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Original Article

Increased levels of miR-210 in human saliva induced by acute hypobaric hypoxia

Yifan Yang[1#], Jianjun Guo[2#], Tong Zhao[2], Lingling Zhu[2], Huiqing Zhao[3,4*], Fei Wang[2,5*]

[1]Department of Pain Medicine, Peking University People's Hospital, Beijing 100044, China; [2]Beijing Institute of Basic Medical Sciences, Beijing 100850, China; [3]Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing 100000, China; [4]China National Clinical Research Center for Neurological Diseases, Beijing 100000, China; [5]National Key Laboratory of Human Factors Engineering, China Astronaut Research and Training Center, Beijing 100094, China. [#]Equal contributors. [*]Co-senior authors.

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Abstract: Objective: This study investigated the effects of acute hypobaric hypoxia (HH) on miR-210 levels in the blood, saliva, and brain, in a high altitude-simulating cabin environment. qRT-PCR was used to detect miR-210 in cerebrospinal fluid, brain tissue, blood and saliva before and after acute HH exposure. This study aimed to evaluate the role of miR-210 as a noninvasive marker of acute HH. Methods: *Sprague-Dawley* rats were randomly assigned to the experimental group and were placed in a hypobaric chamber to mimic a high altitude of 5000 m (12.95% O_2) for 0.5, 1, and 2 h. The control group rats were exposed to corresponding normoxic conditions. At the indicated time points, brain tissue homogenates, cerebrospinal fluid and blood were collected from the rats, and plasma and saliva were collected from the human subjects. Eight healthy human subjects were studied at baseline (80 m), during acute HH at a high altitude (4300 m) for 40 min and upon return to baseline (80 m). All work involving human participants was approved by the Ethics Committee of the Institute of Psychology, Chinese Academy of Sciences (ethics review approval number: H19008). Results: Acute HH led to increased miR-210 levels in the cerebrospinal fluid, plasma and saliva but decreased miR-210 levels in brain tissue and blood cells. Conclusion: This study confirmed that the amount of circulating miR-210 in cerebrospinal fluid, plasma and saliva markedly increases under acute HH, suggesting that circulating miR-210 may be a biomarker of acute HH. In particular, evaluation of salivary miR-210 represents a rapid, noninvasive test of acute HH-related diseases with important clinical significance for diagnosis and disease progression monitoring.

Keywords: Acute hypobaric hypoxia, miR-210, saliva, circulating miRNA, biomarker

Introduction

Hypoxia is not only a characteristic of the tumor microenvironment but also a key pathogenic factor in ischemic diseases, including stroke, myocardial infarction and peripheral arterial disease [1, 2]. miR-210 is a key representative of hypoxia-inducible miRs, also called hypoxamiRs, which are ubiquitously expressed in a wide range of cells and perform diverse functions. Previous studies have shown that miR-210 is a major target gene of hypoxia inducible factor 1 and that hypoxia can significantly upregulate the expression of miR-210 in various normal and transformed cells [3]. High expression of miR-210 is considered a marker of a hypoxic microenvironment *in vivo* and is associated with various solid tumors and ischemic diseases [4]. Moreover, many miR-210 target genes are involved in biological processes, such as cell proliferation, apoptosis, mitochondrial metabolism, DNA damage repair and angiogenesis [5, 6]. Previous studies have shown that miR-210 not only exists in cells but can also be secreted into the extracellular circulatory system, where it is called circulating miR-210. Circulating miR-210 can be used for the rapid and noninvasive diagnosis of diverse diseases [7].

MiR-210 has considerable hallmark effects and therapeutic potential in various hypoxia-induced diseases [8, 9]. Studies have shown that the expression of miR-210 is downregulated in ovarian cancer, human esophageal squamous cell carcinoma and associated derived

cell lines. Marked decreases in the level of miR-210 have been observed, especially in poorly differentiated carcinomas [10, 11]. In contrast, high miR-210 expression has been shown to be related to the occurrence, development and degree of malignancy of clear cell renal cell carcinoma, colon cancer, cervical cancer, head and neck cancer, breast cancer, pancreatic cancer and soft tissue tumors [12-16]. In addition, in cardiovascular diseases, miR-210 is believed to protect the cardiovascular system against potentially lethal damage by inhibiting apoptosis and promoting angiogenesis, thus potentially leading to revascularization [17, 18]. The above research results show that miR-210 plays a clear role in various diseases related to hypoxia. However, few studies have addressed the changes in miR-210 expression in hypobaric hypoxia (HH)-related diseases. Therefore, we used a rat model to study the changes in miR-210 expression under HH exposure and further verified the results in humans.

