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Original Article

Downregulation of miR-27a-3p may increase hematoma volume in rats with intracerebral hemorrhage

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Abstract: Objective: The aim of this study was to investigate expression and mechanisms of action of miR-27a-3p in hematoma tissue of rats with intracerebral hemorrhage (ICH). Methods: Intracerebral injections of 2 μl of bacterial collagen were applied to establish rat models of ICH in control and experimental groups. In the experimental group, miR-27a-3p inhibitors were injected into the right ventricle of rats before initiation of the model. RT-PCR was conducted to measure relative expression of miR-27a-3p in the hematoma of ICH rats. Results: Relative expression of miR-27a-3p in brain hematoma tissue in the experimental group was significantly lower than the control group on days 1 and 3 ($t = 21.140$, $P < 0.001$ on day 1; $t = 12.480$, $P < 0.001$ on day 3). The volume of hematoma in rats of the experimental group was significantly greater than the control group on day 1 ($t = 13.110$, $P < 0.001$). miR-27a-3p inhibitors significantly downregulated miR-27a-3p ($t = 5.061$, $P < 0.001$). In rats with ICH, downregulation of miR-27a-3p increased hematoma volume ($t = 7.453$, $P < 0.001$) and aggravated neurological deficits ($t = 7.992$, $P < 0.001$). Conclusion: miR-27a-3p may be involved in the formation and development of cerebral hematoma after ICH. Therefore, miR-27a-3p could become a biological therapeutic target for ICH.

Keywords: miR-27a-3p, cerebral hemorrhage, volume of hematoma, neurological deficit

Introduction

Intracerebral hemorrhage (ICH) is an acute cerebrovascular disease with a high incidence rate, accounting for 30% of cases of acute cerebrovascular diseases in middle-aged and elderly populations. It also has a rather high mortality rate. Even most survivors have residual disabilities [1]. With changes in lifestyle and dietary patterns, the population with ICH has become younger and younger, resulting in an increased number of cases [2]. After ICH, hematomas rapidly form and last for a long time. They do not subside easily. Hematomas expand peripherally, gradually inducing space-occupying effects and causing intracranial hypertension, cerebral palsy, and secondary cerebral edema. They also lead to changes in coagulation, fibrinolysis, and the microcirculatory system of surrounding tissues, eventually bringing about death [3, 4]. Some studies have shown that when the volume of hematomas has space-occupying effects, these gradually expanding hematomas are closely associated with poor prognosis of patients. Hematoma volume is one of the main factors affecting prognosis of patients with ICH [5, 6]. Therefore, the search for biological indicators closely associated with volume in patients with ICH is important for improving early diagnosis, treatment of ICH, and prognosis of patients.

MicroRNA (miRNA) is a recently discovered highly conserved endogenous noncoding hairpin nucleotide transcript. MicroRNA is abundant in eukaryotic cells, generated by the cleavage by Dicer from endogenous pre-miRNA, and in the range of 18-25 nucleotides in size [7]. MiRNAs play different roles in different physiological processes, including developmental regulation, hematopoiesis, fat metabolism, organ formation, nervous system development, antiviral defense, cell proliferation, and apoptosis. They are extensively distributed in tissues and

cells to participate in a series of processes such as regulation of ontogeny, cell proliferation, apoptosis, and differentiation. miRNAs are closely related to the formation and progression of various malignant tumors [8, 9]. Many studies have revealed abnormal expression levels of some specific miRNAs in ICH. miRNAs have been tightly linked to occurrence and development of ICH. miR-27a-3p, widely distributed in human digestive, nervous, and other organ systems, is closely related to Alzheimer's disease and malignant tumors [10].

In recent years, research on miR-27a-3p has been mainly focused on mechanisms of malignant tumors. Toles of miR-27a-3p in ICH and the relation between the volume of ICH hematomas and miR-27a-3p, however, have not been elucidated. This present study established a rat model of ICH to examine expression of miR-27a-3p and to analyze its involvement and mechanisms of action in ICH.

