

## Review Article

# The role of increased mir-21 in promoting neuronal apoptosis and inducing the inflammatory response in epileptic rats

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**Abstract:** Background/Objective: Epilepsy is a common neurological disease in China, and its pathogenesis is still under investigation at present. Therefore, this study aimed to explore the relationship between miR-21 and epilepsy, so as to provide reliable experimental data for epilepsy treatment. Methods: Rats were randomly assigned to a control group, a model group, an miR-21 antagomir group, and an miR-21 agomir group, with 10 rats in each group. The rats in all the groups except the control group were induced by giving them lithium chloride-pilocarpine to construct epileptic models. After the successful modeling, the miR-21 antagomir group was given miR-21 antagomir, while the miR-21 agomir group was given miR-21 agomir. qPCR was employed to determine the level of miR-21 in each group, and enzyme-linked immuno-sorbent assays (ELISA) and Western blots were adopted to determine the protein levels. The effects of miR-21 on the inflammatory response and neuronal apoptosis in the epileptic rats were evaluated, and a water maze test was used to evaluate the learning and memory abilities of those rats. Results: MiR-21 was highly expressed in the epileptic rats. The increase of miR-21 not only promoted neuronal apoptosis and induced inflammatory responses in the epileptic rats, but it also activated the p53 and NF- $\kappa$ B pathways, but the inhibition of miR-21 contributed to the recovery of the rats' learning and memory abilities. Conclusion: MiR-21 induces the activation of the p53 and NF- $\kappa$ B pathways, thus leading to neuronal apoptosis and an inflammatory reaction.

**Keywords:** Epilepsy, miR-21, neuronal apoptosis, inflammatory response

## Introduction

The hyperexcitability of neurons in the brain leads to epilepsy [1, 2]. Epilepsy is a common brain disease, and it is induced by neural structural variations, gene mutations, infections, and metabolic and immune abnormalities [3]. It may cause serious psychological effects in people, such as anxiety and depression [4], and patients with the disease often have a significant suicidal tendency [5]. Therefore, it is a top priority of brain research to find an effective treatment for epilepsy.

miR-21 is a conserved sequence about 72 bp long on human chromosome 17. Although it does not directly encode genes, it can participate in the post-transcriptional regulation of many genes by binding to mRNA. Because of this regulatory relationship, miR-21 is involved

in the development and progression of various diseases. A large number of studies have shown that the abnormal expression of miR-21 is strongly linked to neurological diseases. MiR-21 acts on neurological diseases in two ways. On the one hand, miR-21 has a neuroprotective effect. It is highly expressed in traumatic brain injury model rats, and miR-21 secreted by HT-22 neuron cells can protect cells from autophagy or apoptosis by inhibiting Rab11a, Bcl2, and Bax in other cells. In addition, it can also promote angiogenesis by regulating VEGF, Ang-1, and Tie-2 [6-8]. In the cerebral hemorrhage model rats, miR-21 improves the cognitive function, neural structure, and blood circulation of the rats with cerebral hemorrhage by inhibiting DUSP8 and FASLG [9, 10]. On the other hand, miR-21 has neurotoxicity. It causes neuronal cell death or immune dysfunction by upregulating the TLR7 pathway or promoting

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potassium efflux in subjects with simian immunodeficiency virus (SIV) infectious neurocognitive disorders [11, 12]. MiR-21 is significantly up regulated during epileptic seizures, and it can aggravate neuronal apoptosis or nerve injuries by inhibiting PTEN and SOX7 [13-15].

This experiment adopted the lithium chloride-pilocarpine method to construct rat epilepsy models, and it upregulated the expression of miR-21 in epileptic rats through antagomir and agomir to explore the relationship between miR-21 and epilepsy, so as to provide a new therapeutic target for epilepsy treatment.

