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Influences of miR-708 on cerebral ischemia-reperfusion injury through targeted regulation of ADAM17

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Abstract. – OBJECTIVE: The purpose of this study was to explore the influences of micro ribonucleic acid (miR)-708 on cerebral ischemia-reperfusion injury by regulating a disintegrin and metalloprotease 17 (ADAM17) in a targeted manner.

MATERIALS AND METHODS: The rat model of middle cerebral artery occlusion (MCAO) was established, and the differentially expressed miRNAs in the cerebral tissues of rats with ischemia-reperfusion injury were detected via sequencing. The research was performed in control group (PC12 cells received no treatment), inhibitor group (the expression of miR-708 in PC12 cells was down-regulated using miR-708 inhibitor), and interference + inhibitor group [PC12 cells were co-treated with miR-708 inhibitor and ADAM17 small interfering RNA (siRNA)]. Then, the expression of ADAM17 in cells, proliferation ability of cells, and number of apoptotic cells were detected in each group.

RESULTS: A total of 225 differentially expressed miRNAs were obtained through miRNA sequencing and bioinformatics analysis, of which miR-708, miR-169, miR-26, and miR-96 were highly expressed, whereas miR-122, miR-118, and miR-177 were lowly expressed in rats in ischemia-reperfusion group. Compared with that in control group, the level of miR-708 declined significantly in inhibitor group after treatment with miR-708 inhibitor. After treatment with miR-708 inhibitor, the protein expression level of ADAM17 in inhibitor group was evidently higher than that in control group, while its protein expression level in interference + inhibitor group was significantly decreased and restored, after interference of ADAM17 siRNA with protein expression. In comparison with control group, inhibitor group had increased apoptotic cells after miR-708 inhibitor treatment ($p<0.05$). Besides, after interference of ADAM17 siRNA with protein expression, there were a smaller number of apoptotic cells in interference + inhibitor group ($p<0.05$), showing mitigated apoptosis. Moreover, the proliferation ability of cells treated with miR-708 inhibitor in inhibitor group was weaker than that in control group ($p<0.05$), whereas the proliferation ability of cells in interference + inhibitor group was restored to a certain degree after ADAM17 siRNA interfered with the protein expression ($p<0.05$).

CONCLUSIONS: MiR-708 can modulate ADAM17 in a targeted manner to affect cellular proliferation and apoptosis in cerebral ischemia-reperfusion injury.

Key Words:
Ischemia-reperfusion, MiR-708, ADAM17.

Introduction

Ischemic cerebrovascular disease is one of the most common diseases in middle-aged and elderly patients, and once it occurs, cerebral tissues will be damaged due to local ischemia and hypoxia[1,2]. Ischemic disease tends to cause secondary damage to tissues *via* reperfusion, which is often the leading cause of irreversible pathological injuries in tissues[3,4]. Cerebral tissues are relatively sensitive to ischemia-reperfusion injury, whose major mechanisms include damage of oxygen and NO radicals to tissues, toxic side effects of amino acids and Ca^{2+} overload[5]. More in-depth research into the pathogenesis of ischemic cerebral injury

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is of great significance for the treatment of cerebral ischemic disease.

Micro ribonucleic acids (miRNAs), as small-fragment non-coding RNAs, are the important components in the cellular regulating networks[6,7]. MiRNAs can regulate the normal physiological activities of cells, and their expressions are often altered in the pathological state to affect cellular functions. Besides, they promote or repress the progression of diseases by modulating the expression of other crucial proteins[8]. Cerebral ischemia-reperfusion injury has been evidenced to be associated with several miRNAs. MiR-146a polymorphism is related to both the risk of cerebral ischemia and reperfusion injury[9], whereas miR-125b is able to target the CK2α/NADPH oxidase signaling pathway to alleviate ischemia-reperfusion injury in cerebral tissues[10].

Therefore, in the present study, the middle cerebral artery occlusion (MCAO) was established in rats, and the influences of ischemia-reperfusion injury on the expression of miRNAs in cerebral tissues were compared. According to the results, miR-708 was differentially expressed in the cerebral ischemia-reperfusion model. With a disintegrin and metalloprotease 17 (ADAM17) predicted as the target gene of miR-708, the expression of miR-708 in cerebral neuronal cell line PC12 was changed using miR-708 inhibitor. Moreover, a rescue experiment was conducted using ADAM17 small interfering RNA (siRNA), and the changes in ADAM17 and cell proliferation and apoptosis were observed. Finally, it was corroborated that miR-708 affects cerebral ischemia-reperfusion injury through the targeted regulation of ADAM17.

