

Neuroprotective Effects of NKN on Focal Cerebral Ischemia in Rats

NKN'nin Fokal Serebral İskemiye Maruz Bırakılmış Ratlardaki Etkisi

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ABSTRACT

AIM: NKN reduces brain edema, neuronal death and neurological deficits in rats after cerebral ischemia. In the present study, we investigated whether NKN was effective on brain injury in cerebral ischemia rats.

MATERIAL and METHODS: The middle cerebral artery occlusion (MCAO) model was used to produce experimental cerebral ischemia in adult male Wistar rats. The activity of SOD and concentration of MDA were determined. We also examined the efficacy of NKN on neurological deficit scores, expression of N-methyl-D-aspartate receptor and endothelin-1 mRNA in brain of focal cerebral ischemia rats.

RESULTS: The results show that NKN significantly increased the activity of SOD, decreased the concentration of MDA, decreased neurological deficit scores, inhibited the expression of N-methyl-D-aspartate receptor and endothelin-1 mRNA in brain of focal cerebral ischemia rats.

CONCLUSION: The results implied NKN could protect brain against injury caused by cerebral ischemia.

KEYWORDS: NKN, Cerebral ischemia, Neurological deficit scores, SOD, MDA, NMDAR, ET-1 mRNA

ÖZ

AMAÇ: Serebral iskemiye maruz kalan ratlarda NKN beyin ödemi, nöronal ölümü ve nörolojik defisiti azaltır. NKN Serebral iskemiye bağlı olarak gelişen beyin hasarında NKN'nin etkili olup, olmadığı araştırılmıştır.

YÖNTEM ve GEREÇ: Erişkin Wistar erkek ratlarda orta Serebral arter oklüzyon modeli ile deneysel olarak Serebral iskemi oluşturulmuştur. SOD aktivitesi ve MDA konsantrasyonu belirlenmiştir. Ayrıca nörolojik defisit ölçütlemesi yanı sıra fokal Serebral iskemi alanlarında N-metil aspartat reseptör ve endotelin-1 mRNA ekspresyonlarına bakılmıştır.

BULGULAR: NKN'nin SOD aktivitesini artırdığı, MDA konsantrasyonunun ve nörolojik defisit skorunu azalttığı görüldü. Bunların yanı sıra fokal serebral iskemi alanlarında N-metil-D- aspartate reseptör ve Endotelin-1 mRNA ekspresyonunun azaldığı da gösterilmiştir.

SONUÇ: Serebral iskemiye bağlı olarak oluşan beyin hasarında NKN'in koruyucu etkisi olduğu gösterilmiştir.

ANAHTAR SÖZCÜKLER: NKN, Serebral iskemi, Nörolojik defisit ölçütleri, SOD, MDA, NMDAR, ET-1 mRNA

INTRODUCTION

Cerebral ischemia or stroke has been recognized as one of the leading causes of death and disability in many countries (3). Previous study demonstrated that the middle cerebral artery occlusion occurred in 10-15% of stroke patients and resulted in the massive cerebral edema with raised intracranial pressure which finally progressed to coma and death (5). Current knowledge regarding the pathophysiology of cerebral ischemia indicates that mechanisms contribute to loss of cellular integrity and tissue destruction. Mechanisms of cell damage include excitotoxicity, oxidative stress, free radical production, apoptosis and inflammation (6). Naokangning (NKN) is a new compound preparation, which consists of ligustrazine, tanshinone IIA, paeonol, and peoniflorin. NKN is mainly used to treat cerebral infarction. To test whether NKN has neuroprotective effects on cerebral ischemia rats,

the effects of NKN on activity of superoxide dismutase (SOD), concentration of malondialdehyde (MDA), and neurological scores were determined. Moreover, we also determined the effect of NKN on the Expression of N-methyl-D-aspartate receptor (NMDAR) and endothelin-1 mRNA (ET-1 mRNA) in brain in cerebral ischemia rats. In this study, we established rat middle cerebral artery occlusion (MCAO) model.

