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Nicotinamide therapy protects against both necrosis and apoptosis in a stroke model

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Abstract

Background and purpose: Nicotinamide protects against brain damage in ischemia–reperfusion. However, the dosage and time of treatment require clarification. It is also not clear if nicotinamide can protect against both necrosis and apoptosis. **Methods:** Dose–response and time–effect studies were designed. Transient focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) for 90 min. Different doses of nicotinamide were injected upon reperfusion. In time–effect studies, 500 mg/kg nicotinamide was administered at different times after the onset of reperfusion. Neurological finding scores were recorded. Infarct volumes were measured. **Results:** In contrast to controls, neurological deficit scores and infarct volumes were greatly reduced by treatment with nicotinamide. The ED_{50} of nicotinamide was 239 ± 79 mg/kg (P=.95). It was found that nicotinamide injected during the first 6 h of reperfusion could effectively inhibit the development of brain damage. The optimal dose of nicotinamide was 500 mg/kg and gave a maximal response. **Conclusions:** Poly(ADP-ribose) polymerase (PARP) plays a key role in DNA repair in stroke. Excessive PARP activity consumes NAD leading to energy depletion and neuronal damage. As an inhibitor of PARP, nicotinamide promotes the supply of energy. The results suggest that early application of nicotinamide at a suitable dosage significantly ameliorates necrotic and apoptotic brain injury after focal ischemia–reperfusion.

Keywords: Nicotinamide; Infarct volume; Limit; Dose–response; Time course; Neurological deficit

1. Introduction

Stroke is induced by a sudden cessation of blood flow to a portion of the brain. Restoration of blood flow can be beneficial and can contribute to further brain injury, as in reperfusion injury. When reperfusion occurs, hydrogen peroxide and radicals such as $HO^{\cdot}$ and $NO^{\cdot}$ are formed in the brain (Morimoto et al., 1996; Patt et al., 1988; Sato et al., 1994). Hydrogen peroxide or $NO^{\cdot}$ enters the nucleus of a brain cell and can damage and fragment the nuclear DNA within minutes of reperfusion (Sandstrom and Marklund, 1990; Yabuki et al., 1997). It has been shown that DNA damage after ischemic insults may activate the nuclear enzyme poly(ADP-ribose) polymerase (PARP). ADP-ribose polymer synthesis comes at a very high-energy cost. It is therefore conceivable that excessive activation of PARP may result in NAD rundown and subsequently ATP depletion (Lo et al., 1998; Zhang et al., 1994). Further depletion of energy stores under already ischemic conditions can lead to necrosis and apoptosis of neurons. Thus, the imbalance between energy supply and consumption is a critical factor in the development of stroke.

Nicotinamide, a soluble B group vitamin (vitamin B_3), is an essential precursor of NAD^+ and a PARP inhibitor (Klaidman et al., 1996). Nicotinamide prevents the depletion of NAD^+ and protects against the decreased production of ATP. Nicotinamide possesses neuroprotective actions against neurochemical toxin-induced lesions in rodent brains (Beal et al., 1994; Klaidman et al., 1996). It has been also found that delayed treatment with nicotinamide improves neurological outcome and reduces infarct volume after transient

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focal cerebral ischemia in Wistar rats (Mokudai et al., 2000). However, questions remain as to the minimum and safe dose of nicotinamide and as to the best time of administration of the medicine in ischemia–reperfusion.

To observe the physical signs and brain parenchymatous lesion in a stroke model comprehensively, we have investigated neurological behavior and infarct volume in a transient middle cerebral artery occlusion (MCAO) rat model. Both a dose–response relation and a time–effect study were designed to examine the possible dose or time-dependent neuroprotection of nicotinamide against brain damage.

