Targeting the Pyk2/MCU Pathway, Ruthenium Red and Nimodipine Alone and in Combination Evoked Neuro-Protective Effect in Experimentally Induced Brain Stroke in Rat

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Abstract

Mitochondrial dysfunction caused by Ca2+ overload plays an essential role in ischemia associated with brain damage. The proline rich tyrosine kinase 2 (pyk2)/Mitochondrial Calcium Uniporter (MCU) pathway is essential in cerebral ischemic stroke, and is responsible for mitochondrial damage and neuronal degeneration. The present study aimed to investigate the neuroprotective potentials of ruthenium red and nimodipine in brain ischemia induced by Middle Cerebral Artery Occlusion (MCAO). Ruthenium Red (RR), Nimodipine (NMD) and their combination effectively decreased the brain ischemic changes and mitigated the neuronal damage evidenced by reversing the brain histopathological aberrations and decreasing percentage of infraction volume. At the same time, both agents and their combination improved cerebral blood flow through down regulation of Pyk2 and MCU gene expression in brain tissue. They also opposed the inflammatory load via attenuation of brain myeloperoxidase activity, lowered lipid peroxides, NAD(P)H dehydrogenase quinine 1 (NQO1) and nitric oxide and boosted the brain glutathione. To the greatest of our knowledge, this is the first study to reveal the neuroprotective effects of RR, NMD and their combination in cerebral brain stroke model.

Keywords: Ruthenium red; Nimodipine; Middle cerebral artery occlusion; Pyk2/MCU pathway

Introduction

Cerebral brain stroke is a universal brain injury. Stroke is a major cause of death over the world. An ischemic stroke caused by either a sudden reduction or complete blockage of cerebral flow of the blood accounts for 85% of all cerebral stroke patients [1]. There are several cerebral ischemia pathophysiology mechanisms, such as the discharge of excitatory neurotransmitters, increase of oxidative stress, intracellular calcium levels, inflammation and apoptosis [2]. Oxidative stress, which plays an essential role in the pathogenesis of cerebral ischemia, is caused by a difference between the manufacturing of ROS and its useful removal by endogenous scavenger enzymes and protective antioxidants [3]. The mitochondrion is the major participant in cell death, and is specifically caught up in ischemia-induction of neuronal damage [4].

In brief, mitochondrial damage and morphological changes include (a) activation of the Mitochondrial Permeability Transition Pore (MPTP); (b) release of cytochrome C and apoptotic inducible factor [5]; (c) decreased production of ATP and increased production of free radicals by dysfunction of mitochondrial respiratory chain [6]; and (d) mitochondrial Ca2+ overload, which activates proteases and finally ends with cell death. The regulatory mechanisms underlying the increase in the concentration of cytoplasmic Ca2+ after brain infarction-induced Ca2+ overload are well understood and include the release of free radicals, activation of calcium-dependent kinases, and start of apoptosis. These processes ultimately result in neuronal death.

Mitochondrial Calcium Uniporter (MCU) is a complex uniporter channel that mediates Ca2+ uptake into the mitochondrial matrix to regulate cell metabolism, cytoplasmic calcium signaling, and cell death [7]. Knockdown of MCU has been proved to reduce mitochondrial Ca2+ influx, protect mitochondrial function, and prevent neuronal death [8]. Proline-rich tyrosine kinase 2 (Pyk2) is a 116-kDa cytoplasmic tyrosine kinase that is a member of the focal adhesion kinase family [9].
Previous studies have confirmed that activation of Pyk2 in cerebral ischemia regulates N-Methyl-D-Aspartate-type (NMAD) glutamate receptor activity and Ca2+ dynamics, which may be responsible for ischemic stroke-induced neuronal death [10,11].

However, further in vivo experiments are required to better recognizing the function of the MCU in neurodegeneration and the application of the MCU antagonist in neurodegenerative disorder therapy.

Ruthenium red has an effect on inhibition of mitochondrial uniporter and selective intracellular Ryanodine Receptor (RyR) antagonist [12]. Ruthenium Red (RR) has been investigated to prevent the brain injury after cerebral ischemic stroke by inhibiting mitochondrial calcium uniporter which participates in ischemic damage [13]. In past reports ruthenium red has been studied to provide beneficial properties by declining brain inflammation and oxidative stress [14,15].

One of well-known calcium channel blocker is the Nimodipine (NMD). Human body naturally responds to hemorrhage by contracting the blood vessels to slow blood flow from the injured site. Conversely, stopping blood flow when the hemorrhage is in the brain sites causes more brain damage. NMD is thought to do its action by providing relaxation in the contracted blood vessels near the area sites causes more brain damage. NMD could be efficient in minimizing ischemia/reperfusion injury in the tissue of the ovary [16].

