphine groups) was taken out and placed in freezing tubes which were pretreated with diethyl pyrocarbonate water and pre-sterilized treatment. Freezing tubes were immediately frozen in liquid nitrogen and then transferred to a −80°C refrigerator. The following operation was performed in a RNA biological purification table using sterile surgical instruments.

**Real-time quantitative polymerase chain reaction**

RNA in lumbar spinal cord was extracted according to the strict requirements of RNA extraction from tissues. Reverse transcription was performed using the PrimeScript®RT reagent Kit. The cDNAs of miR-223 and U6 were synthesized using stem-loop reverse transcription primers (miR-223: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGGGT-3'; U6: 5'-AACGCTTCACGAATTTGCCTAG-3'). According to the gene sequence published in Genbank database, polymerase chain reaction (PCR) reaction primers were designed using Primer 5.0 as follows (Table 3). Primers were synthesized by Shanghai Sangon Company (Shanghai, China). According to instructions on the kit, reaction liquid was prepared, and reaction conditions were set. ABI PRISM 7500 real-time PCR System (ABI, Carlsbad, CA, USA) and SYBR Green I fluorescence kit (Takara, Tokyo, Japan) were used in the PCR reaction. U6/GAPDH served as an internal control. The melting curve was used to evaluate reliability of the PCR results. Threshold cycle (CT) value (the inflection point of the amplification curve) was taken, and the relative expression of target genes was calculated using 2^−ΔΔCt.

**Western blotting**

Tissues in lumbar spinal cord were removed from the −80°C refrigerator, and cell lysis solution containing protease inhibitors was added to the tissues.
Centrifugal tubes containing spinal cord tissues of rats were placed in an ice-water mixture to rewarm tissues. After 30 min of rewarming, tissues were homogenized with an electric homogenizer (Thermo, Holbrook, NY, USA) in ice bath. Refrigerated centrifugation was performed at 15,000 r/min for 10 min after tissues were placed in an ice bath for 30 min, and the supernatant was saved. A small amount of protein samples were used to determine protein concentrations according to instructions on the bicinchoninic acid kit (Wuhan Boster Company, Wuhan, Hubei, China). Loading buffer was added to extracted protein samples and boiled at 95°C for 10 min. Then 10% polyacrylamide gel electrophoresis was performed to extract proteins with 30 μg of samples loaded per well. Wet transfer was performed, protein blotting was transferred onto a polyvinylidene fluoride transfer membrane, and then 5% bovine serum albumin was added for block for 1 h at room temperature. Primary antibodies against NLRP3 (1: 1000), apoptosis-associated speck-like protein (ASC) (1: 1000), Caspase-1 (1: 1000), Interleukin (IL)-1β (1:1000), IL-18 (1:1000), and β-actin (1: 1000) (Abcam Inc., Cambridge, MA, USA) were added, followed by overnight incubation at 4°C. Samples were rinsed in tris-buffered saline for three times/5 min. The appropriate secondary antibodies were added, and then samples were incubated at room temperature for 1 h. The membrane was washed in tris-buffered saline for three times/5 min. Chemiluminescent reagents were then added for color development. A gray value analysis of target bands was conducted using Image J with β-actin as an internal reference.

**Enzyme linked immunosorbent assay**

Tissues in lumbar spinal cord were removed from the −80°C refrigerator, embedded in optimal cutting temperature medium, sectioned to 8 μm thicknesses by a freezing microtome (Cryotome SME, Cambridge, MA, USA), and equilibrated at room temperature for 10 min. Then tissues were fixed with pre-cooled acetone, dried, washed in the phosphate-buffered saline, and treated with 0.3% H2O2 to inactivate endogenous peroxidases. After block, primary antibodies against NLRP3 (1: 500), ASC (1: 500), Caspase-1 (1: 500), IL-1β (1:500), and IL-18 (1:500) were added. After 4°C overnight incubation, tissues were washed with phosphate-buffered saline Tween-20, and secondary antibodies were added. After incubation at room temperature, diaminobenzidine (DAB) (Wuhan Boster Company, Wuhan, Hubei, China) was added to develop colors, and sections were counterstained with hematoxylin. Then the sections were washed in water and mounted by neutral resin. The staining was observed through a microscope (Olympus, Tokyo, Japan), and results were recorded in photos. Positive stain of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 were mainly tan, which were located at cytoplasm. Three sections of each rat were randomly selected for immunohistochemically staining, and the results were gained after analysis of the ratio between the number of positive cells and the total number of cells.

