

Memantine decreases apoptosis and attenuates the activation of caspase-3 and MDA release in rats with ischemia-reperfusion injury

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Abstract: To investigate the effects of memantine on neuron apoptosis and the expression of Caspase-3 and malonaldehyde (MDA) during cerebral ischemia-reperfusion injury in rat. 135 male Wistar rats were randomly divided into 3 groups: sham operation group, cerebral ischemia-reperfusion model group and memantine intervention group. The changes of cell morphology and the expression of caspase-3 in cerebral cortex neurons at 12h, 24h, and 48h after ischemia-reperfusion were observed by Haematoxylin Eosin (HE) and immunohistochemistry staining respectively. The expression of caspase-3 activity and MDA levels at different time points were detected by spectrophotometer. Meanwhile, the apoptosis in situ in the CA1 region of hippocampus of the rats were investigated with TdT-mediated dUTP nicked labeling (TUNEL) method. Results show that in memantine intervention group, the expression levels of caspase-3 and MDA in ischemia-reperfusion injury region increased, in comparison with sham-operated group ($p=0.00$), while lower than that of cerebral ischemia-reperfusion model group ($p=0.00$). Caspase-3 activity remarkably increased in ischemia-reperfusion brain in rats in a time-depended manner. The number of TUNEL positive cells in the CA1 region of hippocampus in the memantine treated rats (7.00 ± 2.04) and model rats (11.57 ± 2.64) were significantly increased compared with the sham operation controls (1.57 ± 4.72) ($p=0.00$), while the number of TUNEL positive cells in the memantine treated rats decreased as compared with that of the model rats ($p=0.00$). Suggesting that memantine may probably have the function of neuroprotection in rats with cerebral ischemia-reperfusion injury by suppressing the expression of caspase-3 activity and MDA and inhibiting the apoptosis of pyramidal neurons in the CA1 region of hippocampus in ischemia-reperfusion rats.

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Key words: Memantine; cerebral ischemia-reperfusion; Caspase-3; MDA; apoptosis

1. Introduction

As one of a leading cause of death in the world, stroke has severely been threatening people's health, with high incidence, prevalence and mortality rates that increased with age. Recently, scientists paid more attention to the ischemia-reperfusion damage, which increased with the application of thrombolysis and interventional techniques for the treatment of acute ischemic stroke. The pathophysiological mechanisms of transient cerebral ischemia are different from that of permanent cerebral ischemia. In the early cerebral ischemia period, the restoration of oxygen and glucose supply may aggravate cerebral injury which occurs in the ischemic penumbra area (Kuroiwa et al, 1988).

It is almost impossible to reverse the harmful outcome of the primary impact on the neural tissue by medical or surgical means. The main target of medical treatment is considered on prevention of secondary injury, which mainly concentrated on the sub-cellular level of organization additional injury death of the peripheral zone caused by the initial damage. (Özsüer et al, 2005). The aim of neuroprotection is to prevent delayed neuron apoptosis in the zone of the ischemic penumbra (Muir, 2002 and Culmsee et al, 2004).

As a nerve protectant, memantine (1-amino-3, 5-dimethyladamantane) is an uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist. In most clinical practice, memantine has been frequently used for the treatment of Parkinson's disease, senile dementia, spastic diseases and viral infectious diseases without serious side effects (Dogan et al, 1999). For the treatment of ischemic stroke, plenty of studies mainly focused on inhibition of excitatory amino acids and calcium influx. While it was founded recently that cell apoptosis and lipid peroxidation may be the underlying mechanisms in ischemia-reperfusion damage (Ikeda and Long, 1990).

As the degradation product of MDA was considered to reflect the degree of lipid peroxidation, and the apoptosis of cells are closely related to caspase-3 reactivity, in this study we investigated the effect of memantine on caspase-3 activity, MDA release and the apoptosis of pyramidal neurons in the CA1 region of hippocampus in rats with cerebral ischemia-reperfusion injury to explore the possible brain protection function(s) of memantine.

2. Material and Methods

Animal treatments: All animal experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986(86/609/EEC). One hundred and thirty-five, 260-280g weights, male Wister rats were bought from the Laboratory Animal Center of Zhengzhou University. Outcome assessments were made by investigators blinded to the experimental group. All experimental procedures have been done according to the regulations for the administration of affairs concerning experimental animals, approved by laboratory animal management and ethical committee of Zhengzhou University.

