Effect of miRNA-146a-mediated TLR4 Signal Pathway on the Pain of Lumbar Disc Herniation

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Abstract

The current experiment was carried out to explore the effect of the miR-146a-mediated TLR4 signaling pathway on the lumbar disc herniation pains. For this aim, a total of 32 rats were divided randomly into 4 groups – the blank group (Group C), Model group (M), miR-146a overexpression group (agomiR-146a group) and negative control group (NC group), with 8 rats in each group. Rats in Group M were prepared for the construction of lumbar disc herniation models, while those in the agomiR-146a group or NC group, in addition to the model construction, would receive the intrathecal injection of agomiR-146a or agomiRNA-146a NC. Thereafter, a series of tests were performed for rats, including the mechanical pain test and heat pain test to measure the pain threshold, RT-PCR to detect the expression of miR-146a, and the transcription of TLR4, IRAK1, TRAF6, IL-6 and TNF-α. Western blot to determine the expression of IRAK1 and TRAF6 and ELISA to determine the expression of IL-6 and TNF-α. Results showed that as compared to the blank group, rats in Group M were more sensitive to the pains, presenting with declines in the thresholds in the pain, and upregulation in the TRL4 signaling pathway (TLR4, IRAK1 and TRAF6) and pro-inflammatory factors, including IL-6 and TNF-α. In comparison with Group M, intrathecal injection of agomiR-146a relieved the pains, with significant upregulation of miR-146a and downregulation of TLR4, IRAK1, TRAF6, IL-6 and TNF-α. Then upregulation of miR-146a could reduce the activity of the TLR4 signaling pathway and the release of pro-inflammatory factors, which may be a potential strategy for the treatment of lumbar disc herniation.

Introduction

Lumbar disc herniation (LDH) is a kind of clinical syndrome caused by the suppression on the nerve root and cauda equina due to the degeneration of the intervertebral disc, disrupted annulus or herniation of nucleus pulposus, mainly presenting symptoms, like neuralgia in the waist or below limb, resulting in the persistent pains that severely affect the life quality of patients\textsuperscript{(1)}. So far, non-surgical treatment has been the top choice for LDH, such as bed rest, pelvic traction, massotherapy, supportive medication of glucosamine sulfate and chondroitin sulfate and antiinflammation therapy of cortical hormones. However, these methods could only relieve the patients from the pains of LDH, but fail to cure them\textsuperscript{(2, 3)}. Surgical treatment is only considered for patients with a long history of LDH, or those who have acute pains that severely affect the life quality or those who do not respond to the conservative treatment\textsuperscript{(4)}. Neither surgical treatment nor non-surgical treatment could eradicate the LDH, so it is quite necessary to search for new methods.

As cell biology develops, genetic therapy has been gradually applied in fundamental research on various diseases. More and more scholars have focused on the genetic therapy for pain, including the efforts to search for the pain-related gene, and they hope to downregulate the pain-related gene expression or upregulate the anti-pain gene expression, so as to relieve the pains. However, the emergence of a variety of gene interference techniques provides the technical support for realizing this objective. MiRNAs are a group of non-coding small RNA in the length of about 20 to 25 nt in eukaryotes that can regulate various biological events. Mature miRNAs could recognize the mRNA of target genes via complementary base pairing to suppress the transcription and translation of target genes to inhibit
the biological functions. More and more evidence has uncovered that miRNA is involved in the development and progression of pains. For instance, miRNA is involved in the development of pain resulting from rheumatoid arthritis (RA) and osteoarthritis, which can be relieved by the upregulation of miRNAs (5-7). Toll-like receptor 4 (TLR4) is a kind of type I transmembrane protein receptor that can induce the release of inflammatory factors. Existing evidence has shown that TLR4 is a critical role in the development of neuropathological pains(8). Current research has shown that miR-146a is downregulated in the chronic constriction injury (CCI)-induced neuropathological pain models, while interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) are the target genes of miR-146a (9). Therefore, we infer that miR-146a may be involved in the pains of LDH via the TLR4 signal pathway.

In this study, we aimed to investigate the effect of the miR-146a-mediated TLR4 signal pathway on the pains of LDH, so as to explore the changes in the pains of rats via interfering on the expression of miR-146a, thereby providing theoretical evidence for gene therapy of LDH pains.

**Materials and methods**

**Ethical statement**

All designs for the animal experiments of this study gained approval from the Ethical Board for Laboratory Animals of Forth Affiliated Hospital of Zhejiang University School of Medicine, and all animal-related operations conformed to the ethical codes.

