Effect of oxidative stress injury on miR-210 expression in spinal neurons and the neuroprotective effect of miR-210 inhibitor

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Objective: To study the effect of oxidative stress injury on miR-210 expression in spinal neurons and the neuroprotective effect of miR-210 inhibitor. 

Methods: Spinal neurons of rats were cultured and divided into control group (group A), H2O2 group (group B), NC group (group C), H2O2+NC group (group D) and H2O2+miR-210 inhibitor group (group E). miR-210 expression levels in group A and group B as well as the cell vitality and expression levels of oxidative stress molecules, apoptotic molecules and function-related molecules in group C, group D and group E were determined.

Results: miR-210 expression level in group B was significantly higher than that in group A; after 12 h, 18 h and 24 h of treatment, OD values of group D were significantly lower than those of group C, and OD values of group E were significantly higher than those of group D; after 24 h of treatment, HO-1, NOX-2, CAT, GAP-43 and synapsin-I levels in group D were significantly lower than those in group C while ROS, 8-OHdG, c-fos, c-jun, AP1, caspase-3, PSD-93 and PSD-95 levels were significantly higher than those in group C; HO-1, NOX-2, CAT, GAP-43 and synapsin-I levels in group E were significantly higher than those in group D while ROS, 8-OHdG, c-fos, c-jun, AP1, caspase-3, PSD-93 and PSD-95 levels were significantly lower than those in group D.

Conclusion: Oxidative stress injury can cause high miR-210 expression in spinal neurons, and miR-210 inhibitor can reduce the neuron injury caused by oxidative stress.

1. Introduction

Spinal cord injury is a type of trauma with high morbidity and mortality rates, it will cause loss of limb sensory function and motor function, and the patients lose labor capacity and bring great burden to the society and families. The pathological basis of spinal cord injury caused by external violence is spinal neuron necrosis, synaptic structure damage and loss of function. Oxidative stress injury is an important pathological link in neuron and synapse damage, and the external violence will cause ischemia hypoxia, enhance intracellular oxygen metabolism, produce a large number of oxygen free radicals and then cause neuron apoptosis via oxidation of oxygen free radicals[1-2]. Therefore, anti-oxidative stress is an important target in treating spinal cord injury and improving nerve function[3]. MicroRNA (miRNA) is a type of non-coding small RNA newly discovered in recent years, it regulates the expression of multiple genes to exert biological effects, and it has been confirmed that miR-210 can resist oxidative stress and inhibit cell apoptosis[4-5]. However, there is not report about the change of miR-210 in oxidative stress injury of spinal cord. In the following study, the effect of oxidative stress injury on miR-210 expression in spinal neurons and the neuroprotective effect of miR-210 inhibitor were analyzed.

2. Materials and methods

2.1. Experimental materials

Spinal neurons of rats and culture medium were bought from San Diego Company, miR-210 inhibitor and negative control sequences were synthesized by GenePharma Company, transfection reagent LipofectamineTM2000 was purchased from Invitrogen Company, miR extraction and reverse transcription as well as PCR kits were purchased from Beijing Tiangen Biotechnology Company, MTS cell vitality kits were bought from Promega
Company, radioimmunoprecipitation kits were purchased from Nanjing Jiancheng Company and enzyme-linked immunosorbent assay kits were bought from Jijin Chemical Technology Company. Multifunctional microplate reader and fluorescence quantitative PCR apparatus were purchased from Bio-rad Company.

2.2 Experimental methods

2.2.1 Cell culture and treatment methods

Spinal neurons of rats were cultured with corresponding medium in 37 °C and 5% CO₂ incubator and routinely digested and sub-cultured with trypsin, the cells that were sub-cultured and in logarithmic growth phase were collected for treatment and divided into control group (group A), H₂O₂ group (group B), NC group (group C), H₂O₂+NC group (group D) and H₂O₂+miR-210 inhibitor group (group E) according to different treatment conditions, and the treatment methods were as follows: group A were treated with culture medium without serum; group B were treated with 100 μmol H₂O₂; group C were transfected with negative control sequences; 24 h after transfected with negative control sequences, group D were treated with 100 μmol H₂O₂; 24 h after transfected with miR-210 inhibitor, group E were treated with 100 μmol H₂O₂.

