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N-methyl-D-aspartate receptor-mediated spinal cord ischemia-reperfusion injury and its protective mechanism

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Abstract

Introduction: *This study investigated the specific mechanism of N-methyl-D-aspartate (NMDA) receptor-mediated spinal cord ischemia-reperfusion by comparing the protective effects of the voltage-gated Ca^{2+} channel blocker nimodipine and the NMDA receptor blocker K-1024 on the spinal cord.*

Material and methods: *In this study, 42 SD rats were divided randomly into four groups: non-blocking (n = 6), normal saline (n = 12), K-1024 (n = 12) and nimodipine (n = 12). The rats in three groups (saline, K-1024, nimodipine) received an intraperitoneal injection 30 minutes before ischemia. In these three groups, 6 out of 12 rats were selected randomly to have their thoracic aorta blocked with a balloon to induce spinal cord ischemia for 10 minutes. Then, the spinal cord tissues were collected. The remaining six rats were evaluated for nerve function at 1, 2, 4 and 8 hours after reperfusion. The lumbar spinal cord was removed for histological examination. The release of neurotransmitter amino acids was observed by high-pressure liquid chromatography, and the protein expression level of neuronal nitric oxide synthase (nNOS) in the spinal cord was determined by immunohistochemistry.*

Results: *All the animals in the normal saline group and five in the nimodipine group were paralysed after ischemia. Compared with the normal saline and nimodipine groups, the rats in the K-1024 group had more normal motor neurons and better behavioural scores. In addition, the histopathology of the rats in the K-1024 group was significantly better than in the normal saline and nimodipine groups. After 10 minutes of ischemia, there was no significant difference in glutamate concentration in each group. The protein expression level of nNOS in the K-1024 group was significantly downregulated compared with the saline and nimodipine groups. At 8 hours after reperfusion, the protein expression level of nNOS in the K-1024 group was significantly upregulated compared with the normal saline group.*

Conclusions: *The specific mechanism of the NMDA receptor blocker K-1024 in protection against spinal cord ischemia-reperfusion injury is related closely to the inhibition of NMDA receptors and the downregulation of the protein expression level of nNOS.*

Key words: *spinal cord ischemia–reperfusion, K-1024, glutamate, neuronal nitric oxide synthase, N-methyl-D-aspartate (NMDA) receptors.*

Introduction

Clinically, spinal cord ischemia during thoracic aortic aneurysm surgery often leads to various degrees of paralysis [4], with the incidence of paralysis of about 2.5-8% [16]. The specific mechanism of spinal cord ischemia is currently unclear. Previous studies [7,12,13,16,17] have found that glutamate can induce large amounts of Ca^{2+} influx to damage nerve cells and lead to cell death by activating N-methyl-D-aspartate (NMDA) receptors. Therefore, the NMDA receptors may be closely related to spinal cord ischemia–reperfusion injury [9].

As an EAA-NMDA receptor antagonist, K-1024 is a hot topic in current medical research. However, there are few studies on the protective effect of K-1024 on

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spinal cord ischemia during the perioperative period of aortic surgery. K-1024 may be a promising therapeutic drug for this common spinal cord injury, improving patients' quality of life. Therefore, this study established a rat spinal cord ischemia–reperfusion model to explore the protective effect of K-1024 on the behaviour and histopathology of ischemia–reperfusion in rats and its influence on the expression of glutamate and nitric oxide synthase (NOS) to explore the specific mechanism of K-1024's protective effect on ischemia–reperfusion injury.