**Subjects**

A total of 32 male, Sprague-Dawley (SD) rats (weight between 250 g and 300 g) provided by Beijing Vital River Laboratory Animal Technology Co., Ltd were selected for this study, with licenses for production [SCXK (Hu) 2012-0002] and use (2015000502794), and maintained in a quiet, well-ventilated environment at 25°C in a 12/12 day/night cycle. All animals had free access to food and water, and the padding was refreshed every day. Following 7 days of adaptive feeding, rats were divided into 4 groups randomly (n = 8 rats for each group): 1) Control group; 2) Model group; 3) AgomiR-146a group; 4) Negative control group.

**LDH models**

Preparation of suspension of autologous nucleus pulposus: Skin preparation was conducted 1 day prior to surgery. On the day of surgery, rats were anesthetized by abdominal injection of 1% ketamine (50 mg/kg) for depilation on the back and sterilization for the caudal and back regions. An incision was made at 1 cm to the root of the tail, where the caudal lumbar disc was also incised to expose the nucleus pulposus in the center. The resulting nucleus pulposus was mixed with the normal saline in appropriate volume and then minced for preparation of suspension.

Model construction: Rats were fixed in the flexion, lateral position for the epidural puncture in the interspace between L4 and L5, followed by the sequential injection of 20 μL suspension of autologous nucleus pulposus and 30 μL 2% lidocaine into the epidural space. Limb activities of rats recovered from the anesthesia were observed: Failed puncture for the four limbs moving freely, a successful puncture for rats with upper limbs moving freely but lower limbs with decline or loss in the motion and sensation which, however, could be restored totally after 20 to 30 min; successful model construction for rats with a successful puncture, but showing allergy to pains in the lower limbs, with no paralysis in lower limbs.

**Intrathecal administration of miR-146a**

Rats were anesthetized by inhalation of 1.5% isoflurane and fixed in the sitting position, with interspace of L5-6 spinous processes as the puncture points. From the site of hip joint that was probed by the left hand, the puncture site was fixed at distance over 3 cm vertical to the highest point of the back. The skin on the back was tightened outward and fixed by the index finger, and the needle of the microsyringe was inserted slowly and vertically into the space. At the emergence of the tail whip, the jugular vein was pressed, and at the sight of cerebral spinal fluid when drawing back the piston, drugs in the volume of 20 μL (5 μg) were injected. Note that tail whip might be seen during the injection.
Test of mechanical pains

Test of mechanical pain was conducted in a quiet room. In brief, rats were placed in a tailor-made glass cage, of which the bottom was made by a metal mesh. After rats were accustomed for 30 min, a Von Frey needle was used to stimulate the foot of rats, and the Von Frey value was recorded at the sight of foot withdrawal. Stimuli were given three times, with an interval of 5 min following one stimulus, and the average of 3 measurements was taken as the mechanical withdrawal threshold of this rat. With the mechanical withdrawal threshold measured one day prior to surgery as the basic threshold, rats with a threshold lower than the basic threshold were allergic to the pains, while those higher than the basic threshold were blunt to pains (10).

Test of heat pains

The test of heat pain was conducted in a quiet environment where the temperature was sustained within 20 and 25°C. Rats were placed in a tailor-made glass cage, of which the bottom was made of glass. Following 30 min of adaptation, planta pedis of rats was exposed to the thermal radiation source, and during the exposure, the time from the start of irradiation to the foot withdrawal was recorded. Each rat received 3 times of irradiation, with an interval of 15 min, and the average of measurements was taken as the escape latency of foot withdrawal (11).

RT-PCR

RNA extraction: Total RNA was extracted from the tissues by using the TRIzol reagent. In brief, tissues were homogenized in 1 mL TRIzol, and 5 min later, the mixture was centrifuged at 12000 rpm for 5 min at 4°C. Then, the supernatant was transferred into a new EP tube to be mixed with 0.2 mL chloroform, followed by centrifugation at 12000 rpm and 4°C for 15 min. The water phase in the upper layer was cautiously transferred into a new EP tube, where it would be mixed with 0.5 mL isopropanol, followed by incubation at room temperature for 10 min and centrifugation at 12000 rpm and 4°C for 10 min, with the supernatant being discarded. Sediment was mixed sufficiently with 1 mL 75% ethanol and then centrifuged at 7500 rpm and 4°C for 5 min, also with the supernatant being discarded, while the sediment was dried for 10 min. RNA was then resolved in DEPC water at 56°C, and the concentration of RNA was determined via using an ultraviolet spectrometer.