Circulating miRNAs have a very stable structure and can resist high temperature and extreme pH environments [19, 20]; thus, circulating miRNAs are suitable for use as diagnostic and prognostic biomarkers. Studies have shown that miR-210 is not only highly expressed in tumor diseases but can also be secreted into the extracellular circulatory system. Therefore, by measuring the miR-210 levels in blood, we can quickly and noninvasively assess the occurrence and prognosis of tumors. Lawrie found that the miR-210 levels in the plasma of patients with B-cell carcinoma were higher than those in normal individuals and could be used as a diagnostic molecular marker of this disease [21]. The studies conducted by Jung and his colleagues showed that miR-210 could be used as a diagnostic molecular marker of breast cancer [22]. Therefore, we focused on analyzing the miR-210 levels in the blood, cerebrospinal fluid and saliva of rats and humans under normal and hypoxic environmental conditions.

Materials and methods

Rat model of HH

Eight-week-old male Sprague Dawley rats with body weights of 220-250 g, purchased from Vital River Laboratory Animal Technology Co. (Beijing, China), were housed in a temperature- and humidity-controlled room. The rats were maintained under a 12-h light-dark cycle and given food and water ad libitum. The rats were randomly selected and divided into the following four groups: acute HH exposure for 0.5 h (n = 10), 1 h (n = 10), and 2 h (n = 10) and control treatment (n = 10). To establish the systemic hypoxia model, the rats randomly assigned to the experimental group were placed in a hypobaric chamber (Fenglei) to mimic a high altitude of 5000 m (12.95% O_2) for 10 min, while the rats in the control group were exposed to the corresponding normoxic conditions. The rats were euthanized, we used 100% CO_2 (tank carbon dioxide) with a pressure-reducing valve and a liter flow meter CO_2 chamber with a lid: (1) Without overcrowding, the animals were placed in the chamber filled with CO_2, and the lid was closed. (2) We introduced CO_2 at a rate equivalent to 10% to 20% of the chamber volume per min (e.g., 1 to 2 liters/min for a 10-liter chamber). (3) The animals remained in the container for several minutes to ensure death. Unconsciousness occurred within 30 sec. (4) Death was verified by the lack of a cardiac pulse and fixed and dilated pupils prior to the carcass disposal. The euthanasia procedures were consistent with the AVMA Guidelines for Euthanasia. All animal experimental procedures fully complied with the regulations of the Institute of Basic Medical Sciences.

Collection of samples

The cerebrospinal fluid, brain tissue, plasma and blood cells of the rats were harvested for further study. For the cerebrospinal fluid sample collection, 100 μL of cerebrospinal fluid were extracted from each rat and then stored at -80°C until required. For the blood sample collection, 2.0 mL of blood was collected in EDTA tubes (BD Vacutainer, K3E 7.2 mg, Plus blood Collection Tubes) from the rat eye socket vein in the morning after the rats fasted for 12 h. To avoid hemolysis, the plasma and red blood cells were separated 45 min after collection by centrifugation (Eppendorf Centrifuge 5424 R; Germany) of whole blood at 1600 rpm for 15 min at 4°C, and aliquots of the plasma and blood cells were stored at -80°C [23].

Recruitment of human subjects

Eight male human participants (mean (± SD) age, 24.39 ± 1.83 years; height, 174.26 ± 5.98

