Abstract
This study examined the microRNA-33a (miR-33a) expression levels in cerebral cortex in rat model of cerebral ischemic tolerance (IT), with 3-nitropropionic acid (3-NPA) preconditioning. Rat model of cerebral IT was established as follows: 14 male Sprague-Dawley rats were randomly divided into two groups, the 3-NPA treated group and the control group. Intraperitoneal injection of 3-NPA or normal saline was performed in 3-NPA treated group and control group, respectively. Middle cerebral artery occlusion (MCAO) was performed in all the rats 3 days after injection and the infarct volume was measured on postoperative day-2. To study miR-33a expression levels in this model, male Sprague-Dawley rats (n = 31) were randomly divided into 4 groups, 3-NPA treatment group (n = 8), 3-NPA treatment + MCAO group (n = 9), MCAO group (n = 9), and control group (n = 5). Surviving rats were sacrificed 4 days after injection and cerebral cortex samples were obtained for total RNA isolation. MiR-33a expression levels were determined by real-time quantitative PCR (qRT-PCR). Infarct volume in 3-NPA treated group decreased significantly in comparison to the control group (P < 0.05). Expression analysis by qRT-PCR revealed that miR-33a expression levels in 3-NPA treated group were significantly higher than that in control group (P < 0.05). However, no statistically significant difference in miR-33a expression levels were observed in 3-NPA + MCAO and MCAO groups (both P > 0.05). Our results present convincing evidence that miR-33a expression levels are significantly reduced in the cerebral cortex in rat model of cerebral IT, which may have significantly impacted the infarct volume.

MicroRNAs (miRNAs) are small non-coding RNAs of 20 to 25 nucleotides in length, with important regulatory functions, widely expressed in animals and plants (6, 7). MiRNAs play indispensable roles in apoptosis, blood cells diversity, homeobox gene regulation, neuronal cell polarity, insulin secretion, brain morphogenesis and late embryonic development (8-11). Abnormal expression of miRNAs significantly impact disease progression, for example, reduced levels of tumor suppressor miRNAs or increased expression of oncogenic miRNAs drive tumor progression, since these miRNAs are at the hub of complex intersecting cellular pathways (12-15). In this context, the miRNA field is largely focused on tumor biology and embryonic development, with very few studies describing the miRNA roles in nervous system development, neural plasticity and neurological responses to environmental factors (16-20). Nevertheless, a study on miR-199a expression and function in cerebral IT in rats suggested that the significant changes in gene expression observed during cerebral IT are directly related to miRNA dysregulation, hence, miRNAs have a critical role in the regulation of gene expression during cerebral IT (2, 21-23). Recent studies also revealed that miR-33a has a major role in IT in myocardial cells and its expression rapidly decreased in hypoxia preconditioning of myocardial cells. Interestingly, although the miR-33a levels are relatively very high in mouse brains and livers, its role in cerebral ischemic injury and IT remains unknown (24-28). In this study, we studied the changes in miR-33a expression level during cerebral IT.

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using a rat model to understand its role and the potential therapeutic applications for cerebral IT.

**Materials and methods**

**Ethics statements**

All animal procedures were approved by the Medical Ethics Review Board of Henan Provincial People’s Hospital and all experiments were carried out on the basis of the recommendations of Guide for the Care and Use of Laboratory Animals of the Chinese National legislation.

**Animals and study design**

A total of 14 male Sprague-Dawley rats weighing 200 to 250g were obtained from the animal center of Henan Provincial People’s Hospital. The rats were allowed free access to food and water and maintained on a 12-h light and dark cycle. The male Sprague-Dawley rats (n = 14) were randomly divided into two groups, 3-NPA treated group (n = 7) and control group (n = 7). The 3-NPA treated group (cerebral IT group) was as follows: rats underwent peritoneal injection of 3-NPA (20mg/kg, approximate volume of 2 ml) on day 0 and middle cerebral artery occlusion (MCAO) on day 3. Infarct volume was measured on day 4 in these rats. Control group (cerebral ischemia group) was as follows: rats underwent peritoneal injection of normal saline (volume, 2 ml) on day 0 and MCAO on day 3. Infarct volume was measured on day 4. Single-blind method was used, therefore, the investigators were blinded to the details on grouping.

