Abstract. – OBJECTIVE: To investigate the effects of hyperkalemia on the brain after I/R in transient middle cerebral artery occlusion (tMCAO) model.

MATERIALS AND METHODS: A total of 120 adult male SD rats were randomly assigned to four groups: (1) hyperkalemia 80 µg/g (HK80) group; (2) hyperkalemia 40 µg/g (HK40) group; (3) normal saline (NS) group; (4) sham (SH) group. The concentration of serum K+ was elevated in HK80 and HK40 groups. The transient middle cerebral artery occlusion (tMCAO) model was used to assess the effect of hyperkalemia on the brain after I/R. After 24 h reperfusion, the infarct volume and cell damage of rat’s I/R brain tissue sections were analyzed. The concentration of K+, Ca2+ and calmodulin (CaM), the activity of Ca-ATPase, the expression of Western blot of Ca2+/calmodulin-dependent protein kinase II (CaMKII) and Na+/Ca2+ exchanger 1 (NCX1), were also measured.

RESULTS: After 24 h reperfusion, compared with NS group, the two-hyperkalemia groups (HK80 and HK40) were with less infarct volume and cell damage, higher concentration of K+ but lower Ca2+ and CaM compared with NS group. The activity of Ca-ATPase was also elevated, the expression of CaMK II and NCX1 were down-regulated in the two hyperkalemia groups.

CONCLUSIONS: Hyperkalemia could also ameliorate the brain I/R injury by alleviating calcium overload inhibiting the activity of NCX1, lowering the concentration of Ca2+.

Key Words: Hyperkalemia, Ischemia/reperfusion injury, Calcium overload.

Introduction

Despite decades of research and clinical practice, acute ischemic stroke is still one of the most frequent causes of death and permanent disability worldwide, as the result of irreversible brain injury and loss of neuronal function. Even if the recanalization is successful poor prognosis often exists because of ischemia/reperfusion (I/R) injury, which mainly involves the release of nitric oxide, excitatory amino acids, cytokine, free radicals, mitochondrial respiratory enzymes damage, calcium overload and induction of programmed cell death.

Among the several factors related to the I/R injury, calcium overload needs to be focused on. Additionally, calcium overload and the function of Na+/Ca2+ exchanger (NCX) have been considered with intimate relationship during the period of ischemia and reperfusion. Researches have indicated that inhibiting the “Ca2+ entry mode” of NCX could have the protective effect during the brain ischemia.

Though the pathophysiologic mechanism of neural injury had been well-documented and several neuroprotective agents had been reported, few human clinical or in vivo trials had produced a positive outcome. However, in the field of cardioprotection, the condition of hyperkalemia had been proven effective on ameliorating I/R injury of myocardium both in vivo and in vitro. In the clinical practice, elevating the concentration of potassium to a certain degree could effectively prevent the reperfusion malignant arrhythmia, with the reasons of preventing or reversing myocardium ischemic contracture, recovering the myocardium contraction diastolic function, alleviating calcium overload and intracellular potassium loss during reperfusion.

Furthermore, as referred by some scholars, we found that the mice administrated with hyperkalemia after a long time of cardiac arrest could still be return of spontaneous circulation and with less nervous system damage. In the clinical practice, we can also find that some
patients (such as acidosis, acute kidney injury and gout) with hyperkalemia suffered from cardiac arrest, and even after a long time of cardiopulmonary resuscitation, some of them could also be return of spontaneous circulation and without nervous system damage.

Since the effect of hyperkalemia has been widely use in the cardioprotection field but seldom has been reported on brain I/R injury, here we suggest that the condition of hyperkalemia might also ameliorate the brain I/R injury during acute ischemia stroke and after reperfusion by alleviating calcium overload. To test our proposal, firstly we elevated the rat’s concentration of potassium on purpose, and then used the transient middle cerebral artery occlusion (tMCAO) model to assess the effect of hyperkalemia on the brain after I/R.