Nicotinamide administration does not alter physiological parameters. After administration of nicotinamide to control animals or animals subjected to brain ischemia and reperfusion, blood pH, pCO_2, hematocrit, blood glucose, blood pressure and heart rate remain unchanged compared to nicotinamide naïve controls for at least 4 h (Ayoub et al., 1999). Ketamine, also used in the current study, does not alter physiological parameters. After ketamine administration, blood pressure, blood pH, pCO_2, pO_2, cranial temperature, blood glucose and hematocrit remain unchanged compared to control animals for at least 2 h (Ridenour et al., 1991).

2. Materials and methods

Wistar male rats were used in this study to examine brain infarctions. Animal experiments conformed to the current " Guide for the Care and Use of Laboratory Animals," and federal laws and regulations, and have been approved by the USC Animal Care and Use Committee.

2.1. Transient focal cerebral ischemia model

Male adult Wistar rats weighing 240–260 g were kept in the USC Vivarium for about 1 day. They had free access to food and water. The procedure of Chan was used to induce MCAO (Yang et al., 1992). Rats were anesthetized with ketamine and xylazine (200 and 2 mg/kg ip). During the operation, anesthesia was maintained with 1% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide. The left common carotid artery was exposed through a midline incision in the neck. The external carotid artery was tied closed. A 3-0 nylon suture with its tip rounded by heating over a flame was introduced into the common carotid artery and advanced into the internal carotid artery for a length of about 22 mm, thereby occluding blood flow through the middle cerebral artery. The suture was left in place for 90 min, then was gently taken out. Reperfusion of the middle cerebral artery could occur immediately. The surgical wound was closed with surgical thread. The animals were returned to their cages and maintained until needed. The neurobehavioral deficits and brains of each rat were examined at 24 or 48 h after the onset of reperfusion.

2.2. Administration of the drug

2.2.1. Dose–response relation study design

Nicotinamide was administered to rats in doses of 1000, 500, 250, 125, 62.5 and 31.25 mg/kg (ip). The drug or vehicle solution in the same volume was injected intraperitoneally at the onset of reperfusion after 90 min of ischemia. At 24 h, the neurological deficit findings were recorded and rats were euthanized.

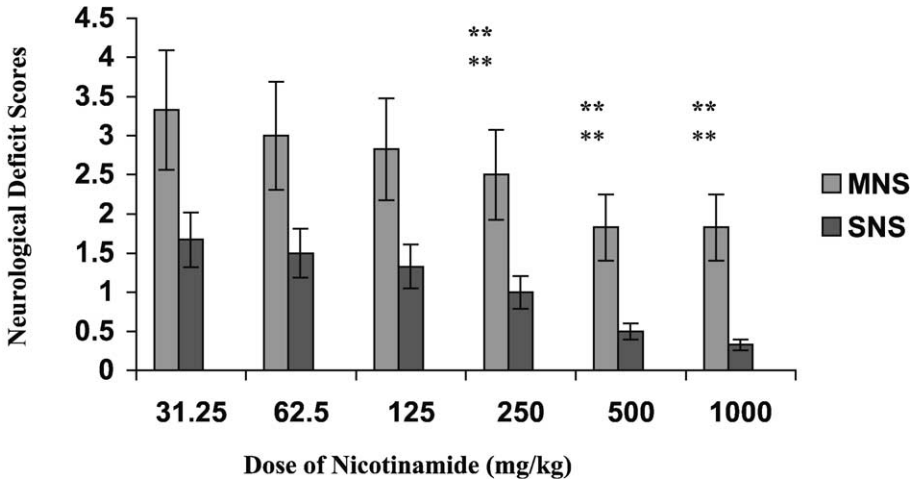

Fig. 1. Effects of various nicotinamide dosages on neurological deficits. Values are means ± S.E.M. Motor neurological scores (MNS) in the saline-treated group were 3.33 ± 0.47. Control sensory neurological scores were 1.83 ± 0.37. There were six rats in each group. Compared with values in the saline-injected (vehicle) group, injection of nicotinamide in different doses intraperitoneally immediately at the onset of reperfusion after 90 min of ischemia in Wistar rats reduced both motor and sensory behavior deficits. $**P<.01$ vs. saline by the Kruskal–Wallis test and the nonparametric multiple comparison test.