In a second experimental set-up, male Sprague-Dawley rats (n = 31) of similar weight were randomly divided into 4 groups. Group 1 (3-NPA treatment group, n = 8): rats underwent peritoneal injection of 3-NPA (20mg/kg; volume, 2 ml) on day 0 and sacrificed on day 4; group 2 (3-NPA treatment + MCAO group, n = 9): rats underwent peritoneal injection of 3-NPA (20mg/kg; volume, 2 ml) on day 0 and MCAO on day 3, and sacrificed on day 4; group 3 (MCAO group, n = 9): rats underwent peritoneal injection of normal saline (volume, 2 ml) on day 0 and MCAO on day 3, and were sacrificed on day 4; group 4 (control group, n = 5): rats underwent peritoneal injection of normal saline (volume, 2 ml) on day 0 and sacrificed on day 4.

**Middle cerebral artery occlusion (MCAO)**

Right MCAO was performed in rats according to the modified technique of Longa et al. Before operation, all rats were provided with only water and fasted overnight. Briefly, rats were anesthetized with 10% chloral hydrate (anesthesia volume, 0.3 ml/100g). After median skin incision on right side of the neck, the right carotid artery was exposed to part the common carotid artery and the external carotid artery. Following an incision made 2 mm to the end of external carotid artery, a 4-0 monofilament nylon suture with a rounded tip was inserted and threaded out of the internal carotid artery up to the middle cerebral artery. The occlusion of middle cerebral artery resulted in focal cerebral ischemia. Three hours later, the monofilament was removed to allow reperfusion.

**Neurological severity scores (NSS) evaluation**

The NSS evaluation on rats was conducted 12h and 24h after surgery (29). Detailed items in NSS (0–18 scores) consisted of exercise, sensation, reflection and balance experiments; 0: normal nerve function; 1–6: mild neurological damage; 7–12 moderate neurological damage; 13–18: severe neurological damage.

**Measurement of cerebral infarction volume**

Twenty-four hours after MCAO, rats were anaesthetized using 10% chloral hydrate, sacrificed with air embolism and the length of the brain was measured. Rats brain was sliced into a total of 6–7 slices that were 2mm thick. Sliced tissues were placed in 1% 2,3,5-Triphenyltetrazolium chloride (TTC) solution immediately, incubated in dark at 37°C for 30 min and fixed in 10% paraformaldehyde. Mitochondrial catalase oxidized TTC and gave the surviving tissues (non-cerebral infarction region) a dark red appearance, while the necrotic tissue (cerebral infarction region) appeared pale. Image Tool image analysis software was used to calculate the cerebral infarction volume in both the groups.

**Quantitative real-time PCR**

Fresh cerebral cortical tissue sample (50–100 mg) was used for quantitative real-time PCR. TRIZOL Reagent (Invitrogen) was used to purify total RNA. The concentration and purity of RNA samples were measured with UV spectrophotometer (Q5000) and agarose gel electrophoresis was used to verify the integrity of RNA samples. Complementary DNA (cDNA) was synthesized from total RNA samples using TaqMan MicroRNA Reverse Transcription kit (ABI, USA), following the manufacturer’s instructions. TaqMan fluorescent probe was employed to detect miR-33a expression levels in rats brain tissue. U6 was amplified as the internal control. Quantitative real-time PCR was performed using real-time fluorescence quantitative PCR instrument (Stratagene MX3005P). The total volume of PCR

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**Table 1.** Comparison of success rates in building model of different groups including 3-NPA group and control group, and the success rate in 3-NPA treated group was significantly higher than the control group.