Materials and Methods

Animals
All procedures involving animals were approved by the Institutional Animal Care Committee and the Ethical Commission of Guangxi Medical University and were performed in accordance with published National Institutes of Health guidelines. Adult male Sprague-Dawley (SD) rats, aged 6-8 weeks and weighing 230-280 g, were obtained from the Experimental Animal Center of Guangxi Medical University (License No: SCXK GUI 2009-0002; Nanning, China), and housed at room temperature in a 12:12 light dark cycle with ad libitum access to food and water.

Experimental Groups and tMCAO
A total of 120 adult male SD rats were randomly assigned to four groups: (1) hyperkalemia 80 µg/g (HK80) group (n = 30): rats were intravenous administrated with 2.5% potassium chloride 80 µg/g then underwent focal I/R; (2) hyperkalemia 40 µg/g (HK40) group (n = 30): rats were intravenous administrated with 1.25% potassium chloride 40 µg/g then underwent focal I/R; (3) normal saline (NS) group (n = 30): rats were intravenous administrated with the equal volume of NS as hyperkalemia groups then underwent focal I/R; (4) sham (SH) group (n = 30); rats underwent the same surgical procedure without intravenous administrated or I/R.

Rats were subjected to perform tMCAO model with the method used by Longa et al25. Briefly, rats were anesthetized with 10% chloral hydrate (30 mg/kg, i.p.). Then, tMCAO was carried out using an intraluminal thread introduced via external carotid artery (ECA). A surgical mid-line incision was made to expose the right common carotid artery (CCA), internal carotid artery (ICA) and ECA. Before ischemia, rats in HK40 and HK80 groups were pumped 3.2 mL/kg 1.25% potassium chloride and 2.5% potassium chloride solution, respectively at the speed of 8 mL/h with infusion pump via right jugular vein. The NS group received equal volume and speed of NS while the SH group without any administration via right jugular vein. After occluding the right CCA by a vascular clamp, a 4-0-mono-filament nylon suture with approximately 0.26 mm round in diameter was inserted into the right ECA lumen and gently advanced into the ICA up to a point approximately 18-20 mm. After 90 min of ischemia26, the nylon suture was slowly removed from the artery and the vascular clamp was removed. After removing the jugular catheter, the incision on the neck was closed in the end. Using an automatic homeothermic blanket control unit, the rat’s body temperature was continually monitored and maintained at 37±0.5°C throughout the surgical procedure and post-surgery recovery.

Infarct Volume Measurement
After 24 h of reperfusion, rats were euthanized by 10% chloral hydrate (300 mg/kg, i.p.). The brains were removed rapidly and frozen at -20°C for 30 min, and dissected. Coronal slices (2 mm in thickness) were acquired from frozen forebrain using a rodent brain matrix slicer. Tissue sections were then immediately stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate-buffered saline (PBS) at 37°C for 20 min in the dark. The sections were soaked in 4% paraformaldehyde phosphate buffer for 1 h and scanned. The percent of infarct area of the entire brain represented the degree of cerebral infarction. Normal brain tissues were stained red, while the unstained area (white) was considered to be the infarct area. Stained sections were imaged using a digital camera (Nikon E5100, Tokyo, Japan) and quantified using ImageJ software (version 1.37c, NIH). The infarct volume was computed as the sum of the measured infarct areas of the evenly sliced (2 mm) brain sections (Simpson’s rule). The percent of infarction is revealed by the equation: % infarct area = infarct area/total area of slice × 100%.
Histopathological Analysis

After 24 h of reperfusion, rats were euthanized by 10% chloral hydrate (300 mg/kg, i.p.) and intracardially perfused with PBS (pH 7.4, 4°C). The brains were removed and fixed in 4% paraformaldehyde overnight at 4°C. After paraffin embedding of coronal sections (6 μm), the sections were stained with hematoxylin and eosin (HE). Pathological and histological changes were observed through a light microscope (Olympus, Tokyo, Japan) at the magnification of ×100 and ×400, and then documented by digital photography.