Material and methods

Establishment and grouping of the spinal cord ischemia model

In this study, healthy adult SD rats were used as experimental animals, and 10% chloral hydrate was used for intraperitoneal injection anaesthesia (300 mg/kg). A polyethylene catheter (PE-50) was inserted into the tail artery to monitor arterial blood pressure, and heparin was injected. After the left femoral artery was separated, a 2-F#Fogarty (Terumo 2.5/3.0) catheter was placed in the thoracic descending aorta until the tip of the catheter reached the level of the left subclavian artery (10.5-11 cm from the insertion point of the catheter). When the PE-50 catheter was inserted 1 cm into the left carotid artery, a blood storage bag (37.5°C) was attached and filled with heparin saline. The height of the loop blood reservoir was adjusted to control the proximal aortic blood pressure. During the occlusion of the aorta, the blood pressure at the proximal end of the occlusion point was controlled at 40 mmHg. After intubation, 200 U of heparin was injected through the tail artery. The balloon catheter was filled with 0.05 ml of normal saline, and the blood was drained into the external blood storage bag. When the pulsation below the blocked aorta disappeared and the pressure decreased, the blocking was complete. After ischemia, the balloon catheter was evacuated and removed, the blood was perfused for 60 seconds, and protamine sulphate (4 mg) was injected subcutaneously. After the perfusion was completed, all the cannulas were removed, and the incision was sutured.

In this study, 42 experimental rats were randomly divided into four groups: the non-blocking group (with a balloon catheter inserted into the thoracic aorta but without balloon filling, n = 6), the normal saline group (2 ml of normal saline was injected intraperitoneally 30 minutes before the occlusion of the descending aorta, n = 12), the K-1024 group (25 mg/kg of K-1024 was injected intraperitoneally 30 minutes before the occlusion of the descending aorta, n = 12) and the nimodipine group (0.5 mg/kg of nimodipine was injected intraperitoneally 30 minutes before the occlusion of the descending aorta, n = 12). Six of the 12 rats in all groups except the non-blocking group were selected randomly to have their thoracic aorta blocked with a balloon to induce spinal cord ischemia for 10 minutes. Then, the spinal cord tissues were collected for subsequent pathological tests. The remaining six rats in each group were evaluated for behavioural scores at 1, 2, 4 and 8 hours after reperfusion. The rats, weighing 350-375 g and of either sex, used in this study were provided by the Experimental Animal Centre of Beijing Anzhen Hospital. K-1024 was from the Shanghai No. 1 Pharmaceutical Factory and nimodipine was from Bayer, Germany.

Behavioural evaluation

This study assessed the degree of recovery of motor function by evaluating lower limb walking and gaits at 1, 2, 4 and 8 hours after reperfusion. The grades were 0 points: normal, 1 point: flat feet and ataxia, 2 points: walking with joints, 3 points: movement of the lower limbs but unable to walk with joints, 4 points: no movement/lower limbs dragging. The rat lift-and-release reflex was evaluated using the following method: the corresponding lift-and-release responses were induced by abducting the dorsal side of the hind paw, with 0 points: normal, 1 point: weak, and 2 points: none. The total scores of the lower limb walking and leg-lifting reflexes were recorded.

Histopathological evaluation

In this study, L3-L5 spinal cord tissues were collected and stored in 4% paraformaldehyde (containing 0.1% DEPC), fixed for 6 hours and embedded in slices. Five typical sections were removed, each with a thickness of 3 μm, from each rat's L3, L4 and L5 segments. After sectioning, haematoxylin-eosin staining was used.

Determination of glutamate content in spinal cord tissue

In this study, high-performance liquid chromatography was used to detect the content of glutamate in the spinal cord tissue: 0.1 mol/l phosphate-buffered saline (PBS) (pH = 6.8) was used to prepare the spinal cord tissue homogenate on ice, and 20 μl of the supernatant was added to 180 μl of 0.4 mol/l perchloric acid. They were mixed well and centrifuged for 45 minutes (37°C, 12,000 r/min). Then, 10 μl of the supernatant was added to 10 μl of internal standard solution (30-pmol/l homoserine) and 100 μl of o-phthalaldehyde. After 2 minutes, 10 μl of the sample was injected, and gradient elution was performed according to the chromatographic conditions.