**Statistical analysis**

All data analysis was conducted using SPSS (version 18.0) (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (x ± s). For normally distributed measurement data, comparisons between two groups were examined using the t test, while multiple comparisons among groups were examined using the single-factor analysis of variance (one-way analysis of variance). P < 0.05 was considered statistically significant.
Results

Morphine analgesic tolerance in rat model of CCI after drug intervention

On D1, D3, D5, D7, D9, and D11 after CCI surgery, PWT and %MPE were measured. The results are as follows: there were significant differences on PWT and %MPE in all time points (except for D1) in the CCI group ($P < 0.05$), while there were no significant differences between the sham operation and normal control groups at each time point (Figure 1(a) and (b)). These results indicated that the rat model of CCI was established successfully. On D1, D3, D5, D7, D9, and D11 after drug intervention following CCI surgery, behavioral tests were performed on rats. There were no significant differences between the CCI group without intrathecal catheter insertion and the normal saline group in which rats received saline injection through intrathecal catheter in the PMT and %MPE over time ($P > 0.05$). After drug intervention, compared with the normal saline group, the PMT and %MPE in the morphine and miR-223 + morphine groups were decreased, and on the D9 after drug intervention, they were closed to the normal saline group (both $P > 0.05$); while the PMT and %MPE in the miR-223 group were enhanced first and decreased on the D5 after drug intervention. Compared with the normal saline group, the NLRP3 group had lower PMT and %MPE (both $P < 0.05$). The miR-223 + morphine group maintained good analgesic effects, and significant differences were found in the PMT and %MPE between the normal saline and miR-223 + morphine groups (both $P < 0.05$). The PMT and %MPE in the NLRP3 + morphine group were decreased, which were closed to the normal saline group on the D5 (all $P > 0.05$). Compared with the morphine group, the miR-223 + morphine group had higher PMT and %MPE in all time points (except for D1), while the NLRP3 + morphine group had lower PMT and %MPE in all time points (except for D1) (all $P < 0.05$). No significant difference was found in comparisons of the PMT and %MPE between the morphine and miR-223 + NLRP3 + morphine groups (both $P > 0.05$). Compared with the miR-223 group, the miR-223 + morphine group had higher PMT and %MPE in all time points (both $P < 0.05$). Besides, the NLRP3 + morphine group had higher PMT and %MPE in all time points compared with the NLRP3 group, and the PMT and %MPE in the NLRP3 + morphine group were lower than those in the miR-223 + NLRP3 + morphine group (all $P < 0.05$) (Figure 1(c) and (d)). These results suggested that miR-223 and NLRP3 influence the morphine analgesic tolerance.

![Figure 1](image_url)

Figure 1. Comparisons of paw withdrawal threshold (PWT) and percentage maximum possible effect (%MPE) of rats in each group at different time points. (a) Comparisons of PWT of rats in each group after CCI. (b) Comparisons of %MPE of rats in each group after CCI. (c) Comparisons of PWT of rats in each group after drug intervention. (d) Comparisons of %MPE of rats in each group after drug intervention. N: the normal control group, $n = 10$; S: the sham operation group, $n = 10$; C0: the CCI group, $n = 10$; C1: the normal saline group, $n = 10$; C2: the morphine group, $n = 10$; C3: the miR-223 group, $n = 10$; C4: the NLRP3 group, $n = 10$; C5: the miR-223 + morphine group, $n = 10$; C6: the NLRP3 + morphine group, $n = 10$; and C7: the miR-223 + NLRP3 + morphine group, $n = 10$. *$P < 0.05$, compared with the normal control group; #$P < 0.05$, compared with the sham operation group.
**NLRP3 is a target gene of miR-223**