Materials and methods

Animals

Experimental animals included 87 purebred and inbred Sprague-Dawley (SD) rats, aged 3 to 7 weeks, with body weights of 250-300 g. SD rats were purchased from Bioray Laboratories Inc. and maintained in a clean stable environment, with an indoor temperature of 22-26°C and humidity of 52% to 58%. This animal experiment was approved by the Hospital Ethics Committee of Renming Hospital of Wuhan University. Experimental procedures were performed in compliance with the "Guide for the Care and Use of Laboratory Animals by the National Institutes of Health" [11]. Pentobarbital sodium was applied as anesthesia during the surgical procedure to minimize the suffering of experimental animals.

Creation of the rat ICH model

According to the principle of similar body weight among groups, rats were subdivided into an experimental group and control group, with 35 rats in the experimental group and 30 in the control group. As reported by Lv et al. [12], the rat ICH model was established in the following manner: SD rats were anesthetized with pentobarbital sodium (40 mg/kg) and fixed on a locator with the head fully exposed. The scalp was isolated through a midline incision and a small hole ~1.5 mm in diameter was drilled in the skull. A microneedle was introduced into the right basal ganglia area under the guidance of the locator. Rats in the experimental group were injected with 2 μl of bacterial collagen into the basal ganglia area, while rats in the control group were injected with 2 μl of normal saline. Injections lasted 3 minutes. After 10 minutes, the microneedle was removed, skull incision of rats was closed, and scalp incision was sutured. Next, 0.3 mL of gentamicin was injected to prevent infection. On days 1, 3, and 7 after initiation of the model, the rats were euthanized. Intact brain tissue was collected and hematoma tissue was isolated and preserved at -80°C for later use.

Intraventricular injection of mimic inhibitors

According to a study by Liang et al. [13], miR-27a-3p inhibitors were prepared and corresponding NCs (Beijing Bioneeds Biotechnology Co., Ltd.) were injected, respectively, into the right ventricle of the remaining 22 rats (11 rats for miR-27a-3p and 11 rats for NC). The stereotaxic technique was chosen, using a Harvard pump (11 plus microinjection pump) to inject RNA into right ventricles of anesthetized rats, with the bregma as the base point, 1 mm backwards, 2 mm on the right side, and 4.5 mm in depth. The rat model was established within 50 minutes after injection.

Hematoma volume measurement in rats

After washing, hematoma tissue was fixed in a 4% paraformaldehyde solution to measure the width and length of the largest section of the hemorrhage area of the rats. Coronal equidistant sections were made and hematoxylin and eosin (HE) staining was performed. XSP-8C/8CA trinocular biological microscope (Shanghai Dianying Optical Instrument Co., Ltd.) was applied to examine and take pictures of tissues. Image analysis software was used to measure hematoma volume.

Neurological deficit scores of rats

One day after initiation of the ICH model, determination of neurological deficit scores (NDS) was carried out with reference to a study by Muengtaweepongsa et al. [14]. A total of 5 grades were employed, including non-neurolog-

Table 1. miR-27a-3p and GAPDH gene sequence primers

Gene	Upstream primer sequences	Downstream primer sequences
miR-27a-3p	5'-GGGTTCACAGTGGCTAAG-3'	5'-CAGTGcGTGTCGTGGA-3'
GAPDH	5'-GGTGAAGGTCGGTGTGAACG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'

Table 2. General information on the three groups of rats [n (%)] ($\bar{x} \pm s$)