Concentration of K⁺, Ca²⁺ and Calmodulin (CaM), and the Activity of Ca-ATPase

Before ischemia, the serum in rats (n=6 per group) was collected at 5 min after administration by the right jugular vein. Then, the serum was centrifuged and the supernatant was collected to detect the concentration of serum K⁺ and Ca²⁺ (mmol/L). After 24 hours of reperfusion, rats (n = 6 per group) were euthanized with 10% chloral hydrate (300 mg/kg, i.p.). Then, the rats were decapitated, right cerebral cortex was made into homogenate and the supernatant was collected to detect concentration of K⁺, Ca²⁺ and CaM, and the activity of Ca-ATPase. The concentration of K⁺ (mmol/gprot), Ca²⁺ (mmol/gprot) and CaM (mmol/gprot) were detected with K⁺ kit, Ca²⁺ kit and CaM kit (Nanjing JianCheng Bio Company, Nanjing, China); the activity of Ca-ATPase (U/mgprot) was detected by Ca-ATPase kit (Nanjing JianCheng Bio Company, Nanjing, China); the protein content (mg/L) in the corresponding tissue was detected with Coomassie Brilliant Blue protein assay kit (Nanjing JianCheng Bio Company, Nanjing, China).

Western Blot Analysis and the Expression of Ca²⁺/Calmodulin-Dependent Protein Kinase II (CAMKII) and NCX1

The rat’s I/R hemispheres (n=6 per group) were removed rapidly after saline perfusion and rinsed in NS (4°C) to wash away the blood and blood clot. After that, parts of cortex were homogenized in radioimmunoprecipitation assay (RIPA) buffer. The lysate was centrifuged at 12000 g at 4°C for 20 min, and the supernatant was collected. The protein concentration was estimated by the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Bioengineering Inc., Shanghai, China). Next, per the manufacturer’s instructions, 200 µg samples were subjected to sodium dodecyl sulphate (SDS) Loading Buffer (Solarbio; Beijing, China) at a proportion of 3:1 and boiled at 100°C for 5 min, loaded into sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, with 50 µg/well. PAGE was conducted at 60 V for spacer gel and 100 V for separation gel, and the protein samples were transferred to a polyvinylidene difluoride (PVDF) membranes (pore size, 0.22 mm; Immobilon-PSQ) membrane at 200 mA for 60 min in a tank transfer system. Membranes were blocked using 5% nonfat dry milk (0.5 g milk is dissolved into 10 mL TSBT solution), then washed three times in Tris-buffered saline and Tween (TBS-T, 0.5%). The primary antibodies used in the study were CAMKII (1:2,000; Abcam, Cambridge, MA, USA), NCX1 (1:1,000; Abcam, Cambridge, MA, USA) and GAPDH (1:1,000; Abcam, Cambridge, MA, USA); β-actin (1:1,000; CST, Danvers, MA, USA). The membrane was incubated at 4°C overnight. The membrane was washed with TBS-T and the corresponding secondary antibodies were added and incubated with the membrane at room temperature for 1 h, followed by another TBS-T washing 3 times with 5 min. Binding of the protein bands was visualized using LI-COR Odyssey Infrared Imaging System. The gray value was analyzed using ImageJ software with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. The gray ratio of the target band and internal reference band was regarded as the relative expression of the protein.

Statistical Analysis

The data were expressed as means ± SD or percentage. Statistics were performed using the software package statistical product and service solutions (SPSS 21.0, Armonk, NY, USA). The difference among four groups was compared using One-way ANOVA followed by Bonferroni test or Dunnett’s test for post-hoc test. p < 0.05 was considered statistically significant.

