

Acute meningitis in rats is associated with decreased levels of miR132 and miR146a

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Abstract

Introduction: The pathogenesis of bacterial meningitis due to *Streptococcus pneumoniae* is still unclear. Despite early treatment with antibiotics, its morbidity and mortality is still high.

Material and methods: *Streptococcus pneumoniae* induced rat meningitis models were taken and divided into 2 groups; control (C) and meningitis (M). Western blot was used to detect toll like receptor 4 (TLR4), tumor necrosis factor α (TNF- α), interleukin 1B (IL1B), nuclear factor κ B (NF κ B) and real time polymerase chain reaction were used to detect the expression of miR146a, miR132, respectively.

Results: We found that the expressions of TLR4, TNF- α , IL1B, NF κ B were all up-regulated in the acute stage of bacterial meningitis when compared to the control group. While for the post transcription factors, miR146a and miR132, the opposite was observed. They were down-regulated in the meningitis group.

Conclusions: miR146a and miR132 may take part in the pathogenesis of SP bacterial meningitis as well as the TLR4- NF κ B- TNF- α /IL1B signal transduction pathway.

Key words: miR146a, miR132, bacterial meningitis, mechanism, inflammation.

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Introduction

Bacterial meningitis, caused by *Streptococcus pneumoniae*, commonly leads to a significant number of deaths of approximately 700,000 to 1 million deaths per year globally [1], and is associated with the highest case fatality rate and neurological sequelae [2] even in patients being treated with appropriate antibiotics [3]. It is hence imperative to explore the pathogenesis mechanism of *S. pneumoniae* meningitis, in order to diagnose and give proper treatment to these children as early as possible [4]. It has been seen that the pathogenesis of bacterial meningitis is due to the host reaction with the capsule, cell wall and cytoplasm and also to the nucleus of the Gram-positive cocci [5-8]. During inflammatory responses, the toll like receptors (TLRs) recognize molecules broadly shared by streptococcus [7]. Stimulation of the TLRs due to the pathogen associated microbial patterns causes the initiation of the signaling cascade and activates transcription factors such as nuclear factor κ B (NF κ B) and induces some pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) and IL1B [7]. These pro-inflammatory cytokines are considered to be related to acute meningitis [9]. While, identifying the post regulatory transcription factors that regulate the pro-inflammatory cytokines in a negative manner, can bring a novel understanding of the meningitis' pathogenesis.

miRNAs are a family of 18-24 single stranded nucleotide molecules which modulate the post-transcriptional expression of specific genes by controlling the stability and/or translation of target miRNAs [10]. Among them, miR146a and miR132, are present in elevated amounts in central nervous diseases [11, 12] acting as a bridge in linking both the immune and the neuronal functions in the case of an acute infection [13-15]. For example, miR146a was approved to be a pro-inflammatory marker in relation to IL1B [15] and was also seen to be down-regulated in numerous autoimmune diseases such as systemic lupus erythematosus [16]. The expression of miR132 is also decreased in CD4+ cells in the experimental autoimmune encephalitis model, suggesting a novel anti-inflammatory effect [17]. However, there are much more studies about these miRNAs in carcinogenesis and autoimmune diseases, making it hard to be found in infectious diseases.

Thus, in this research work, we studied the inflammatory markers of the signaling cascade from the receptor (TLR4) to the cytokines level (TNF- α , IL1B), then targeting the nucleus at transcription steps for NF κ B, and finally at the post transcriptional level – the miRNAs. Our aim was that these different levels could bring some light in the pathogenesis of acute *S. pneumoniae* bacterial meningitis.