## Materials and methods

### *Epilepsy model construction*

The rats in all groups except the normal group were given lithium chloride-pilocarpine to induce epilepsy and were intraperitoneally injected with 125 mg/kg lithium chloride. After 20 h, they were intraperitoneally administered 1 mg/kg hyoscine butylbromide, and then after 0.5 h, they were intraperitoneally injected with 1% 30 mg/kg pilocarpine. According to the Racine scale, epilepsy lasting for 0.5 h was regarded as prolonged epilepsy, and the rats that suffered from prolonged seizures and survived were regarded as successful models. If a rat suffered no epileptic symptoms or had epileptic symptoms not reaching level IV, it was injected with pilocarpine every 0.5 h (note: 10 mg/kg each time) until it showed prolonged epilepsy. After the successful modeling, the rats in the miR-21 antagomir group were injected with miR-21 antagomir via their tails, and the rats in the miR-21 agomir group were injected with miR-21 agomir via their tails.

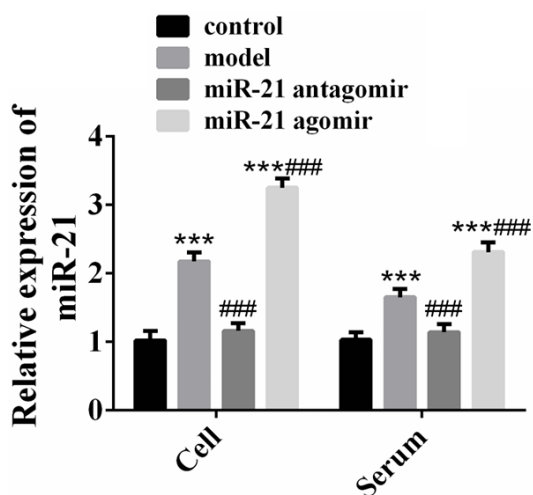
### *Water maze test*

An orientation navigation experiment was carried out as follows: before the experiment, the rats were familiarized with the environment in the Morris system and made to learn to find the platform by swimming around. Those who could not find it within 2 min were helped to climb onto the platform and rest on it for 3 s. At the beginning of the experiment, the rats were randomly placed into the water with their backs to the pool wall, and the time (escape latency) and swimming distance they took to find the fixed platform were recorded. If a rat failed to find

the platform for more than 2 min, its escape latency was recorded as 2 min. Subsequently, a spatial probe test was carried out as follows: The platform was taken out of the water, and the rats were placed into the water from any point in a quadrant corresponding to the platform area, and the time the rats stayed in the target quadrant and the number of times they accurately crossed the platform within 2 min were recorded. The water maze test lasted for 6 days.

### *qPCR*

Venous blood was sampled from the rats, placed in anticoagulative tubes, and centrifuged at  $3 \times 10^3$  r/min at low temperature for 30 min to collect the supernatant. The rat brains were taken, and the hippocampi were isolated out from them to sample the neuronal cells. The total RNA of the serum samples was extracted using a miRNeasy Serum/Plasma Kit (QIAGEN, Germany, 217184), and the total RNA of the neuronal cells in the rats was extracted using the Trizol method. The optical density (OD) of the total RNA at 260-280 nm was measured using ultraviolet spectrophotometry, and the RNA with OD<sub>260</sub>/OD<sub>280</sub>>1.8 was used for next experiment. Reverse transcription, and PCR amplification and quantification were conducted on the RNA using a FastKing one-step reverse transcription-fluorescence quantitative kit (Tiangen Biotech (Beijing) Co., Ltd., FP314) and ABI PRISM 7000 (Applied Biosystems, United States). The primers of miR-21 were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (F: 5'-GACAAGCTTGCGCCGCCCTTTAGGAGCATTATGAGCAT3'; R: 5'-ATCCTCTAGAGTCGACGAAGGTCAAGTAACAGTCATAC-3'). qPCR was performed under a reaction system following the kit's specifications. The system consisted of 50 mL of total volume containing 1.25 mL of upstream primer, 1.25 mL of downstream primer, 1.0 uL of probe, 10 pg/ $\mu$ g of RNA template, 5  $\mu$ L of 50 $\times$ ROX Reference Dye ROX, and RNase-Free ddH<sub>2</sub>O added to adjust the volume. The reaction process included reverse transcription at 50°C for 30 min (one cycle) and pre-denaturation at 95°C for 3 min (one cycle) followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s. The results were analyzed using an ABI PRISM 7000 instrument with the internal reference gene of U6, and the normalization was carried out using the 2<sup>- $\Delta\Delta$ Ct</sup> method.