Main instruments and reagents: WFZ UV-2000 UV visible spectrophotometer(Yu nika, China); micro camera system (Lecia, Germany); analytical system (Biosens Digital Imaging System v1.6, China); Caspase-3 immunohistochemical antibody (Epitomics, U.S.A.); Caspase-3, MDA activity detection kit, TUNEL detection kit (Germany); Bradford protein concentration determination kit (Beyotime, China); Memantine hydrochloride tablet (Lundbeck A/S, Bafch number: 843101, Each 10 mg H., Denmark).

Grouping and model preparation: All rats were randomly divided into three groups: sham operation group, ischemia-reperfusion model group and memantine intervention group (n=45). Middle cerebral artery (MCA) occlusion was performed on rats in ischemia-reperfusion group and memantine intervention group using an intraluminal thread embolism method as described by Zea Long et al (Longa et al, 1989). After 90 minutes of cerebral ischemia, pull out the filament slowly. The successful cerebral-reperfusion model is that animals have left Honer sign and hemiplegia on right limbs when awaked. In sham group, animals were only operated by isolating blood vessels, without thread embolism. Rats in memantine intervention group were given 20 mg/kg/d dose of amantine through a gastric tube immediately after cerebral ischemia-reperfusion and 24h later. In contrast, rats in the other two groups received physiological saline of the same volume.

HE staining and immunohistochemistry: After 12 h, 24 h and 48 h ischemia-reperfusion, 5 rats in each group were randomly selected and killed. Then perform 4% paraformaldehyde perfusion and fixation followed by ethanol dehydration, xylene and embedding, coronal slices as thin as 5 μ m for HE and immunohistochemistry staining.

The expression of caspase-3 in cerebral cortex of was detected by SP immunohistochemical staining, strictly in accordance with the instruction in kit. The apoptosis of hippocampal cells were investigated in situ in the CA1 region of hippocampus of the rats by TdT-mediated dUTP nicked labeling (TUNEL), 48 h after cerebral ischemia-reperfusion (n=5).

Image Analysis: The average integrated optical density of the positive area was analyzed by Lecia microscope camera system and Biosens Diosens Digital Imaging System v1.6 analysis system. Five visions in the same area of each slice were randomly selected and tested, then the average integrated optical density of the positive area of the target area were calculated.

MDA, Caspase-3 Levels in hemisphere cortex homogenate: 12 h, 24 h and 48 h after ischemia-reperfusion, another 5 rats in each group were randomly picked out and taken the left cerebral hemisphere cortex homogenate. The levels of MDA, Caspase-3 of cerebral tissue were assessed respectively in accordance with operating instructions in MDA, Caspase-3 detection kits and Bradford protein concentration assay kit.

Statistical Analysis: All data were expressed as the mean \pm SD and were analyzed by a repeated measures analysis of variance (ANOVA) followed by contrasts in repeated measures design and Student's t test, and LSD method with a comparison of between two groups with SPSS 13.0.

3. Results

HE staining of rat cortical neurons

The neurons in rat brain tissue of sham operation group were well arranged, uniform and neat. Nuclei were round or oval, chromatin was uniform; While the size of neurons in rat brain of model group were relatively large, the nucleoli were disappeared and empty halo was found around the cytoplasm; In the memantine intervention group the normal neurons were less than that of sham group, but more than that of model groups (Figure 1).

Caspase-3 positive area integrated optical density of cortex by immunohistochemistry

Caspase-3 positive cells appeared brown granules within the cytoplasm. The color of Caspase-3 positive cells cytoplasm in model group was most deep, brownish yellow. The density of Caspase-3 positive cells in memantine intervention group was less than that of model group, but higher than that of sham group. There were significant differences in density. At different time points the Caspase-3 positive area average integral optical density of intervention group was significantly reduced compared with that of ischemia group ($p < 0.05$), but significantly increased than that of sham group ($p < 0.01$) (Table 1).

MDA, Caspase-3 Levels of Cortex homogenate

At different time points the Caspase-3 level and MDA activity unit generated content of cortex homogenate in Memantine treatment group were significantly reduced compared with that of model group respectively ($P < 0.05$), but significantly increased

than that of sham group ($P < 0.01$) (Table 2, Table 3).