Results

Concentration of Serum K⁺ and Ca²⁺ Before Ischemia

Before ischemia, the rat’s concentration of serum K⁺ both in HK80 and HK40 groups were higher than those in NS group (n = 6) (p < 0.05). The higher dosage of potassium chloride administration group (HK80) was with higher
concentration of serum K⁺ ($p < 0.05$). However, the concentration of serum Ca²⁺ was with no significant difference between groups ($p > 0.05$) (Table I).

**Infarct Volume and Cell Damage**

The rat’s infarct volume in the SH group did not exhibit deficit. After 24 h reperfusion, the infarct volume stained with TTC in the two hyperkalemia groups ($n = 6$) were less than the result in NS group ($n = 6$) HK80 vs. NS: 8.72 ± 1.62 vs. 20.71 ± 3.04, $p < 0.05$. HK40 vs. NS: 13.29 ± 2.07 vs. 20.71 ± 3.04, $p < 0.05$). Additionally, compared with HK40 group, HK80 group has less infarct volume (HK80 vs. HK40: 8.72 ± 1.62 vs. 13.29 ± 2.07, $p < 0.05$). The TTC results and the percentage of infarct volume are showed in Figure 1. By observing the HE stain of brain tissue, the two hyperkalemia groups were with more regulated cell and less karyopyknosis compared with the NS group (Figures 2 and 3).

**The Brain Concentration of K⁺, Ca²⁺ and CaM, and the Activity of Ca-ATPase**

Compared with SH group, other three I/R groups were with lower concentration of K⁺ in I/R brain tissue, but with higher concentration of CaM and Ca²⁺ ($n = 6$) ($p < 0.05$). Meanwhile, the two hyperkalemia groups were with higher K⁺ (HK80 vs. NS: 3.88 ± 0.03 vs. 3.67 ± 0.03, $p < 0.05$). HK40 vs. NS: 3.88 ± 0.03 vs. 3.67 ± 0.03, $p < 0.05$). However, HK80 group was with lower concetration of CaM and Ca²⁺ (HK80 vs. NS: 4.72 ± 0.14* vs. 4.19 ± 0.06*, $p < 0.05$). HK40 vs. NS: 4.19 ± 0.06* vs. 3.67 ± 0.03, $p < 0.05$). HK80 vs. HK40: 4.19 ± 0.06* vs. 4.19 ± 0.06*, $p < 0.05$). HK80 vs. HK40: 4.19 ± 0.06* vs. 4.19 ± 0.06*, $p < 0.05$). HK40 vs. NS: 4.19 ± 0.06* vs. 3.67 ± 0.03, $p < 0.05$).

The activity of Ca-ATPase in I/R brain tissue was significantly elevated both in HK80 and HK40 groups compared with NS group ($n = 6$) (HK80 vs. NS: 0.971 ± 0.001 vs. 0.971 ± 0.001, $p < 0.05$). HK40 vs. NS: 0.971 ± 0.001 vs. 0.971 ± 0.001, $p < 0.05$). HK80 vs. HK40: 0.971 ± 0.001 vs. 0.971 ± 0.001, $p < 0.05$) compared with NS group. The results between the two hyperkalemia groups were with no difference ($p > 0.05$).

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**Table I.** Concentration of serum K⁺ and Ca²⁺ before ischemia.

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<thead>
<tr>
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<th>K⁺ [mmol/L]</th>
<th>Ca²⁺ [mmol/L]</th>
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<tbody>
<tr>
<td>SH</td>
<td>3.88 ± 0.03</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>HK80</td>
<td>4.72 ± 0.14*</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>HK40</td>
<td>4.19 ± 0.06*</td>
<td>1.04 ± 0.01</td>
</tr>
<tr>
<td>NS</td>
<td>3.67 ± 0.03</td>
<td>1.04 ± 0.01</td>
</tr>
</tbody>
</table>

SH: sham group; NS: normal saline group; HK80: hyperkalemia group-rats were administrated with 2.5% potassium chloride 80 µg/g; HK40: hyperkalemia group-rats were administrated with 1.25% potassium chloride 40 µg/g. (*Compared with HK40, $p < 0.05$; *Compared with HK40, $p < 0.05$).