2.2.2 miR-210 expression level detection methods

Control group (group A) and H₂O₂ group (group B) of cells were collected, inoculated in 12-well cell plate and continuously treated for 24 h, the supernatant was abandoned, the cells were collected, 100 μL of protein lysis buffer in 12-well cell plate and treated for 24 h, the supernatant was abandoned, the cells were collected, inoculated in 12-well cell plate and continuously treated for 24 h, the supernatant was abandoned, the cells were collected, inoculated in 12-well cell plate and treated for 24 h, the supernatant was abandoned, the cells were collected.

2.2.3 Cell viability detection methods

NC group (group C), H₂O₂+NC group (group D) and H₂O₂+miR-210 inhibitor group (group E) of cells were collected, inoculated in 96-well cell plate and treated for 18 h, 12 h and 24 h, then 20 μL of MTS test fluid was added in each cell well, cells were continuously incubated for 4h, and then the absorbance at 490nm wavelength was detected from microplate reader.

2.2.4 Detection methods of oxidative stress, apoptosis and nerve injury molecules in cells

NC group (group C), H₂O₂+NC group (group D) and H₂O₂+miR-210 inhibitor group (group E) of cells were collected, inoculated in 12-well cell plate and treated for 24 h, the supernatant was abandoned, the cells were collected, 100 μL of protein lysis buffer was added in each cell well, scraper was used to fully break cells and obtain protein suspension, enzyme-linked immunosorbent assay kits were used to determine HO-1, NOX-2, CAT, c-fos, c-jun, AP1, caspase-3, GAP-43, synapsin-I, PSD-93 and PSD-95 levels, and radioimmunoprecipitation kits were used to detect ROS and 8-OHdG levels.

2.3 Statistical methods

SPSS 20.0 software was used to input and analyze data, measurement data analysis was by t test and P<0.05 indicated statistical significance in differences.

3. Results

3.1 miR-210 expression levels in spinal neurons after H2O2 treatment

miR-210 expression level in group A was (1.04±0.14) and miR-210 expression level in group B was (3.57±0.62). t test analysis of the differences in miR-210 expression levels between group A and group B showed that miR-210 expression level in group B was significantly higher than that in group A, and differences in miR-210 expression levels were statistically significant between two groups (P=17.832, P<0.05).

3.2 Spinal neuron viability after miR-210 inhibitor combined with H2O2 treatment

Analysis of spinal neuron viability of group C, group D and group E after 12 h, 18 h and 24 h of treatment was as follows: OD values of three groups were significantly different after 12 h, 18 h and 24 h of treatment, OD values of group D after 12 h, 18 h and 24 h of treatment were significantly lower than those of group C, OD values of group E after 12 h, 18 h and 24 h of treatment were significantly higher than those of group D, and differences in pairwise comparison of OD values were statistically significant after 12 h, 18 h and 24 h of treatment (P<0.05).

Table 1. Comparison of spinal neuron viability.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Repeated batches</th>
<th>After 12 h of treatment</th>
<th>After 18 h of treatment</th>
<th>After 24 h of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>5</td>
<td>0.74±0.11</td>
<td>1.05±0.14</td>
<td>1.42±0.18</td>
</tr>
<tr>
<td>Group D</td>
<td>5</td>
<td>0.45±0.07</td>
<td>0.58±0.08</td>
<td>0.89±0.12</td>
</tr>
<tr>
<td>Group E</td>
<td>5</td>
<td>0.68±0.09</td>
<td>0.83±0.10</td>
<td>1.25±0.16</td>
</tr>
<tr>
<td>F</td>
<td>6.938</td>
<td>8.303</td>
<td>7.658</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

*: compared with group C, P<0.05; #: compared with group D, P<0.05.