It was concluded that NLRP3 is a downstream target gene of miR-223 by TargetScan. Plasmid vectors containing the wild-type loci of miR-223 binding NLRP3 (NLRP3 wild) and plasmid vectors containing the mutant loci of miR-223 binding NLRP3 (NLRP3 mut) were designed and expressed (Figure 2(a)). Luciferase results showed that the transcriptional activity of NLRP3 decreased significantly ($P < 0.05$) after instantaneous transfer miR-223 in the NLRP3 wild group in comparison with the control group ($P < 0.05$), that the transcriptional activity of NLRP3 increased significantly after instantaneous transfer miR-223 in the miR-223 inhibitor group in comparison with the miR-223 group ($P < 0.05$), and that no factors influenced transcriptional activities in the NLRP3 mutant group and normal control group, indicating that miR-223 targeted and regulated the NLRP3 expression negatively (Figure 2(b)). RNA and protein were extracted from HEK293 cells in the transfection groups. The results of real-time quantitative

| Position 439-445 of NLRP3 3’UTR | Predicted consequential pairing of target region (top) and miRNA (bottom) | seed match |
|----------------------------------|---------------------------------------------------------------------|------------|
| mmu-miR-223                      | 5’ ... UCUUGUCUCCUGUAACUGACC... 3’ ACACGUAACUGU                   | 7mer-m8    |

**Figure 2.** MicroRNA-223 negatively regulates the NLRP3 expression. (a) MiR-223 binding sites in the NLRP3-3’UTR predicted by TargetScan online database. (b) Dual-luciferase reporter gene assay showed that miR-223 negatively regulates the expression of NLRP3. (c) qRT-PCR assay confirmed that miR-223 could regulate the mRNA expression of NLRP3. (d) Western blotting indicated that miR-223 negatively regulates the protein expression of NLRP3. (e) The gray value analysis of Western blotting results. *$P < 0.05$, compared with the normal control (NC) group. # $P < 0.05$ as compared with the miR-223 mimic group.
polymerase chain reaction and Western blotting were similar to the results of luciferase reporter gene assay: transfected miR-223 inhibited the NLRP3 expression, and the NLRP3 expression increased in the miR-223 inhibitor group after transfection (Figure 2(c) and (d)).

Figure 3. The expressions of miR-223, NLRP3, ASC, Caspase-1, IL-1β, and IL-18 mRNA in the spinal cord tissues of rats in each group after drug intervention. (a) The relative expression of miR-223 in the spinal cord tissues of rats in each group. (b) The mRNA expression of NLRP3 in the spinal cord tissues of rats in each group. (c) The mRNA expression of ASC in rat lumbar spinal cord tissues. (d) The mRNA expression of Caspase-1 in rat lumbar spinal cord tissues. (e) The mRNA expression of IL-1β in rat lumbar spinal cord tissues. (f) The mRNA expression of IL-18 in rat lumbar spinal cord tissues.

Note: NLRP3: nucleotide-binding and oligomerization domain-like receptor 3; ASC: apoptosis-associated speck-like protein; IL-1β: interleukin-1β; IL-18: interleukin-18; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; C0: the CCI group, n = 10; C1: the normal saline group, n = 10; C2: the morphine group, n = 10; C3: the miR-223 group, n = 10; C4: the NLRP3 group, n = 10; C5: the miR-223 + morphine group, n = 10; C6: the NLRP3 + morphine group, n = 10; C7: the miR-223 + NLRP3 + morphine group, n = 10.

*p < 0.05, compared with the CCI and normal saline groups, #p < 0.05, compared with the morphine group.

The mRNA expressions of miR-223, NLRP3 inflammasomes, and inflammatory cytokines in the spinal cord tissues of rats

Real-time quantitative polymerase chain reaction results of all groups (as shown in Figure 3) showed that there
were no significant differences among the CCI, normal saline, morphine, NLRP3, and NLRP3 + morphine groups in the miR-223 expression, and no significant differences among the miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine groups in the miR-223 expression (all \( P > 0.05 \)). The miR-223, miR-223 + morphine, and miR-223 + NLRP3 + morphine groups had higher miR-223 expression compared with that in the CCI, normal saline, and morphine groups (all \( P < 0.05 \)). Besides, no significant difference was found among the CCI, normal saline, morphine, and miR-223 + NLRP3 + morphine groups in the expressions of NLRP3, ASC, Caspase-1, IL-1\( \beta \), and IL-18 (all \( P > 0.05 \)). Compared with the CCI, normal saline and morphine groups, the miR-223, and miR-223 + morphine groups had decreased mRNA expressions of NLRP3, ASC, Caspase-1, IL-1\( \beta \), and IL-18 (all \( P < 0.05 \)), while the NLRP3 and NLRP3 + morphine groups had increased mRNA expressions of NLRP3, ASC, Caspase-1, IL-1\( \beta \), and IL-18 (all \( P < 0.05 \)). Compared with the CCI, normal saline and morphine groups, no significant differences were found among those mRNA expressions in the miR-223 + NLRP3 + morphine group (all \( P > 0.05 \)). These results suggested that miR-223 could negatively regulate the expressions of NLRP3, ASC, Caspase-1, IL-1\( \beta \), and IL-18.