Reverse transcription of miRNA: A reaction system in the volume of 11 μL was prepared according to the instruction of the kit and well mixed, while the reaction parameters were set as follows: 70°C for 10 min, ice for 2 min and centrifugation for a short time. The resulting solution was then mixed with 25 μL reaction system in the second part in following conditions: 42°C for 15 min and 85°C for 5 s, followed by the following experiment or preservation at -20°C.

Reverse transcription of mRNA: The first part of the reaction system was prepared in a 20 μL system, and the reaction parameters were set as follows: 42°C for 2 min and brief centrifugation. Then, the second part of the reaction system (40 μL) was added, while the parameters were set as follows: 37°C for 15 min and 85°C for 5 s, followed by the following experiment or preservation at -20°C.

A 25 μL reaction system was then prepared according to the instructions, while the reactions were carried out by using a fluorescent quantitative PCR apparatus in following conditions: pre-denaturation at 95°C for 15 min, and 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. Relative quantification analysis was performed by using the method of 2^ΔΔCT.

Western Blot

With the proteins extracted from the tissues, we detected the concentration of proteins by using the BCA kit. According to the protein concentration, the same amount of protein samples was loaded with the loading buffer after being boiled for 5 min, with 40 μg proteins in each well, followed by electrophoresis in ice bath at 300 mA for 75 min. The unoccupied sites on the membrane were then blocked by 5% non-fat milk diluted by TBST for 1 h, and the proteins on the membrane were probed by incubation with the primary anti-β-actin(1:1000), IRAK1(1:200) and TRAF6(1:1000) antibodies at 4°C overnight, followed by 3 washes in TBST, 5 min/wash. Thereafter, the resulting immunoblots were further detected by the secondary antibodies
Enzyme-linked immunosorbent assay (ELISA)

Tissues (50 mg) were placed in a 1.5 mL enzyme-free centrifuge tube, where tissues would be homogenized with 0.5 mL enzyme-free water. The resulting mixture was centrifuged with 0.5 mL enzyme-free water at 4°C and 12000 r/min to obtain the supernatant. Standard solutions were then prepared according to the instructions. Samples were then loaded, except for the blank wells, into the corresponding wells, with 40 μL sample dilution and 10 μL samples to be tested, while the standard solutions in varying concentrations were added into the corresponding wells, with 50 μL each well. The plate was then sealed and incubated in a 37°C water bath for 30 min, followed by 5 washes in washing buffer, 30 s/wash, with the washing buffer being discarded. Then, the enzyme-conjugated reagent was added into each well, with 50 μL in each well, followed by the addition of color-developing reagent, with 50 μL in each well, and incubation without light at 37°C for 15 min. Incubation was then terminated by the terminating solution, with 50 μL in each well. The optical density of each well was determined at 450 nm.

Statistical methods

SPSS 21.0 software was utilized to perform the statistical analysis. Measurement data were expressed in form of mean ± standard deviation (SD). Pairwise data in normal distribution were compared by using the t-test, while the difference of data in comparison between groups was testified using the one-way analysis of variance (One-way ANOVA). *P < 0.05 suggested that the difference had statistical significance.

Results and discussion

miRNA-146a mitigates the pains of LDH

As shown in Table 1, as compared to the normal rats, LDH rats presented significant declines in the threshold of mechanical pain at the 3rd, 5th and 7th days (all *P < 0.05), suggesting that LDH models were constructed successfully, and LDH rats are more sensitive to mechanical pains than the normal rats. In comparison with the LDH rats, intrathecal injection of miR-146a would result in an increase in the threshold of mechanical pains at the 3rd, 5th and 7th days (all *P < 0.05), while intrathecal injection of miR-146a-NC showed no significant differences from the LDH rats (all *P > 0.05), suggesting that miR-146a could alleviate the LDH pains. Similar results were also seen in the heat pain test (Table 2 and Figure 1). Thus, LDH models are sensitive to pains, which could be relieved by the intrathecal injection of miR-146a, thereby partially restoring the threshold of mechanical pain and heat pain.