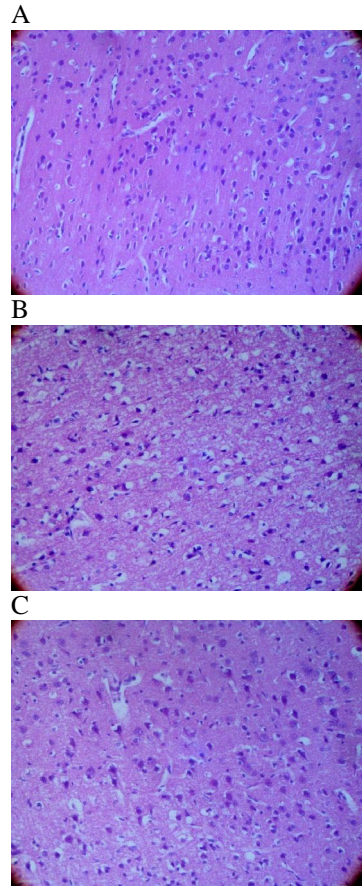


Figure 1 HE staining of rat cortical neurons(48h): A.. Sham group; B. model group; C. Intervention group

TUNEL positive cell in CA1 region of hippocampus

The number of TUNEL positive cells in the CA1 region of hippocampus in the memantine treated rats (7.00 ± 2.04 , figure 2C) and model rats (11.57 ± 2.64 , figure 2B) were significantly increased compared with the rats in sham operation control group (1.57 ± 4.72 , figure 2A) ($P < 0.01$), while the number of TUNEL positive cells in the memantine treated rats was decreased as compared with that of the model rats ($P < 0.05$) (Figure 2).

Table 1. The caspase-3 positive area integrated optical density of cerebral cortex at different time points ($\bar{x} \pm s$)

group	Sham operation (n=10)	Memantine (n=10)	Model (n=10)
12 h	96.07±5.08	125.77±7.94	134.28±10.00
24 h	98.51±3.41	139.92±6.69	147.36±5.35
48 h	97.38±3.48	145.14±5.24	151.36±4.15

Note : compared with sham operation group $P < 0.01$;
compared with model group $p < 0.05$, $P < 0.01$

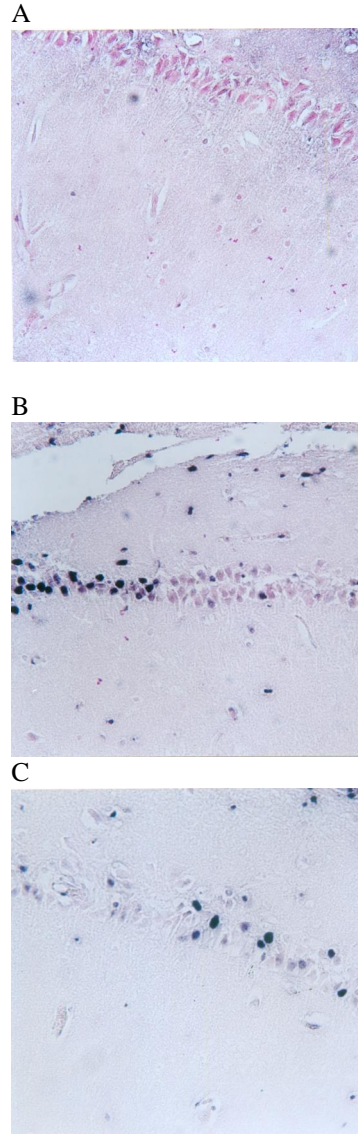


Figure 2. The apoptosis of hippocampal cells in situ in the CA1 region of the rats by TUNEL(48h): A., Sham group; B. model group; C. Intervention group

Table 2 The Caspase-3 expression enzyme activity unit of cerebral cortex at different time points (U/mg, $\bar{x} \pm s$)

group	Sham operation (n=5)	Memantine (n=5)	Model (n=5)
12 h	0.175±0.062	0.289±0.013	0.397±0.0215
24 h	0.175±0.063	0.454±0.045	0.520±0.043
48 h	0.176±0.068	0.523±0.016	0.595±0.049

Note: compared with sham operation $P < 0.01$;
compared with model $P < 0.05$, $P < 0.01$

Table 3 The expression of MDA of cerebral cortex at different time points ($\mu\text{mol}/\text{mg}$, $\bar{x} \pm s$)

group	Sham operation (n=5)	Memantine (n=5)	Model (n=5)
12 h	0.047±0.007	0.394±0.025	0.440±0.033
24 h	0.049±0.006	0.664±0.079	0.850±0.080
48 h	0.049±0.008	0.469±0.037	0.591±0.033

Note : compared with the sham operation group $P < 0.01$; compared with model group $P < 0.05$, $P < 0.01$