Material and methods

Preparation of the animal model

Twenty healthy Sprague Dawley (SD) rats, aged 3 weeks old, corresponding to 6 months in the human infant, were purchased from the Experimental Animal Centre of Xiangya Medical College at the Central South University. They were kept in germ free circumstances and allowed free access to standard pellet diet and water. Animals were cared for according to the guidelines of the China Council on animal care and all procedures carried out in accordance in the rules and regulations of the Institutional Animal Care and Use Committee of the Central South University. The animals were kept at specific room temperature of $20 \pm 2^\circ\text{C}$ and humidity around 50-60%. The animals were housed on an alternating 12-hour day/night cycle (lights on from 07:00 to 19:00). They were divided randomly into 2 groups: control (C = 10) and meningitis (M = 10). The mortality in each group was as follows: in the control group C = 2, and in the meningitis group M = 4.

Bacterial strain

Streptococcus pneumoniae serotype 3 was commercially bought from ATCC, American Type Culture Collection. (catalog number 6303).

Solid media

The bacteria were diluted in a dose of 0.2 mg/ml, then cultured on solid blood agar medium. The blood agar was prepared according to the manufacturer's instructions, in brief: for each 45 g of solid agar base dissolved in 1000 ml of distilled water, 50 ml of fresh goat's blood must be added. In this experiment, 200 ml of distilled water was mixed with 9 grams of solid agar base. The bottle was carefully sealed and sent for sterilization for a minimum of 4 hours. Later, 10 ml of goat's blood was added to that mixture. Sterilized plates were used on which blood agar base, distilled water and goat's blood were poured onto. The plates were allowed to set overnight at 4 degrees. On the next day, a bacterial inoculator was used to spread the bacteria on the chocolate agar. On the solid blood agar, glistening colonies were formed after 18 hours, measuring about 1 mm each. The bacteria were diluted in a dose of 0.2 mg/ml, then cultured on solid blood agar medium. The blood agar was prepared according to the manufacturer's instructions. On the solid blood agar, glistening colonies were formed after 18 hours, measuring about 1 mm each.

Liquid media

Streptococcus pneumoniae strains were grown on Trypticase soy agar (TSA II) supplemented with 5% sheep's blood at 37°C in 5% CO_2 in liquid media. 4.6 grams of agar base was mixed in 200 ml of distilled water. Yeast extract was added at a ratio of 0.2% in 1000 ml. It was sent for sterilization

for at least 4 hours. 5-10% of goat's blood was added; about 10 ml of blood was added each time to the liquid media. The bacteria, bought commercially from ATCC, were dissolved in 500 ml of normal saline 0.9%. 2 ml was removed each time and added to 7 ml of the liquid media. The tubes along with a control, were centrifuged for at least 12 hours at 220 rpm at 37 degrees. The growth was observed at 6, 12 and 18 hours. The bacteria were grown on a serial of 4 subsequent cultures on liquid blood agar. With initial growth beginning in optimal conditions, autolysis usually begins within 18-24 hours, with colonies collapsing in the centers. They were examined after 15 hours for solid residues in the tubes. The red blood cells have been lysed and the cultures compared to the 0.5-5 McFarland bacterial comparator.

In both solid and liquid media, the culture plates were carefully labeled as control, positive results and negative results.

Animal model

The 3-week-old healthy rats were weighed being on average 100 mg each. They were all given chloral hydrate as anesthesia in a dose of 10%, 3 ml/kg intra-peritoneally and placed on the stereotactic frame. Intracisternal injection of 10^6 colony-forming unit (CFU) *S. pneumoniae* in 10 μl of 0.85% sterile saline. While the control group ($n = 10$) were injected with normal saline. The rats were allowed to recover in their cages with free access to food and water. Each group was carefully labeled. After 8 hours, the rats were observed for signs of meningism, piloerection, irritability, decreased spontaneous activity and a hunched position. 10 μl CSF was then obtained from the experimental ($n = 10$) and control ($n = 10$) rats by puncture of the cisterna magna, 5 μl CSF quantitatively cultured on TTC-nutrient agar for bacterial counts, another 5 μl CSF was used to count WBC. CSF leukocyte counts showed a significant difference between bacterial infected rats and those of the control group, $4582 \pm 16.31 \times 10^6/\text{l}$ vs. $18.4 \pm 7.19 \times 10^6/\text{l}$ ($p < 0.05$), thus confirming the presence of meningitis in our rat model. Then, 3 of the infected rats were sacrificed under deep anesthesia by intra-peritoneal injection of chloral hydrate (10%, 3 ml/kg) 24 h after intracisternal injection of the *S. pneumoniae* inoculums and 4 of the control rats also included.