MATERIAL and METHODS

All procedures were approved by Shandong University Animal Care and Use Committee, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Adult male Wistar Rats (weighing 300-350g, from Shandong University Laboratory Animal Center) were used throughout the study, and kept under controlled light and dark conditions and given food and water ad libitum.

Throughout the study, the investigators and veterinarian staff closely monitored the rats' health status. All chemicals and reagents were of reagent grade unless otherwise specified.

Stroke Model and Neurological Examination

The proximal right middle cerebral artery occlusion (MCAO) model was used as previously described (17). A total of 40 animals were divided randomly into 5 groups: (1) sham-operated group, (2) model group, (3) NKN 1 group, (4) NKN 2 group, (5) venoruton group. All animals received treatments: (1) sham-operated group rats received equal volumes of saline solution; (2) model group rats received equal volumes of saline solution too; (3) NKN 1 group rats received intragastric administration of 100 mg/kg NKN; (4) NKN 2 group rats received intragastric administration of 50 mg/kg NKN; (5) Venoruton group rats received intragastric administration of 35 mg/kg venoruton. At one hour after being administrated, all animals were anesthetized by 2% pentobarbital sodium (40 mg/kg) injected i.p., and fixed. An incision was performed in the scalp and the musculus temporalis between eye and ear on the right side. Then, the head of the animal was turned to the left for easy access to MCA proximally to its origin at the circulus arteriosus. The temporalis muscle was sectioned and the zygomatic arch was carefully removed using a bone forceps. A subtemporal craniotomy was carried out using a dental drill and the dura mater was locally incised with a fine needle. Then the MCA was carefully elevated from brain surface by a hook mounted on a stereotactic frame. For permanent vessel blocking an electrically heated wire loop was used to disrupt and immediately occlude the MCA (MCA was not occluded in sham-operated group). The incisions were sutured using absorbable surgical threads and the wound surface was temporarily anaesthetized by Lidocaine Hydrochloride Gel (2%, Zhizupharma Ltd, Beijing, China). Following the administration of 0.05 mg/kg i.m. penicillin, and then animals were taken back to their home cages to recover. Above procedures were performed at 25°C.

Neurological examination was performed carefully at 24 hr after MCA occlusion by an examiner who was blinded to the identity of these rats according to the Sederson's method with modifications. Neurological scale reads as follows: (1) The tail of the rat was lifted to observe the flexibility of the forelimbs, with both forelimbs symmetrically stretching forward scored as 0, and occurrence of left shoulder inflexion, elbow inflexion, shoulder intorsion in the contralateral forelimb opposite to the operation side scored as 4. (2) The animal was placed on a plane, and its shoulders were pushed to the opposite direction to check resistance, Equivalency and strong resistance in both sides was scored as 0, and reduction in resistance in the contralateral side was scored as 1-3 according to extent of reduction. (3) Both forelimbs of the rat were placed on wire gauze to observe the muscular tension. Equivalency and strong tension in both sides was scored as 0, and reduction in muscular tension in the contralateral forelimb was scored as 1-3 according to extent of reduction. According to the criteria above, the full score is 10, and the

higher the score is, the more serious the behavior disorder of the animal is.

Measurement activity of SOD and concentration of MDA

Finally, the rats were anesthetized by sodium pentobarbital, then were killed by decapitation, and the brains were removed rapidly without cerebral and olfactory bulb. Brain homogenate was prepared. The concentration of MDA and activity of SOD in cerebral tissue homogenate were measured using an assay kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's protocol.

Histopathological examination

A total of 30 animals were divided into five groups: (1) sham-operated group, (2) model group, (3)NKN 1 group, (4) NKN 2 group, (5) venoruton group. All animals received treatments as mentioned above, at one hour after being administrated, they were anesthetized, and fixed. As mentioned above, all rats' MCA were occluded except sham-operated group. the rats were killed by decapitation, and the brains were dissected rapidly from the skull. The hippocampus was removed and sliced at 400 µm thickness. Slices were prepared from the middle third of the hippocampus in a plane perpendicular to the septotemporal axis. Slices were placed in 40mL neutral buffered formalin and fixed. Slices were dehydrated and embedded in paraffin (slice thickness 5 µm).