The purpose of the present study is to investigate the neuro-protective potentials of ruthenium red, nimodipine and their combination in brain ischemia induced by Middle Cerebral Artery Occlusion (MCAO).

**Material and Methods**

**Animals and surgical preparation**

All the study protocols were accepted by The Ethical Committee of Faculty of Pharmacy, Alazhar University for girls, Egypt. Adult male Sprague-Dawley rats weighing 240 g to 270 g were used. Left cerebral artery occlusion was done to induce cerebral ischemia, as reported by Cummins & his co-workers [17].

Chloral hydrate was used for anesthesia in dose of 350 mg/kg, intraperitoneally; middle neck incision was done followed by left carotid artery occlusion using 0.5 cm stainless steel bulldog [18]. The occlusion lasted for one hour and the skin was closed in this period for induction of ischemia then reperfusion was done for this artery for 24 hours. During the operation, the body temperature was kept at 37°C ± 0.5°C with a heating lamp and blanket. The sham-operated rats were handled similarly, except the left cerebral artery was not occluded after the neck incision.

**Chemicals**

Ruthenium red and nimodipine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nimodipine was dissolved in 50% dimethyl sulfoxyl but ruthenium was dissolved in physiological saline.

**Design of the work**

Sixty four male rats were randomly divided into 8 groups (n=8): (1) sham group, (2) vehicle group, (3) MCAO group, (4) NMD group alone (3 mg/kg, i.p.), (5) NMD with MCAO group, (6) RR group alone (3 mg/kg, i.p.) (7) RR with MCAO group, (8) NMD and RR combination with MCAO group.

**Determination of infarct volume**

Twenty four hour after reperfusion animals were scarified and brains were collected rapidly and frozen at -80°C for 5 min. Brain slices were done at 2 mm from the frontal tips, and slices were dipped in 2% 2,3,5-Triphenyltetrazolium Chloride (TTC) at 37°C for 20 min [19]. After the staining, high quality images of these slices were taken and the software image J was used to calculate the size of infarction. Percentage infarct volume was calculated using the equation: [(VC-VL)/VC]*100, where VC is the volume of control hemisphere and VL is the volume of non-infarcted tissue in thelessoned hemisphere [19].

**Determination of NAD(P)H dehydrogenase (quinone 1)**

Animals were killed followed by brains collection out of the skull. Immediately after removal, the brain from each rat was cleaned with cold normal saline and used to make a 10% (w/v) homogenate in ice-cold 0.05 M potassium phosphate buffer (pH 7.4) using Glas-Col motor driven homogenizer (Glas-Col Co., CA, USA). This kit uses a monoclonal antibody that binds competitively to NQO1 in the standard or samples. After incubations at room temperature immediately the excess reagents were removed and substrate was added. When a short incubation time was finished the enzymatic reaction was blocked and the yellow color generated is read on BioTek micro plate reader at 405 nm [20]. The intensity of the yellow color is inversely proportional to the concentration of NQO1 in either standard or samples. The measured optical density is used to calculate the concentration of NQO1.

**Determination of gene expression of MCU and pyk2**

RNA was extracted using (NucleoSpin®) kit. Total RNA was then extracted by using RNA Purification Kit NucleoSpin Filter (violet ring according to the manufacturer’s instructions. NucleoSpin® RNA Column placed into a collection tube (1.5 ml, supplied with kit) then RNA eluted in 60 µl DNase -free H2O, and centrifuged at 11,000 rpm for 1 min. The clarity (A260/A280 ratio) and the RNA concentration were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The purified and extracted DNA samples were stored at -80°C for further use [21]. Target genes were detected by real-time quantitative (q) PCR (Table 1).

**Determination of malonaldehyde MDA**

Malondialdehyde (MDA) content was according to the method of Satoh [22].

**Determination of brain Myeloperoxidase (MPO) content**

The method described by Bradley et al. [23] was used for the determination of myeloperoxidase formation in Brain homogenate.

**Determination of Nitric Oxide (NO)**

The Bio-diagnostic Nitric Oxide reagent kit was used for the determination of NO in brain homogenate according to the method described by Miranda et al. [24].

**Determination of Glutathione (GSH)**

The level of glutathione was determined in brain homogenate using Biodiagnostic kit (Cairo, Egypt) according to the method described by Beutler [25].

**Histopathological analysis**

It is done according to Banchoft et al. [26]. The brain of rats in...
different groups is used to make autopsy samples followed by fixation step using 10% formal saline for 24 h.

Statistical analysis
The data were expressed as means ± SEM. Statistical analysis was done by GraphPad Prism software (version 5, San Diego, CA, USA); the accepted probability level was less than 0.05 as statistically significant. One-way analysis of variance ANOVA was used for comparison between means followed by Tukey-Kramer multiple comparison tests.