Category	Research group (n = 33)	Control group (n = 30)	t/χ^2	P
Gender			1.395	0.496
Male (%)	18 (54.55)	7 (70.00)		
Female (%)	15 (45.45)	3 (30.00)		
Weekly age			0.831	0.660
≤ 8 weeks	11 (33.33)	4 (40.00)		
> 8 weeks	22 (66.67)	6 (60.00)		
Body mass			2.578	0.282
≤ 180 g	8 (24.24)	5 (50.00)		
> 180 g	25 (75.76)	5 (50.00)		
Room temperature (°C)	24.26 ± 1.19	23.76 ± 1.01	1.514	0.226
Indoor humidity (%)	54.19 ± 1.47	54.87 ± 1.28	1.317	0.099

ical deficit scored as 0, paralysis with asymmetry in the activity of the limbs scored as 1, paralysis with a failure to climb or turn around scored as 2, paralysis with one paralyzed side scored as 3, and no activity and loss of consciousness scored as 4. Rats were scored in an awakened state after model establishment. A score of 1-2 implied a successful model, while 0 or 4 represented a failure of modeling.

RT-PCR analysis

For total RNA extraction, this study processed myocardial tissue cryopreserved at -80°C by means of TRIzol Reagent (Thermo Fisher Scientific [China] Co., Ltd.) with vortexing at room temperature for 30 minutes for thorough lysis. The extraction process strictly followed manufacturer instructions. The absorption value of RNA was measured on an Ultrospec III ultraviolet spectrophotometer (Takara, Japan) and the purity of total RNA was evaluated by agarose gel electrophoresis. An RT-PCR assay was performed with the miR-27a-3p fluorescence quantitative PCR kit (Invitrogen, USA). Reverse transcription (RT) reaction (reaction volume 18.6 μl) was prepared as follows: added RNA (10 μl) and oligo-dT (1.3 μl) into thin-walled tubes, mixed them well, heated at 65°C for 30 minutes, and added 7.3 μl of the MIX solution to the mixture. Components were reacted at 37°C for 2.5 hours and heated at 65°C for 30 minutes; Diluted synthesized cDNA (20 to 100 μl) with deionized water and stored the solutions at -20°C for later use. PCR used *GAPDH* as an internal reference. Primer sequences are shown in **Table 1**. Primers were synthesized by Shanghai Gefan Biotechnology Co., Ltd. PCR reaction conditions: predenaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 minutes, extension at 72°C for 1 minute, and final elongation at 72°C for 10 minutes, with a total of 35 cycles of amplification. Software supplied by the manufacturer was used for analysis of amplification data and *GAPDH* served as the internal reference gene. Results were processed by the $2^{-\Delta Ct}$ method. Primers for miR-27a-3p and *GAPDH* gene sequences are shown in **Table 1**.

Statistical analysis

SPSS 18.0 software (Yijun [Shanghai] Information Technology Co., Ltd.) was used for statistical analysis. Measurement data are expressed as mean ± standard deviation and comparison of measurement data between groups was performed by *t*-test. Chi-squared test was carried out to compare data between groups, while one-way analysis of variance was conducted to compare multiple groups. $P < 0.05$ implies statistical significance.

Results

General condition of rats

After model establishment, two rats in the experimental group showed failed modeling. Thus, the success rate was 94.29% (33/35). Both the control group and inhibition group were set up successfully. Gender, age, body weight, indoor temperature, and indoor humidity of rats in the three groups had no influence on this experiment ($P > 0.05$) (**Table 2**).

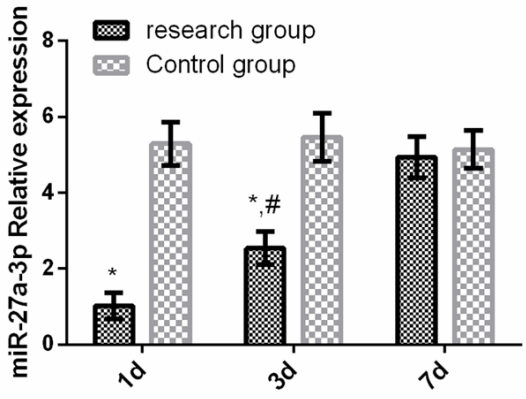

Figure 1. Changes of miR-27a-3p expression in cerebral hematomas of the ICH Rat Model. Expression levels of miR-27a-3p in cerebral hematomas of the ICH rat model on days 1, 3, and 7 were determined by RT-PCR. *P < 0.01 compared with day 7; #P < 0.01 compared with day 1.