Materials and Methods

Establishment of Rat MCAO Model

A total of 20 male Sprague-Dawley rats were randomly assigned into control group (n=10) and ischemia-reperfusion group (n=10), and there were no statistically significant differences in general data of rats, such as age and weight, between the two groups. After anesthesia *via* 10% chloral hydrate (0.3 mL/100 g) and shaving, each group of rats was disinfected using 75% alcohol. Then, sham operation was performed in control group. In ischemia-reperfusion group, the left common carotid artery and external carotid artery were separated and ligated at the proximal end of the heart, and the thread was inserted into the clamped internal carotid artery, whereas the common carotid artery was fastened at the distal end of the heart. Following 2 h of ischemia, blood supply of cerebral tissues was restored, and the cerebral tissues in control and ischemia-reperfusion groups were surgically resected for subsequent analyses. This investigation was approved by the Animal Ethics Committee of The Affiliated Hospital of Qingdao University Animal Center.

Detection of MiRNA Expression Profiles in Ischemia-Reperfusion Cerebral Tissues

Total RNAs were extracted from the cerebral tissues in control and ischemia-reperfusion groups using TRIzol method (Invitrogen, Carlsbad, CA, USA), and the qualified samples with the optical density (OD) value of 1.8-2.2 were sent to Shanghai Biotechnology Co., Ltd. (Shanghai, China) for sequencing. Finally, differentially expressed miRNAs were obtained through bioinformatics analyses.

Prediction of MiR-708 Target Gene and Grouping

ADAM17 was predicted as the target gene of miR-708 using miRNA Data Integration Portal (mirDIP) database. PC12 neuronal cell line was taken as the tool cells for subsequent experiments and grouped into control group (PC12 cells received no treatment), inhibitor group (the expression of miR-708 in PC12 cells was down-regulated using miR-708 inhibitor), and interference + inhibitor group (PC12 cells were co-treated with miR-708 inhibitor and ADAM17 siRNA).

Real-Time Fluorescence Quantitative Polymerase Chain Reaction (qPCR)

Total RNAs were extracted from each group of cells using TRIzol reagent and reversely transcribed through RT-PCR into complementary deoxyribonucleic acids (cDNAs). Then, Prime Primer was used to design primers (Table I), and the expression of miR-708 was detected *via* qPCR, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. After that, the PCR was conducted in the total PCR system (25 μL) containing 1 μL each of forward and reverse primers, 0.5 μL of cDNA template, 12.5 μL of 2× SYBR premix Taq, and 10 μL of dH_2O under the conditions of 95°C for 5 min and (94°C for 30 s, 59°C for 40 s, and 72°C for 30 s) ×45 cycles.

Western Blotting (WB)

The protein expression of ADAM17 was detected *via* WB in each group, with glyceraldehyde

Table I. QPCR primer sequences.

Gene	Primer sequences
MiR-708	F: 5'-GTGGATGGTAAAAACGAAAGCG-3' R: 5'-GGCTAGAACCCTAGAGTCAGG-3'
GAPDH	F: 5'-CACAAAACTTGAGAGTCGTGGT-3' R: 5'-GCTAGAACCCTAGAGTCAGGC-3'

3-phosphate dehydrogenase (GAPDH) as internal reference. The cells were first lysed using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) supplemented with 1% phenylmethylsulfonyl fluoride (PMSF) protease inhibitor for 15 min, inverted and mixed evenly for 3 times, and centrifuged at 1,400 rpm for 20 min, and the whole process was completed on ice. Next, the resulting proteins were added with loading buffer, bathed in boiling water, and stored at -20°C. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked in 5% skim milk for 1 h and incubated with antibodies at 4°C overnight. Finally, the washed proteins were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h, washed again, and exposed on an imager.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Apoptosis Assay

The cells in each group were harvested, grown on glass slides, and fixed using 4% paraformaldehyde. Then, the sections were detected using the TUNEL cell apoptosis assay kit strictly according to the instructions of the kit. After addition of color developer, TUNEL-positive cells were obtained under 488 nm excitation light and counted and photographed in 5-10 fields.