<table>
<thead>
<tr>
<th></th>
<th>3-NPA treated group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Death toll</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Number of successful models</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Success rate</td>
<td>85.7%</td>
<td>82.8%</td>
</tr>
<tr>
<td>NSS scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 hours after surgery</td>
<td>9.26±1.68</td>
<td>11.93±1.62</td>
</tr>
<tr>
<td>24 hours after surgery</td>
<td>7.18±0.73</td>
<td>9.39±1.12</td>
</tr>
</tbody>
</table>

Note: 3-NPA, 3-nitropropionic acid; NSS, neurological severity scores.
Results

Neurologic deficit scores and success rates in building model

A total of three rats died due to accidental anesthetic overdose, with 1 from the 3-NPA treated group and 2 rats from the control group. After excluding these 3 rats, a total of 6 IT rats remained in the 3-NPA treated group and 5 rats in the control group. The IT was successfully established in these rats and the success rates for 3-NPA treated group and control group were 85.7 % and 82.8 %, respectively. There was no significant difference in the success rate between the 3-NPA treated group and control group (P > 0.05) (Table 1). Further, 12h and 24h after surgery, the NSS evaluations in the 3-NPA treated group were 11.93±1.62 and 9.39±1.12, respectively, exhibiting statistically significant differences between the time points (P = 0.026 and P =0.003, respectively).

Infarction volume 24h after MCAO

Staining results of the fixed rat brain tissues revealed infarction in both the 3-NPA treated group and control group mainly in the lateral striatum, lateral frontal and parietal cortex (as shown in Fig. 1). The infarction volume was analyzed in the successful IT mice models and the results revealed that the infarction volume in 3-NPA treated group [(243.64 ± 22.21) mm³] was dramatically reduced compared to the control group [(321.79 ± 21.19) mm³], with a decrease rate of 21.76% (P< 0.05) as shown in Table 2.

Quality of miR-33a and expression of miR-33a

The PCR-amplified products of miR-33a and U6 were 70bp and 89bp in size, respectively. The solubility curve of both miR-33a and U6 displayed a single sharp peak, confirming the high specificity of the primers (Fig. 2).

Statistical analysis

Statistical analysis was performed using software SPSS 18.0. The results of cerebral IT model were analyzed using adjusted cerebral infarction volume. ΔCT (target gene Ct value – internal control gene Ct value) was employed to analyze the result of real-time PCR. 1-K-S test was used to check normal distribution of the data. Nair test was used to identify and exclude outliers. The average of adjusted cerebral infarction volume and ΔCT were expressed as mean ± standard deviation (SD) and comparisons of the average were analyzed with t-test. Data were considered statistically significant at P < 0.05.
The results of the MCAO group indicated no difference in miR-33a expression compared to the control group, revealing that miR-33a expression level is not influenced by severe and fatal IT. However, 3-NPA preconditioning downregulated miR-33a expression and miR-33a levels returned to the normal level within 3 days after MCAO, accompanied by decrease of infarction foci. This results observed in the 3-NPA+MCAO group confirmed the preconditioning effect of 3-NPA.

Our results also indicated that miR-33a expression in cerebral cortex decreased significantly. MiR-33a regulates the dynamic equilibrium of high-density lipoprotein cholesterol (HDL-C) and HDL-C is an anti-atherosclerosis cholesterol. When co-expressed with SREBPs, miR-33a regulates the cholesterol load in macrophages (35). In addition, miR-33a controls cholesterol release from macrophages, which is a vital anti-atherosclerosis mechanism (36). Recent study detected that miRNA expression in cerebral cortex, striatum and hippocampus decreased during IT induced by 3-NPA (37,38). IT is a transient elevation of resistance to ischemic stress that is activated by endogenous protective mechanisms in IPC. IT is attributed multiple factors including EAA, inflammation, stress protein response, adenosine levels, signal transduction pathways and apoptosis (1). Accordingly, miRNA pathways may constitute a general mechanism of IT regulation, with no correlation to organ-specific tolerance and the methods of inducing tolerance include hypoxia, transient ischemic and 3-NPA. Consistent with this, our study shows that miR-33a expression is down-regulated during myocardial hypoxia preconditioning indicating that alteration in miR-33a level is an impor-