Protein expressions of NLRP3 inflammasomes in the spinal cord tissues of rats

Proteins were extracted from the spinal cord tissues of rats. Western blotting was adopted to detect protein expressions of NLRP3 inflammasomes, and the results showed that there were no significant differences among the CCI, normal saline, and morphine groups in protein expressions of NLRP3, ASC, and Caspase-1 (all \( P > 0.05 \)). Compared with the CCI, normal saline and morphine groups, protein expressions of NLRP3, ASC, and Caspase-1 in the miR-223, and miR-223 + morphine groups significantly reduced, while protein expressions of NLRP3, ASC, and Caspase-1 in the NLRP3 and miR-223 + morphine groups significantly increased (all \( P < 0.05 \)). No significant differences were found among those protein expressions in the miR-223 + NLRP3 + morphine group compared with the CCI, normal saline and morphine groups (all \( P > 0.05 \)) (Figure 4(a) and (b)). These results indicated that miR-223 could negatively regulate the expression of NLRP3 and inhibit the activities of inflammasomes (NLRP3, ASC, and Caspase-1).

Protein expressions of IL-1\( \beta \), and IL-18 in the spinal cord tissues of rats

Western blotting and ELISA were adopted to detect protein expressions of IL-1\( \beta \) and IL-18. Results in Figure 5 showed that there were no significant differences among the CCI, normal saline, morphine, and miR-223 + NLRP3 + morphine groups in protein expressions of IL-1\( \beta \) and IL-18 (all \( P > 0.05 \)). Compared with the normal saline group, protein expressions of IL-1\( \beta \) and IL-18 in the miR-223 and miR-223 + morphine groups significantly reduced, while protein expressions of IL-1\( \beta \) and IL-18 in the NLRP3 and NLRP3 + morphine groups significantly increased. Our results indicated that miR-223 affects the processing and secretion of IL-1\( \beta \) and IL-18 through negatively regulating the expression of NLRP3.

Figure 4. The protein expressions of NLRP3, ASC, and Caspase-1 in the spinal cord tissues of rats in each group. (a) Protein expressions of NLRP3, ASC, and Caspase-1 in rat lumbar spinal cord tissues were detected by Western blotting. (b) The gray value analysis of Western blotting results.

Note: NLRP3: nucleotide-binding and oligomerization domain-like receptor 3; ASC: apoptosis-associated speck-like protein; C0: the CCI group, \( n = 10 \); C1: the normal saline group, \( n = 10 \); C2: the morphine group, \( n = 10 \); C3: the miR-223 group, \( n = 10 \); C4: the NLRP3 group, \( n = 10 \); C5: the miR-223 + morphine group, \( n = 10 \); C6: the NLRP3 + morphine group, \( n = 10 \); C7: the miR-223 + NLRP3 + morphine group, \( n = 10 \).

*\( P < 0.05 \), compared with the CCI and normal saline groups, \#\( P < 0.05 \), compared with the morphine group.
Immunohistochemical staining results of spinal cord tissues of rats

The results of positive expression rates of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 were in Figure 6. There were no significant differences among the CCI, normal saline, morphine, and miR-223 + morphine groups in NLRP3, ASC, Caspase-1, IL-1β, and IL-18 expressions (all $P > 0.05$). Compared with the normal saline group, positive expression rates of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 in the miR-223 and miR-223 + morphine groups significantly reduced, while positive expression rates of NLRP3, ASC, Caspase-1, IL-1β, and IL-18 in the NLRP3 and NLRP3 + morphine groups significantly increased (Figure 6). These results indicated that miR-223 could negatively regulate the expression of NLRP3 and inhibit the activities of inflammasomes and affect the expressions of IL-1β and IL-18.

