Trehalose Attenuates Learning and Memory Impairments in Aged Rats via Overexpression of miR-181c

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Abstract
MicroRNAs have been recognized as important regulators of the aging process. Trehalose, a natural disaccharide, displays protective effects against neuronal impairment through several mechanisms. However, little is known about the interactive effects of aging and trehalose on behavioral function and underlying miRNA expression patterns in the hippocampus of young and old rats. Male Wistar rats were divided into four groups. Two groups of aged (24 months) and young (4 months) rats were administered 2% trehalose solution for 30 days. Two other groups of aged and young rats received regular tap water. At the end of treatment, rats were assessed for cognitive behavior using the Morris water maze test. The expression level of miR-181c and mir-34c was also measured by qRT-PCR. We found that trehalose treatment reduced learning and memory impairment in old rats compared to control old animals (p < 0.05). In contrast, cognitive performance was not significantly improved in trehalose-treated young rats in comparison with young controls (p > 0.05). We also showed that the expression level of miR-181c was significantly increased in trehalose-treated rats (p < 0.01). However, analysis of miR-34c expression level indicated no significant difference between trehalose-treated old rats and non-treated old animals (p > 0.05). Our results indicated that trehalose treatment improved learning and memory function in aged rats by targeting miR-181c. Therefore, trehalose administration may provide a therapeutic strategy to ameliorate age-associated cognitive impairment.

Keywords Aging · Cognitive function · miR-181c · miR-34c · Trehalose

Abbreviations
Mir MicroRNA
AD Alzheimer’s disease
PD Parkinson’s disease

Introduction
Aging has been accepted as a natural and inevitable aspect of life in all organisms. The biology of the aging process is complex and has not been fully characterized. Although the precise definition of aging remains controversial, it can generally be viewed as the gradual accumulation of harmful biological changes that accompany a progressive loss of function [1]. The decline in performance with age occurs in all vital organs of the body, particularly in the brain. Brain aging can be manifested by the gradual deterioration of the brain’s capacity to use information. The brain starts to go through structural and functional changes during normal aging. These alterations include loss of synapses, decreased brain size, a decline in perceptual abilities, a moderate degree of cognitive impairment, and an enhanced incidence of developing neurological diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD) [2].

MicroRNAs (miRNAs) have increasingly been recognized as important regulators of the aging process and modulators of longevity [3]. Several studies have indicated that the brain’s miRNAs profile changes with normal aging...
in mammals [2, 3]. MiRNAs represent a class of highly conserved small, non-coding RNA molecules that negatively regulate mRNA stability and protein expression [4]. MiRNAs are numerous in the brain and play an important role in synaptic plasticity, neurological development, and pathogenesis of neurodegenerative diseases [5]. They are also known to play an important role in memory loss and reduced cognitive functions associated with aging [6–8]. An increasing number of reports have shown that miRNAs change the expression of local proteins at synapses rapidly and spatially through destroying, inhibiting, or stabilizing specific mRNAs without altering the transcriptional profile of the entire cell [2, 9]. In addition, a deep comparison of brain sequencing data from young and old mice revealed that miRNAs expression patterns alter with aging [9]. Among the long list of miRNAs identified to be associated with the process of aging, miR-181c and miR-34c are of special interest [10, 11]. These miRNAs are abundant in the nervous system and have been shown to play important roles in the context of neurological disorders, and normal aging through targeting several genes such as SIRT1 [12, 13].