**Figure 1.** High expressions of miR-21 in epileptic rats (\*\*\*) indicates in comparison with the control group,  $P < 0.001$ , and ### indicates in comparison with the model group,  $P < 0.001$ .

#### Western blot

The hippocampus of each rat was separated out by breaking their craniums, and they ground to homogenate them. The homogenate was lysed and taken out through 1 ml cell protein, and repeatedly pipetted until the cells were completely lysed. The homogenate was centrifuged at  $1.2 \times 10^4$  r/min for 15 min to take the supernatant, and the protein was separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to an NC membrane. The membrane was allowed to stand at room temperature for 1 h (blocked by 5% skim milk -phosphate buffer saline (PBS) solution). Subsequently, Caspase 3, Caspase 9, Bax, Bcl-2, p50, p52, p65, p53, mdm2, p21, and  $\beta$ -actin primary antibodies were added into the homogenate, and allowed to stand at 4°C overnight. The NC membrane was cleaned with PBS solution three times, mixed with goat anti-rabbit secondary antibody (HRP conjugant), and then allowed to stand for 1 h at room temperature. Finally, the NC membrane was washed with PBS solution and visualized using the enhanced chemiluminescence method. The internal reference protein was  $\beta$ -actin, and the relative expression level of the protein to be detected = the gray value of the band to be detected/the gray value of  $\beta$ -actin protein band.

Caspase 3, Caspase 9, Bax, Bcl-2, p50, p52, p65, p53, mdm2, p21, and  $\beta$ -actin primary antibodies and goat anti-rabbit secondary antibody (HRP conjugant) were all purchased from the Shanghai Abcam Company.

#### ELISA

Venous blood was sampled from the rats, placed in anticoagulative tubes, and centrifuged at  $3 \times 10^3$  r/min at low temperature for 30 min to collect the supernatant. The contents of the serum TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-10 were determined using enzyme-linked immunosorbent assays (ELISA) with the corresponding ELISA kits purchased from the Shanghai Abcam Company.

#### Statistics and analysis

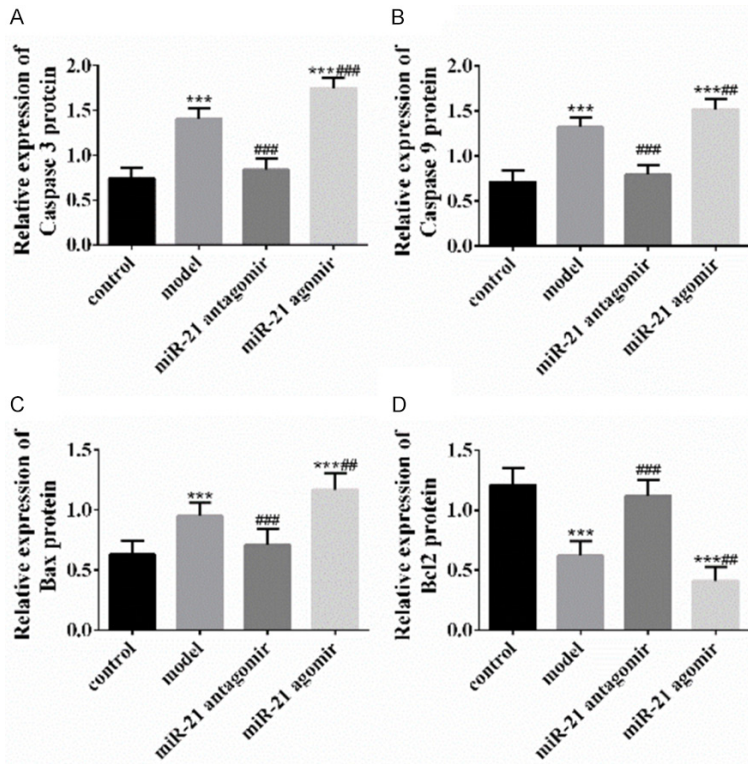
The above index data were input into SPSS 20.0 (Asia Analytics Formerly SPSS China) and GraphPad Prism 6.0 for the statistical analysis. The experiment was repeated three times. The measurement data were expressed as the mean  $\pm$  SD. The comparison among multiple groups were carried out using one-way ANOVA, and the post hoc pairwise comparisons were performed using LSD-t tests. All the data were analyzed using two-tailed tests. Ninety-five percent was used as the confidence interval.  $P < 0.05$  indicates a significant difference.