Rat tissue preparation for ribonucleic acid isolation

After decapitation, the brain of the animals of the other 3 infected group rats and remaining 4 controls, were immediately removed using RNase free instruments and frozen in liquid nitrogen, stored at -80 degrees until further use.

RNA isolation

Total RNA was isolated using 1 ml of Trizol Reagent (Invitrogen, Carlsbad CA, USA), 1 ml for every 50 μg of cortical tissue. After adding 0.2 ml of chloroform, the

aqueous phase was separated using Phase Lock tubes (Eppendorf, Hamburg, Germany). RNA was then precipitated using 0.5 ml of isopropyl alcohol, washed twice with 75% ethanol and then dissolved in 50 μ l of nuclease free water. The optical density and concentration of each RNA group was measured using the nanodrop spectrophotometer at 260/280 nm (Ocean Optics, Dunedin, FL, U.S.A.).

Quantitative PCR (qPCR)

cDNA synthesis was performed from the RNA extracted from the different prepared astrocyte groups using the One Step PrimeScript[®] miR cDNA Synthesis Kit (TAKARA, Dalian, China) which includes three mixes (2 \times miRNA Reaction Buffer Mix, miR PrimeScript[®] RT Enzyme Mix and 0.1% BSA). A 10- μ l reaction contained 5 μ l 2 \times miR reaction buffer mix, 1 μ l miR PrimeScript[®] RT enzyme mix and 1 μ l 0.1% BSA. RNA 100 pg, and DEPC-treated water up to 10 μ l. The tubes were incubated at 37°C for 60 min, the reaction was terminated at 85°C for 5 sec, and then the reaction was held at 4°C. qPCR were performed using the SYBR[®] Premix Ex Taq[™] II (TAKARA, Dalian, China) kit in triplicate. The 10- μ l PCR contained the following: 5 μ l SYBR[®] Premix Ex Taq[™] II, 0.4 μ l Uni-miR qPCR Primer, 2 μ l miR specific primer for the tested miRNA (GeneCopoeia, USA), 1 μ l cDNA, and 1.6 μ l DEPC-treated water. The PCRs were incubated at 50°C for 2 min (UDG incubation) and 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by melting curve analysis from 65.0 to 95.0°C (increment 0.5°C, 0:05). The relative expression levels for brain-enriched miR132, and inflammation-related miR146a were calculated using the comparative CT method. miR146a and miR132 gene expression were analyzed using Takara miRNA assays, which were run on the Applied Biosystems 7900HT (Foster City, CA, U.S.A.) according to the instructions and manufacturer's set conditions. The relative quantitative level of miR146a and miR132 was determined using the 2^{- $\Delta\Delta$ CT} method from the software data analysis, provided by the manufacturer. Values were normalized.

Protein extraction

About 100 mg of cerebral cortex tissue was crushed into powder in liquid nitrogen and 1 ml of lysis buffer added (970 μ l of NP40, 10 μ l of phenylmethylsulfonyl fluoride (PMSF), 10 μ l of cocktail (protease inhibitor) and 10 μ l of LY (phosphatase inhibitor). The mixture was vortexed, and incubated at 4°C in ice for one hour. The materials for protein extraction were bought from Amresco (Solon, U.S.A.). Centrifugation was done at 12,000 rpm for 10 minutes at 4°C. The nucleoproteins were extracted for the western blot of NF κ B, which were operated followed by the instruction manual of NE-PER (R) Nuclear and Cytoplasmic Extraction kit (Pierce, U.S.A.).