MiR-181c expression levels were found to be down-regulated in the hippocampus of AD mouse models [14, 15]. In addition, a recent study has indicated that the miR-181c level decreased in the brain and blood of AD patients. These findings suggest that miR-181c plays a significant role in the pathogenesis of AD, and it could be considered as a therapeutic target in aging and age-related neurodegenerative diseases [16]. MiR-34c represents an important role in regulating synaptic networks plasticity, neuronal activity, and cognitive processes. It was demonstrated that miR-34c is dysregulated in the hippocampus of aged animals, and increased expression level of miR-34c is correlated with age-associated cognitive decline [10]. Moreover, miR-34c overexpression exhibited hippocampal dendritic spine loss and significant memory impairment [17]. Trehalose, a disaccharide made of glucose, is a safe natural sugar [18]. Trehalose displays multiple biological activities, including the ability to suppress inflammatory responses and oxidative stress in cells [19, 20]. It also act as an autophagy activator and a protein stabilizer [21]. Furthermore, trehalose can hamper the accumulation of toxic proteins and subsequently postpones the development of neurodegeneration in several transgenic mouse models [22, 23]. In particular, trehalose is suggested to improve cognitive function in the transgenic Tg2576 mouse model of AD [24].

Given a large amount of data regarding the protective effects of trehalose, we conducted this study to investigate the influence of trehalose administration on the expression of miR-34c and miR-181c in the hippocampus of aged rats and further, to determine their association with cognitive outcomes following trehalose treatment.

### Materials and Methods

#### Animals and Treatment

This animal study was approved by the Institutional Animal Care and Use Committee of Kerman University of Medical Sciences (IR.KMU.REC.1399.350). In this research, 31 male Wistar rats, including 16 aged (24 months) and 15 young (4 months) rats were used. All animals were housed 2/cage with the same age. They were kept in air-conditioned rooms with a constant temperature of 24 °C and a 12-h light/dark cycle, and they had free access to regular food and water. Animals were divided into four groups. Two groups including one aged and one young with eight animals in each group were orally administered 2% trehalose (Merck, Germany) solution for 30 days [20]. A fresh trehalose solution was prepared in regular drinking water twice a week. Trehalose dissolved in water completely and the taste of water was not bitter and unpleasant. Each cage was daily checked for trehalose intake by weighing trehalose solution and calculating the average amount consumed by each rat. Two other groups of aged with eight rats and young animals with seven rats received regular tap water. At the end of treatment, rats were assessed for cognitive behavior using the Morris water maze test. Then, animals were sacrificed under deep anesthesia via intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylazine, and their hippocampus tissues were collected and stored frozen at -70 °C until analysis.

#### Morris Water Maze Test

Spatial learning and memory in various groups were evaluated by the Morris Water Maze (MWM) test. The MWM consists of a black circular pool (183 cm wide and 47 cm height) filled with water. The pool temperature was maintained at 24 ± 0.5 °C by a heater. The platform (17 cm wide and 35 cm height) was placed in the pool approximately 1.5 cm below the water surface in one of the four quadrants. Different shapes of visual cues were placed on the walls of the MWM apparatus room so that the rats could see them. All the experiments were done between 8:00 a.m. and 11:00 a.m. The pool was divided into four quadrants (compass locations: NE, NW, SE, and SW). The swimming animal was captured and recorded using a video camera placed above the center of the pool which was connected to a computer system and tracking software (Noldus Ethovision system, version 7, Netherland). Briefly, each group of animals was trained for three days in the MWM, with four trials per day beginning from various locations in the pool. Each block per day consisted of four
successive trials with 60 s duration and about 60 s intervals. In each trial, the rats were randomly released into the water from one of the four quadrants of the maze, facing the wall of the quadrant where they were released. Each rat had four different releasing points. During acquisition, the location of the platform remained constant, and the rat was allowed to swim to the hidden escape platform. After finding the platform, the animal was allowed to remain there for 20–30 s and then it was located in an animal cage to wait for 20–30 s until the next trial. However, if a rat failed to find the platform in 60 s, the experimenter guided it toward the platform and after 20–30 s of staying on the platform, the rat was placed in an animal cage to wait for 20–30 s until the next trial. The time and distance to find the hidden platform were collected and analyzed later. On the probe trial session test, the hidden platform was removed, and each rat was released into the pool from the start position of the opposite quadrant, facing the tank wall, and allowed to swim for 60 s to find the platform. The time and distance spent in the trigger zone and the number of platform-site crossovers were also recorded [25, 26].