Immunohistochemistry

Paraffin slides were deparaffinized. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 10 min, followed by rinsing in distilled water for 3 times. Tissue was then blocked in 5% goat serum, followed by incubation for 20 min at room temperature, and then the anti-human NMDAR1 monoclonal antibody at a 1:100 dilution was added and allowed to incubate at 4°C overnight. The next day the slides were rinsed 3 times for 3 min each in PBS. Biotin-goat anti-mouse IgG was added and then allowed to incubate at 37°C for 30min, followed by rinsing 3 times for 3 min each in PBS. Peroxidase streptavidin was added, followed by incubation for 20 min at 37°C. The slides were washed 3 times for 3 min each in PBS and diaminobenzidine (DAB) solution was applied and allowed to incubate for 20 min. The DAB was drained and the slides were placed in water for 5 min. Slides were counterstained in hematoxylin. The slides were then dehydrated and then mounting medium and coverslips were applied. The slides were scanned with image analyzer, and optical density values were determined.

In Situ Hybridization

Paraffin slices were routinely deparaffinized, and rehydrated. Endogenous peroxidase was blocked with 3% H₂O₂ solution for 10 min. The slices were digested with pepsase containing 3% citric acid dilution at 37°C for 30 min, washed with PBS 3 times for 5 min each, rinsed with distilled water. Hybridization was carried out by applying the diluted probe to each slide section. Each slice was covered with a cover glass and

incubated in a humidified chamber overnight at 37°C. On the following day, slices were washed 3 times in washing solution (2x SCC) at 37°C for 5 minutes each. The mixture (20 µl stabilizing buffer of hybridized) was dropped on every slice, which was placed for 6 hours in a wet cabinet, washed 3 times in washing solution (2x SCC) at 37°C for 5 minutes each. After being blocked with blocking solution, the slices were incubated with an rabbit anti-digoxin for 1 hour at room temperature, washed in 0.5mol/L PBS (3 x 2 min). Biotin-goat anti-mouse IgG was added and then allowed to incubate at 37°C for 30min, washed in 0.5mol/L PBS (3 x 2 min). SABC was added and then allowed to incubate at 37°C for 20min, washed in 0.5mol/L PBS (4 x 5 min). DAB solution was applied and allowed to incubate for 20 min. The DAB was drained and the slices were placed in water for 5 min. Slices were counterstained in hematoxylin. The slides were then dehydrated with alcohol and then mounting medium and coverslips were applied. The slides were scanned with image analyzer, and optical density values were determined.

Statistical analysis

All results were presented as mean ± standard deviation, and Statistical significance of differences was determined using unpaired t-test, with P<0.05 considered statistically significant.

RESULTS

Neurological Examination

The results of neurological examination for the cerebral ischemia study groups are reported in Table I and Figure 1. Neurological deficit score of model group increased. Compared with sham-operated group, there was significant difference in neurological deficits score between sham-operated group and model group; Neurological deficit score of NKN group obviously decreased, there was significant difference in neurological deficits score between NKN group and model group.

Measurement activity of SOD and concentration of MDA

The results were shown in Table II, Figure 2 and Figure 3. Compared with Sham-operated group, the activity of SOD of model group decreased, the contents of MDA of model group increased. There were significant differences between the 2 groups (P<0.01) through the statistics analysis; Compared with model group, the activity of SOD of NKN groups increased, the contents of MDA of NKN groups decreased, and there were significant differences between NKN groups and model group (P<0.01) through the statistics analysis.

Histopathological examination

Sham-operated group: Image showed that morphology and structure of neuronal cell with abundant, uniform and light-stained endochylema was normal, nuclei were spherical or elliptical, and obviously visible, without degenerative and necrotic morphological changes of neuron, gliocyte, vascular endothelial cell, and changes of hyperaemia, edema and cerebromalacia focus.

