Isoflurane-induced expression of miR-140-5p aggravates neurotoxicity in diabetic rats by targeting SNX12

Dongyi Fan, Simin Yang, Yuxiang Han, Ru Zhang and Lukun Yang

Department of Anesthesiology, the Fifth Affiliated Hospital of Sun Yat-Sen University, Zhuhai City, Guangdong Province, 519000, China

(Received September 20, 2019; Accepted November 2, 2019)

ABSTRACT — MicroRNAs (miRNAs) are widely known as critical regulators in isoflurane-induced neurotoxicity during the development of brain. Moreover, isoflurane could aggravate cognitive impairment in diabetic rats. The present study was designed to investigate the role and mechanism of miR-140-5p on isoflurane-induced neurotoxicity in diabetic rats. Firstly, a diabetic rat model was established by injection of streptozotocin (STZ) and identified by Morris water maze test. The result indicated that isoflurane treatment exacerbated STZ-induced cognitive impairment, as demonstrated by increase of the latency to the platform and decrease of the proportion of time spent in the target quadrant. Secondly, miR-140-5p was up-regulated in diabetic rats treated with isoflurane. Functional assays revealed that knockdown of miR-140-5p attenuated neurotoxicity in diabetic rats, which was shown by a decrease of the latency to the platform and an increase of the proportion of time spent in the target quadrant. Mechanistically, we demonstrated that miR-140-5p directly bonded to SNX12 (sorting nexin 12). At last, the neuroprotective effect of miR-140-5p knockdown against isoflurane-aggravated neurotoxicity in diabetic rats was dependent on up-regulation of SNX12 and inhibition of cell apoptosis. In summary, these meaningful results demonstrated the mitigation of miR-140-5p knockdown against isoflurane-aggravated neurotoxicity in diabetic rats via SNX12, suggesting a novel target for neuroprotection in diabetes under isoflurane treatment.

Key words: Isoflurane, Cognitive impairment, Streptozotocin, Neurotoxicity, miR-140-5p, SNX12

INTRODUCTION

Diabetes is a progressive metabolic disease that endangers people’s health (Bahouth et al., 2018; Olokoba et al., 2012). The prevalence rate of diabetes is increasing rapidly (Piero et al., 2015). In addition, due to the unsatisfactory treatment, diabetes and its chronic complications, such as cardiovascular disease and neuropathy, are the third leading cause of death in humans (Kubota et al., 2017; Zhang et al., 2018b). Generally, diabetic patients tend to show a decline in cognitive ability (Verdile et al., 2015). For example, diabetes is related to increased risk of dementia (McMillan et al., 2018). The cognitive dysfunction always has a negative impact on patients’ life and significantly increases medical costs (Rehman and Kemp, 1999).

The occurrence of postoperative cognitive dysfunction is closely related to anesthesia during the operation (Ramaiah and Lam, 2009). As a commonly used inhaled anesthetic, isoflurane can affect the development of the central nervous system (Lemkuil et al., 2011) and induce cognitive dysfunction in rats (Rammes et al., 2009). Recently, isoflurane has been found to aggravate cognitive dysfunction in diabetic rats (Yang et al., 2014). Therefore, investigation of the effects of anesthetic drugs on patients with diabetes during surgery is urgently needed.

MicroRNAs (miRNAs) bind to the 3'-non-transcription region (3'-UTR) of the transcript of their homologous genes to regulate the expression of target genes (MicroRNAs: target recognition and regulatory functions.). Studies have shown that miRNAs are involved in regulation of diabetes via impairment of glucose metabolism (Feng et al., 2016). Moreover, anesthetics have certain effects on the expression level of miRNAs in the brain (Lu et al., 2015). Further studies have shown the correlation between the expression of several miRNAs and isoflurane-related cognitive disorders, such as miRNA-153 (Shao et al., 2019), miR-124 (Yang et al., 2019) and miR-
MiR-153 protected against neurotoxicity induced by isoflurane via inhibition of neurocyte apoptosis and promotion of cell proliferation (Shao et al., 2019). MiR-124 promoted cell proliferation and inhibited cell apoptosis of hippocampal neurons via regulation of early growth response 1, thus improving spatial learning and memory ability damaged by isoflurane (Yang et al., 2019). Decrease of miR-448 attenuated learning and memory impairment induced by isoflurane (Wu et al., 2017). However, whether miRNAs are associated with isoflurane-related cognitive dysregulation in diabetes has not been reported yet. MiR-140-5p was identified as a tumor suppressor in gastric cancer (Fang et al., 2017), and regulates odontoblastic differentiation (Lu et al., 2019). More recently, miR-140-5p was shown to protect against hypoxic-ischaemic brain damage via promotion of learning and memory abilities (Han et al., 2018). In addition, a study has shown that miR-140-5p was increased 6 hr after isoflurane treatment (Luo et al., 2015). However, the possible mechanism remains completely unknown.