Western blot technique to detect TLR4, TNF- α , IL1B, NF κ B

Different concentrations of sodium dodecyl sulphate-polyacrylamide gels were made overnight according to the molecular weight of the protein targeted. A total of 30 μ g of protein was loaded in each well. The polyvinylidene fluoride (PVDF) membrane was blocked with milk, was incubated overnight at 4°C with specific primary antibodies against each targeted protein at specified concentrations, then incubated again with the corresponding second antibody as shown in Table 1. β -actin was used each time as a loading control. Enhanced chemiluminescence (ECL) detection system was used to visualize each reaction. Autography detected the signals on the blots. The film was digitally scanned and then quantified using FluorChem software, (Alpha Innotech, U.S.A.). Quantification was performed by densitometry of Western blot bands using the Image J software.

Statistical analysis

All experimental data were expressed as mean \pm SD. Student t test using SPSS software (version 13.0 SPSS) was used to compare the difference between the 2 groups. Statistical significance was defined as *p* less than 0.05.

Table 1. The different concentration ratios for the respective primary and secondary antibodies in the following inflammatory markers

Protein	Molecular weight/KDA	Gel concentration	First antibody	Second antibody
TLR4	93	8%	monoclonal mouse antibodies (Abcam Ltd. Hong Kong, ab22048) 1 : 50	goat anti mouse IgG (Jackson, Immuno Research, U.S.A.) at 1 : 10 000
IL1B	31	10%	polyclonal antibodies (Novus Biologicals, Litterton, CO, U.S.A.) at 1 : 500	horseradish peroxidase (HRP) conjugated sheep anti-rabbit IgG (Amersham Biosciences, Piscataway, Nj, U.S.A.) 1 : 10 000
TNF- α	17	10%	monoclonal mouse antibodies (Abcam Ltd. Hong Kong, ab22048) 1 : 1000	goat anti mouse IgG (Jackson, Immuno Research, U.S.A.) at 1 : 5000
NF κ B	64	10%	polyclonal antibodies (Abcam, Hong Kong) 1 : 500	horseradish peroxidase (HRP) conjugated sheep anti-rabbit IgG (Amersham Biosciences, Piscataway, Nj, U.S.A.) 1 : 10 000

Results

In Fig. 1 to Fig. 4, the upper bands demonstrate the different immunoblots for TLR4, IL1B, TNF- α and NF κ B, placed above β -actin, which was used as control throughout the experiment.

TLR4

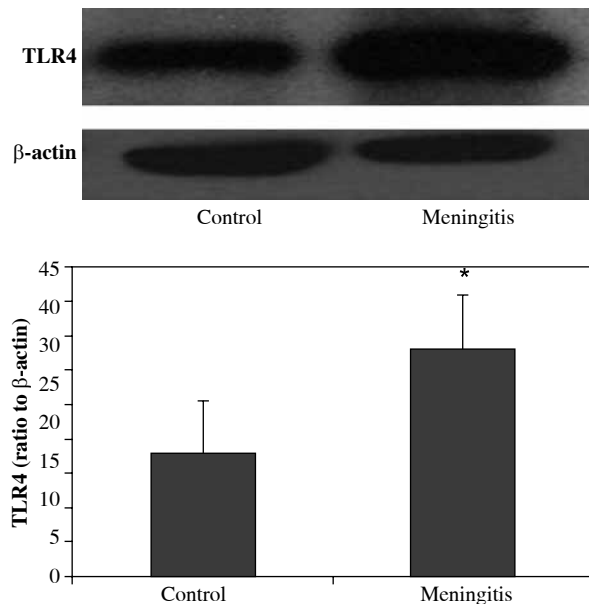
Using the Western Blot method, TLR4, originating from the surface receptors of dendritic cells, showed an escalation in the meningitis group (mean = 33.24 \pm 0.70) as compared to the control group (mean = 18.01 \pm 1.51), as shown in Fig 1. The results were compared each time with β -actin as control. To get the different expressions of TLR4, 30 nanograms of extracted protein was placed in the protein wells in Western Blot. An 8% gel was used. The primary antibody and secondary antibody are at a concentration of 1 : 150 and 1 : 10 000, respectively.