Table I: The Effect of NKN on Neurological Deficit Score

groups	n	neurological deficit score
sham-operated	8	1.9±0.57
model	8	9.8±0.48 [#]
NKN1	8	6.0±0.67 ^{**}
NKN2	8	6.9±0.74 ^{**}
venoruton	8	7.5±0.53 ^{**}

compared with Sham-operated group, [#]P<0.01; compared with model group, ^{**}P<0.01.

Table II: The effects of NKN on the Activity of SOD and Concentration of MDA in Brain Issue after Cerebral Ischemia n=8

groups	SOD (NU/mg.prot)	MDA (nmol/mg.prot)
sham-operated	164.7±19.2	3.0±0.43
model	75.7±16.4 [#]	7.4±1.2 [#]
NKN1	123.6±25.1 ^{**}	3.8±0.3 ^{**}
NKN2	107.2±19.8 ^{**}	4.4±0.3 ^{**}
venoruton	91.2±10.7 [*]	5.4±0.5 ^{**}

compared with Sham-operated group, [#]P<0.01; compared with model group, ^{*}P<0.05, ^{**}P<0.01

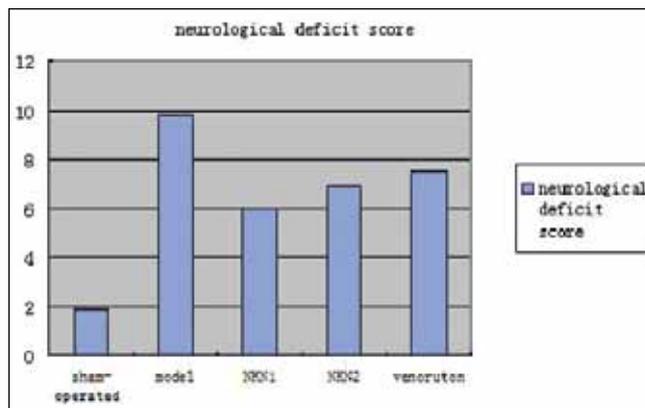


Figure 1: Neurological deficit score was significantly lower in NKN and venoruton groups (p<0.01).

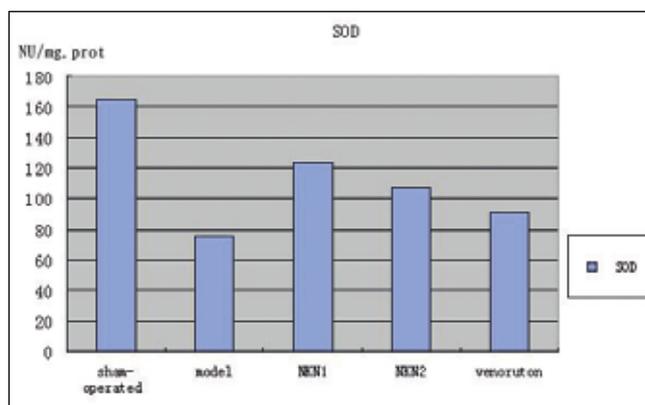


Figure 2: SOD levels were significantly higher in both NKN 1 and NKN 2 groups (p<0.01).

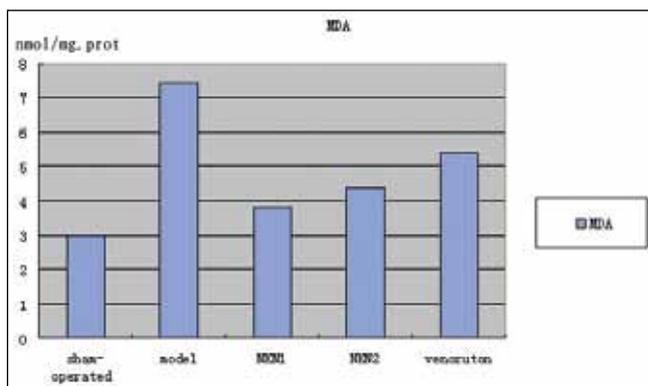


Figure 3: MDA levels were significantly lower in NKN and venoruton groups ($p < 0.01$).