Neuronal nitric oxide synthase

In this study, immunohistochemical staining (ABC method) was used to detect the neuronal NOS (nNOS) protein expression levels: a 1 : 50 concentration of normal goat serum was incubated at room temperature for 30 minutes. Rabbit anti-nN0S antibody was added dropwise (working concentration 1 : 80 [Sigma]). After 72 hours of incubation (4°C), it was rinsed thoroughly with 0.01 mol/l PBS (pH = 7.4), and the goat anti-rabbit antibody (working concentration 1 : 100 [Boster]) was added and incubated for 30 minutes (37°C). After a complete rinse, the ABC complex was added dropwise and incubated at 37°C for 30 minutes. DAB-H_20_2 was used to colour (room temperature, 5-10 minutes) after a complete rinse before finally, it was dehydrated, made transparent and sealed with neutral gum. In this study, PBS was used as a blank control instead of a primary antibody.

Image analysis

A microscopic image analyser (TJTY-400 true colour cell image analysis) was used for the image analyses of the slices, and all slices were magnified 100 times. The number of positive reaction cells was sampled, and the field of view was 261632.00 μm^2. Three slices were selected for each rat, and the average value was obtained to reduce errors.

Statistical processing

SPSS 25.0 software was used for the data analysis, and the results were expressed as mean ± standard deviation (± s). A one-way analysis of variance was used to compare continuous variables (behavioural scores, glutamate content and nNOS activity) among multiple groups. A value of $p < 0.05$ indicated that the difference was statistically significant.

Results

Behavioural evaluation

After 8 hours of reperfusion, no neurological insufficiency was found in any rats in the non-blocking group. Five rats developed acute scleroplegia before perfusion in the normal saline group, and there was no change after 8 hours of reperfusion. The motor function of the one remaining rat recovered after 2 hours of reperfusion but worsened after 8 hours. In the K-1024 group, four rats were scored as level 4 after 1 hour of reperfusion. The other two rats were scored as level 2 after 1 hour of reperfusion, and they recovered to level 2 after 2 hours of reperfusion. However, after 8 hours of reperfusion, all six rats had slight muscle weakness, and the sports injury classification remained at level 2. All of them could walk and lift their feet, and their behaviour scores were similar to those of the normal saline group. The six rats in the nimodipine group all showed severe motor-damaged scleroplegia after 2 hours of reperfusion, and two rats recovered after 4 hours. After 8 hours of reperfusion, two of the six rats showed no change, two rats recovered to level 3 motion damage (unable to walk with joints), and two rats recovered to moderate motion damage (ataxia but walking with joints) (Table I, Fig. 1).

Histopathological changes

In this study, the degree of grey matter damage (necrosis or dark neurons) was determined from the assessment of three areas: in the first area, the damage involved layers I-VI; in the second area, the damage involved layers VII and X; and in the third area, the damage involved layers VIII and IX. The results of this study showed that the motor and sensory functions of the rats in the non-blocking group were completely restored, and there were no histopathological changes in the L2-L5 spinal cord segmental slices (Fig. 2A). There was no necrosis in the small and medium-sized contact and large α motor neurons. There were no pathological changes in the nerve fibres. The rats in the nimodipine group were similar to those in the normal saline group; all of them had scleroplegia. Histopathological changes were characterised by extensive necrosis and irregular cavities in the grey matter at layers III-VII of the spinal cord L2-L5 (Fig. 2B, C), but the layers located at the periphery of the grey matter, such as layers I-II, X and VIII-IX, were all close to the normal structures, and the α motor neurons and nuclear structures were

Table I. The effect of drugs on behavioural scores in the spinal cord of rats with ischemia-reperfusion (±*s*)

Variable	Reperfusion time			
	1 h	2 h	4 h	8 h
Normal saline group	5.83 ±0.41	5.33 ±0.52	5.83 ±0.41	5.83 ±0.41
K-1024 group	3.33 ±1.03	2 ±0	2 ±0	2 ±0
Nimodipine group	6 ±0	6 ±0	5.67 ±0.52	5 ±0.89
F value	32.568	310.000	195.000	75.690
P value	< 0.01	< 0.01	< 0.01	< 0.01

normal. The degradation of neurons by 'darkening' or 'shrinking' was discovered accidentally in the rats of the K-1024 group during the recovery of neurological function. These neurons were typically distributed in the middle of the mid-inner zone (layer VII) and the middle and posterior parts of the posterior horn. However, most contact and α motor neurons did not change (Fig. 2D).