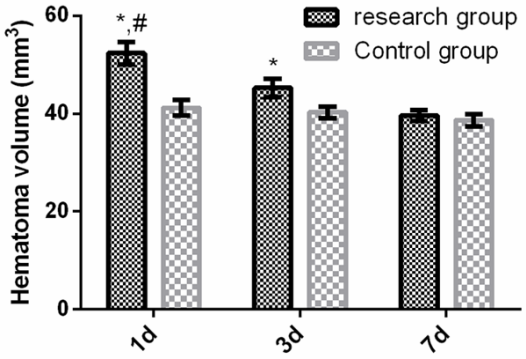

Figure 2. Changes of brain hematoma volume in the rat ICH model on days 1, 3, and 7. *P < 0.01 compared with day 7; #P < 0.01 compared with day 3.

Changes in miR-27a-3p expression in cerebral hematoma on days 1, 3, and 7 after initiation of the ICH model

miR-27a-3p levels in the cerebral hematoma of the experimental group were 1.018 ± 0.346, 2.536 ± 0.431, and 4.935 ± 0.539, respectively, on days 1, 3, and 7. Levels in the control group were 5.287 ± 0.564, 5.459 ± 0.633, and 5.137 ± 0.497, respectively. Relative expression of miR-27a-3p in the brain hematoma in the experimental group was significantly lower than the control group (t = 21.140, P < 0.001) on the first day. On the third day, it was significantly lower than the control group (t = 12.480, P < 0.001) and significantly increased compared with the first day (t = 9.109, P < 0.001). On the seventh day, no significant differences were observed between the experimental group and control group (t = 0.913, P = 0.371), and levels were significantly higher than on the third day (t = 11.530, P < 0.001) (**Figure 1**).

Changes in brain hematoma volume in the rat model of ICH on days 1, 3, and 7

Volumes of the hematoma in brains of rats in the experimental group were 52.38 ± 2.26, 45.26 ± 1.81, and 39.61 ± 1.16 mm^3 on days 1, 3, and 7, respectively, while those in the control group were 41.22 ± 1.53, 40.26 ± 1.19, and 38.67 ± 1.24 mm^3. Volumes of the hematoma in rats of the experimental group were significantly larger than the control group on day 1 (t = 13.110, P < 0.001). On day 3, they were significantly greater than the control group (t = 7.394, P < 0.001), significantly smaller volumes on day 1 (t = 8.156, P < 0.001). On day 7, no significant differences were observed between the experimental group and control group (t = 1.795, P = 0.088) and levels were significantly greater than on the third day (t = 8.717, P < 0.001) (**Figure 2**).

Effects of miR-27a-3p on hematoma volume and neurological function of rats with ICH

To analyze the effects of miR-27a-3p on hematoma volume in rats with ICH, miR-27a-3p mimic inhibitors or corresponding NCs were injected into the right ventricle of rats to assess change of hematoma volume and neurological function of rats with ICH on day 1. Results showed that miR-27a-3p inhibitors significantly downregulated miR-27a-3p (t = 5.061, P < 0.001). Downregulation of miR-27a-3p appeared to increase hematoma volume after ICH (t = 7.453, P < 0.001). Downregulation of miR-27a-3p aggravated neurological deficits of rats after initiation of ICH (t = 7.992, P < 0.001) (**Figures 3-5**).