2.2.2. Time–effect relation study design

Nicotinamide (500 mg/kg) was given to rats at 0, 1, 3, 6, 12 or 24 h after the onset of reperfusion. A control group was treated at 0 h with vehicle only. All rats were examined at 48 h of reperfusion.

2.3. Neurological deficit status evaluation

The neurological deficit status was evaluated at 24 or 48 h after surgery by two examiners. A method published previously was used to evaluate the motor and sensory deficits (Belayev et al., 1996). Five categories were scored to evaluate motor neurological deficit: Grade 0 (*no observable deficit*), Grade 1 (*forelimb flexion*), Grade 2 (*forelimb flexion with decreased resistance to lateral push*), Grade 3 (*forelimb flexion with decreased resistance to lateral push and unilateral circling*), Grade 4 (*forelimb flexion and difficulty or inability to ambulate*). To score the sensory neurological deficit, the affected forelimb received forward and lateral visual tests, which were scored as follows: Grade

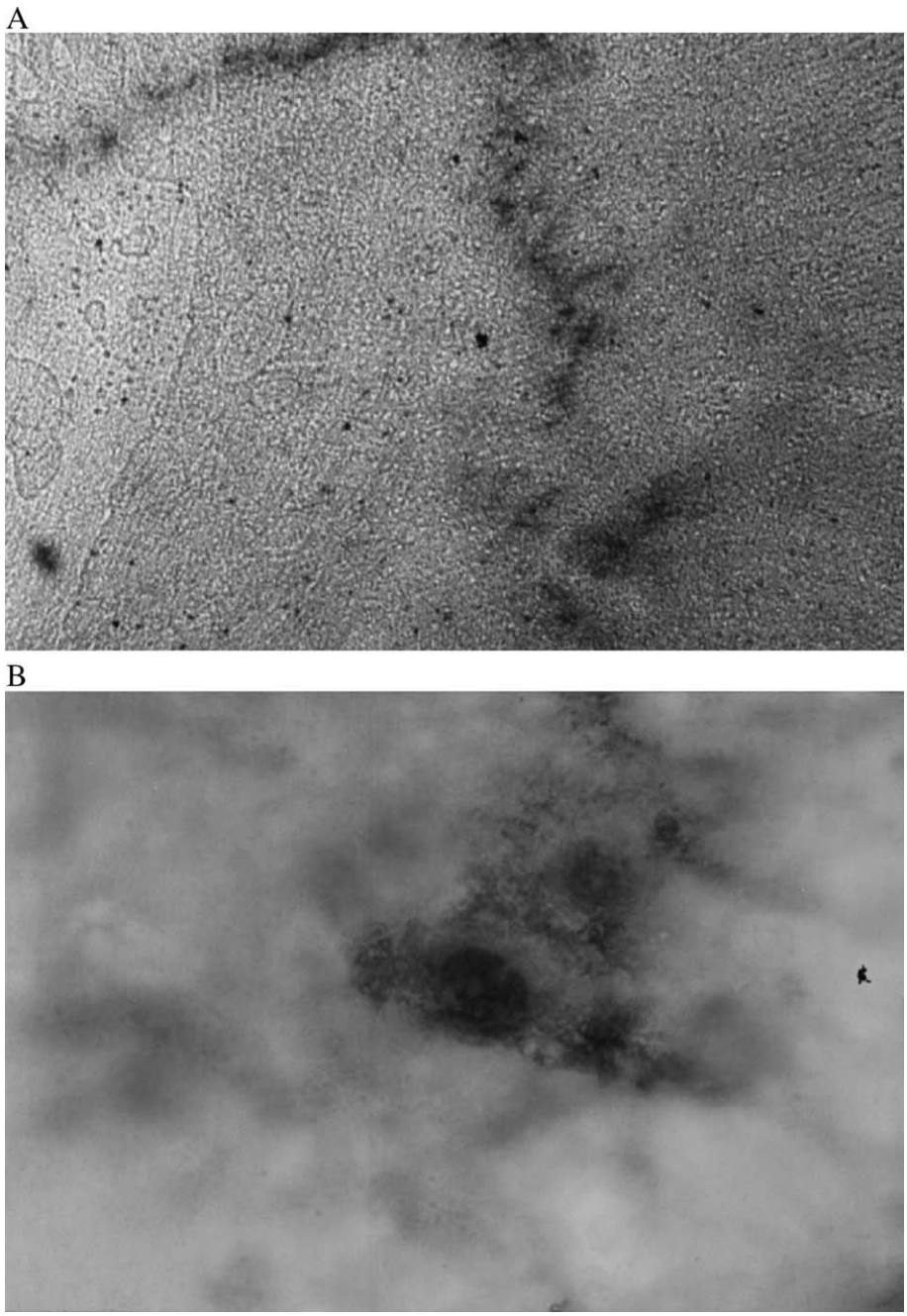

Fig. 2. Cells in the ‘‘limit’’ area of the striatum: in (A), TUNEL-stained cells at the ‘‘limit’’ area border surrounding the core at 24 h of reperfusion (× 240). In (B), a closer view of two apoptotic cells in the ‘‘limit’’ showing condensed nuclei and condensed cytoplasms with vacuoles (× 600). In (C), cresyl violet stained cells at the ‘‘limit’’ area border (× 240). In (D), a closer view of an apoptotic neuron in the ‘‘limit’’ with a condensed nucleus and cytoplasm (× 600).