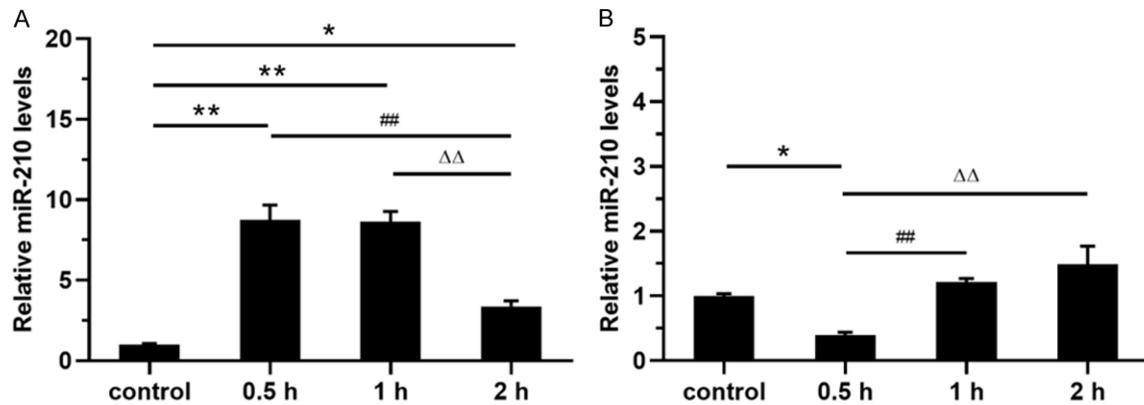

Figure 1. Exposure to HH increases the miR-210 levels in cerebrospinal fluid. (A and B) *Sprague-Dawley* rats were exposed to 5000 m (12.95% O_2) for 0.5, 1, and 2 h. The content of miR-210 in cerebrospinal fluid (A) and brain tissue (B) was analyzed by qRT-PCR. All experiments were performed in triplicate, and the values are presented as the means ± SEMs; **P* < 0.05 and ***P* < 0.01 versus the control group, ##*P* < 0.01 versus the 0.5-h group, ΔΔ*P* < 0.01 versus the 1-h group, as determined by a one-way ANOVA (A, B).

cm; body mass, 66.56 ± 8.42 kg) were recruited to participate in this study. The participants were healthy, did not experience high-altitude conditions after birth, and did not have any history of cardiovascular, cerebrovascular, or respiratory diseases. The participants were asked to refrain from strenuous exercise for at least 48 h before each experiment. All participants provided written informed consent to participate. Constant assistance was provided throughout the study from the accompanying doctor. After the experiment was completed, if the subjects had any queries, problems, or feedback related to the experiment, they were told that they could contact the researchers via phone, WeChat, e-mail, or in person to obtain answers immediately. The subjects received follow-up dietary advice and counseling individually from the accompanying doctor. Additionally, when asked whether they experienced any adverse effects after the stimulation session, the subjects reported no adverse effects. All work involving human participants was approved by the Ethics Committee of the Institute of Psychology, Chinese Academy of Sciences (ethics review approval number: H19008).

Experimental procedures

The experiment was performed from 9:00 AM-12:00 AM on four nonconsecutive days. The whole experimental process consisted of 5 consecutive phases as shown in **Figure 1**. On the day of the experiment, the participants rested in a lounge for 30 min (Phase I) and then went downstairs to an underground area and rested in the room outside a large cabin for 30 min (Phase II). Ten minutes after entering the large low oxygen cabin, the participants were told that the simulated elevation increases were starting (false increases) (Phase III). After approximately 20 min, the simulated elevation increases actually started (at 3 m per sec to 4300 m above sea level), and the simulated elevation was maintained at 4300 m (14.10% O_2) (Phase VI); after approximately 30 min, simulated elevation decreases started (3 m per sec) to the plain elevation (Phase V), and the participants remained in the cabin for 30 min after the experiment. Serum samples were collected from the volunteers. Morning fasting blood was collected into 10 mL EDTA tubes (SST; containing a clot activator and serum separator gel) (BD Bioscience, Franklin Lakes, NJ, USA) to prepare plasma. The samples were centrifuged at 3000 rpm for 10 min to obtain plasma and collect saliva.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted with TRIzol (Invitrogen, Carlsbad, California, USA). An Ambion miRNA Isolation Kit (Ambion, Austin, Texas, USA) was used to isolate miRNAs according to the protocol. Stem-loop RT-PCR was used to evaluate the expression of mature miR-210. The stem-loop RT primer for miR-210 was designed as shown in **Table 1**. qRT-PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to

Table 1. Primer sequences

The stem-loop RT primer for miR-210 was designed as follows	
5'-CTCGTATGGAGTGCAGGGTCCGAGGTATTCGCACTCCATACGAGTCAGCCGC-3'	
The PCR primers for mature miR-210 were designed as follows	
Forward	5'-CTGTGCGTGTGACAGC-3'
Reverse	5'-GTGCAGGGTCCGAGGT-3'
The PCR primers for U6 were designed as follows	
Forward	5'-AACGCTTCACGAATTTGCGT-3'
Reverse	5'-CTCGCTTCGGCAGCACA-3'.