### Discussion

In this study, 3-NPA was used to delineate pathways involved in IT. The toxin 3-NPA is derived from plant and fungi and inhibits the activity of mitochondrial succinate dehydrogenase and complex II to reduce ATP levels and leads to oxidative stress (30). In addition, 3-NPA promotes release of reactive oxygen species from mitochondria, and induces mitochondrial DNA damage and loss of mitochondrial function (31). In the present study, cerebral IT model was established with 3-NPA to simulate hypoxic preconditioning and we examined the changes in miR-33a expression during IT. Our findings suggested that the volume of cerebral infarction in 3-NPA treated rats decreased significantly compared to the control group. A possible explanation might be that a high dose of single intraperitoneal injection of 3-NPA used in rats or a continuous 30-day intraperitoneal injection of small dose (20mg/kg) of 3-NPA result in selective degeneration of striatum neurons (32). A small single dose of 3-NPA simulates mild, transient ischemia or hypoxia, which confers resistance to subsequent severe cerebral ischemia without causing histoneurology abnormalities. Consistent with our results, previous studies showed that cerebral infarction volume in rat cerebral cortex decreased with 3-NPA treatment, compared to the controls (33,34). Evaluation of corresponding neurological deficit results indicated that the NSS parameters tested were significantly improved in 3-NPA treated group compared to the control group during the same time. The above result indicated that 3-NPA is highly effective in preconditioning for IT. Further, the

### Table 2. Comparison of the infarction volume (S) and average infarction volume in rats between the 3-NPA treated group and control group, and average infarction volume of the 3-NPA treated group was lower than the control group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>S (mm³)</th>
<th>Average S (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NPA treated group</td>
<td>-1</td>
<td>242.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-2</td>
<td>238.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3</td>
<td>242.15</td>
<td>243.64 ± 22.21</td>
</tr>
<tr>
<td></td>
<td>-4</td>
<td>252.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-5</td>
<td>254.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-6</td>
<td>232.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-7</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-8</td>
<td>323.91</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-9</td>
<td>354.73</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>-10</td>
<td>342.51</td>
<td>321.79 ± 21.19</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>289.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>298.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

Note: S, infarction volume; 3-NPA, 3-nitropropionic acid.

### Table 3. The 2-ΔΔCt method for calculating relative change in gene expression as determined from real-time fluorescence quantitative PCR, ΔCt value of miR-33a expression in 3-NPA treated group was significantly higher than the control group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control group</th>
<th>3-NPA treated group</th>
<th>3-NPA+MCAO group</th>
<th>MCAO group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 4)</td>
<td>(n = 7)</td>
<td>Left side (n =9)</td>
<td>Right side (n =9)</td>
</tr>
<tr>
<td>ΔCt</td>
<td>10.53 ± 0.28</td>
<td>14.50 ± 0.38</td>
<td>11.27 ± 0.21</td>
<td>12.00 ± 1.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11.57 ± 0.49</td>
<td>10.98 ± 0.49</td>
</tr>
</tbody>
</table>

Note: 3-NPA, 3-nitropropionic acid; MCAO, middle cerebral artery occlusion.
tant physiological mechanism during IT.

The present study had several limitations. On one hand, gene regulation is a dynamic process, especially at different time points of cerebral ischemia, and the level of gene expression may be significantly different. On the other hand, our study did not discuss the biological effect of miR-33a in cerebral IT, only speculating that miR-33a may play a significant role in preconditioning by 3-NPA and that its down-regulation may have a protective effect on cerebral ischemic injury (37). Importantly, we only detected the miR-33a expression level during cerebral IT within cerebral cortex, and striatum or hippocampus was not examined, which might influence the general application of our results, and require further studies in this direction.

In conclusion, the present study demonstrated that miR-33a expression in the cerebral cortex is dramatically reduce in rats during cerebral IT. However, due to the above limitations, further studies are required to better understand the role of miR-33a in cerebral IT to design effective strategies for optimal brain tolerance to ischemia.

Acknowledgements

We would like to acknowledge the reviewers for their helpful comments on this paper.

References

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miR-33a expression and ischemic tolerance.