4. Discussion

Reperfusion after a certain period of cerebral ischemia may aggravate the progression of cerebral damage. During ischemia-reperfusion injury process, oxidative stress occurs in cortical neurons, resulting in a large number of oxygen free radicals, which contribute to a large number of excitatory amino acids. It has been shown that there was a linear correlation between the severity of ischemic damage and the amounts of glutamate (Rothman et al, 1986). In addition, intracellular calcium overload leading to harmful signal transduction pathway activated, that eventually cause neuronal death and delayed apoptosis. NMDA receptor blockers are proved having the function of suppress neurotoxicity damage caused by excitatory amino acids (Blanpied et al, 2005, Davis et al, 2000 and Lees et al, 2001). One of the underlying mechanisms for the neuroprotection function of memantine lies in blocking of Ca^{++} influx through the NMDA-operated Ca channel (Dogan et al, 1999). Apoptosis develops in a certain period of ischemia while excitotoxic cell damage arises within a few minutes, and accumulated in over subsequent hours (Krivonos OV et al, 2010). Neuronal death occurs in the core area of ischemia, while apoptosis occurs in ischemia peripheral areas. For apoptosis cells can be saved, rescuing apoptotic cells as therapeutic targets may become a reality. The aim of neuroprotection is to prevent delayed apoptosis in the zone of the ischemic penumbra (Dávalos et al, 2006 and Suslina et al, 2000). Ischemia-reperfusion brain injury induced neuron apoptosis. As a key enzyme involved in apoptosis, the activity of caspases can be inhibited to hinder apoptosis process. Among the protease family related to apoptosis, Caspase-3 plays a crucial role in the cascade reaction. The damage of ischemia reperfusion cell is also closely related to the degree of lipid peroxidation. MDA is a degradation product of lipid peroxidation, which can reflect the degree of neuronal lipid peroxidation. Thus, it is feasible to explore the potential protective effect of memantine on neurons by detecting caspase-3 activity and MDA release in cerebral ischemia-reperfusion injury rats.

In this study, we still use memantine at a dose of 20 mg/kg after ischemia-reperfusion injury, which

has been previously showed it is the necessary dose to exhibit the significant protective of memantine after permanent focal cerebral ischemia (Krieglstein et al, 1997). NMDA receptors are widely distributed in the brain, especially densely distributed in hippocampus and cerebral cortex (Monaghan et al, 1985) that makes it possible for memantine to protect the cortical neurons. Our results showed that, during ischemia-reperfusion injury process, in memantine intervention group, the increase of expression level of caspase-3 in cortex neuron and MDA release in cortex homogenate were inhibited significantly on 12 h, 24 h, and 48 h time points, compared to that of rats in ischemia-reperfusion model group. Such results indicate that memantine can alleviate ischemia-reperfusion injury and has neuron protective function, which is supported by the recent studies that memantine can attenuate staurosporine-induced or isoflurane-induced neuronal apoptosis by inhibiting the expression of high activity of caspase-3 in mouse (Zhang et al, 2008 and Jantas-Skotniczna et al, 2006). In ischemia-reperfusion injury process, the main excitatory neurotransmitter in central nervous system, glutamate as well as aspartate, releases in large amounts, that resulting in excessive activation of NMDA receptors. Glutamate or aspartate binding to receptors induce excitation of postsynaptic neurons, which leading to followed excitatory postsynaptic potential and develop cell damage eventually. Excess excitatory neurotransmitters lead to over activation of NMDA receptors and the influx of calcium ions, and then activate the lipase and protease, that cause cell damage (Bormann et al, 1989 and Dogan et al, 1999). Memantine, as an uncompetitive NMDA receptor antagonist, can inhibit the toxic effects of excitatory neurotransmitters (Matsumoto et al, 1996) and reduce lipid peroxidation levels after closed head trauma in rats (Özsüer et al, 2005). In our study, memantine can inhibit the caspase-3 activity expression and MDA release amounts, suggesting a new mechanism of neural protection effect of memantine to prevent delayed apoptosis.

In conclusion, memantine may suppress the Caspase-3 expression level and MDA release, thus inhibit the neuron apoptosis and lipid peroxidation in ischemia-reperfusion injury, as well as resist intracellular calcium overload and relieve neurotoxicity caused by excitatory neurotransmitter to play neural protection role in ischemia-reperfusion injury. Our study drives the further development of clinical application of uncompetitive NMDA open channel blockers in the treatment of ischemia-reperfusion injury in brain. Further study is needed to explore such neural protective mechanisms in-depth.