Here, the expression of miR-140-5p in diabetic rats under isoflurane anesthetic was firstly detected, and thereafter, the effect of miR-140-5p on cognitive abilities was then determined. Moreover, the downstream targets of miR-140-5p were also explored. The meaningful results might provide new evidence for development of novel treatment for diabetes under isoflurane treatment.

**MATERIALS AND METHODS**

**Animal model**

All animal experiments were approved by the Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-Sen University. Thirty-six male Sprague-Dawley rats weighing 220-300 g were purchased from Harlan Laboratory (Indianapolis, IN, USA). Rats were randomly divided into 6 groups (n = 6 each). Nondiabetic groups comprised sham and isoflurane (ISO) groups, and diabetic groups comprised streptozotocin (STZ), STZ+ISO, STZ+ISO+NC antagonmir, and STZ+ISO+miR-140-5p antagonmir groups.

For diabetic groups, male rats were intraperitoneally injected with 65 mg/kg STZ (Sigma, St. Louis, MO, USA) suspended in 50 mM citrate buffer (pH 4.0). For nondiabetic groups, rats were injected with equal volume of citrate buffer. Blood glucose levels were then determined after injection, and one week later, rats with blood glucose levels > 250 mg/dL were determined as the diabetic rat model.

One month later, for ISO and STZ+ISO groups, rats were exposed to 2% isoflurane in 100% O₂ for 2 hr at a flow rate with 2 L/min. The sham and STZ groups were exposed to 100% O₂ for 2 hr at 2L/min. Both of the two groups of rats were returned to their home cages after anesthesia.

For STZ+ISO+NC antagonmir and STZ+ISO+miR-140-5p antagonmir groups, the antagonmir were purchased from Dharmaco (Lafayette, CO, USA). Rats were lateral cerebroventricular injected with 2 nmol antagonmir 30 min before ISO anesthesia. One week later, the rats were sacrificed immediately by decapitation, and the hippocampus tissues were harvested for analysis.

**Morris water maze test**

A maze (80 cm deep; 100 cm diameter) was separated into four equal quadrants on the monitor screen of a computer. The maze was filled with water to a depth of 30 cm and maintained at 24-25°C. The swimming paths of the rats for 4 consecutive days were recorded and analyzed by VideoMot software version 2.4.50923 (TSE Systems GmbH, Bad Homburg, Germany). The escape latency and time spent in the quadrant were recorded.

**Cell culture**

Rat neuroblastoma B104 cell line (American Type Culture Collection; Rockville, MD, USA) was maintained in complete medium (Dulbecco’s modification of Eagle’s medium with 10% FBS; Mediatech; Manassas, VA, USA) at 37°C with 5% CO₂.

**Cell transfection**

MiR-140-5p mimics, inhibitor and the respective negative controls (NC mimics, NC inhibitor) were synthesized by GenePharma (Pudong, Shanghai, China). B104 cells were transfected with miR-140-5p mimics/inhibitor or NC mimics/inhibitor (20 nM) via Lipofectamine 2000.