Table 2. The reaction system and qRT-PCR conditions

miR-210 reverse transcription		
5 × Reaction Buffer		4 μL
$MgCl_2$		2.5 μL
dNTP		5 μL
RNase Inhibitor		0.5 μL
Reverse Transcriptase		1 μL
DEPC		7 μL
Reaction condition: 42°C, 60 min; 70°C, 5 min.		
RT-PCR		
2 × SYBR MIX		25 μL
H_2O		21 μL
Primer-F (20 μM)		1 μL
Primer-R (20 μM)		1 μL
cDNA Template		2 μL
Reaction conditions:		
95°C	10 min	
95°C	15 s	40 ×
60°C	15 s	40 ×
72°C	30 s	40 ×
95°C	15 s	
60°C	1 min	

the manufacturer's instructions (**Table 2**). A StepOne Plus RT-PCR System (Applied Biosystems, Frederick, Maryland, USA) and the supplied analytical software were used. The relative quantification of the RT-PCR data was based on the expression ratio of the target gene to the reference (housekeeping) gene. The U6 snRNA level was used for normalization. The PCR primers for mature miR-210 and U6 were designed as shown in **Table 1**.

Quantitative and statistical analyses

All data are expressed as the mean ± SEM values, and the statistical analysis was carried out using GraphPad Prism (version 8.0, GraphPad Software Inc., California, USA). The comparisons between two groups were assessed using a one-way analysis of variance, followed by Tukey's multiple comparisons test. A *p*-value < 0.05 was considered indicative of significance throughout this study.

Results

Acute HH increased the miR-210 levels in the cerebrospinal fluid of rats

First, we measured the miR-210 levels in the cerebrospinal fluid and brain homogenates from rats under acute HH. The qRT-PCR results showed that the miR-210 levels in the cerebrospinal fluid were significantly increased after the acute HH exposure for 0.5 h and 1 h. The miR-210 content in the 2 h group was significantly decreased but was still higher than that in the control group (**Figure 1A**), indicating that acute HH can significantly increase the content of miR-210 in the cerebrospinal fluid of rats. In addition, the level of miR-210 in brain tissue was decreased in the 0.5 h group compared with that in the control group but was significantly increased in the 1 h and 2 h groups compared with that in the 0.5 h group (**Figure 1B**), indicating that acute HH can cause a transient decrease in the miR-210 levels in rat brain tissue. We surmised that acute HH may promote the secretion of miR-210 into the extracellular matrix by brain cells and that a portion of this secreted miR-210 can enter the cerebrospinal fluid, thus increasing the content of miR-210 in cerebrospinal fluid.

Acute HH increased the miR-210 levels in the plasma of rats

Next, we measured the miR-210 levels in the plasma and blood cells of rats. The miR-210 levels in plasma were significantly increased in the groups exposed to HH for 0.5 h, 1 h and 2 h compared with those in the control group. The