**RNA Isolation and cDNA Synthesis**

MiRNA isolation was performed using the Trizol-based method with some modifications to increase miRNA isolation yield. Briefly, 900 µl of cold Trizol was added to 30 mg of hippocampus tissues. Then tissues were homogenized by sonication. The homogenized samples were then incubated at room temperature for 20 min. After adding 200 µl chloroform, samples were vortexed and centrifuged at 12,000 g for 20 min at 4 °C, and the upper aqueous phase was carefully collected. Then 100 µl of chloroform was added, and the previous step was repeated. An equal volume of cold 100% ethanol was added to the supernatant, and the tubes were stored at − 20 °C overnight. The following day, the tubes were centrifuged at 12,000 g for 60 min at 4 °C. After centrifugation, the supernatant was completely discarded. Then, the RNA pellet was washed with 1.5 ml of 70% ethanol and centrifuged at 12,000 g for 20 min at 4 °C. The supernatant was then removed, and this step was repeated. Finally, RNA pellets were air-dried at room temperature for 20 min and resuspended in 50 µl of RNase-free DEPC water. The concentration and purity of isolated total RNA were measured by a NanoDrop Spectrophotometer (Thermo Fisher, USA), at an optical density (OD) ratio of A260/280 and A260/230, and the integrity of samples was checked by 2% agarose gel electrophoresis. Extracted microRNAs were stored at -70 °C until further analysis.

An equal amount of isolated RNA was applied to synthesize cDNA using a stem-loop primer designed specifically for each miRNA (Table 1). In brief, 1.5 µl of 10 pmol stem-loop RT primer, 100 ng total RNA, and RNase-free water with a final volume of 10 µl were mixed and incubated at 65 °C for 5 min and then placed on ice for 2 min. Next, 2 µl dNTP (10 mM), 1 µl M-MLV reverse transcriptase (25 U/µl), 4 µl of 5 × first strand buffer containing 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2, and 50 mM DTT were added and the mixture was incubated at 25 °C for 15 min, 37 °C for 15 min, and 42 °C for 45 min. Finally, the reaction was stopped by incubating at 95 °C for 2 min. Then cDNAs were stored at -70 °C until analysis.

**REAL-TIME PCR**

Reverse transcription-quantitative PCR analysis of miRNAs was performed using a Universal TaqMan probe and reverse primer in addition to a specific forward primer. The sequences of primers used in this study are shown in Table 1. The qPCR analysis was carried out on Rotor-Gene 6000 (Corbett Research, Germany). Real-time PCR reactions of the samples were performed in a mixture consisting of 6.5 µl 2 × probe qPCR Master Mix, 1 µl of each specific

| Table 1 | Sequences of primers used for reverse transcription-quantitative PCR |
|---------|---------------------------------------------------------------|
| **Primer** | **Sequence** |
| miR-34c | 5'-AGGCAGTGTAGTTAGCTGATTC-3' |
| Forward | 5'-GTATGCCGGCTACCTCGGACCCTGCTTAGTGCCATGCCCATGCCCCGACGATACTAC-3' |
| RT | 5'-AACATTCAACCTGTCGATGTAG-3' |
| miR-181c | 5'-GTATGCCGGCTACCTCGGACCCTGCTTAGTGCCATGCCCATGCCCCGACGATACTAC-3' |
| Forward | 5'-GCAAGGATGACAGCAAAATTCG-3' |
| RT | 5'-GTATGCCGGCTACCTCGGACCCTGCTTAGTGCCATGCCCATGCCCCGACGATACTAC-3' |
| miR-U6 | 5'-FAM-AGTGCCCATGCTGCTGAGGC-BHQ1-3' |
| Forward | 5'-CTACCCCTCGGACCCTGCT-3' |
| PROB | 5'-AGAGGTACTATGAGAACTACAC-3' |
forward and reverse primers (10 pmol), 0.2 µl TaqMan Probe (10 pmol), 2 µl of RNA specific cDNA, and 2 µl of H2O, with a final volume of 12.7 µl. The program of Real-Time PCR was as follows: initial denaturation at 95 ºC for 5 min, followed by 45 cycles of denaturation at 94 ºC for 30 s, and annealing and extension at 60 ºC for 40 s. MiRNA expression levels were normalized to U6 as endogenous control, and the fold change was determined using $2^{-\Delta\Delta Cq}$ method [27].