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References

1. Kuroiwa T, Shibutani M, Okeda R. Blood-brain barrier disruption and exacerbation of ischemic brain edema after restoration of blood flow in experimental focal cerebral ischemia. *Acta Neuropathol* 1988; 76: 62-70.
2. Özsüer H, Görgülü A, Kırı T, Cobano lu S. The effects of memantine on lipid peroxidation following closed-head trauma in rats. *Neurosurg Rev* 2005; 28: 143-147.
3. Muir KW. Heterogeneity of stroke pathophysiology and neuroprotective clinical trial design. *Stroke* 2002; 33: 1545-1550.
4. Culmsee C, Junker V, Kremers W, Thal S, Plesnila N, Kriegstein J. Combination therapy in ischemic stroke: synergistic neuroprotective effects of memantine and clenbuterol. *Stroke* 2004; 35: 1197-1202.
5. Dogan A, Eras MA, Rao VL, Dempsey RJ. Protective Effects of Memantine Against Ischemia-Reperfusion Injury in Spontaneously Hypertensive Rats. *Acta Neurochir (Wien)* 1999; 141(10): 1107-1113.
6. Ikeda Y, Long DM. The molecular basis of brain injury and brain edema: the role of oxygen free radicals. *Neurosurgery* 1990; 27(1): 1-11.
7. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 1989; 20(1):84- 91.
8. Rothman SM, Olney JW. Glutamate and the pathophysiology of hypoxic ischemic brain damage. *Ann Neurol* 1986; 19(2):105-111.
9. Blanpied, TA, Clarke R J. and Johnson JW. Amantadine inhibits NMDA receptors by accelerating channel closure during channel block, *J. Neurosci* 2005; 25(13), 3312-3322.
10. Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, Norris J, Selfotel in acute ischemic stroke: possible neurotoxic effects of an NMDA antagonist, *Stroke* 2000; 31(2), 347-354.
11. Lees K. R, Dyker A, G. Sharma A, Ford GA, Ardron ME, Grosset DG. Tolerability of the low affinity, use-dependent NMDA antagonist AR-R15896AR in stroke patients: a dose-ranging study. *Stroke* 2001; 32(2): 466-472.
12. Dogan A, Eras MA, Rao VL, Dempsey RJ. Protective Effects of Memantine Against Ischemia-Reperfusion Injury in Spontaneously Hypertensive Rats. *Acta Neurochir (Wien)* 1999; 141(10): 1107-1113.
13. Krivonos OV, Amosova NA, Smolentseva IG. Use of the Glutamate NMDA Receptor Antagonist PK-Merz in Acute Stroke. *Neuroscience and Behavioral Physiology* 2010;40(5):529-32.
14. Rogalewski A, Schneider A, Ringelstein EB, Schäbitz WR. Toward a multimodal neuroprotective treatment of stroke. *Stroke* 2006; 37(4): 1129-1136.
15. Suslina ZA, Fedorova TN, Maksimova MIu, Riasina TV, Stvolinski SL, Khrapova EV, Boldyrev AA. Antioxidant treatment in ischemic stroke. *Zh Nevrol Psikhiatr Im S S Korsakova*. 2000; ffa100(10):34-8.
16. Seif el Naser M, Peruche B, Rossberg C, Mennel HD, Kriegstein J, Neuroprotective effect of memantine demonstrated in vivo and in vitro. *Eur J Pharmacol*. 1990;21:185(1): 19-24.
17. Monaghan DT, Cotma CW, Distribution of N-methyl-D-aspartate-sensitive L-[3H] glutamate-binding sites in rat brain. *Journal of Neuroscience* 1985; 5(11): 2909-2919.
18. Zhang G, Dong Y, Zhang B, Ichinose F, Wu X, Culley DJ, Crosby G, Tanzi RE, Xie Z. Isoflurane-Induced Caspase-3 Activation Is Dependent on Cytosolic Calcium and Can Be Attenuated by Memantine. *Neuroscience* 2008; 28(17):4551- 4560 • 4551.
19. Jantas-Skotniczna D, Kajta M, Laso W, Memantine attenuates staurosporine-induced activation of caspase-3 and LDH release in mouse primary neuronal cultures. *Brain research* 2006; 1069: 145-153.
20. Bormann J. Memantine is a potent blocker of N-methyl- D-aspartate (NMDA) receptor channels. *Eur J Pharmacol* 1989; 166(3): 591-592.
21. Dogan A, Eras MA, Rao VL, Dempsey RJ. Protective effects of memantine against ischemia-reperfusion injury in spontaneously hypertensive rats. *Acta Neurochir (Wien)* 1999; 141(10): 1107-1113.
22. Matsumoto K, Lo EH, Pierce AR, Halpern EF, Newcomb R. Secondary elevation of extracellular neurotransmitter amino acids in the reperfusion phase following focal cerebral ischemia. *J Cereb Blood Flow Metab* 1996; 16(1): 114-124.

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