Discussion

The current situation of NP treatment is not ideal with some NP patients unable to adequately relieve pain. Morphine is considered as first-line drugs in NP treatment, but it was easy to develop morphine tolerance during the use of morphine. The mechanisms underlying morphine analgesic tolerance are not fully understood. If an effective therapeutic target which prevents morphine tolerance can be found, it is of great significance for the treatment of NP.

Experimental results have shown that miR-223 and NLRP3 could negatively regulate the expression of NLRP3 and inhibit the activities of inflammasomes and affect the expressions of IL-1β and IL-18. Because chronic pain and morphine tolerance have similar signaling pathways, miRs are possible to participate in the formation of morphine analgesic tolerance. Morphone tolerance results from changes in gene transcription levels of the second messenger and neurotransmitter levels because of a series of compensatory reactions caused by prolonged use of morphine. Previous evidence showed that the functions of NLRP3 inflammasome were crucial for the regulation of neuroinflammation mediated by microglia, thus, compounds that inhibited the activation of NLRP3 inflammasome may contribute to the improvement of morphine.
tolerance.\[^{28,29}\]\) Besides, Bauernfeind et al.\[^{30}\] found that miR-223 could target the 3′UTR area of NLRP3 mRNA to inhibit NLRP3 expression.

It is found that miR-223 could negatively regulate NLRP3, ASC, Caspase-1, IL-1β, and IL-18 expressions. Inflammasomes contain high-molecular weight signaling platforms that resulted in the activation of inflammatory caspases.\[^{31}\] Activated Caspase-1 plays an important role in the proteolytic processing and, thus, maturation of the cytokine precursors of IL-1β and IL-18.\[^{32}\]\) Besides, morphine also can modulate immune responses, including the inflammatory response, by affecting the pro-inflammatory cytokines production such as IL-1β.\[^{33}\] Furthermore, morphine-tolerance suppresses immune activation, thereby decreasing levels of proinflammatory cytokines, and studies have even reported that IL-1β treatment reduces morphine-induced analgesia, thereby contributing to the development of tolerance.\[^{34,35}\]\)

A study has showed that overexpression of miR-223 inhibits NLRP3 protein expressions and inhibits IL-1β production from the inflammasome.\[^{17}\] Yang et al.\[^{18}\] found that miR-223 could downregulate NLRP3 to inhibit inflammation through Caspase-1 and IL-1β after intracerebral hemorrhage. NLRP family proteins, proteins scattered in the cell, were able to quickly identify inflammation induced by a pathogen and involved in the processing of IL-1β and IL-18. In addition, they can secrete IL-1β which may lead to apoptosis.\[^{36}\]\) Precise and ordered protein oligomers of NLRP3 are formed by polymerizing ATP, and the oligomers through combining with ASC containing a caspase recruitment domain and cysteine-aspartic proteases (caspase) form a multi-protein complex called the inflammasome, which controls Caspase-1 activation.\[^{37,38}\] Caspase-1 is a converting enzyme of IL-1β. Activated Caspase-1 can form a mature and activated IL-1β through reactions, which is then secreted outside the cell.\[^{39,40}\]\) Apart from participation in inflammasome-related signaling pathways, NLRP3 can also activate NF-κB, starting the transcription of IL-1β precursor.\[^{41}\] Lamkanfi and coworkers\[^{42}\] found that Caspase-1 can activate NF-κB, and that NF-κB activation and IL-1β maturation occur simultaneously. The latest study in 2015 found that miR-223 can negatively regulate the activity of NLRP3 inflammasomes, and that activated inflammasomes promote the body to produce IL-1β and IL-18 and have an automatic negative feedback regulation on IL-1β and IL-18, inhibiting specific pathological damage and maintaining normal physiological activities.\[^{43}\]

In summary, the experimental results in this study showed that miR-223 could suppress the activities of NLRP3 inflammasome (NLRP3, ASC, and Caspase-1) to relieve morphine analgesic tolerance in rats by down-regulating NLRP3, which is expected to become a new target for the treatment of morphine analgesic tolerance. However, the mechanism of miR-223 and NLRP3 in the occurrence, development, and prognosis of NP needs further confirmation, there is no clinical validation of effects of targeted regulation of NLRP3 by miR-223 on morphine analgesic tolerance, and there is no in situ hybridization for detecting the miR-223 expression and the expression position in cells due to limitation in funding. Continuous improvement and relevant research are still needed.

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Author Contributions

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Declaration of Conflicting Interests

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