Glutamate content in spinal cord tissue

The results of this study showed that the glutamate content of each group was different after 10 minutes of ischemia. The neurotransmitter glutamate level of the normal saline group was three times higher than the non-blocking group, but the glutamate content of the K-1024 group was not significantly different from the normal saline group (p = 0.801). The glutamate content of the nimodipine group was slightly lower than the normal saline group, but there was no significant difference (p = 0.450). There was no significant difference between the K-1024 and nimodipine groups (p = 0.609).

The results of this study showed that 8 hours after reperfusion, there was no significant difference in the glutamate content among the three groups (p = 0.375). Furthermore, multiple comparisons showed that the glutamate content of each group was similar

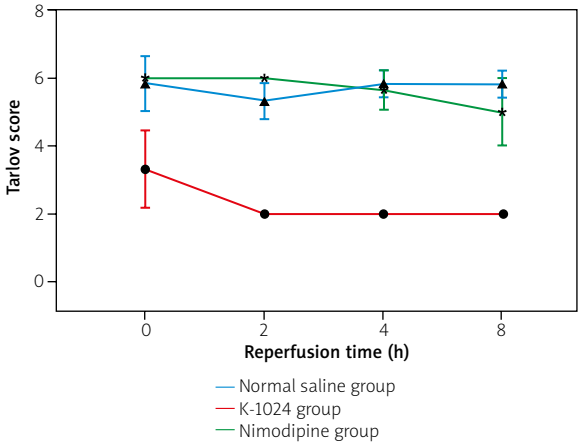

Fig. 1. The effect of different drugs on the spinal cord behaviour of rats with ischemia-reperfusion.

to that of the non-blocking group (13.73 ±4.22 μmol/l). Although the glutamate content of the K-1024 group was slightly lower than the normal saline group (p = 0.789), the glutamate levels of the nimodipine and normal saline groups were not significantly different (p = 0.196), and there was no significant difference between the K-1024 and nimodipine groups (Table II, Fig. 3).

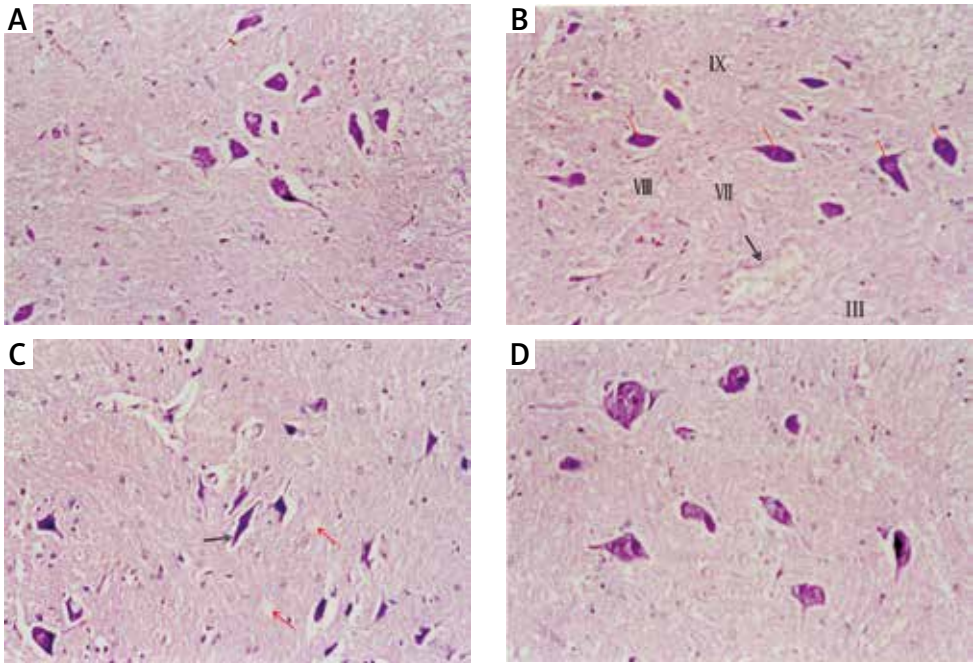

Fig. 2. A-D) HE staining of spinal cord of different groups of rats (10×). **A)** Non-blocking group: complete spinal cord structure; **B)** Normal saline group: normal temperature ischemia for 10 minutes, the black arrow shows necrosis of grey matter layers III-VII in the spinal cord, and the red arrow shows normal α motor neurons; **C)** Nimodipine group: the black arrow shows the neuron "shrinking", and the red arrow shows the formation of grey matter vacuoles; **D)** K-1024 group: the spinal cord structure is basically complete.