Discussion

ICH is a very destructive disease. Treatment methods for ICH, however, remain limited. The 30-day mortality rate of spontaneous ICH has reached 50%, with approximately 80% of survivors suffering neurological function disorders. Only 20% of surviving patients have been restored to living independently [15, 16]. In most patients with spontaneous ICH, hemorrhages are rapidly stopped in the brain. Re-

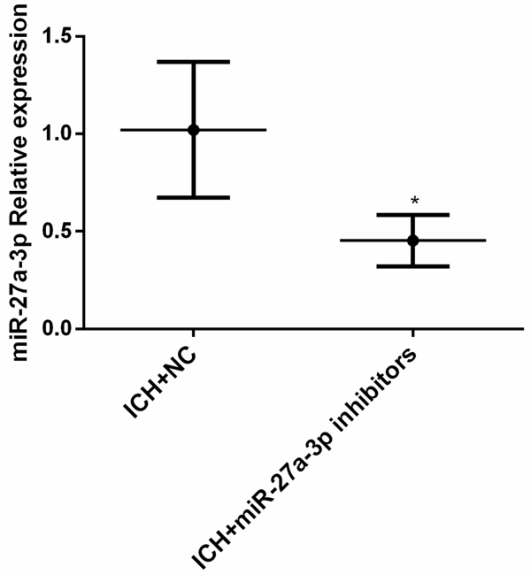

Figure 3. Changes of relative expression of miR-27a-3p in the inhibition group after intraventricular injection of a miR-27a-3p inhibitor. *P < 0.01 compared with the ICH+NC group.

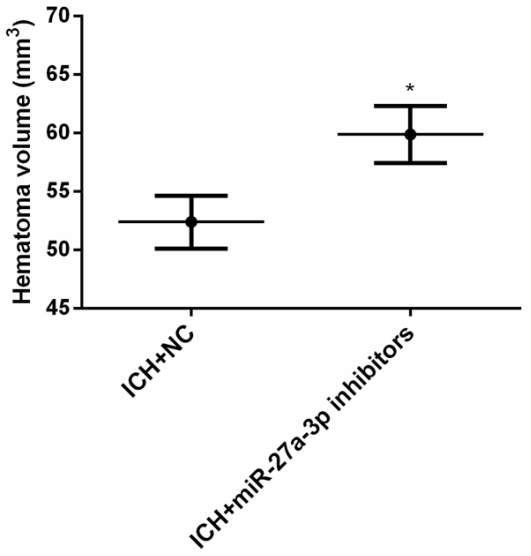

Figure 4. Effects of downregulation of miR-27a-3p on hematoma volume in ICH rats. *P < 0.01 compared with the ICH+NC group.

bleeding exacerbates the deterioration of neurological function. Intracranial hemorrhages occur within 24 hours in 30% of patients with spontaneous ICH [15]. The mechanism of brain injury caused by ICH is mainly related to mechanical occupancy effects of hematoma. Researchers are currently trying to determine how to reduce or reverse the occupancy effects,

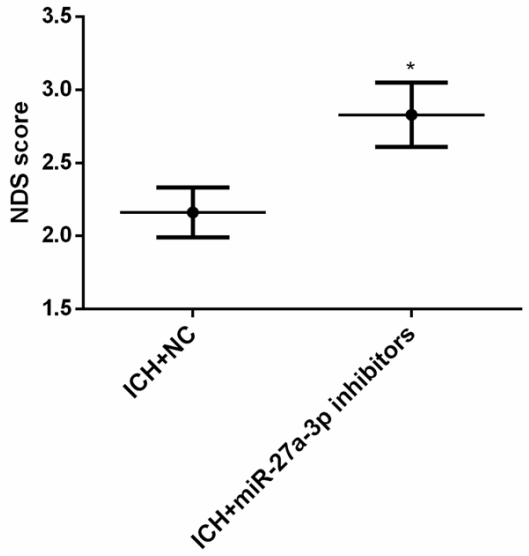

Figure 5. Influence of downregulation of miR-27a-3p on neurological function in ICH rats. *P < 0.01 compared with group ICH+NC.

limit expansion to the periphery, and to eliminate hematomas altogether [17, 18]. The volume of ICH hematomas reflect the severity of the disease, while control of hematoma volume often determines the prognosis of patients [19]. Therefore, finding biological markers closely associated with volume of hematomas in patients with ICH may be a key to successful treatment of ICH.