Results

NMD and RR decreased the over expression of MCU and pyk2 caused by MCAO

As shown in Figure 1A, 1B the MCAO operation in rats caused significant increases in MCU by (48.19%) and pyk2 (57.63%) after one hour of reperfusion as compared to sham group. In contrast, administration of NMD (10 mg/kg, i.p.) for three days before MCAO significantly reduced the elevated levels of MCU and pyk2 in brain tissue by (64.15%) and (42.478%) respectively in comparison with MCAO inducted group. Similarly, treatment with RR (3 mg/kg; i.p.) for three days before MCAO significantly reduced the elevated levels of MCU and pyk2 in brain tissue by (41.16%) and (25.53%) respectively as compared to MCAO inducted group. Interestingly the combination of RR (1.5 mg/kg; i.p.) and NMD (5 mg/kg; i.p.) provides synergistic effects by reducing MCU and pyk2 content to (42.89%) and (52.75%) respectively in comparison with MCAO inducted groups.

NMD and RR decreased NQO1 and MPO expression induced by MCAO

The MCAO operation in rats resulted in significant increases in NQO1 by (66.6%), and MPO by (37.89%) after one hour of reperfusion as compared to sham group. In contrast, administration of NMD (10 mg/kg, i.p.) for three days before MCAO significantly reduced the elevated levels of NQO1 and MPO in brain tissue by (55%) and (30.95%) respectively in comparison with MCAO inducted group. Similarly, treatment with RR (3 mg/kg; i.p.) for three days before MCAO significantly reduced the elevated levels of NQO1 and MPO in brain tissue by (43.14%) and (18.5%) respectively as compared to MCAO inducted group. Interestingly their combination of RR (1.5 mg/kg; i.p.) and NMD (5 mg/kg; i.p.) provides synergistic effects by reducing NQO1 and MPO content by (65.87%) and (29.9%) respectively in comparison with MCAO inducted groups (Figure 2A, 2B).

NMD and RR reversed MDA, GSH and NO attenuation induced by MCAO

The MCAO operation in rats caused significant increases in MDA by (47.12%), and NO (37.89%) but significant decrease in GSH by (37.26%) after one hour of reperfusion as compared to sham group.
In contrast, administration of NMD (10 mg/kg, i.p.) for three days before MCAO significantly reduced the elevated levels of MDA and NO in brain tissue by (28.79%) and (34.70%) respectively and significantly increased the reduced level of GSH by (36.68%) in comparison with MCAO induced group. Interestingly their combination of RR (1.5 mg/kg, i.p.) and NMD (5 mg/kg, i.p.) provide synergistic effect by reducing percentage of infraction volume by (99.04%) in comparison with MCAO induced groups (Figure 3). The RR, NMD, and their combination decreased the infraction volume when the brain sections are stained using TTC stain showing higher intensity of red color (Figure 4).

**Effect of MCAO inducted group on histopathological findings of brain tissue**

Sections in the brain of animals in the sham group and Dimethyl Sulfoxyl (DMSO) control group revealed normal tissue structure in cerebral cortex of brain tissues respectively (Figure 5A, 5B). On the other hand, induction of MCAO revealed nuclear pyknosis, degeneration and congestion in blood vessels in some neurons at cerebral cortex (Figure 5E). Treatment with Nimodipine (NMD) (10 mg/kg i.p. 3 days; Figure 5C) and Ruthenium Red (RR) (3 mg/kg; i.p. 3 days; Figure 5D) showed normal histopathological structure at cerebral cortex of the brain tissues compared to MCAO inducted group.

Sections in the brain of animals that were injected with NMD (10 mg/kg, i.p.) for three consecutive days then were inducted with MCAO revealed nuclear pyknosis and degeneration in some neurons in cerebral cortex (Figure 5F). In addition to treatment with Ruthenium Red (RR) (3 mg/kg; i.p) for three consecutive days then were inducted with MCAO revealed normal histopathological structure in all parts of the brain (Figure 5G). Treatment with combination of RR (1.5 mg/ kg; i.p.) and NMD (5 mg/kg; i.p.) for three consecutive days then induction of MCAO revealed normal histopathologic structure in most of the brain parts (Figure 5H).

**Discussion**

Brain ischemia is one of the top causes of losing the life and the third important cause of disability all over the world [27]. In brain stroke, there is a leakage of blood delivery to brain areas which cause degeneration in cerebral neuronal tissues [28]. MCAO is a classical model of cerebral ischemia that causes regional brain infarction and neurological deficits in rats by blocking the middle cerebral artery. Middle Carotid Artery Occlusion (MCAO) is highly effective model in stroke induction by unilateral or bilateral occlusion [29,30].