**Data Presentation and Statistics**

Results are presented as mean ± SEM. IBM SPSS software package, version 20.0 was used for statistical analysis. Acquisition scores of latency (days 1 to 3) in MWM were analyzed using two-way repeated measures ANOVA followed by Bonferroni post hoc test. For other experiments, statistical analysis was carried out with two-way ANOVA test. Spearman’s rank correlation analysis was also used to evaluate the correlation between memory data and gene expression. The criterion for significance was determined using p < 0.05.

**Results**

**Spatial Learning**

As shown in Fig. 1, animals in all groups except the old group, learned to find the location of the hidden platform as evidenced by the reduction in their swimming distance and their escape latency across blocks of training. Two-way analysis of ANOVA with repeated measures test revealed that the path length and escape latency of old rats significantly increased on day 2 (Fig. 1a, p < 0.05 & Fig. 1b, p < 0.05) and day 3 (Fig. 1a, p < 0.05 & Fig. 1b, p < 0.05) compared to young rats in these days. This clearly indicated the spatial learning impairment in old animals. However, treatment with trehalose progressively improved the ability of aged rats to find the hidden platform on days 2 and 3. This was demonstrated by a significant reduction in their swimming distance on day 2 (Fig. 1a, p < 0.05) and day 3 (Fig. 1a, p < 0.01), as well as a significant decrease in the escape latency on day 2 (Fig. 1b, p < 0.05) and day 3 (Fig. 1b, p < 0.05), in contrast to rats in the non-treated old group in the MWM test. However, further analysis in young control and trehalose-treated rats showed that there

![Fig. 1](image-url)
was no discernible difference in the path length and escape latency to find the hidden platform in the MWM test. Taken together, escape latency and path length decreased in all groups over three days, but the reduction was significant in trehalose-treated aged rats compared to the old control on the second and third days.

**Spatial Long‑Term Memory**

A probe trial was given to assess reference memory at the end of learning. In this study, the probe test was done 24 h after the acquisition phase to examine long-term spatial memory retention. The obtained results included the mean percentage (%) of time as well as distance and the number of crossings in the target quadrant. The probe test results demonstrated that old rats significantly spent less time and distance in the target quadrant than young animals (Fig. 2a, p < 0.05 for time & Fig. 2b, p < 0.05 for distance), indicating long-term memory impairment. However, memory deficit was significantly prevented in the old rats treated with trehalose since they spent more time and distance in the target quadrant compared to the old control rats (Fig. 2a, p < 0.05 vs control old, (#) p < 0.01 vs control old). We also analyzed the number of entrances into the target quadrant for all animals. However, no considerable difference was observed among various groups (Fig. 2c, p > 0.05).

**Swimming Speed and Escape Latency to a Visible Platform**

Analysis of swimming speed and escape latency to find the visible platform indicated no significant difference between young and old groups (p > 0.05) (Table 2). In addition, our results indicated that trehalose administration had no effect on the visible test and swimming speed. Thus, visual and motor functions were not different among experimental groups (p > 0.05).