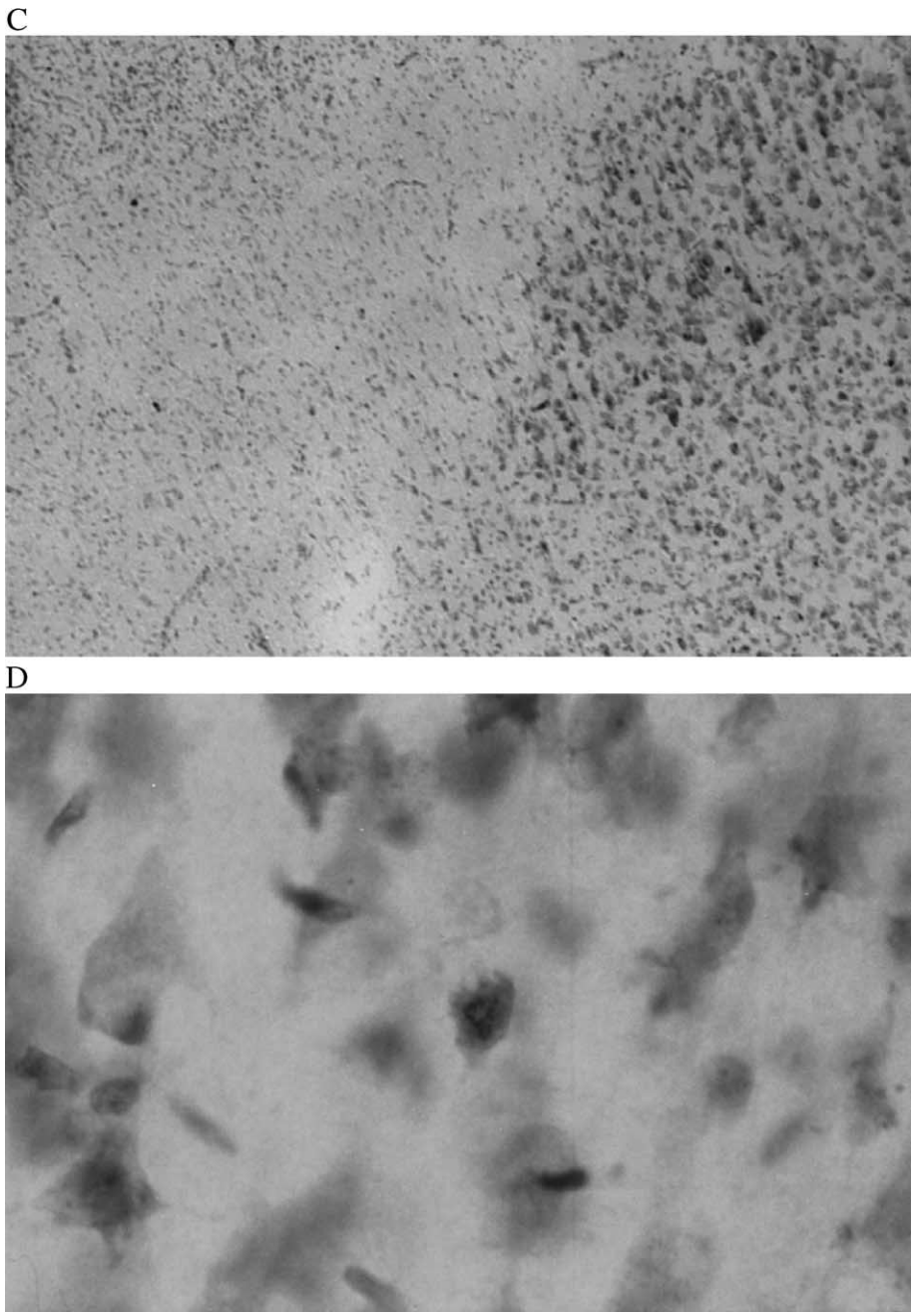

Fig. 2 (*continued*).

0 (*complete immediate placing*), Grade 1 (*incomplete and/or delayed placing*, <2 s), Grade 2 (*absence of placing*).

2.4. Morphometric measurement of infarction volume

After 24 or 48 h of reperfusion, brains were removed and washed with saline. The reperfusion injury was visually confirmed. This was followed by cutting the brain into seven coronal brain sections, each 2 mm thick with a rodent matrix. Then, the slices were immersed in saline containing 1% 2,3,5-triphenyltetrazolium chloride (TTC) and kept at 37 °C in the dark for 15 min and turned every 5 min (Sun and Cheng, 1998). The stained slices were fixed with 10% phosphate-buffered formalin. Images of each slice were scanned and stored on a computer. The infarction area of each slice was traced and calculated using the NIH image analysis program for both color and black and white images. Each color picture indicated a light red area, designated the ‘‘limit’’ area, which surrounded the white core area. The ‘‘limit’’ volume was calculated as the difference between the infarct volume in color and that in black and white. The infarction volume from the black and white images was the core infarction volume. Total infarction volume for each brain was calculated by summation of the core infarction areas and ‘‘limit’’ areas of all

slices (total area × thickness). Infarct volume or "limit" volume was corrected for swelling using the formula, corrected infarct volume = infarct volume × contralateral hemishpere volume/ipsilateral hemisphere volume.

In order to clearly describe the "limit" area, some brain samples were stained with cresyl violet or the TUNEL technique (terminal deoxynucleotidyl transferase). Rats were perfused with 0.175 M sodium phosphate in 0.155 M saline (pH 7.5, PBS), then with 4% formaldehyde and 0.2% glutaraldehyde in PBS. Brains were sliced with a microtome to make 50-μm slices. These slices were stained with 0.5% cresyl violet to visualize nissl substance or the TUNEL technique to visualize DNA fragments.

2.5. Statistical analysis

Group size for the assessment of neurological deficits and infarct volumes from the various nicotinamide treatment groups and the group treated with vehicle was six rats at least. The data were expressed as means ± S.E.M. ANOVA, Kruskal–Wallis and Student's *t* tests were used. The ratios of positive TTC staining between groups were analyzed with the exact probabilities test. *P* values less than .05 were considered significant.