Figure 1. The TTC-stained and infarct volume results of each group after 24 h reperfusion (SH: sham group; NS: normal saline group; HK80: hyperkalemia group-rats were administrated with 2.5% potassium chloride 80 µg/g; HK40: hyperkalemia group-rats were administrated with 1.25% potassium chloride 40 µg/g. (*Compared with HK40, $p < 0.05$; *Compared with HK40, $p < 0.05$).
Hyperkalemia on focal cerebral ischemia/reperfusion injury

Western Blot Results of CaMK II and NCX1
The expression of CaMK II and NCX1 in I/R brain tissue was significantly down-regulated both in HK80 and HK40 groups compared with NS group (n = 6) (\(p < 0.05\)). The difference of the expression of CaMK II and NCX1 between the HK80 group and HK40 group was not statistically significant (n = 6) (\(p > 0.05\)). The results of the ratio of CaMK II/\(\beta\)-actin and NCX1/\(\beta\)-actin of each group are showed in Figure 3.

Figure 2. The HE stain of I/R brain tissue of each group after 24h reperfusion at the magnification of \(\times 100\) and 400 (SH: sham group; NS: normal saline group; HK80: hyperkalemia group-rats were administrated with 2.5% potassium chloride 80 µg/g; HK40: hyperkalemia group-rats were administrated with 1.25% potassium chloride 40 µg/g. Normal neurons were orderly arranged with normal morphology and evident nucleus and nucleolus. Abnormal neurons were shrunken and deeply stained).

Figure 3. The results of the ratio of CaMK II/\(\beta\)-actin and NCX1/\(\beta\)-actin of each group (SH: sham group; NS: normal saline group; HK80: hyperkalemia group-rats were administrated with 2.5% potassium chloride 80 µg/g; HK40: hyperkalemia group-rats were administrated with 1.25% potassium chloride 40 µg/g; CaMK II: Ca\(^{2+}\)/calmodulin-dependent protein kinase II; NCX: Na\(^+\)/Ca\(^{2+}\) exchanger. \(p^*\) vs. SH < 0.05; \(p^*\) vs. NS < 0.05).
In this work, we sought to verify our proposal whether hyperkalemia could also ameliorate the brain I/R injury. Our results showed that, by elevating the concentration of potassium to a certain degree before ischemia, the two-hyperkalemia groups were with less brain injury compared with NS group. Additionally, the two-hyperkalemia groups were with lower brain concentration of Ca²⁺ and less expression of NCX1, and the activity of Ca-ATPase was elevated compared with NS group. Furthermore, rats with higher dosage administration of potassium (HK80 group) showed a better trend of prognosis between the two-hyperkalemia groups.

Referring to the previous researches about NCX, we could know that the NCX, which had three subtypes (NCX1, NCX2 and NCX3) was a membrane protein, which involved in regulating the concentration of Ca²⁺. To regulating the cellular environmental homeostasis in neuron and neuroglial cell, the function and distribution of the three subtypes were with selectivity and contributed. The two modes of regulation by NCX termed “Ca²⁺ exit mode” (Ca²⁺ efflux and Na⁺ influx) and “Ca²⁺ entry mode” (Ca²⁺ influx and Na⁺ efflux) depend on the concentration gradient between Ca²⁺ and Na⁺. The activity of NCX partly depends on Na⁺-K⁺-ATPase. When in the ischemia or hypoxia condition, injury occurs in Na⁺-K⁺-ATPase and Ca-ATPase, affecting the function of NCX, leading to the disorder of intracellular concentration of Ca²⁺ and Na⁺, and also resulting in the injury and death of the neuron⁹.