IL1B

IL1B is an inflammatory cytokine found in the early stages of inflammation. It has a molecular weight of 64KDA. Hence, a 10% sodium dodecyl gel was used. The concentration of the primary and the secondary antibodies were 1 : 500 and 1 : 10,000, respectively. In the meningitis group (mean = 35.04 \pm 0.75), the expression of IL1B was upregulated as compared to the control group (mean = 11.81 \pm 0.67).

Tumor necrosis factor α

TNF- α , the primary antibody was monoclonal mouse antibody at a concentration of 1 : 1000 and the second-



* $p < 0.05$ on comparing with Control

Fig. 1. The expression of TLR4

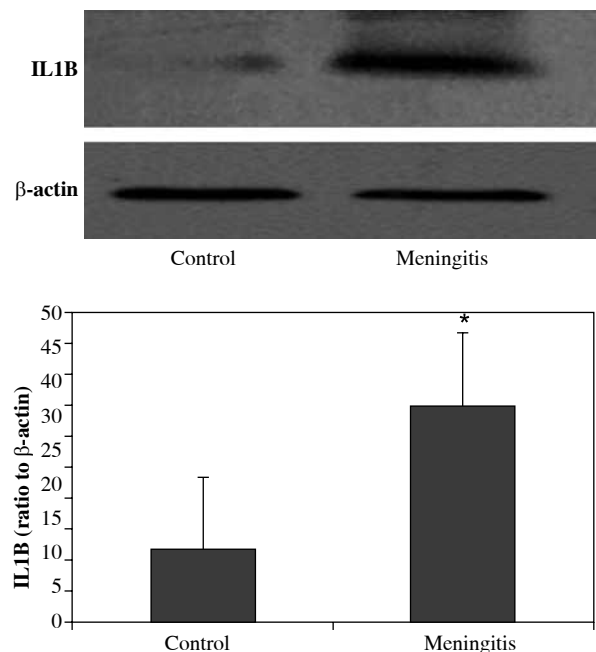
ary antibody was at a concentration of 1 : 5000. Being of molecular weight of 17KDA, a 10% sodium dodecyl gel was used. Fig. 3 shows that in the control group, when only normal saline has been injected, this had a result of mean = 21.31 \pm 3.40, compared to the meningitis group with a (mean = 34.37 \pm 1.26).

Nuclear factor κ B

NF κ B is found in the nucleus of cells enabling transcription, which showed that if the NF κ B in the nucleus is increased, it would be up-regulated. The result showed a rise in the level of NF κ B in the meningitis group. The Fig. 4 shows the expression of NF κ B being as follows: control group (mean = 10.71 \pm 0.57), meningitis group (mean = 32.77 \pm 1.73). The results tally with the other inflammatory markers described earlier, where an increased expression is seen in the meningitis group.

Real time PCR results of miR146a

The miRNA expression of miR146a was detected using the SYBEX (Biorad) protocol which is as follows: 95.0°C for 30 min, 95.0°C for 0.05 s, 60.0°C for 30 min. The melt curve was set at a temperature of 65.0°C to 95.0°C with an increment of 0.5°C at 0.05 s. Real time PCR results showed a completely different result as compared to the other inflammatory markers. A significant down-regulation of miR146a in the meningitis group (mean = 0.19 \pm 0.04) compared to the control group (mean = 0.78 \pm 0.32) was noted in Fig. 5.



* $p < 0.05$ on comparing with Control

Fig. 2. The expression of IL1B

Real time PCR results of miR132

The miRNA expression of miR132 was detected using the same SYBEX (Biorad) protocol as for miR146a. Real time PCR results showed a completely different result as compared to the other inflammatory markers. A significant down-regulation of miR146a in the meningitis group (mean = 0.19 ± 0.04) compared to the control group (mean = 0.78 ± 0.32) was noted in Fig. 6.