**MicroRNA‑181c Expression**

A growing body of evidence indicates an age-associated alteration in microRNAs expression, which may be
involved in learning and memory impairment in aging [3]. MiR-181c has been demonstrated to contribute to brain aging thus we measured miR-181c expression levels in young and old rats. Our results showed that miR-181c levels were significantly reduced in the hippocampus of aged rats when compared to young animals (p < 0.05), indicating an age-related decrease in the expression of miR-181c. We also measured the expression level of miR-181c in young and old rats treated with trehalose. The results showed that the expression of miR-181c was up-regulated in trehalose-treated old rats compared with non-treated old animals (p < 0.001). In addition, our data revealed that the expression of miR-181c in the hippocampus of trehalose-treated old rats compared with non-treated old animals (p < 0.001). In addition, our data revealed that the expression of miR-181c in the hippocampus of trehalose-treated young rats was substantially increased compared with non-treated young animals (p < 0.01). Therefore, these findings demonstrate that trehalose can induce miR-181c expression in the hippocampus of both young and old animals (Fig. 3a).

Our data also detected a significant positive correlation between the expression level of miR-181c and the time spent in target quadrant (%) in total old animals (r = 0.707, p = 0.004) (Fig. 3b).

**MicroRNA-34c Expression**

MiR-34c appears to play a less beneficial role in brain aging and may contribute to memory impairment associated with old age [2]. To examine the age-associated differential expression of miR-34c in the hippocampus, we assessed its levels in four groups by qPCR. Our results indicated that the expression level of miR-34c in the hippocampus of old rats was not statistically significant compared to young animals (p > 0.05). Treatment with trehalose reduced the level of miR-34c expression in both old and young rats in comparison with non-treated animals. However, statistical analysis showed that the difference was significant just between young groups (p < 0.01), suggesting that trehalose may have a role in the regulation of miR-34c in the hippocampus of young rats (Fig. 4).

![Fig. 3](image-url) Relative transcription levels of miR-181c among experimental groups (n = 7–8); (A) Two-way ANOVA test was used for the analysis of data. Data represents mean ± SEM. (*) p < 0.05 vs control young, ** P < 0.01 vs control young, (##) p < 0.001 vs control old. (B) Correlation between the expression level of miR-181c and the time spent in target quadrant (%) in total old rats (r = 0.707, p = 0.004)

![Fig. 4](image-url) Relative transcription levels of miR-34c among experimental groups (n = 7–8); Two-way ANOVA test was used for the analysis of data. Data represents mean ± SEM. (*) p < 0.05 vs trehalose-treated young, ** P < 0.01 vs control young
Discussion

The aging process is characterized by a progressive loss in cognitive and motor functions due to complex biochemical processes [28–30]. The most important signs of normal brain aging in several model organisms include gradual declines in learning and memory ability in the MWM task [31–33]. In agreement with these observations, our results revealed that old rats took longer to find the location of the hidden platform, and their behavioral performance showed to be slower than that observed in young rats in the MWM test. In the present study, we investigated the effect of trehalose on cognitive function and age-associated microRNAs 34c and 181c expression in hippocampal tissue of biologically aged rats. We found that oral administration of trehalose significantly improved learning and memory ability of old rats compared to non-treated old animals. Analysis of the expression level of miR-34c and 181c in the hippocampus of aged rats demonstrated that the level of miR-34c was not significantly changed following treatment with trehalose. In addition, we found that old rats displayed cognitive impairment along with a reduction in miR-181c level. However, the expression of miR-181c was considerably upregulated following trehalose administration, suggesting that miR-181c might be involved in trehalose-induced cognitive improvement in old rats.

Trehalose is a sugar made up of two glucose molecules, which has recently been demonstrated to have neuroprotective effects in several neurodegenerative mouse models [34, 35]. The beneficial influence of trehalose on learning and memory has also been reported, but the underlying mechanism remains to be elucidated [24, 36, 37]. Our results revealed that trehalose treatment could effectively reverse spatial learning and memory impairment in old rats, as evidenced by reduced escape latency and increased time spent in the original quadrant during the MWM test. Moreover, cognitive performance was improved in trehalose-treated young rats compared to young animals. However, the difference did not reach statistical significance. In a similar study, Sun et al. assessed the effects of trehalose on learning and memory ability in d-galactose-induced aging mice [19]. Consistent with our findings, they found that trehalose improved learning and spatial memory performance in aged animals. Additionally, several studies showed that trehalose improved cognitive deficits in various neurodegenerative disorders, such as Huntington’s disease (HD), Parkinson’s disease (PD) and AD [24, 34, 35].