Ischemia model group: Image demonstrated that there was severe damage in ischemic area, cell body and nuclei showed severe pyknosis and were darkly stained, and it isn't easy to distinguished nuclei from endochylema, the neurons in hippocampus obviously degenerated, intercellular space was 1 time larger than cell body.

NKN 1 Groups: Image showed that morphology and structure of neuronal cell with abundant endochylema was normal on the whole, nuclei were spherical or oval, and nucleoli were relatively clear, a few neurons in hippocampus had degenerated, part of cell body and nuclei showed pyknosis, intercellular space appeared and was nearly 50% cell body.

NKN 2 Groups: Image showed that morphology and structure of neuronal cell with abundant endochylema was relatively normal, nuclei were spherical or oval, and nucleoli were slightly clear, some neurons in hippocampus had degenerated, part of cell body and nuclei showed pyknosis, intercellular space appeared and was 50% cell body.

Venoruton group: Image demonstrated that morphology and structure of neuronal cell was abnormal, nucleoli were not clear, a few neurons in hippocampus had degenerated, cell body and nuclei showed dark stained pyknotic, intercellular space was 50%-100% cell body (Figur 4).

Immunohistochemistry and In Situ Hybridization

The results were shown in Table III, Figure 5, and Figure 6. Compared with Sham-operated group, level of express of NMDAR and ETmRNA-1 of model group obviously increased, and there were significant differences between the two groups ($P < 0.01$) through the statistics analysis; compared with model group, level of express of NMDAR and ETmRNA-1 of NKN groups obviously decreased, and there were significant differences between the two groups ($P < 0.05$, $P < 0.01$) through the statistics analysis. The results are shown in Table III.

DISCUSSION

The brain consumes a large quantity of oxygen because of the high metabolic rate of neurons, making it particularly susceptible to oxidative stress. Under normal physiological

Table III: Effects of NKN on the Expression of NMDAR and ET-1mRNA in Brain Tissue in Rats

groups	n	NMDAR	ET-1mRNA
sham-operated	6	5.08±0.82	9.07±2.02
model	6	9.91±1.00##	13.35±1.69##
NKN 1	6	7.88±1.20**	11.18±1.11**
NKN 2	6	8.10±0.83**	11.22±1.23*
venoruton	6	8.03±0.85**	10.61±1.53**

compared with Sham-operated group, ## $P < 0.01$; compared with model group, * $P < 0.05$, ** $P < 0.01$.

conditions, there is equilibrium between the antioxidants and oxidants produced by aerobic cellular systems. A number of studies documented that ischemic stroke was associated with increased production of free radicals in animal and human models (8, 15, 1, 16, 4, 14, 13, 19). There is a consensus of opinion that the brain ischemia-induced free radicals are capable mediating neuronal degeneration and death and are possibly involved in the pathogenesis of neuronal death in neurodegenerative diseases. Lipid peroxidation readily decomposes to liberate carbonyl fragments, the most prominent being MDA, which are highly reactive and responsible for cytotoxic effects and neuronal death. MDA level is widely used as a marker of lipid peroxidation in states of elevated oxidative stress. SOD is considered to be a key constituent in oxidative stress. The reported abnormal alteration in SOD activity in cerebral ischemia may further aggravate disorders of the brain. In the present study, the activity of SOD in the ischemic hippocampus and cerebral cortex markedly elevated, which is consistent with some studies (9, 10).

In cerebral ischemia, energy failure induces membrane depolarization and release of various neurotransmitters, such as glutamate and aspartate (2), which cause a marked influx of Ca^{2+} into postsynaptic neurons. A series of these events provokes the catastrophic enzymatic process leading to irreversible neuronal damage (12), such as degradations of DNA, protein and phospholipid.