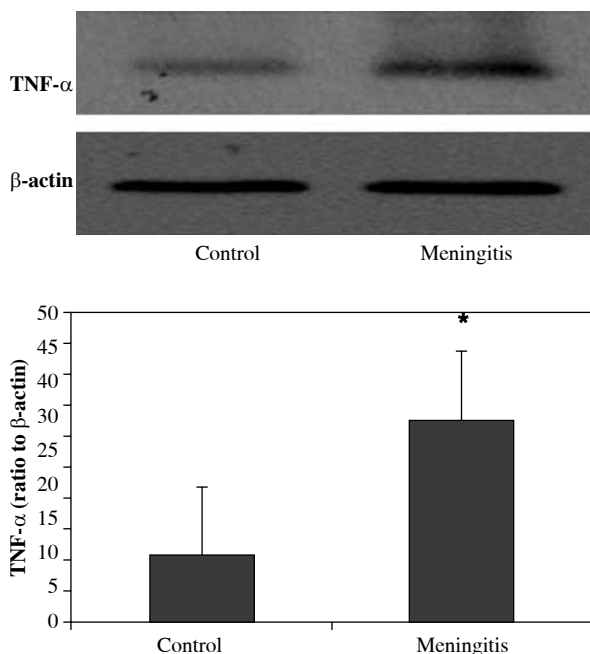
Discussion

Bacterial meningitis no doubt leads to significant mortality and disability if left untreated early. There are many retrospective and prospective studies showing the importance of preventing major adverse outcomes in the early administration of antibiotics [18, 19]. But still, it seems that antibiotics alone, are not sufficient to approach its serious consequences. The final outcome and prognosis in acute bacterial meningitis depend mainly on the inflammatory response in the subarachnoid space [9].

It has been found that TLR4, NFκB and TNF-α, IL1B form a part in the pro-inflammation signal transduction cascade of meningitis [8]. Toll like receptors could initiate specific immune responses due to their pattern recognition receptors which allow them to detect both self and non-self-antigens [20] and then recruit the myeloid differentiation primary response gene (MyD) 88, which in turn mediates the degradation of NFκB inhibitor IκB. Activated

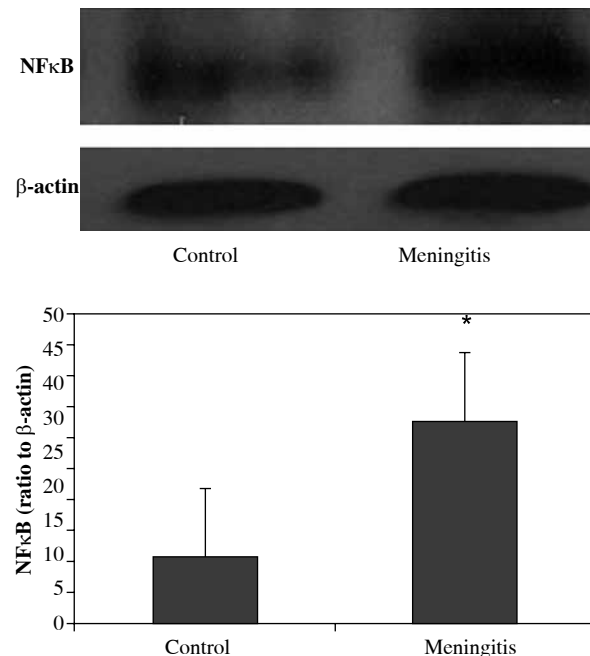
NFκB migrates to the nucleus and up-regulates the pro-inflammatory cytokines like TNF-α and IL1B, which trigger a cascade of inflammatory mediators including other cytokines, chemokines, arachidonic acid metabolites, reactive nitrogen and oxygen intermediates and proteases, and then cause blood brain barrier damage, hence resulting in brain edema [21]. It is hinted that TLR4-NFκB-TNF-α/IL1B pathway could be a potential treatment strategy for regulating the inflammation in meningitis patients, which would decrease the brain edema and reduce the neology squeals/neurological sequelae. In our study, the expression of the receptor (TLR4), nucleus for transcription (NFκB) and inflammatory cytokines (TNF-α and IL1B) shoot up in the acute stage of bacterial meningitis compared to the control, which supported that TLR4- NFκB-TNF-α/IL1B pathway is important in the regulation of the inflammatory process in *S. pneumoniae* bacterial meningitis. However, it is not completely known how to regulate this pathway.