Detection of Cell Proliferation Function Via Cell Counting Kit 8 (CCK-8)

The proliferation function of cells in each group was determined using CCK-8 assay and CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) as follows. On the evening before the assay, each group of cells was paved onto a 96-well plate at 4×10^3 cells/100 μL/well, with quintuplicate wells set. At 1, 2, 3, and 4 d, each well was added with 10 μL of CCK-8 reagent, and 3 h later, the OD was measured at a wavelength of 450 nm using a micro-plate reader. Finally, the proliferation results were obtained through conversion.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Intergroup differences were analyzed using *t*-test and analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference), and $p<0.05$ suggested statistically significant differences.

Results

Differentially Expressed MiRNAs in the Rat Ischemia-Reperfusion Model

According to the miRNA sequencing and bioinformatics results, there were 225 differentially expressed miRNAs, of which miR-708, miR-169, miR-26, and miR-96 were highly expressed, while miR-122, miR-118, and miR-177 were lowly expressed in rat brain tissues in ischemia-reperfusion group (Figures 1, 2).

Level of MiR-708 Determined in Control Group and Inhibitor Group

As shown in Figure 3, after treatment with miR-708 inhibitor, the level of miR-708 in inhibitor group was significantly lower than that in control group, and the difference was statistically

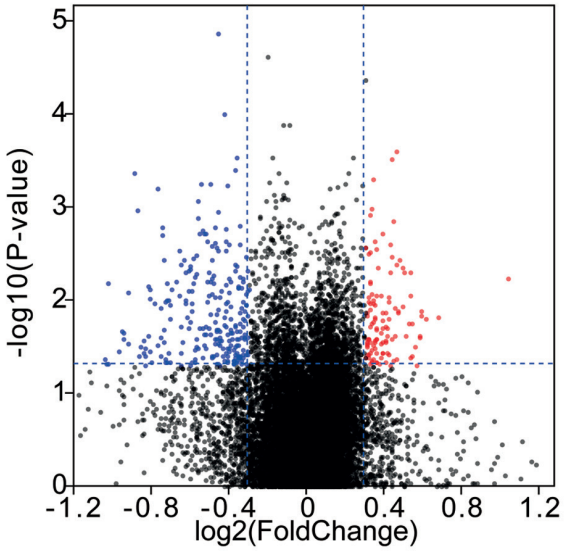

Figure 1. Volcano map of differentially expressed miRNAs in the rat ischemia-reperfusion model.

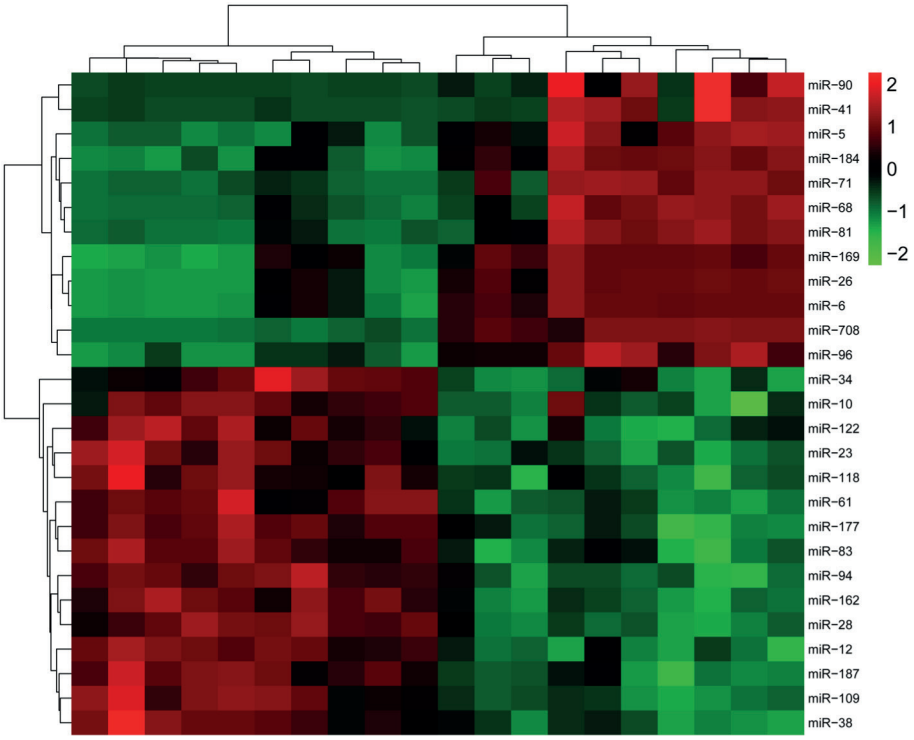

Figure 2. Heat map of differentially expressed miRNAs in the rat ischemia-reperfusion model.

significant, suggesting that miR-708 inhibitor has an evident inhibitory effect on the expression of miR-708 in PC12 cells.