## Results

#### High expression of miR-21 in epileptic rats

In order to study the role of miR-21 in epilepsy, this study employed the lithium chloride-pilocarpine method to construct rat epilepsy models, and it also used the miR-21 antagomir and the miR-21 agomir to regulate the miR-21 levels in epileptic rats. **Figure 1** shows the results of the qPCR quantification of miR-21 in each group. Compared with the control group, the model and miR-21 agomir groups showed upregulated miR-21 in the neuronal cells and serum, while the miR-21 antagomir group showed no difference in the neuronal cells or serum. Compared with the model group, the miR-21 antagomir group showed downregulated miR-21 in the neuronal cells and serum, while the miR-21 agomir group showed upregulated miR-21 in them. The above results indi-

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**Figure 2.** The role of miR-21 in promoting neuronal apoptosis in epileptic rats (A: MiR-21 up regulated Caspase 3; B: MiR-21 up regulated Caspase 9; C: MiR-21 up regulated Bax; D: MiR-21 down regulated Bcl2. The above data were about the rats' neuronal cells. \*\*\* indicates in comparison with the control group,  $P < 0.001$ ; ## and ### indicate in comparison with the model group,  $P < 0.01$  and  $P < 0.001$ , respectively).

cate that miR-21 in epileptic rats is significantly increased.

### *The role of miR-21 in promoting neuronal apoptosis in epileptic rats*

This part of the experiment employed Western blot to determine Caspase 3, Caspase 9, Bax, and Bcl2 in the neuronal cells of the rats in each group to study the effects of miR-21 on apoptosis. The results are shown in **Figure 2**. Compared with the control group, the model and the miR-21 agomir groups showed significant increased expressions of Caspase 3, Caspase 9, and Bax, and a decreased expression of Bcl2, while the miR-21 antagonist group did not show a great change in the expression of those factors. Compared with the model group, the miR-21 antagonist group showed decreased expressions of Caspase 3, Caspase 9, and Bax, and an increased expression of Bcl2, while the miR-21 agomir group showed increased expressions of Caspase 3, Caspase

9, and Bax, and a decreased expression of Bcl2. The above results indicated that miR-21 can promote neuronal apoptosis in epileptic rats by upregulating the expressions of Caspase 3, Caspase 9, and Bax and downregulating the expression of Bcl2.