**Dual luciferase reporter assay**

The wildtype binding sequence of miR-140-5p in SNX12 and the corresponding mutants were subcloned into pGL3 (Promega, Madison, WI, USA). B104 cells (3 x 10⁶/well) were seeded in 24-well plates for overnight. Cells were then co-transfected with 2.5 ng/µL pGL3 luciferase reporter vectors, 12.5 pg/µL pRL-TK with 100 nM miR-140-5p mimics (5’-uaccacaggguagaaccacgg-3’) or NC-mimics (sense: UUCUCCGAACGUGUCACGUTT; anti-sense: ACGUGACACGUUCGGAGAATT). Two days later, the luciferase activities were performed via Lucifer Reporter Assay System (Promega) and normalized to Renilla luciferase activity of pRL-TK.
qRT-PCR

Total RNAs or miRNAs were isolated via Trizol (Invitrogen, Carlsbad, CA, USA) or miRNeasy MiniKit (Qiagen, Hilden, Germany), respectively. cDNAs were synthesized by PrimeScript RT Reagent (Takara, Shiga, Japan) with the following conditions:

70°C for 10 min, ice bath for 2 min, 42°C for 60 min and 70°C for 10 min, and qRT-PCR analysis was conducted on ViiA 7 (Applied Biosystems, Austin, TX, USA) under 95°C pre-denaturation for 10 min, 40 cycles with at 95°C for 10 sec, 60°C for 60 sec and 72°C for 30 sec. GAPDH or U6 were used as endogenous control for mRNAs or miRNAs, respectively. The relative expression levels of target genes were calculated by 2^−∆∆Ct method. The primer sequences were as shown below in Table 1.

Western blot

Twenty µg proteins from hippocampus tissues were separated by SDS-PAGE, and then transferred to PVDF membrane. After blocking with 5% BSA, the membranes were incubated overnight with primary antibodies, including anti-SNX12 (1:1500, Abcam, Cambridge, MA, USA), Bcl-2, Cleaved Caspase-3 (1:2500, Abcam), GAPDH (1:3000, Abcam) antibodies at 4°C. Then, after washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 hr at room temperature. At last, the immunoreactivities were detected by enhanced chemiluminescence (KeyGen, Nanjin, China) after incubating with HRP labeled secondary antibody (1:5000; Abcam).

Statistical analysis

The statistical analyses were performed via GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA) and one-way analysis of variance (ANOVA) was used for the comparison among more than two groups. Student’s t test for comparison between two groups. The data was shown as mean ± SEM. A p < 0.05 was regarded as statistically significant difference.

RESULTS

MiR-140-5p was up-regulated in diabetic rats after isoflurane anesthesia

The effect of isoflurane on cognitive abilities of diabetic rats were identified via Morris water maze test. Results demonstrated that streptozotocin injection caused cognitive impairment in rats by the increase of escape latency (Fig. 1A) and decrease of time spent in the target quadrant (Fig. 1B) compared to the sham rats, confirming the establishment of the diabetic rat model. In addition, isoflurane-anesthetized rats further aggravate cognitive impairment in diabetic rats (Fig. 1A, 1B), revealing that isoflurane anesthesia further impaired learning and memory abilities of diabetic rats. The expression of miR-140-5p was significantly increased in diabetic rats post-anesthesia compared to diabetic rats pre-anesthesia (Fig. 1C), suggesting the possible association between miR-140-5p and isoflurane-induced cognitive impairment in diabetic rats.

Table 1. Primers sequences.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence(5′- 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH F</td>
<td>ACCACAGTCCATGCCATCAC</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TCCACCACCTGTTGCTGTA</td>
</tr>
<tr>
<td>miR-140-5p F</td>
<td>TCGGCGATGGTTTTAACCCTATG</td>
</tr>
<tr>
<td>miR-140-5p R</td>
<td>CCAATGACGGTGTCAGAGGT</td>
</tr>
<tr>
<td>SNX12 F</td>
<td>AACCTGAAGCCCAAGGACCTGAC</td>
</tr>
<tr>
<td>SNX12 R</td>
<td>TTGCTCTCTGTGGCAAGAACATGTG</td>
</tr>
<tr>
<td>U6 F</td>
<td>CTCGTTGCCAGCACACA</td>
</tr>
<tr>
<td>U6 R</td>
<td>AACGTTCACGAAATTTGCGT</td>
</tr>
</tbody>
</table>