After an ischemic stroke the levels of ET-1 is increased in plasma, cerebrospinal fluid and cerebral tissue (7, 18). In addition, exogenous ET-1 is able to decrease cerebral blood flow to levels that induce ischemia (11).

The present study showed that NKN improved activity of SOD and decreased content of MDA in in cerebellar tissue in cerebral ischemia rats, which alleviated oxidative damage. It was unequivocal from this study that NKN can decrease the number of NMDAR in brain tissue in rats after cerebral ischemia by inhibiting expression of NMDAR, thus reduced neuron damage induced by excitatory amino acids (EAA), accordingly, decreased toxicity of EAA. In addition, it was obvious from this study that NKN can reduce production of ET-1 by inhibiting expression of ET-1mRNA, thus increased blood supply in ischemia area. In conclusion, NKN can reduce

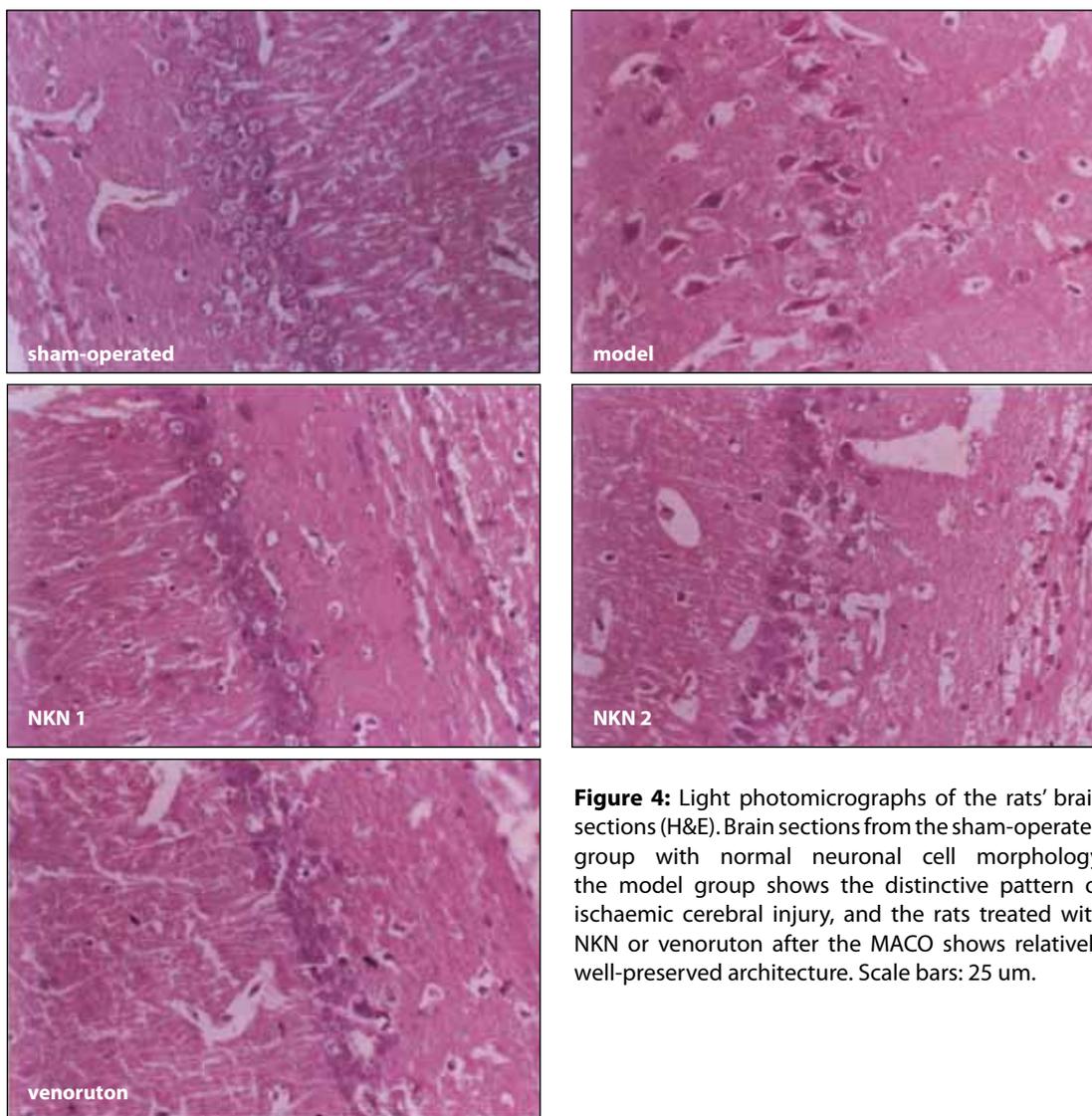