Our results showed that by elevating the concentration of potassium to a certain degree, the expression of NCX1 in the two-hyperkalemia groups was significantly down-regulated compared with NS group. Theoretically, hyperkalemia could alleviate the exchange of Na⁺-K⁺ and the Na⁺-Ca²⁺. Additionally, our data confirmed that the concentration of Ca²⁺ in the I/R brain tissue was lower, and the expression of CaMK II and NCX1 were down-regulated in hyperkalemia groups. It was indicated that the administration of potassium chloride to a certain degree could alleviate the brain I/R injury by inhibiting the activity of NCX1 and the calcium overload.

The researches¹⁴-¹⁷ about I/R injury in cardioprotection field have already indicated the protective effect of hyperkalemia, which has been widely used in clinical practice. However, the protection of hyperkalemia on the brain in vivo was seldom reported. As the hyperkalemia could alleviate the I/R injury in the myocardium, we suggested the protective effect of hyperkalemia could also benefit to the brain I/R injury. Our results confirmed the hypothesis. Therefore, we suggest that elevating the concentration of potassium to a certain degree could also benefit to the I/R brain by inhibiting the activity of NCX1, lowering the concentration of Ca²⁺ and alleviating the brain I/R injury. It is worthy of attention that, whether the benefit of hyperkalemia is also available for other I/R organs besides heart and brain, needs to be further study to be confirmed.

Our investigation had some limitations: (1) our protocol was mainly about elevating the concentration of potassium and then using the tMCAO mode to test our hypothesis. Though there was no any more intervention, it still could not completely equal to the real condition of ischemia stroke in clinical practice. (2) Our work was more like a qualitative research, mainly testing the hypothesis that whether hyperkalemia could also alleviate the brain I/R injury. Though there were two concentrations of potassium, the optimal therapeutic dose of potassium was still unknown. In our pre-

<table>
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<tr>
<th>K⁺ (mmol/gprot)</th>
<th>Ca²⁺ (mmol/gprot)</th>
<th>CaM (mmol/gprot)</th>
<th>Ca-ATPase (U/mgprot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH</td>
<td>1.3818 ± 0.0225*</td>
<td>0.0712 ± 0.001*</td>
<td>43.6561 ± 3.1111*</td>
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<td>HK80</td>
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<td>59.5432 ± 4.6084</td>
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<td>0.1121 ± 0.001</td>
<td>62.5883 ± 4.5471</td>
</tr>
<tr>
<td>NS</td>
<td>0.9789 ± 0.0368*</td>
<td>0.1406 ± 0.001*</td>
<td>72.3386 ± 3.5612*</td>
</tr>
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SH: sham group; NS: normal saline group; HK80: hyperkalemia group-rats were administrated with 2.5% potassium chloride 80 ug/g; HK40: hyperkalemia group-rats were administrated with 1.25% potassium chloride 40 ug/g; CaM: calmodulin (*Compared with SH, p < 0.05; #Compared with NS, p < 0.05).
liminary experiment, we chose 3.2 mL/kg 2.5% potassium chloride (80 µg/g) solution to pump into the rats via vein at the speed of 8 ml/h, which did not affect the rat’s hemodynamic stability. Given the higher concentration of potassium solution (120 µg/g), arrhythmia that resulted in hemodynamic instability would occur. Therefore, the potassium solution which concentration was more than 80 µg/g was excluded in the present study, although it might exert better protection to the rats subjected to the tMCAO. (3) Our study didn’t involve longer prognosis of the I/R rats. We only could confirm that the hyperkalemia would ameliorate the rats ischemia brain injury after 24 h reperfusion. The exact mechanism of the protection of hyperkalemia to the I/R injury needs to be further studied.

Conclusions

We showed that hyperkalemia could ameliorate the brain I/R injury by alleviating calcium overload-inhibiting the activity of NCX1 and lowering the concentration of Ca2+-.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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