Fig. 1. MiR-140-5p was up-regulated in diabetic rats after isoflurane anesthesia. (A) The effect of isoflurane and streptozotocin on escape latency of rats. ** represents p < 0.01. (B) The effect of isoflurane and streptozotocin on time spent in the target quadrant of rats. * ** represents p < 0.05, p < 0.01. (C) The effect of isoflurane and streptozotocin on miR-140-5p expression detected by qRT-PCR. ** represents p < 0.01.
Knockdown of miR-140-5p attenuated isoflurane-induced neurotoxicity in diabetic rats

To explore the influence of miR-140-5p on cognitive abilities of diabetic rats with isoflurane anesthesia, miR-140-5p antagonir was delivered into rats. A down-regulation of miR-140-5p was observed in hippocampus tissues (Fig. 2A). Moreover, injection with miR-140-5p antagonir dramatically decreased escape latency (Fig. 2B) and increased time spent in the target quadrant (Fig. 2C) in diabetic rats post-anesthesia, revealing that knockdown of miR-140-5p attenuated isoflurane-induced neurotoxicity in diabetic rats.

SNX12 was a target of miR-140-5p

SNX12 was predicted to be the putative binding target of miR-140-5p via Targetscan (Fig. 3A) and confirmed by dual luciferase reporter assay (Fig. 3B). MiR-140-5p mimics significantly decreased the luciferase activity of SNX12 wildtype luciferase reporter vector in B104 cells compared to NC mimics, while had no impact on SNX12 mutant luciferase reporter vector (Fig. 3B). The effect of miR-140-5p on SNX12 expression was then detected in B014 cells transfected with miR-140-5p mimics or inhibitor, which was shown in Fig. 3C. The mRNA (Fig. 3D) and protein (Fig. 3E) expression of SNX12 were decreased by miR-140-5p mimics while increased by miR-140-5p inhibitor, further indicating the binding ability between miR-140-5p and SNX12.

Effects of miR-140-5p on SNX12 and cell apoptosis-associated protein expression

Isoflurane treatment decreased protein expression of SNX12 in non-diabetic rats (Fig. 4A), and significantly reduced protein expression of SNX12 in diabetic rats compared to non-diabetic rats (Fig. 4A). Western blot analysis showed that injection with miR-140-5p antagonir dramatically increased the expression of SNX12 in hippocampus (Fig. 4B). Since isoflurane anesthesia could induce the apoptosis in hippocampal neuron and lead to reduced learning and memory abilities, the expression of apoptotic-related proteins was also examined. The results demonstrated that the expression of Bcl2 was increased, while Cleaved Caspase3 was decreased in diabetic rats post-anesthesia with miR-140-5p antagonist injection compared NC antagonist injection (Fig. 4B), suggesting that knockdown of miR-140-5p increased the expression of SNX12 and inhibited isoflurane-induced cell apoptosis in diabetic rats.

DISCUSSION

Spatial and temporal coordination during brain development ensures the precise neuronal connection (Huang et al., 2017), while impairment of spatial and temporal coordination shows profound effects on brain function (Possin, 2010). Some clinical studies have shown that isoflurane anesthesia can result in cognitive dysfunction (Hudson and Hemmings, 2011; Liu et al., 2014). Therefore, isoflurane anesthesia has been associated with brain dysfunction (Stratmann et al., 2009; Zhang et al., 2013). In addition, isoflurane anesthesia can aggravate cognitive impairment in diabetic rats (Yang et al., 2014). Therefore, there is an urgent need to study the effects of isoflurane anesthesia on cognitive dysfunction in diabetic patients and its underlying mechanisms. In this study, we found a significant increase of miR-140-5p in diabetic rats with isoflurane anesthesia, and then elucidated the functional role of miR-140-5p on isoflurane-induced neurotoxicity.

First, we established a diabetic rat model by streptozotocin induction (Vatandoust et al., 2018). Consistent with a previous study (Liu et al., 2016), streptozotocin caused cognitive deficits, manifested by increased escape latency and decreased time spent in the target quadrant. In addition, isoflurane anesthesia did aggravate impairment of learning and memory abilities of diabetic rats, as deciphered by longer escape latency and less time spent in the target quadrant. However, the underlying mechanism remains unclear. Previous studies have shown that inhalation anesthetics may cause functional or structural damage to hippocampal neurons, leading to cognitive deficits (Jevtovic-Todorovic et al., 2003; Kong et al., 2011). Moreover, isoflurane can result in neuroapoptosis and impairments in synaptic plasticity (Jevtovic-Todorovic et al., 2003). The mechanism of isoflurane-mediated cognitive impairments in diabetic rats should be explored in a future study.