Figure 4: Light photomicrographs of the rats' brain sections (H&E). Brain sections from the sham-operated group with normal neuronal cell morphology, the model group shows the distinctive pattern of ischaemic cerebral injury, and the rats treated with NKN or venoruton after the MACO shows relatively well-preserved architecture. Scale bars: 25 um.

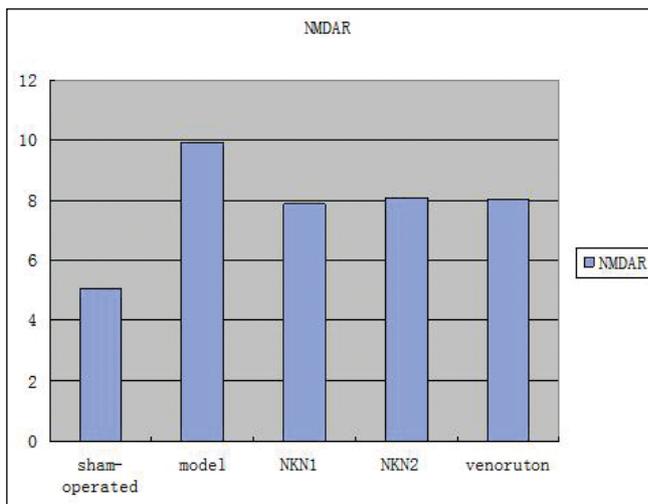


Figure 5: In the levels of expression of NMDAR, there was statistically meaningful difference in NKN and model groups ($p < 0.01$).

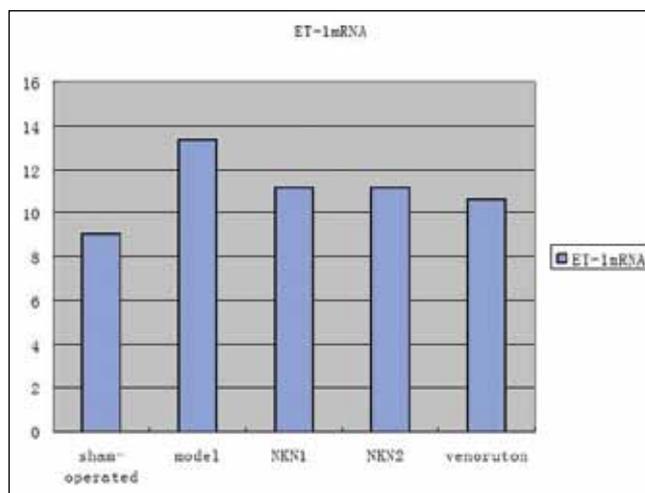


Figure 6: In the levels of expression of ET-1mRNA, there was statistically meaningful difference NKN and model groups ($p < 0.01$).

cerebral damage from cerebral ischemia, thus had protective effects on cerebral ischemia.

CONCLUSIONS

In light of the findings of the present study, we think that NKN could obviously protect brain against injury caused by cerebral ischemia.

ACKNOWLEDGMENTS

This work was supported by Health Department of Shandong Province.