Expression of ADAM17 in Control, Inhibitor, and Interference + Inhibitor Groups

Compared with that in control group, the protein expression level of ADAM17 was evidently elevated in inhibitor group after treatment with miR-708 inhibitor, and significantly decreased and restored in interference + inhibitor group after interference of ADAM17 siRNA with protein expression (Figure 4).

Changes In Cell Apoptosis In Control, Inhibitor, and Interference + Inhibitor Groups

As displayed in Figure 5, in comparison with control group, inhibitor group had increased apoptotic cells after miR-708 inhibitor treatment ($p<0.05$). Following interference of ADAM17 siRNA with protein expression, there were a smaller number of apoptotic cells in interference + inhibitor group ($p<0.05$), showing mitigated apoptosis.

Changes In Cell Proliferation Function In Control Group, Inhibitor Group, and Interference + Inhibitor Group

The proliferation ability of cells treated with miR-708 inhibitor in inhibitor group was weaker than that in control group ($p<0.05$), while the proliferation ability of cells in interference + inhibitor group was recovered to a certain degree after ADAM17 siRNA interfered with the protein expression ($p<0.05$; Figure 6).

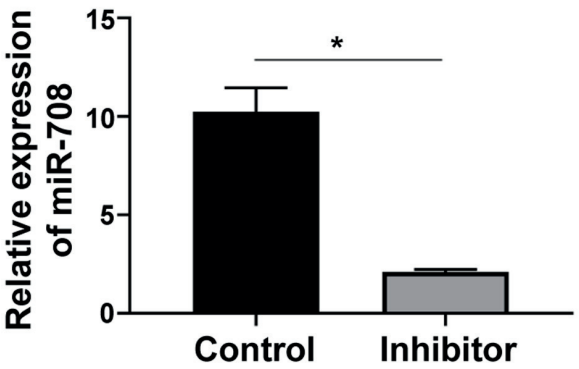

Figure 3. MiR-708 level determined in control group and inhibitor group (*$p<0.05$).

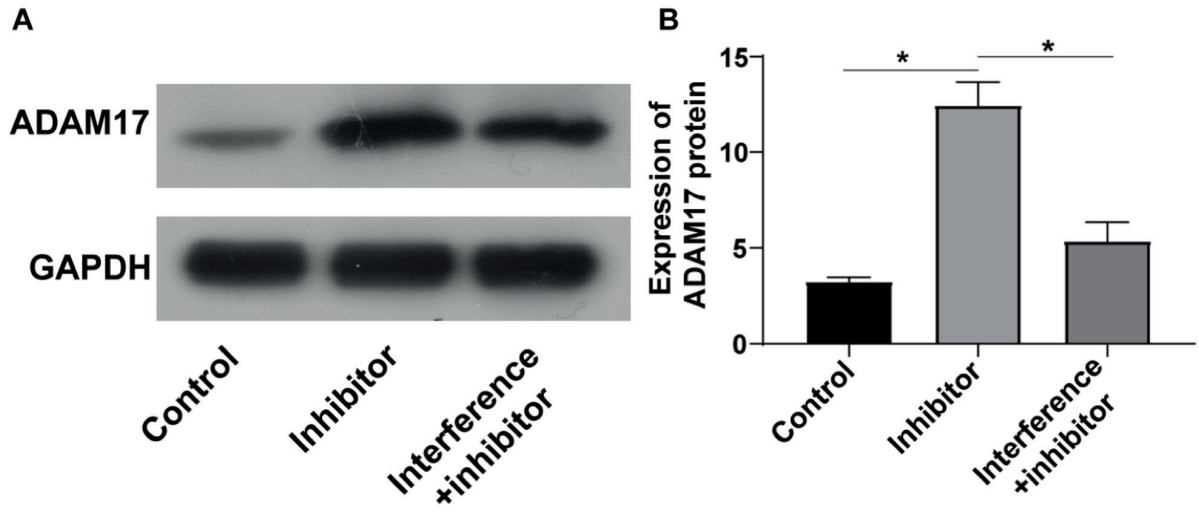

Figure 4. Expression of ADAM17 in control, inhibitor, and interference + inhibitor groups (*p<0.05).