Table II. The effect of drugs on the content of glutamate in the spinal cord of rats with ischemia-reperfusion (μmol/l) (±*s*)

Variable	Ischemia for 10 min	Reperfusion for 8 h
Normal saline group	50.15 ±5.03	14.08 ±3.70
K-1024 group	49 ±2.05	13.4 ±1.59
Nimodipine group	46.65 ±9.40	10.65 ±4.45
F value	0.324	1.095
P value	0.731	0.375

The non-blocking group was 13.73 ±4.22 μmol/l.

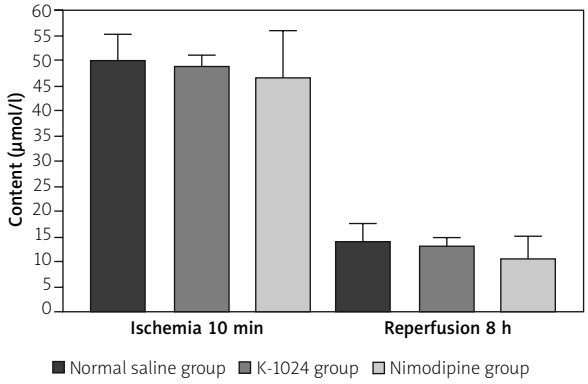

Fig. 3. Glutamate content of ischemia-reperfusion spinal cord in mice.

Effect of neuronal nitric oxide synthase activity in the spinal cord tissue of ischemic rats

The results of this study showed that the number of nNOS-positive cells in the K-1024 group was significantly downregulated compared with the normal saline group after 10 minutes of ischemia (11.18 ±2.18 vs. 22.31 ±3.79, $p < 0.01$), and the nimodipine group was also significantly lower than the normal saline group (12.61 ±3.74 vs. 22.31 ±3.79, $p < 0.01$). The results of the K-1024 group were slightly better than the nimodipine group ($p = 0.283$).

The results of this study showed that 8 hours after reperfusion, the number of nNOS-positive cells in the K-1024 group was significantly upregulated compared with the normal saline group (19.12 ±4.55 vs. 14.75 ±3.34, $p < 0.05$), and there was no significant difference between the nimodipine and normal saline groups ($p = 0.096$). There was no significant difference between the K-1024 and nimodipine groups ($p = 0.441$) (Table III, Figs. 4 and 5).

Discussion

Previous animal experimental studies [18,21] found that NMDA receptor antagonists can block the cascade of injury after cerebral spinal cord ischemia and hypoxia and reduce brain and spinal cord injuries. Li *et al*. [8] found that the NMDA receptor antagonist ginsenoside has an anti-neuronal damage effect. De Miranda *et al*. [3] confirmed that the NMDA receptor antagonist MK-801 could significantly reduce the spinal cord infarct area and reduce neuronal damage. At present, K-1024, as a non-competitive NMDA receptor antagonist, is widely used in clinical intravenous anaesthesia. However, there are few studies on the protective effect of K-1024 on spinal cord ischemia during the perioperative period of aortic surgery and few reports on ischemia–reperfusion spinal cord protection therapy [1,6,14].