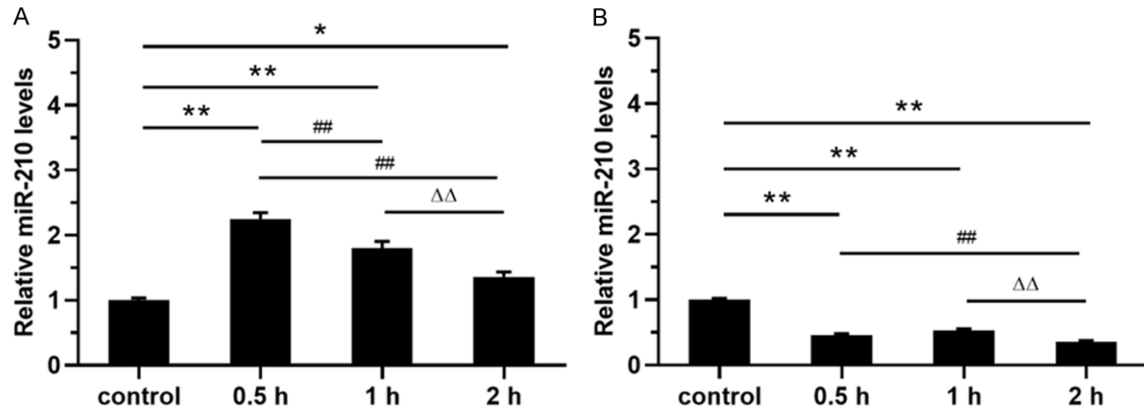

Figure 2. Exposure to HH increases the miR-210 levels in plasma. (A and B) *Sprague-Dawley* rats were exposed to 5000 m (12.95% O_2) for 0.5, 1, and 2 h. The miR-210 levels in plasma (A) and blood cells (B) were analyzed by qRT-PCR. All experiments were performed in triplicate, and the values are presented as the means ± SEMs; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the 0.5-hour group, $^{\Delta\Delta}P < 0.01$ versus the 1-h group, as determined by a one-way ANOVA (A, B).

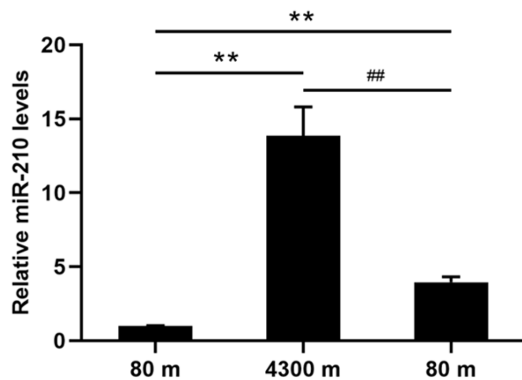

Figure 3. Exposure to HH increases the miR-210 levels in human plasma. Subjects were exposed to HH for 30 min. The miR-210 levels in plasma were analyzed by qRT-PCR. All experiments were performed in triplicate, and the values are presented as the means ± SEMs; $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the 4300 m group, as determined by a one-way ANOVA (A, B).

miR-210 levels in the 1 h and 2 h groups were decreased compared with those in the 0.5 h group (**Figure 2A**), which is similar to the trend in the miR-210 levels observed in cerebrospinal fluid (**Figure 1A**). In contrast, the expression of miR-210 in blood cells gradually decreased over time. After 2 h of acute HH exposure, the miR-210 levels were lower than those in the control and 1 h groups (**Figure 2B**).

Acute HH increased the level of miR-210 in human plasma

The above studies indicate that acute HH exposure affects the miR-210 levels in rat plasma, but whether similar changes occur in humans is unclear. Therefore, we further explored whether the miR-210 levels in human plasma change under HH. The miR-210 levels in plasma were significantly increased after the human subjects entered the acute HH environment but decreased when the subjects returned to the plain environment. However, even in the plain environment, the plasma miR-210 levels in the acute HH-exposed subjects were still significantly higher than those in the control subjects (**Figure 3**).

Exposure to an acute HH environment increased the miR-210 levels in saliva

MiR-210 is a key molecule that has been found to be involved in the response to hypoxia, and the level of miR-210 was found to be increased in many body fluids; thus, we further evaluated the miR-210 levels in saliva. Interestingly, the miR-210 levels in human saliva were similar to those in plasma. The level of miR-210 in saliva was significantly increased when the subjects entered the acute HH environment and significantly decreased when the subjects returned to the plain environment, but the levels remained higher than those in the control subjects (**Figure 4**).

Discussion

Millions of people worldwide live at a high altitude, and a significant number of these individuals are at risk of developing altitude sickness. In addition, the therapeutic options for altitude

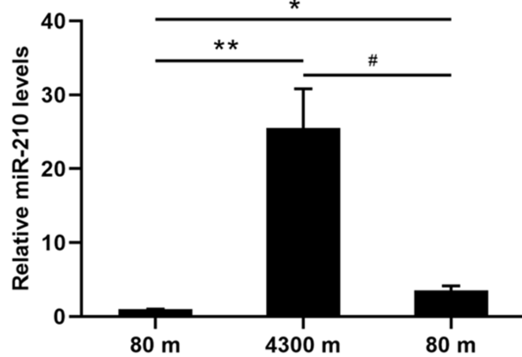

Figure 4. Exposure to HH increases the miR-210 levels in human saliva. Subjects were exposed to HH for 30 min. The miR-210 levels in human saliva were analyzed by qRT-PCR. All experiments were performed in triplicate, and the values are presented as the means ± SEMs; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group, $^{\#}P < 0.05$ versus the 4300 m group, as determined by a one-way ANOVA (A, B).