Discussion

Ischemic disease can cause certain damage to normal tissues in human bodies, and cerebral tissues with the largest demand of oxygen and blood nutrients suffer from more damage[11,12]. In addition to ischemia causing cerebral tissue destruction, reperfusion is considered to induce secondary injury in cerebral tissues, resulting in severer consequences, such as local accumulation of harmful substances in cerebral tissues, edema, inflammation, and even irreversible cerebral injury. Cerebral ischemia-reperfusion injury can lead to cell membrane damage largely due to the increase in local oxygen free radicals. Meanwhile, mitochondrial membranes in cerebral cells are damaged because of intracellular Ca^{2+} overload, further reducing the energy supply to cerebral tissues and accelerating the progression of the disease[13]. Hence, studying the specific mechanism of cerebral cell injury in cerebral ischemia-reperfusion can provide a new idea of treating cerebral reperfusion injury.

As a part of important non-coding RNAs in cells, miRNAs are able to regulate various physiological and pathological processes and play pivotal roles in the pathogenesis and progression of diseases. Moreover, miRNAs can serve as potent regulating tools to modulate the expression of about 30% of human genes, affect gene transcription and translation, and interfere with protein expression, thereby influencing cellular functions and a variety of disease processes. It has been reported that miRNAs are able to affect the development and progression of multiple diseases. For example, miR-214 can decrease the malignancy of cervical cancer cells through the targeted inhibition on MKK3[14], miR-1296-5p is able to regulate the proliferation, migration and invasion abilities of osteosarcoma cells[15], and miR-514a-5p is differentially expressed in pulmonary thromboembolism and can exert a certain regulatory effect therein[16]. As such, miRNAs may play crucial regulatory roles in various diseases, and affect the progression of the diseases.

The main novelty of this study was that we screened differentially expressed miRNAs in ischemia-reperfusion rat models for the first time and found that miR-708 was significantly overexpressed in brain tissues of ischemia-reperfusion rats. We also explored the possible mechanism of action of miRNA-708. It was found that miR-708 may regulate the proliferation and apoptosis of brain cells during cerebral ischemia-reperfusion injury via targeting ADAM17. In the present study, the differences in the expression of miRNAs in rat brain tissues between ischemia-reperfusion group and control group were analyzed, and 225 differentially expressed miRNAs were obtained *via* bioinformatics analysis. Among them, miR-708, miR-169, miR-26, and miR-96 were highly expressed, while miR-122, miR-118, and miR-177 were lowly expressed in rat brain tissues in ischemia-reperfusion group. Moreover, there have been reports that miR-708 is associated with the onset of some diseases, such as colorectal cancer[17], Angelman's syndrome[18], pancreatic cancer[19], and gastric cancer[20]. It can be inferred