### *The role of miR-21 in aggravating the inflammatory response in epileptic rats*

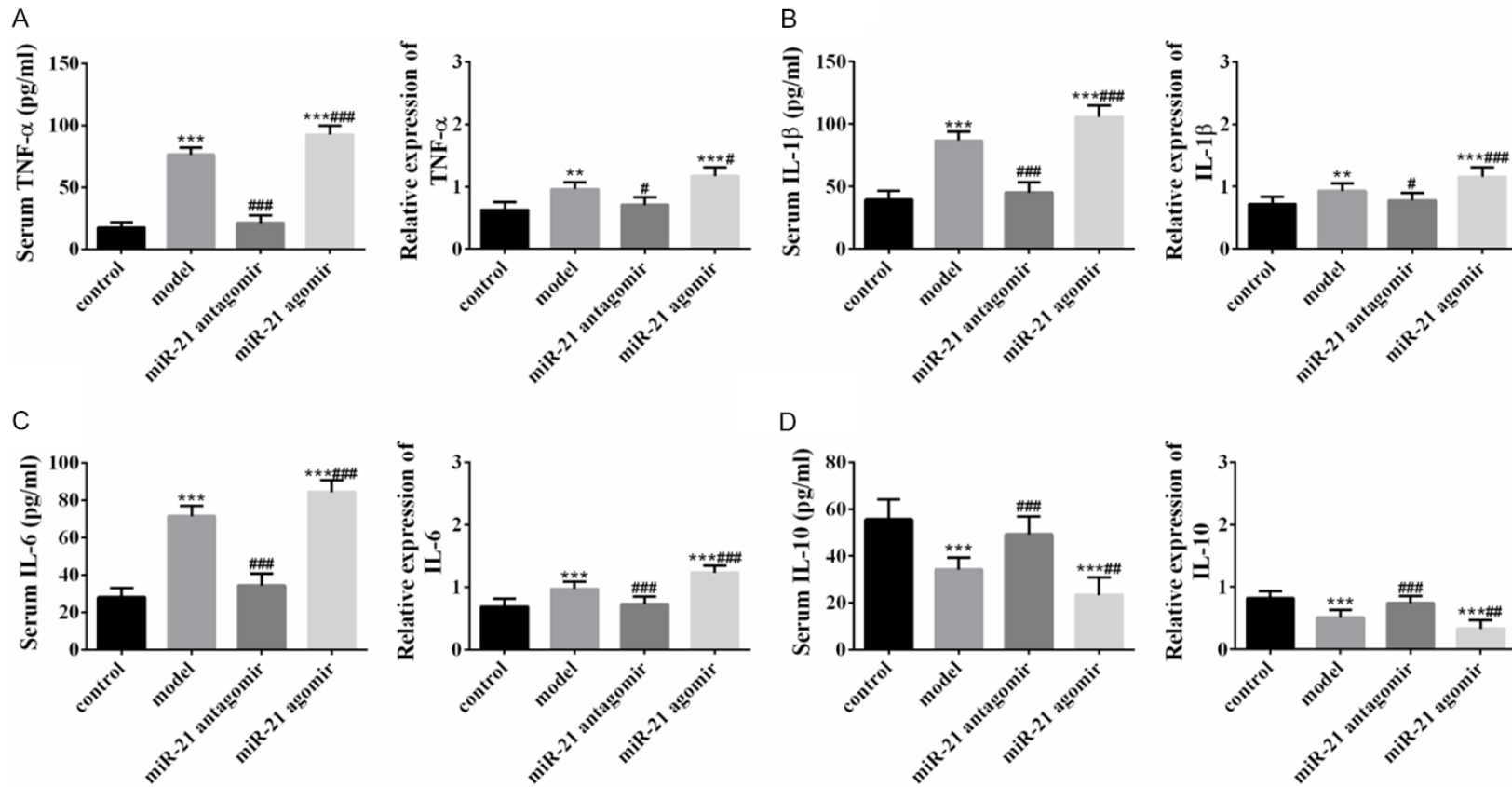
This part of the experiment used Western blot and ELISA to determine the TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and IL-10 levels in the serum and neuronal cells of the rats in each group to study the effects of miR-21 on the inflammatory response in rats. The results are shown in **Figure 3**. Compared with the control group, the model group and the miR-21 agomir group showed significantly increased expressions of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the serum and neuronal cells and decreased expressions of IL-10 in them, while the miR-21 antagonist

group did not show a big difference in them. Compared with the model group, the miR-21 antagonist group showed significantly decreased expressions of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in the serum and neuronal cells and an increased expression of IL-10 in them, while the miR-21 agomir group showed increased expressions of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in them and a decreased expression of IL-10 in them. The above results suggest that miR-21 can intensify the inflammatory response in epileptic rats by upregulating TNF- $\alpha$ , IL-6, and IL-1 $\beta$  and downregulating IL-10.