The mechanism of cognitive decline in normal aging is not entirely understood. Currently, microRNAs have been suggested to be involved in neurological cognitive disorders in aged subjects [3]. Previous studies have shown that miR-34c and miR-181c are highly expressed in the hippocampus, and their modulation has beneficial physical outcomes in aging [10, 11, 38]. Therefore, we initially measured the expression level of miR-34c and miR-181c in the hippocampal tissue of rats following treatment with trehalose. We subsequently assessed the possible correlation between these microRNAs and cognitive performance in young and aged animals. Our data revealed an increasing trend in the levels of miR-34c with aging. Recently, several reports have demonstrated that dysregulation of miR-34c is associated with aging and several neurodegenerative disorders. For example, Zovoilis et al. [10] showed that miR-34c was significantly elevated in the hippocampus of aged mice, causing memory impairment in these animals whereas reduced miR-34c level was critical for restoring memory formation in old mice. In contrast to their results, we found that miR-34c expression level in the hippocampus of normally aged rats was not significantly changed when compared with young animals. The discrepancies found in these two studies are possibly due to the differences in control group age, animal species or epigenetic modifications. On the other hand, treatment with trehalose reduced the level of miR-34c expression in both old and young rats in comparison with non-treated animals which was significant just between young groups, suggesting that trehalose may play a role in the regulation of miR-34c in the hippocampus of young rats.

Analysis of miR-181c in the brain and blood of patients and animal models of neurodegenerative disorders showed that miR-181c is one of the critical regulators of learning and memory ability and may be involved in the brain aging process [38]. The implication of miR-181c in AD has previously been assessed by Schonrock et al. showing that miR-181c was downregulated in β-amyloid treated primary neurons and APP23 mouse hippocampus as well as human AD cortex [39, 40]. Moreover, recent findings identified an association between the low level of miR-181c and increased level of β-amyloid in serum of normal elderly subjects [41]. In line with these results, in the present study, we demonstrated that the expression of miR-181c was remarkably downregulated in the hippocampus of aged rats compared to young animals. Moreover, old rats showed a decline in learning and memory functions, suggesting that the low level of miR-181c was associated with brain aging. This finding was further confirmed by the positive correlation detected between miR-181c transcripts and the time spent in target quadrant in old animals. In addition, we analyzed the expression level of miR-181c in the hippocampal tissue of trehalose-treated rats. Our data showed that administration of trehalose significantly increased the expression level of miR-181c in trehalose-treated rats. Moreover, trehalose treatment improved learning and memory ability of animals, indicating the involvement of miR-181c in the molecular mechanism underlying the observed improvement in cognitive function.
This finding was further supported by a recent study which showed that overexpression of miR-181c in a rat model of progressive spatial memory deficits could effectively alleviate cognitive decline. Their results also demonstrated that miR-181c overexpression promotes neuron remodeling and synaptogenesis to ameliorate cognitive impairment [38]. In this sense, more studies are required to explore the role of miR-181c in neuronal function and cognitive behavior.

Conclusions

In conclusion, our results revealed that trehalose improved learning and memory function through targeting miR-181c in aged animals. However, miR-34c was not implicated in improving cognitive outcomes induced by trehalose. Collectively, our data suggest that trehalose can be used as a potential dietary supplement to delay cognitive impairments associated with aging.

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Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethical Approval This animal study was approved by the Institutional Animal Care and Use Committee of Kerman University of Medical Sciences (IR.KMU.REC.1399.350).

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