miRNAs are noncoding RNA molecules that participate in biological processes such as cell proliferation, apoptosis, differentiation, metabolism, and death by regulating gene expression through transcription or after transcription [20]. miRNAs perform a key function in the development of various cell types and are closely linked to cell differentiation, morphogenesis, and tumorigenesis [21]. Studies have revealed that miRNAs may play cancer-promoting or tumor-suppressing roles in the development of various human tumors and can serve as an effective molecular biological indicators for early diagnosis, treatment, and prognostic assessment of tumors [22]. One study by Xu et al. [23] indicated that when a neuroglioma is under the conditions of hypoxia, miR-27a-3p regulates the adaptability of breast cancer cells to the hypoxic state through hypoxia-inducible factor (HIF). Results of this present study showed that miR-27a-3p in brain hematomas of rats in the experimental group was significantly lower than

that in the control group and peaked. On day 3, it was significantly lower than that of the control group and significantly increased compared with the control group on day 1. On day 7, no significant differences from the control group was observed and levels were significantly higher than on day 3. Volume of hematomas in rats with ICH in the experimental group was significantly greater than the control group on day 1. Volume was significantly greater on day 3 than that in the control group, while significantly decreasing on day 3. No significant differences were observed on day 7 compared with the control group and levels significantly increased relative to day 3. Accordingly, it was speculated that miR-27a-3p may be involved in the occurrence and development of cerebral hematomas after ICH. With downregulation of miR-27a-3p, the volume of cerebral hematomas in rats gradually increased. Therefore, miR-27a-3p may participate in the regulation of volume of ICH hematomas.

miRNAs can bind to the 3' untranslated region (UTR) of a target gene to form a corresponding RNA-induced silencing complex, inhibiting translation of mRNA and downregulating expression of the target gene. A study by Zhao et al. [24] revealed that miR-27a-3p had multiple target genes, including *BCLAF* and *BBC3*, closely associated with apoptosis-related gene *Bcl-2,* according to software analysis of target genes. A study by Yuan et al. [25] showed that relative expression of Bcl-2 in hematomas of ICH rats was higher compared with that of the control group, suggesting that hematomas of ICH may cause brain damage by increasing expression of apoptosis-related gene *Bcl-2*.

By injecting miR-27a-3p inhibitors into the lateral ventricle of rats, this study found that miR-27a-3p inhibitors may significantly downregulate miR-27a-3p. This downregulation may increase hematoma volume of rats after ICH and aggravate neurological deficits of rats after ICH. These data suggest that downregulation of miR-27a-3p may aggravate brain injuries caused by ICH. Thus, miR-27a-3p may regulate hematoma volume in rats and it is possible to relieve ICH brain damage through regulation of miR-27a-3p expression.

This present study considered the reproducibility and reliability of animal experiments. All purchased rats were rigorously screened. Gender, age (weeks), body weight, indoor temperature, and indoor humidity had no effect on the results, thereby ensuring the scientific rigor of this study. In this study, the mechanisms of action of miR-27a-3p in a ICH rat model was preliminarily discussed. More detailed verification of mechanisms was not provided. Therefore, this present study had certain limitations. Due to the complexity of an *in vivo* environment, whether observed miR-27a-3p mechanisms exist in the human body and whether they are affected by peripherally related genes requires further clinical study.

Moreover, miR-27a-3p may be involved in the occurrence and development of cerebral hematomas after ICH. With downregulation of miR-27a-3p, the volume of cerebral hematomas in rats gradually increased. Thus, miR-27a-3p may play a part in the regulation of the volume of hematomas. It may be possible to alleviate brain injuries due to ICH by regulating expression of miR-27a-3p. This miRNA could become a biological therapeutic target for brain injuries caused by ICH.

Disclosure of conflict of interest

None.

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