The results of this study show that the neurological function of the ischemia–reperfusion spinal cord was closely related to histopathological changes. When the aorta was blocked for 10 minutes, the rats in the normal saline and nimodipine groups both developed scleroplegia in the acute phase and could not resume

Table III. Effects of drugs on the number of nNOS-positive cells in the spinal cord of rats with ischemia-reperfusion (±s)

Variable	Ischemia for 10 min	Reperfusion for 8 h
Normal saline group	22.31 ±3.79	14.75 ±3.34
K-1024 group	11.18 ±2.18$^{\Delta}$	19.12 ±4.55*
Nimodipine group	12.61 ±3.74$^{\Delta}$	17.6 ±4.88
F value	40.597	3.409
P value	< 0.01	0.046

*The non-blocking group was 9.12 ±1.83/261632.00 μm^2; $^{\Delta}p < 0.01$ compared with the normal saline group; *$p < 0.05$ compared with the normal saline group.*

joint walking after 8 hours. The rats in the K-1024 group showed muscle weakness in the acute phase, and all animals resumed walking and lifting their feet after 8 hours. In terms of histopathology, the nimodipine group was similar to the normal saline group, and the α motor neuron and nucleus also showed normal structures, which was consistent with the occurrence of scleroplegia. Although the K-1024 group had occasional degradation of neurons by 'darkening' or 'shrinking', most of the contact and α motor neurons did not change. Therefore, K-1024 may have a certain protective effect on the recovery of ischemic spinal cord function and tissue structure. Notably, K-1024 seems to provide a delayed protective effect on muscle function and significantly improve behavioural scores after the acute phase. However, there is a lack of evidence on the exact mechanism underlying this phenomenon. Further experimental studies are required to reveal the long-term efficacy of K-1024 in the treatment of spinal cord injury.

The glutamate-Ca^{2+} overload theory proposed by Coyle *et al*. [2] is one of the important pathological mechanisms of cerebral and spinal cord ischemic injuries [11]. However, it is currently believed that the expression and functional changes of glutamate transporters are the primary mechanisms for the abnormal release of excitatory glutamate during spinal cord ischemia [5]. In this

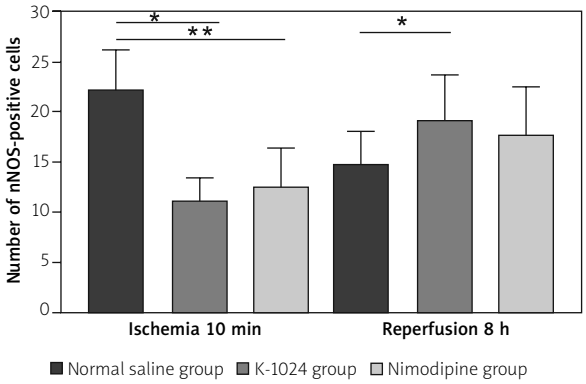

Fig. 4. Number of nNOS-positive cells in mice after ischemia-reperfusion.