### *The role of miR-21 in upregulating the NF- $\kappa$ B pathway-related proteins*

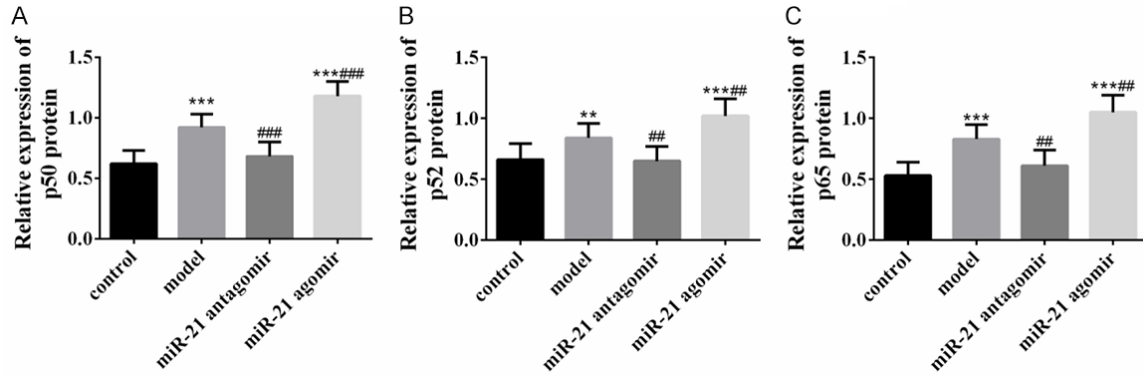
The NF- $\kappa$ B pathway is a pathway related to the inflammatory response, so this study determined the expressions of the NF- $\kappa$ B pathway-related proteins, p50, p52, and p65, in the neuronal cells of rats in each group to analyze the effects of miR-21 on the NF- $\kappa$ B pathway. The

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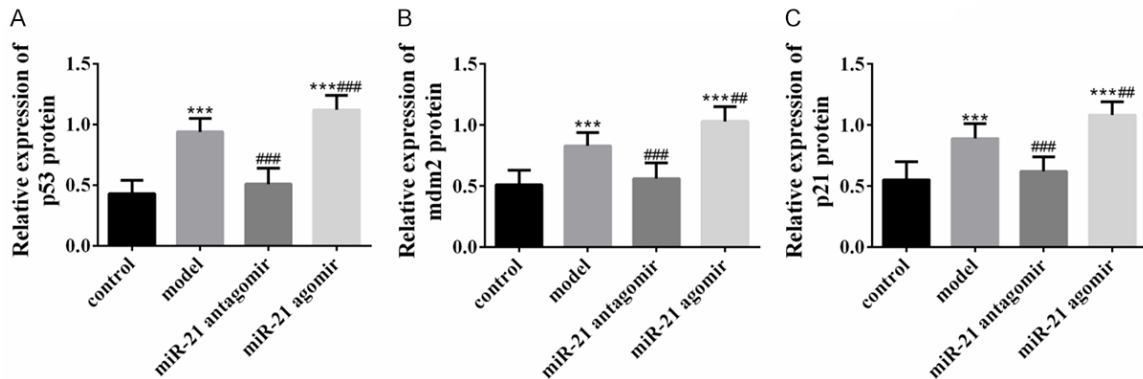


**Figure 3.** The role of miR-21 in aggravating the inflammatory response in epileptic rats (A: MiR-21 upregulated TNF-α in the serum and neuronal cells; B: MiR-21 up-regulated IL-1β in them; C: MiR-21 up regulated IL-6 in them; D: MiR-21 up regulated IL-10 in them. \* and \*\*\* indicate in comparison with the control group, P<0.05 and P<0.001, respectively, and #, ##, and ### indicate in comparison with the model group, P<0.05, P<0.01, and P<0.001, respectively).

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**Figure 4.** MiR-21 activates the NF-kB pathway (A: MiR-21 up regulated p50; B: MiR-21 up regulated p52; C: MiR-21 up regulated p65; \*\* and \*\*\* indicate in comparison with the control group,  $P < 0.01$  and  $P < 0.001$ , respectively, and ## and ### indicate that in comparison with the model group,  $P < 0.01$  and  $P < 0.001$ , respectively).