### Table 1: Primers used in the study.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence from 5'-3'</th>
<th>Gene bank accession number</th>
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</thead>
</table>
| PYK2 | F: AGAGCCCCAAGGTGACCAT  
R: GATACGATTGTAGTAGG | AF063890.1 |
| MCU | F: ATTCGATAGCCGGTTGCCC  
R: GTTGGTGCAAGTGTCGCCCTC | NM_001106398.1 |
| β-actin | F: ATGGATGACGATATCGCTGC  
R: CTTCTGCCACATCCACCA | NM_031144.3 |

### Table 2: The effects of RR, NMD, and their combination on brain content of MDA, GSH, NO in MCAO inducted group. Data are expressed as mean ± S.E. *p<0.05 vs. sham group, 'p<0.05 vs. MCAO group, 'p<0.05 vs. combination group.

<table>
<thead>
<tr>
<th>Parameters/gps</th>
<th>MDA &quot;nmol/g tissue&quot;</th>
<th>GSH &quot;mg/g tissue&quot;</th>
<th>NO &quot;nmol/g tissue&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10.56 ± 0.63</td>
<td>118.30 ± 7.82</td>
<td>40.1 ± 0.87</td>
</tr>
<tr>
<td>DMSO</td>
<td>12.32 ± 0.41</td>
<td>120 ± 4.99</td>
<td>40.84 ± 1.42</td>
</tr>
<tr>
<td>NMD</td>
<td>12.683 ± 0.46</td>
<td>121.2 ± 6.22</td>
<td>41.55 ± 1.29</td>
</tr>
<tr>
<td>RR</td>
<td>13.68 ± 0.79</td>
<td>133.9 ± 5.96</td>
<td>41.37 ± 1.18</td>
</tr>
<tr>
<td>MCAO</td>
<td>19.97 ± 0.39*</td>
<td>74.21 ± 4.45*</td>
<td>64.57 ± 2.78*</td>
</tr>
<tr>
<td>MCAO+NMD</td>
<td>14.22 ± 0.59**</td>
<td>118.5 ± 5.20 b</td>
<td>42.16 ± 1.14 b</td>
</tr>
<tr>
<td>MCAO+RR</td>
<td>15.92 ± 0.44**</td>
<td>117.2 ± 5.07 b</td>
<td>44.85 ± 1.05 b</td>
</tr>
<tr>
<td>Combination</td>
<td>14.82 ± 1.01**</td>
<td>124.8 ± 7.73b</td>
<td>41.92 ± 1.10b</td>
</tr>
</tbody>
</table>
In this study, a significant damage for neuronal cells, increase in inflammatory and oxidative stress markers, increase in expression of MCU/pyk2 pathway and marked histopathological alterations in the brain tissue were observed in MCAO induced rats, findings that are in agreement with previous work who confirmed that there was an activation in Pyk2/MCU pathway, increase infarction volumes of brain and reactive oxygen species in an in vivo model of rat cerebral ischemia [30].

These results may be explained as Ca\textsuperscript{2+} overload plays an important role in ischemia-induced brain damage, MCU, located on the mitochondrial inner membrane, is the major channel responsible for mitochondrial Ca\textsuperscript{2+} uptake. Pyk2 can directly phosphorylate MCU, which enhances mitochondrial Ca\textsuperscript{2+} uptake. Thus Pyk2/MCU pathway may play a vital role in cell apoptosis and neuronal death [30].

In addition the MCU functions as an enhancer of inflammation by increasing the calcium concentration of mitochondria and up-regulation of endoplasmic reticulum stress [31].

In this study it is observed that RR, NMD and their combination specifically attenuated neuronal oxidative stress, inflammation and decreased expressions of MCU/pyk2 genes molecules.

These results were augmented by that showing nimodipine; regulator of calcium homeostasis has neuroprotective effect against oxidative stress and iNOS induction in acute model of cerebral ischemia in hippocampal slices, increasing antioxidant and anti-inflammatory enzyme heme-oxygenase [32].

The effects of RR on neurons are still under debate. Some studies discovered the toxicity of neurons from it, while other studies found it to have a neuroprotective effect [33,34].

Zhao et al. [35] proved that RR has neuroprotective actions and led to a significant reduction in cerebral infarction during MCAO that may be attributed to its blocking action of MCU which has crucial role in brain ischemia as illustrated previously [35].

This study proved that the treatment with RR alone, NMD alone or their combination are effective as a protective treatment in case of cerebral brain stroke through several mechanisms. The mechanisms involved in this study are decreasing the inflammation, oxidative stress, infraction volume targeting MCU/pyk2 pathway.

**Conclusion**

According to our results we can conclude that, RR, NMD and their combination could ameliorate MCAO-induced brain injury; that might be attributed to their potent antioxidant and anti-inflammatory activities via MCU/pyk2 pathway.

**References**