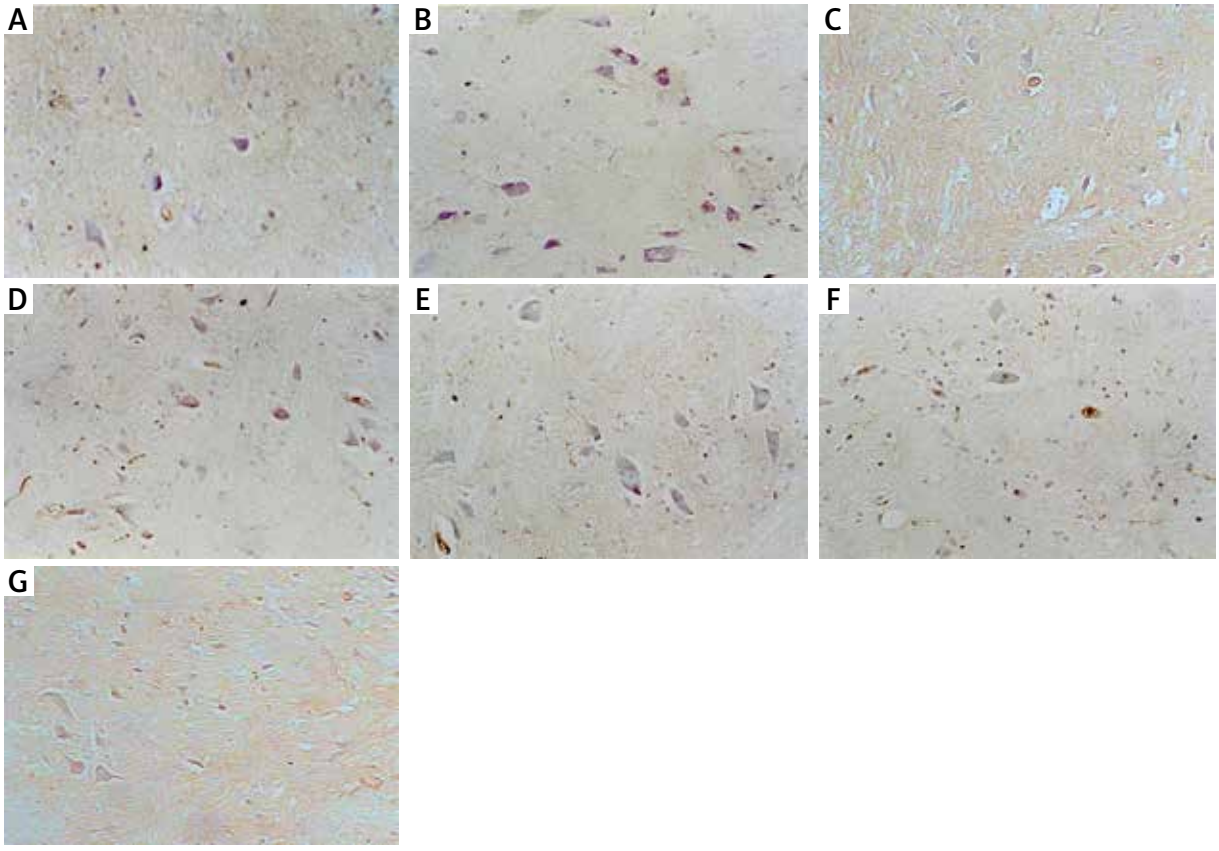

Fig. 5. The results of ischemia-reperfusion nNOS-positive cells in different groups of mice. **A**) Non-blocking group; **B**) Normal saline group: ischemia for 10 minutes; **C**) Normal saline group: ischemia-reperfusion for 8 hours; **D**) K-1024 group: ischemia for 10 minutes; **E**) K-1024 group: ischemia-reperfusion for 8 hours; **F**) Nimodipine group: ischemia for 10 minutes; **G**) Nimodipine group: ischemia-reperfusion for 8 hours.

study, it was found that the neurotransmitter glutamate level was three times higher than the baseline value at 10 minutes of ischemia and returned to the baseline level after 8 hours of reperfusion, although there was no significant difference in the glutamate content between the groups. Therefore, the NMDA receptor pathway did not achieve spinal cord protection by changing the level of glutamate release [22].

Several previous studies revealed an inconsistent role of different NMDA receptor antagonists, such as memantine and MK-801, in treating spinal cord injury, suggesting that the affinity property of drugs may also be a key factor in their effectiveness [10,19]. In the future, with continuous advancements in drug processing technology, it is expected that drugs will be transformed into preparations with better therapeutic effects.

The neurotoxicity induced by spinal cord ischemia may be largely related to NMDA receptors. In this experiment, both K-1024 and nimodipine could reduce the expression of nNOS at 10 minutes of ischemia, but only K-1024 maintained better spinal cord biological activity after 8 hours of reperfusion. This may be because both K-1024 and nimodipine can block the Ca^{2+} influx, but their pathways of action are different. Although nimodipine can block the Ca^{2+} influx, it cannot inhibit the Ca^{2+} influx mediated by NMDA receptors. K-1024 can block the excessive activation of NMDA receptors to avoid intracellular Ca^{2+} overload, thereby reducing the activation of nNOS and the production of NO and oxygen free radicals to reduce mitochondrial damage [15,20].

Conclusions

K-1024 has a certain protective effect on spinal cord ischemia–reperfusion injury. Its mechanism is closely related to the inhibition of NMDA receptors and the downregulation of the protein expression level of nNOS.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Beijing Anzhen Hospital.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Disclosure

The authors report no conflict of interest.

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