sickness are limited, and the outcomes are poor. Therefore, appropriate medical knowledge regarding altitude sickness is necessary for providing an adequate diagnosis and healthcare to inhabitants of high-altitude environments. Moreover, minimally invasive or noninvasive biomarkers specific to altitude sickness are crucially needed. MiRNA-210 has been identified as a major microRNA induced by hypoxia and is associated with diverse diseases associated with hypoxia [24-26]. MiR-210 was identified as either up- or downregulated in multiple oxygen-limited models and has been implicated in central hypoxia-inducible processes. Some groups performed miRNA expression profiling in patients with diverse tumors and investigated miRNAs, including miR-885-5p, miR-541, miR-765, miR-139-3p, miR-483-5p, miR-15a, and miR-16 [27-29]. However, few studies evaluated miR-210, and these studies focused on miR-210 extracted from tumor and/or adrenal tissues but not from cerebrospinal fluid or saliva. Using a univariate analysis, Samantha et al. identified low serum miR-210 levels as markers of pheochromocytoma or paraganglioma [30]. Wen Xie et al. reported that an upregulation of miR-210 in synovial fluid may occur during the early stage of osteoarthritis and that this upregulation may be a useful biomarker for the early diagnosis of osteoarthritis [31]. Many studies reported that circulating miR-210 in the blood can be used as a diagnostic molecular marker of various solid tumors and tissue injuries. In addition, several studies have shown that the levels of miR-16 and miR-320 in the plasma of patients with acute renal injury are decreased, while the level of miR-210 is increased, and the amount of circulating miR-210 was found to be highly related to patient mortality [32]. In addition, some studies found that circulating miR-210 in plasma can be used as a diagnostic molecular marker of renal cell carcinoma and pancreatic cancer [14, 33], and other studies have shown that miRNA-210 in cerebrospinal fluid and serum may be used as an indicator of Alzheimer's disease [34]. Similarly, our study shows that acute HH can cause an increase in the miR-210 levels in the cerebrospinal fluid and plasma of rats and that the expression of miR-210 was significantly increased and maintained at a high level after 0.5 h of exposure to HH. These results suggest that circulating miR-210 can be used as a molecular marker of acute HH in rats. Therefore, it is conceivable that the upregulation of miR-210 may be induced by HIF-1α and regulated by vascular endothelial growth factor (VEGF) under hypoxic conditions [35]. In contrast, the levels of miR-210 are downregulated in hypoxic naked mole rat brains, and such reduced expression of miR-210 may be related to the induction of cell cycle arrest and apoptosis [36, 37].

Salivary miRNAs have been proven to be potential biomarkers of pancreatic cancer in patients. Four miRNA biomarkers (miR-21, miR-155, miR-196, and miR-210) have been found to be consistently dysregulated in pancreatic ductal adenocarcinoma [38]. Similar to this finding, we further observed that the level of miR-210 in human saliva was significantly increased under HH. When the subjects were removed from the HH environment, the salivary level of miR-210 was decreased but remained higher than that in the control group. These results show that the amount of miR-210 in saliva can be used as an indicator of acute HH. MiR-210 exhibiting a strong response to low oxygen stress inhibits apoptosis and regulates neuroinflammation, thereby likely providing neuroprotection, and may become a tool for early diagnosis [39, 40].

How can we build upon these findings to improve our understanding of the mechanisms used to survive extreme stress and develop an

effective treatment plan by detecting changes in the expression of miR210? Unfortunately, our sample size was small, the mechanism of miR-210 elevation in cerebrospinal fluid and saliva under HH conditions is unclear, and further investigation is needed. In summary, miR-210 plays an important role in HH, and an evaluation of miR-210 expression in saliva may be a convenient approach for detecting altitude sickness. Our study provides evidence of a correlation between miR-210 and HH. Although the sample size was small, our cohort can serve as a robust pilot cohort for future studies.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huiqing Zhao, Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing 100000, China; China National Clinical Research Center for Neurological Diseases, No. 119 South Fourth Ring West Road, Fengtai District, Beijing 100000, China. E-mail: xiaoqing0926@sina.com; Dr. Fei Wang, Beijing Institute of Basic Medical Sciences, Beijing 100000, China. E-mail: wfamms@126.com

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