As to the anti-inflammatory process, some miRNAs, especially for the miR146a, are considered to participate as the post-transcriptional factor. It could target TAB2, IRAK1 and TRAF6, thereby preventing NFκB activation [22]. The latest studies found that miR146a inhibited endothelial activation by dampening the activation of pro-inflammatory transcriptional programs, including AP-1 and MAPK/EGR pathways, likely through regulation of IL1B signaling pathway adaptor proteins [19]. As we know, endothelial activation would contribute to blood brain barrier disruption and would lead to brain edema resulting in the



* p < 0.05, on comparing with the Control

Fig. 3. The expression of TNF-α



* p < 0.05 on comparing with Control

Fig. 4. The expression of NFκB

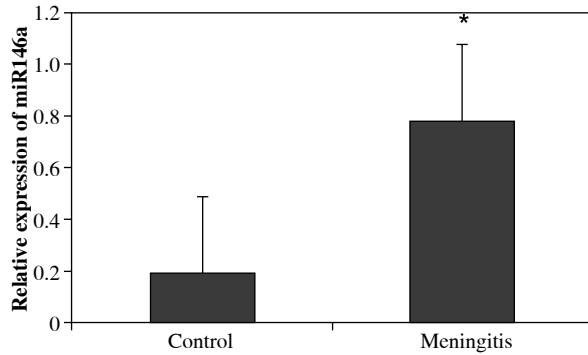


Fig. 5. The relative expression of miR146a

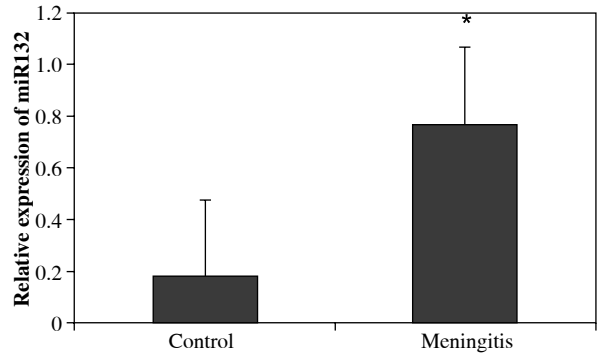


Fig. 6. The relative expression of miR132

long sequelae of meningitis. Thus, it would be hypothesized that miR146a negatively regulates inflammation in meningitis. miR132 is another post transcription factor which is brain enriched and also modulates inflammation. Its transcription is stimulated by LPS and up-regulated at an early stage of HCMV infection, and it has been approved to be involved in the regulation of CNS inflammation induced by *A. cantonensis* [23] and of ocular infection induced by herpes simplex virus [24]. Thus, we speculated that both miR146a and miR132 could be considered as the possible anti-inflammatory genes in *S. pneumoniae* meningitis. There is still no research being done about the different pathogenic steps of miR146a and miR132 in the *S. pneumoniae* animal model. In our study, we found that, corresponding with the activation of TLR4- NF κ B-TNF- α /IL1B pathway in acute *S. pneumoniae* meningitis, the results of real-time PCR detected significant down-regulation of both miR146a and miR132 in the same animal model, putting these miRNAs as potential candidates to understand the pathogenesis of *S. pneumoniae* meningitis at the posttranscriptional level.

Conclusions

We are the first to reveal that the noncoding small miRNA, miR146a and miR132 are novel regulators of inflammation of *S. pneumoniae* meningitis. The modulation of the anti-inflammatory process mediated by these miRNAs was likely to be related to the TLR4-NF κ B-TNF- α /IL1B signal transduction pathway. However, further loss-of-function and gain-of-function studies of these miRNAs should be implemented in order to clearly understand its mechanisms in regulating gene expression and to evaluate its potential as a biomarker or therapeutic target in *S. pneumoniae* meningitis diseases.

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