**Figure 5.** MiR-21 activates the p53 pathway (A: MiR-21 up regulated p53; B: MiR-21 up regulated mdm2; C: MiR-21 up regulated p21; \*\*\* indicates in comparison with the control group,  $P < 0.001$ , and ## and ### indicate in comparison with the model group,  $P < 0.01$  and  $P < 0.001$ , respectively).

results are shown in **Figure 4**. Compared with the control group, the model group and the miR-21 agomir group showed increased expressions of p50, p52, and p65, but the miR-21 antagonist group did not show a difference in their expressions. Compared with the model group, the miR-21 antagonist group showed decreased expressions of p50, p52, and p65, but the miR-21 agomir group showed increased expression of them. The above results indicate that miR-21 can activate the NF-kB pathway.

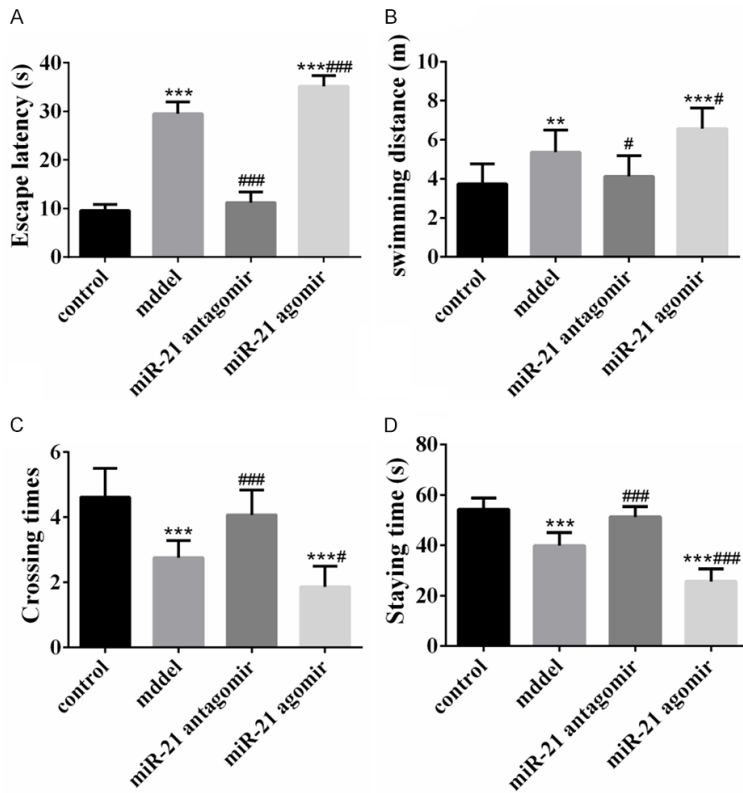
### *The role of miR-21 in up regulating the p53 pathway-related proteins*

The p53 pathway is a pathway related to apoptosis, so this study determined the expression of the p53 pathway-related proteins, namely p53, mdm2, and p21, in the rats' neuronal cells in each group to analyze the effects of

miR-21 on the p53 pathway. The results are shown in **Figure 5**. Compared with the control group, the model group and the miR-21 agomir group showed increased expressions of p53, mdm2, and p21, but the miR-21 antagonist group did not show a difference in their expressions. Compared with the model group, the miR-21 antagonist group showed decreased expressions of p53, mdm2, and p21, while the miR-21 agomir group showed increased expression of them. The above results indicated that miR-21 can activate the p53 pathway.

### *Water maze test*

In order to understand the learning and memory abilities of the experimental rats, this paper selected the water maze test to evaluate them. The results are shown in **Figure 6**. Compared with the control group, the model and miR-21



**Figure 6.** Water maze test (A: Escape latency; B: Swimming distance; C: The number of times they crossed the target quadrant; D: The resistance time on the platform; \*\*\* indicates in comparison with the control group,  $P < 0.001$ , and # and ### indicate in comparison with the model group,  $P < 0.05$  and  $P < 0.001$ , respectively. The data were recorded based on the water maze test at the 6th day).

agomir groups experienced extended escape latencies and swimming distances, shorter times to cross the target quadrant, and shorter residence times on the platform, and the miR-21 antagomir group did not show a large difference in those aspects. Compared with the model group, the miR-21 antagomir group experienced a shortened escape latency and swimming distance, longer times to cross the target quadrant, and a longer residence time on the platform, while the miR-21 agomir group did not show any large difference in those aspects. The above results suggest that miR-21 is helpful in the recovery of epileptic rats' learning and memory abilities.