MiR-140-5p was significantly increased under isoflurane anesthesia, consistent with a previous study (Luo et al., 2015). Interestingly, streptozotocin treatment had a slight effect on the up-regulation of miR-140-5p. Functional assays showed that injection of miR-140-5p antagonist into diabetic rats attenuated isoflurane-induced neurotoxicity, as manifested by decrease of escape latency and increase of time spent in the target quadrant. Given that isoflurane anesthesia and streptozotocin treatment are associated with cognitive deficits, miR-140-5p may be a potential therapeutic target for cognitive deficits diseases.

Studies have indicated that isoflurane anesthesia can result in hippocampal neuron apoptosis (Chen et al., 2016), leading to reduced learning and memory abili-
miR-140-5p/SNX12 in diabetic rats

Fig. 2. Knockdown of miR-140-5p attenuated isoflurane-induced neurotoxicity in diabetic rats. (A) Expression of miR-140-5p in miR-140-5p antagomir or NC antagomir delivered diabetic rats with isoflurane anesthesia detected by qRT-PCR. ** represents \( p < 0.01 \). (B) The effect of miR-140-5p antagomir on escape latency of rats. ** represents \( p < 0.01 \). (C) The effect of miR-140-5p antagomir on time spent in the target quadrant of rats. ** represents \( p < 0.01 \).
ties (Li et al., 2015). Apoptosis can be achieved via activation of caspases and endogenous pathways, including down-regulation of Bcl-2 family proteins (Pan et al., 2011). In our study, knockdown of miR-140-5p decreased the expression of Cleaved Caspase-3 and increased the expression of Bcl-2 to inhibit hippocampal neuronal apoptosis in diabetic rats with isoflurane anesthesia.

Wnt1 was identified as a target of miR-140-5p in neonatal rats for cerebral protection (Han et al., 2018) or in dental pulp stem cells for odontoblastic differentiation (Lu et al., 2019). In the present study, SNX12 was identified as a new target of miR-140-5p. SNXs participate in membrane trafficking and protein sorting (Xu et al., 2001), to regulate pathway of endocytic trafficking (Gallon and Cullen, 2015). Dysregulation of SNXs may lead to impaired homeostatic responses, as well as disease states, including neurodegenerative diseases (Zhang et al., 2018a). For example, BAR-SH3 sorting nexins are involved in neurotransmission (Ukken et al., 2016). SNX27 contributes to the dynamic nature of neuronal networks for learning (Hussain et al., 2014). Mutation in SNX3 impairs development of nervous system (Vieira et al., 2018). SNX12 has been shown to contribute to pathology of Alzheimer’s disease (Zhao et al., 2012), and mediated neurite formation during cerebral cortical development (Mizutani et al., 2012). Here, we found that miR-140-5p could directly target SNX12, and knockdown of miR-140-5p increased the expression of SNX12 in hippocampus of diabetic rats under isoflurane anesthesia. However, the effect and mechanism of miR-140-5p/SNX12 axis on cognitive deficits induced by isoflurane anesthesia in diabetic rats needs to be further investigated. Moreover, other diabetic rat model studies should also be conducted to investigate ISO-induced cognitive impair-

Fig. 4. Effects of miR-140-5p on SNX12 and cell apoptosis-associated protein expression. (A) The effect of isoflurane and streptozotocin on protein expression of SNX12 detected by western blot. (B) The effect of miR-140-5p antagomir on SNX12, Bcl-2 and Cleaved Caspase-3 expression in diabetic rats post-anesthesia. ** represents $p < 0.01$.
ment as well as the effect and mechanism of miR-140-5p/SNX12 axis on cognitive deficits.

In conclusion, miR-140-5p protected against neurotoxicity and apoptosis via the regulation of SNX12, suggesting the potential application of miR-140-5p in the therapeutic target for neurotoxicity of diabetes following isoflurane anesthesia.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES


miR-140-5p/SNX12 in diabetic rats