Table 2. Comparison of oxidative stress molecule levels in spinal neurons.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Repeated batches</th>
<th>Anti-oxidative stress molecules (ng/mL)</th>
<th>Oxidative stress products (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HO-1</td>
<td>NOX-2</td>
</tr>
<tr>
<td>Group C</td>
<td>5</td>
<td>103.62±13.52</td>
<td>57.39±7.24</td>
</tr>
<tr>
<td>Group D</td>
<td>5</td>
<td>35.57±4.62*</td>
<td>21.38±3.35*</td>
</tr>
<tr>
<td>Group E</td>
<td>5</td>
<td>64.21±8.79†</td>
<td>37.59±5.49†</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*: compared with group C, P<0.05; †: compared with group D, P<0.05.
3.3 Oxidative stress molecule levels in spinal neurons after miR–210 inhibitor combined with H₂O₂ treatment

Analysis of anti-oxidative stress molecules HO-1, NOX-2 and CAT among group C, group D and group E after 24 h of treatment was as follows: HO-1, NOX-2 and CAT levels in group D were significantly lower than those in group C while HO-1, NOX-2 and CAT levels in group E were significantly higher than those in group D; analysis of oxidative stress products ROS and 8-OHdG was as follows: ROS and 8-OHdG levels in group D were significantly higher than those in group C while ROS and 8-OHdG levels in group E were significantly lower than those in group D. Differences in pair-wise comparison of HO-1, NOX-2, CAT, ROS and 8-OHdG levels among three groups were statistically significant (P<0.05).

3.4 Apoptotic molecule expression levels in spinal neurons after miR–210 inhibitor combined with H₂O₂ treatment

Analysis of apoptotic molecules c-fos, c-jun, AP1 and caspase-3 among group C, group D and group E after 24 h of treatment was as follows: c-fos, c-jun, AP1 and caspase-3 levels in group D were significantly higher than those in group C while c-fos, c-jun, AP1 and caspase-3 levels in group E were significantly lower than those in group D. Differences in pair-wise comparison of c-fos, c-jun, AP1 and caspase-3 levels among three groups were statistically significant (P<0.05).

3.5 Injury marker molecule expression levels in spinal neurons after miR–210 inhibitor combined with H₂O₂ treatment

Analysis of nerve injury marker molecules GAP-43, synapsin-I, PSD-93 and PSD-95 among group C, group D and group E after 24 h of treatment was as follows: GAP-43 and synapsin-I levels in group D were significantly lower than those in group C while PSD-93 and PSD-95 levels were significantly higher than those in group C; GAP-43 and synapsin-I levels in group E were significantly higher than those in group D while PSD-93 and PSD-95 levels were significantly lower than those in group D. Differences in pair-wise comparison of GAP-43, synapsin-I, PSD-93 and PSD-95 levels among three groups were statistically significant (P<0.05).

4. Discussion

Oxidative stress is an important pathophysiological change in local spinal cord injury, and the oxygen free radicals massively produced during the process of oxidative stress are the key molecules causing neuron damage and synaptic structure damage. Oxygen free radicals generated in local spinal cord injury can not only directly result in tissue and cell damage, but can also cause the changes in expression of apoptotic molecules, anti-apoptotic molecules and neural functional molecules[6,7]; miRNA is a type of small non-coding RNA discovered in recent years, and it regulates the expression of multiple genes so as to affect the biological behavior and function of cells. MiR-210 is a kind of miRNA associated with oxidative stress, cell apoptosis, energy metabolism and other cell behaviors[7–9], but there is no clear report about the change in miR-210 expression in local tissue after spinal cord injury. In the study, H₂O₂ treatment of spinal neurons was adopted to simulate the oxidative stress injury of spinal cord, and the analysis of miR-210 expression levels showed that miR-210 expression level in group B was significantly higher than that in group A. This means that oxidative stress injury will increase the expression of miR-210 in spinal neurons. Thus it is speculated that the up-regulated miR-210 in spinal neurons may affect the cell viability, apoptosis and oxidative stress so as to cause nerve damage, and inhibition of miR-210 may ease neuron damage.