REFERENCES

1. Adachi T, Nakamura M, Yamada H, Futenma A, Kato K, Hirano K: Quantitative and qualitative changes of extracellular-superoxide dismutase in patients with various diseases. *Clin Chim Acta* 229: 123-131, 1994
2. Benveniste H, Drejer J, Schousboe A, Diemer NH: Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J Neurochem* 43: 1369-1374, 1984
3. Bonita R, Mendis S, Truelsen T, Bogousslavsky J, Toole J, Yatsu F: The global stroke initiative. *Lancet Neurol* 3: 391-393, 2004
4. Gruener N, Gross B, Gozlan O, Barak M: Increase in superoxide dismutase after cerebrovascular accident. *Life Sci* 54:711-713, 1994
5. Hacke W, Schwab S, Horn M, Spranger M, De Georgia M, Von Kummer R: 'Malignant' middle cerebral artery territory infarction: Clinical course and prognostic signs. *Arch Neurol* 53: 309-315, 1996
6. Bramlett HM, Dietrich WD: Pathophysiology of cerebral ischemia and brain trauma: Similarities and differences. *J Cereb Blood Flow Metab* 24:133-150, 2004
7. Lampl Y, Fleminger G, Gilad R, Galron R, Sarova-Pinhas I, Sokolovsky M: Endothelin in cerebrospinal fluid and plasma of patients in the early stage of ischemic stroke. *Stroke* 28: 1951-1955, 1997
8. Leinonen JS, Ahonen JP, Lonnrot K, Jehkonen M, Dastidar P, Molnar G, Alho H: Low plasma antioxidant activity is associated with high lesion volume and neurological impairment in stroke. *Stroke* 31: 33-39, 2000
9. Liao Y, Wang R, Tang XC: Centrophenoxine improves chronic cerebral ischemia induced cognitive deficit and neuronal degeneration in rats. *Acta Pharmacol Sin* 12:1590-1596, 2004
10. López F, Hernández-Palazón J, López R, Alarcón E, Martínez-Lage JF: Activity of copper-zinc superoxide dismutase in a global ischemic brain lesion model without arterial hypotension. *Neurocirugia (Astur)* 2:151-158, 2004
11. Macrae IM, Robinson MJ, Graham DI, Reid JL, McCulloch J: Endothelin-1-induced reductions in cerebral blood flow: Dose dependency, time course, and neuropathological consequences. *J Cereb Blood Flow Metab* 13: 276-284, 1993
12. Mitani A, Takeyasu S, Yanase H, Nakamura Y, Kataoka K: Changes in intracellular Ca^{2+} and energy levels during in vitro ischemia in the gerbil hippocampal slice. *J Neurochem* 62: 626-634, 1994
13. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351-358, 1979
14. Okabe T, Hamaguchi K, Inafuku T, Hara M: Aging and superoxide dismutase activity in cerebrospinal fluid. *J Neuro Sci* 141:100-104, 1996
15. Spranger M, Krempien S, Schwab S, Donneberg S, Hacke W: Superoxide dismutase activity in serum of patients with acute cerebral ischemic injury. Correlation with clinical course and infarct size. *Stroke* 28: 2425-2428, 1997
16. Strand T, Marklund SL: Release of superoxide dismutase into cerebrospinal fluid as a marker of brain lesion in acute cerebral infarction. *Stroke* 23: 515-518, 1992
17. Tamura A, Graham DI, McCulloch J, et al: Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 1: 53-60, 1981
18. Viossat I, Duverger D, Chapelat M, Pirotzky E, Chabrier PE, Braquet P: Elevated tissue endothelin content during focal cerebral ischemia in the rat. *J Cardiovasc Pharmacol, Suppl* 8: S306-309, 1993
19. Yamamoto M, Shima T, Uozumi T, Sogabe T, Yamada K, Kawasaki T: A possible role of lipid peroxidation in cellular damages caused by cerebral ischemia and the protective effect of alpha-tocopherol administration. *Stroke* 14: 977-982, 1983