Table 1. Comparison of the thresholds of mechanical pains of rats among groups (x±s, n=8); Control group (A), Model group (B), AgomiR-146a group (C), NC group (D)

<table>
<thead>
<tr>
<th>Time</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D5</th>
<th>D7</th>
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<tbody>
<tr>
<td>A</td>
<td>17.23±16.83 17.17±17.52 17.48±17.48 0.34 0.14 0.20 0.18 0.23</td>
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<tr>
<td>B</td>
<td>17.41±15.56 10.57±11.94 11.58±13.99 0.28 0.19 0.33 0.27 0.32</td>
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<tr>
<td>C</td>
<td>16.88±15.78 14.57±14.01 13.99±14.01 0.15 0.17 0.24 0.26 0.19</td>
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<tr>
<td>D</td>
<td>17.34±16.14 11.48±10.79 11.34±14.55 0.32 0.25 0.31 0.29 0.16</td>
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Table 2 Comparison of the thresholds of heat pains of rats among groups (x±s, n=8)

<table>
<thead>
<tr>
<th>Time</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D5</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>14.35±15.01 14.97±14.55 14.82±14.55 0.16 0.14 0.18 0.19 0.23</td>
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<tr>
<td>Model group</td>
<td>14.66±14.46 8.49±8.36 8.36±8.36 0.19 0.07 0.08 0.07 0.05</td>
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<tr>
<td>AgomiR-146a group</td>
<td>15.03±14.42 11.97±10.88 11.32±10.88 0.20 0.21 0.25 0.19 0.32</td>
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<tr>
<td>NC group</td>
<td>14.67±14.21 9.42±9.03 8.33±9.03 0.21 0.17 0.16 0.18 0.20</td>
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miRNA-146a is involved in the LDH pains via the TLR4 signal pathway

RT-PCR (Figure 2) and Western blot (Figure 3) were carried out to determine the expression of miR-146a and key proteins in the TLR4 signal pathway. As
shown in Figure 2A, we found that the expression of miR-146a in the LDH rats was much higher than that in the normal rats ($P < 0.05$), while intrathecal injection of miR-146a could upregulate the expression of miR-146a, nearly 2.83 times that of normal rats ($P < 0.05$). In Figure 2B, TLR4 expression in LDH rats and miR-146a-treated rats was upregulated when comparing to the normal rats (all $P < 0.05$), while the difference between LDH rats and miR-146a-treated rats was not significant ($P > 0.05$). Besides, as compared to the normal rats, IRAK1, as shown in Figure 2C, was also upregulated in the LDH rats ($P < 0.05$), but down-regulated in the miR-146a-treated rats ($P < 0.05$). Similar changes were also noted in the results of RT-PCR for TRAF6 (Figure 2D) for TRAF6 and Western blot (Figure 3), suggesting that miR-146a is involved in the LDH pains through the TLR4 signal pathway.

miR-146a-mediated TLR4 signal pathway regulates the LDH pains through proinflammatory factors

RT-PCR (Figure 4) and ELISA (Figure 5) were performed to detect the expression of proinflammatory factors. As shown in Figure 4A, we found that as compared to the normal rats, IL-6 level increased in the LDH rats ($P < 0.05$), while compared to the LDH models, rats, after intrathecal injection of miR-146a, had a sharp decrease in the level of IL-6 ($P < 0.05$). RT-PCR results of TNF-α (Figure 4B) were similar to that of IL-6, while the results of Western blot were consistent to those of RT-PCR (Figure 5), indicating that miR-146a-mediated TLR4 signaling pathway participates in the LDH pains via proinflammatory factors, including IL-6 and TNF-α.

Figure 1. Thresholds of mechanical pains of rats in different groups. A: Time-dependent changes of thresholds of mechanical pains of rats in different groups; B: Time-dependent changes of thresholds of heat pains of rats in different groups; * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model group

Figure 2. Results of RT-PCR for the expression of miR-146a and mRNAs in the TLR4 signaling pathway. RT-PCR to detect the expression of miR-146a (A) and mRNA expression of TLR4 (B), IRAK1 (C) and TRAF6 (D) in TLR4 signaling pathway in Control group, Model group, agomiR-146a group and NC group. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model group

Figure 3. Results of Western Blot for protein expressions in TLR4 signaling pathway in different groups. Western blot to detect the protein expression of IRAK1 (B) and TRAF6 (C) in TLR4 signaling pathway in Control group, Model group, agomiR-146a group and NC group. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model group

Figure 4. Results of RT-PCR for the mRNA expression of proinflammatory cytokines in different groups. RT-PCR to detect the mRNA expression of IL-6 (A) and TNF-α (B) in the Control group, Model group, agomiR-146a group and NC group. * $P < 0.05$ vs. Control group; # $P < 0.05$ vs. Model group
The most prominent hallmarks of LDH are the pains in varying degrees, which also represent the pains in lower limbs, severely affecting the life quality of patients. Currently, mechanical pressing and non-bacterial inflammatory stimulation are the major mechanisms of LDH pains. Conventional opinions hold that the herniation of lumbar disc suppressing the nerve root is the major cause, while the subsequent studies have shown that radiative pains may exist even without suppressing, so the mechanical suppressing hypothesis is not the sole mechanism for LDH pains (12). However, according to the hypothesis of non-bacterial inflammatory stimulation, it is believed that the massive release of inflammatory cytokines by the herniated nucleus pulposus, including IL-6, TNF-α, COX-2 and p38 MAPK, may induce nervous radiating pains (13). Therefore, in this study, we prepared the non-suppressing LDH models on rats to investigate the underlying mechanism of inflammatory pains in LDH.