### Discussion

Neuronal apoptosis and the inflammatory response are common cellular biological manifestations in epileptic patients. The regulatory mechanism of neuronal apoptosis involves multiple signal pathways and non-coding RNA.

Liu et al. [16] revealed that the PI3K/Akt pathway in epileptic rats can suppress neuronal apoptosis in them after being activated by Trem2, and Wang et al. [17] confirmed that miR-181b can protect cells from autophagy and apoptosis by down regulating the p38/JNK pathway. In addition, Zhao et al. [18] revealed that the deletion of miR-145 induced the down-regulation of Caspase 9, thus inhibiting neuronal apoptosis and improving the cognitive function of epileptic rats. The inflammatory response plays an important role in epilepsy, and an increase of the inflammatory factors in the brain leads to the persistence of epilepsy and neuronal cell death [19, 20]. Therefore, it is a hot topic in the field of epilepsy research to find therapeutic targets to inhibit neuronal apoptosis and the inflammatory response.

The results of this study revealed that miR-21 promotes cell apoptosis by upregulating

the pro-apoptotic proteins Caspase 3, Caspase 9, and Bax and by downregulating the anti-apoptotic protein, Bcl2, and also by upregulating the p53 pathway. The p53 pathway can induce cell senescence and apoptosis [21]. Previous studies have shown that the p53 pathway induces neuronal apoptosis in epileptic model animals [22, 23]. The above results suggest that miR-21 induces the expression of the pro-apoptotic proteins, Caspase 3, Caspase 9 and Bax, by up regulating the p53 pathway, thus causing neuronal apoptosis.

In order to find out whether miR-21 affects the inflammatory response in epileptic rats, this study analyzed the effects of miR-21 on the inflammatory response by regulating the expression of miR-21. It turned out that miR-21 inhibits the anti-inflammatory factor, IL-10, by up regulating the pro-inflammatory factors, TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , which indicated that miR-21 can intensify the inflammatory response in epileptic rats. In addition, in order to clarify the

specific regulatory mechanisms of the miR-21 on inflammatory response, this study also analyzed the NF- $\kappa$ B pathway related to the inflammatory response. It turned out that miR-21 upregulated the NF- $\kappa$ B pathway proteins (p50, p52, and p65). Previous studies pointed out that the up-regulation of NF- $\kappa$ B would promote the expression of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  in epileptic model rats [24, 25]. Therefore, we speculated that for epileptic rats, miR21 with a high expression up regulated the NF- $\kappa$ B pathway, thus activating the downstream proinflammatory factors, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  and suppressing the anti-inflammatory cytokine, IL-10, which eventually caused the severe inflammatory response.

This study explored the regulatory mechanism of miR-21's involvement in epilepsy progression. miR-21 induces apoptosis and the inflammatory response by regulating a series of signal pathways, which aggravates epilepsy. Although the miR-21's mechanism of action on epilepsy was described from the perspective of molecular biology in this paper, it only qualitatively confirms the influence of miR-21 on signal pathways and did not involve a specific target protein regulation mechanism. Therefore, the specific target protein and upstream regulatory factors of miR-21 can be further explored in the subsequent experimental design, so as to supplement the regulatory network of miR-21 in epilepsy.

To sum up, this study has found a possible molecular mechanism of epilepsy by analyzing the protein changes caused by miR-21 in epileptic rats, and it is believed that miR-21 induces the activation of the downstream p53 pathway and the NF- $\kappa$ B pathway, and the activation of these two pathways directly leads to neuronal apoptosis and the inflammatory response in epileptic rats. The deletion of miR-21 can alleviate neuronal apoptosis and the inflammatory response and promote the recovery of learning and the memory abilities of epileptic rats. Therefore, targeted regulation of miR-21 may be beneficial to epileptic patients.

## Disclosure of conflict of interest

None.

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