In order to further confirm the role of miR-210 in spinal neuron injury and whether inhibition of miR-210 had neuroprotective effect, transfection of miR-210 inhibitor was adopted to treat the spinal neurons, then cell viability was analyzed, and the results showed that after H₂O₂ treatment combined with negative control sequence transfection, the spinal neuron viability decreased significantly; after H₂O₂ treatment combined with miR-210 inhibitor transfection, the spinal neuron viability was recovered to a certain degree. This means that H₂O₂ can cause spinal neuron damage, and the use of miR-210 inhibitor can antagonize the damage effect of H₂O₂ and protect spinal neurons. H₂O₂ mainly simulates oxidative stress injury and can cause increased generation of reactive oxygen species and DNA oxidative damage product (8-OHdG) in local tissue[10]. Antioxidant enzyme HO-1/NOX2 in neurons can directly scavenge reactive oxygen species, CAT can directly scavenge hydrogen peroxide, and in the process of massive production of reactive oxygen species, HO-1/NOX2 and CAT are constantly consumed[11,12]. In the study, the analysis of above oxidative stress products and antioxidant molecules showed that HO-1, NOX-2 and CAT levels in group D were significantly lower than those in group C while ROS and 8-OHdG levels were significantly lower than those in group C, and the analysis of miR-210 expression levels showed that miR-210 expression level in group B was significantly higher than that in group A. This means that oxidative stress injury will increase the expression of miR-210 in spinal neurons. Thus it is speculated that the up-regulated miR-210 in spinal neurons may affect the cell viability, apoptosis and oxidative stress so as to cause nerve damage, and inhibition of miR-210 may ease neuron damage.

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levels were significantly higher than those in group C; HO-1, NOX-2 and CAT levels in group E were significantly higher than those in group D while ROS and 8-OHdG levels were significantly lower than those in group D. This means that H$_2$O$_2$ can increase the generation of reactive oxygen species and enhance peroxidation, and the use of miR-210 inhibitor can reduce the peroxidation caused by H$_2$O$_2$.

Oxidative stress injury caused by H$_2$O$_2$ can cause spinal neuron apoptosis and the corresponding neural function damage[13]. c-fos and c-jun are the key genes adjusting the neuron apoptosis, and both belong to the mys family and are lowly expressed under physiological state; when the neurons are affected by oxidative stress and other factors, c-fos and c-jun are activated and massively expressed, then increase the expression of Caspase-3 and induce neuron apoptosis through the AP-1 complex[14,15]. In the study, analysis of the above apoptosis-related molecule expression levels in cells proved that after H$_2$O$_2$ treatment combined with negative control sequence transfection, c-fos, c-jun, AP1 and caspase-3 expression levels significantly increased; after H$_2$O$_2$ treatment combined with miR-210 inhibitor transfection, c-fos, c-jun, AP1 and caspase-3 expression levels were significantly suppressed. This means that H$_2$O$_2$ can cause spinal neuron apoptosis, and the use of miR-210 inhibitor can alleviate the cell apoptosis caused by H$_2$O$_2$.

Neuron apoptosis can affect its function, the performance is the change in expression of a variety of functional molecules, GAP-43 and synapsin-I are directly related to the neuron function, the former is a marker molecule of neuron growth and axon regeneration, and the latter has the function of protein kinase and can induce the presynaptic membrane depolarization and vesicle release[16,17]; both PSD-93 and PSD-95 belong to postsynaptic density, and the excessive expression can generate nervous excitotoxicity and cause synaptic function damage[18]. In the study, analysis of above neuron function-related molecules confirmed that GAP-43 and synapsin-I levels in group D were significantly lower than those in group C while PSD-93 and PSD-95 levels were significantly higher than those in group C; GAP-43 and synapsin-I levels in group E were significantly higher than those in group D while PSD-93 and PSD-95 levels were significantly lower than those in group D. This means that H$_2$O$_2$ can cause the spinal neuron function damage, and the use of miR-210 inhibitor can reduce the cell function damage caused by H$_2$O$_2$.

To sum up, oxidative stress injury can cause high miR-210 expression in spinal neurons, and miR-210 inhibitor can reduce the neuron injury caused by oxidative stress.

References