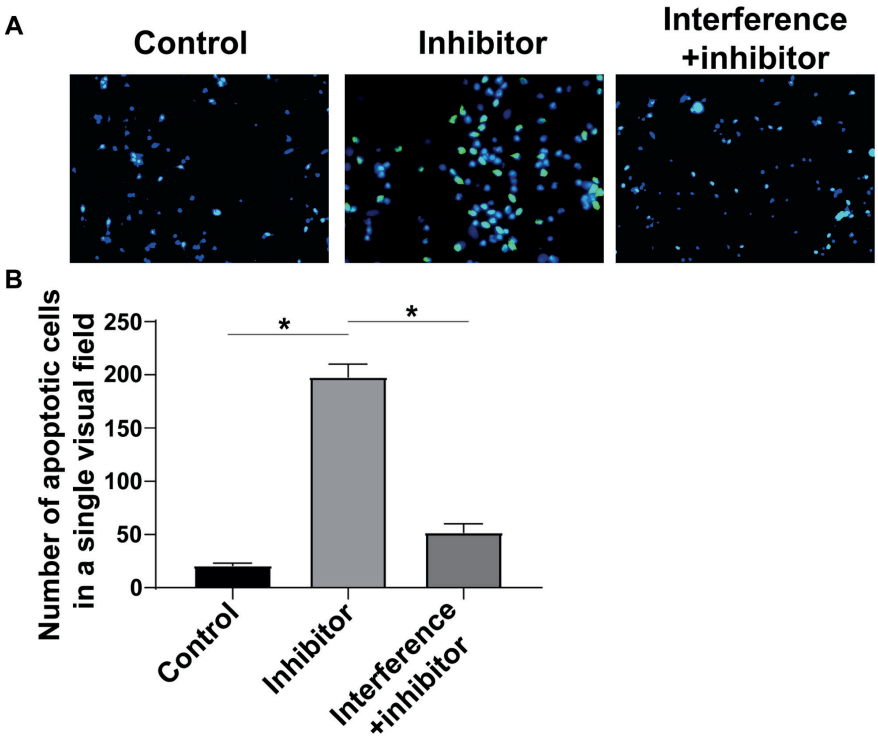

Figure 5. Changes in the number of apoptotic cells in control, inhibitor, and interference + inhibitor groups (magnification: 400×) (*p<0.05 *vs.* control group and interference + inhibitor group).

that miR-708 plays a vital role in cerebral ischemia-reperfusion injury as well. In this study, therefore, miR-708 was taken as the direction of subsequent investigations. According to the data of the present study, the level of miR-708 was distinctly lowered in inhibitor group after miR-708 inhibitor treatment, suggesting that miR-708 inhibitor can affect the expression of miR-708 indeed, with a relatively ideal interference effect, thus ensuring the quality of the subsequent experiments.

Moreover, the prediction based on the mirDIP database revealed that ADAM17 was the target gene of miR-708, so it was speculated that miR-708 may regulate ADAM17 to exert effects in cerebral ischemia-reperfusion, and the targeted regulatory effect of miR-708 on ADAM17 was detected. The rescue experiment was performed by interfering with miR-708 and ADAM17 expressions. According to the results, compared with that in control group, the protein expression level of ADAM17 was evidently elevated in inhibitor group after treatment with miR-708 inhibitor, and significantly decreased and rescued in interference + inhibitor group after interference of ADAM17 siRNA with protein expression. The above findings imply that miR-708 can regulate ADAM17 in a targeted manner indeed and exert effects in cerebral ischemia-reperfusion.

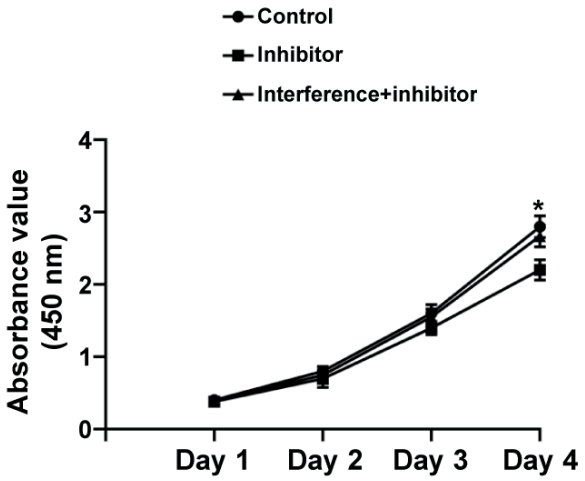

Figure 6. Changes in cell proliferation function in control, inhibitor, and interference + inhibitor groups.

Additionally, the changes in the number of apoptotic cells and proliferation ability were detected in each group. According to the results, inhibitor group had more apoptotic cells than control group after miR-708 inhibitor treatment ($p<0.05$), whereas there were a smaller number of apoptotic cells in interference + inhibitor group after ADAM17 siRNA interfered with the protein expression ($p<0.05$), showing mitigated apoptosis. Besides, the proliferation ability of cells treated with miR-708 inhibitor in inhibitor group was weaker than that in control group ($p<0.05$), while the proliferation ability of cells in interference + inhibitor group was recovered to a certain degree after ADAM17 siRNA interference with the protein expression ($p<0.05$).

Conclusions

In summary, miR-708 can affect cerebral cell proliferation and apoptosis functions through the targeted regulation of ADAM17 and may exert an important regulatory effect in cerebral ischemia-reperfusion injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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