Results of our study also demonstrated that as compared to the normal rats, the levels of IL-6 and TNF-α in LDH models increased. Genevay et al. reported that as compared to the non-LDH patients with back pains, LDH patients may have a higher level of TNF-α in the periradicular fat tissues (14). Enormous evidence has shown the pivotal role of proinflammatory cytokines in the generation and maintenance of pains, and the contact of epidural injection of nucleus pulposus with the blood, as reported, can induce the immune responses to release the inflammatory cytokines, including IL-6 and TNF-α, with the resulting inflammation as one of the mechanisms of pains (15). Some scholars also prepared the LDH models and found that TNF-α impairs the DRG neurons, thereby inducing the radiating pain (16). IL-6 and TNF-α can activate the expression of various inflammatory cytokines, thereby being taken as the major indicators reflecting the severity of inflammation and tissue injury. Proinflammatory cytokines can trigger the action potential of the peripheral cells of the nociceptor to induce the influx of sodium and calcium, thus forming pains (17). Inflammatory cytokines are involved in the development and progression of pains, so it is quite necessary to explore the upstream mechanism to increase the levels of IL-6 and TNF-α.

miRNAs, as one of the small RNA families, can regulate the post-transcriptional gene expression and modulate the development and progression of pains. Our work demonstrated that LDH rats, after the injection of miR-146a, could upregulate the expression of miR-146a in the dorsal root ganglion, with alleviation in the pain sensation in a behavioral test, including the robust increase in the threshold of mechanical pain and heat pain; the results of the molecular test showed that expression of IL-6 and TNF-α was downregulated. Hence, miR-146a did inhibit the expression of proinflammatory cytokines, thereby being involved in the development of pains. Existing data have shown the involvement of miR-146a in the pain mechanism. MiR-146a is expressed abnormally in patients with rheumatoid arthritis and systemic lupus erythematosus (18,19). Downregulation of miR-146a was also reported in the rats with bilateral sciatic nerve chronic constriction injury (20). Besides, miR-146a is associated closely with the development and progression of inflammation (21). In the blood and urine samples of diabetic nephropathy patients, expression of miR-146a is in a negative correlation with the levels of proinflammatory interleukins (22). However, in the pain models, the effect of miR-146a on the expression of proinflammatory factors remains to be clarified in future studies.

TLR4 signal pathway is one of the most currently found inflammatory pathways, and associates with the release of proinflammatory cytokines, resulting in the neuronal changes and sensation of CNS, thereby triggering the long-term pains (23). Our findings supported that the intrathecal injection of miR-146a into the LDH rats increased the expression of miR-

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Figure 5. Results of ELISA for the expression of proinflammatory cytokines in different groups. ELISA to detect the protein expression of IL-6 (A) and TNF-α (B) in the Control group, Model group, agomiR-146a group and NC group. * P < 0.05 vs. Control group; ** P < 0.05 vs. Model group.
miR-146a in the dorsal nerve ganglion, with downregulation of IRAK1 and TRAF6, the key molecules in the TLR4 signaling pathway, and IL-6 and TNF-α. Similarly, it has been suggested that miR-146a can suppress the expression of IRAK1 and TRAF6 to reduce the expression of IL-6 and TNF-α, thereby negatively regulating the inflammatory responses(24, 25). In the hippocampus and cortex of patients with Alzheimer’s disease, the upregulation of miR-146a is found to be associated with the downregulation of IRAK1(26,27). Hence, in pain models, miR-146a can inhibit the expression of proinflammatory cytokines via the negative regulation of the TLR4 signaling pathway.

In summary, our work demonstrates that miR-146a, TLR4 and IRAK1 (key molecules in TLR4 signaling pathway), and IL-6 and TNF-α (proinflammatory cytokines) are upregulated in the LDH models, while the further upregulation of miR-146a could inhibit the TLR4 signaling pathway and the expression of proinflammatory cytokines to varying degrees. Thus, the results of this study provide theoretical evidence for genetic therapy of LDH pains.

Acknowledgments

None

Conflict interest

The authors declare no conflict of interest.

References