3. Results

3.1. Experiment 1: dose–response relation study

3.1.1. Neurological deficit

After 24 h of reestablishment of blood flow, all rats in the group treated with vehicle solution developed serious neurological deficits. Compared with vehicle-treated rats, both motor neurological scores and sensory scores in the rats treated with nicotinamide were improved gradually with increasing dosage. The motor neurological scores in the rats administered 250 mg/kg or above of nicotinamide were decreased significantly, as were the sensory neurological scores (Fig. 1).

3.1.2. Infarct volume

All rats treated with vehicle solution after 90 min of ischemia developed visible infarctions. The damaged areas included both the cortex and the striatum. The necrotic core was found to be surrounded by an area of apoptotic and necrotic cells, designated the "limit" (Fig. 2). The "limit" appears pink in color images of TTC stained slices. Apoptotic cells in the "limit" have cytoplasms that are shrunken, condensed and vacuolated (Fig. 2). The nuclei of these cells are condensed. Many apoptotic bodies (small condensed bodies) can be seen in the "limit." The core also contains some apoptotic bodies. Some cells in the "limit" area are not apoptotic but have taken up DNA fragments into their cytoplasms from neighboring apoptotic and necrotic cells. In these cells, the nuclei and cytoplasms appear normal.

The results of brain infarction volumes conformed to the change in behavioral deficits. After treatment with nicotinamide, the total infarction volumes were drastically reduced. The damaged areas in the groups which were treated with nicotinamide (125 mg/kg or above) were mainly found in portions of the striatum. With increasing doses of nicotinamide, the infarct volumes in MCAO rats were decreased significantly. At the same time, the ratio of the "limit" volume to the total infarction volume in all groups treated with nicotinamide was obviously increased. Doses of 500 or 1000 mg/kg of nicotinamide also reduced total "limit" volume significantly. This suggests that nicotinamide can protect cortical and striatal areas from further damage. The neuroprotection of nicotinamide reached its apparent maximum at a dose of about 500 mg/kg (Table 1).

3.1.3. Dose–response curve

In contrast to rats treated with saline, the brain damage in rats in all groups treated with nicotinamide was lessened to some extent (Fig. 3). There was obvious improvement with nicotinamide doses in the range of 31.25 to 1000 mg/kg. Protection increased as dosage increased up to 500 or 1000 mg/kg. The ED_{50} of nicotinamide was 239 ± 79 mg/kg (P=.95). The optimal dose of nicotinamide appears to be 500 mg/kg, since this gives maximal protection against infarction. There was no sign of nicotinamide toxicity, in terms of mortality, in any rat after 24 or 48 h. Upon gross examination, all internal organs, such as the liver and kidneys, in nicotinamide treated rats appeared normal.

3.2. Experiment 2: time–effect study

3.2.1. Neurological deficit

There was no obvious difference in the neurological deficits between groups treated with saline that underwent

Table 1
Effects of nicotinamide on total infarct volume and limit volume in MCAO rats

Group	Dose (mg/kg)	Total infarct volume (mm^3)	Ratio of limit volume (%)	Limit volume (mm^3)
Saline		337.0 ± 59.7	30.3 ± 13.7	102.0 ± 47.0
NAM	31.25	305.4 ± 116.9	42.5 ± 18.4	129.9 ± 62.4
NAM	62.5	258.1 ± 79.1	43.8 ± 11.2	113.1 ± 44.9
NAM	125.0	232.8 ± 121.4 *	46.5 ± 11.6 *	108.3 ± 64.0
NAM	250.0	186.2 ± 82.2 **	48.2 ± 12.2 **	89.7 ± 38.6
NAM	500.0	69.5 ± 70.1 **	73.2 ± 19.1 **	50.9 ± 40.3 *
NAM	1000.0	53.0 ± 53.6 **	92.5 ± 6.3 **	49.0 ± 48.2 *

Values are mean ± SEM. There were six rats in each group. Compared with values in the saline-injected (vehicle) group (after 24 h of reperfusion), injection of nicotinamide (NAM) in a dose of 125 mg/kg or above immediately at the onset of reperfusion after 90 min of ischemia in rats reduced total infarct volumes and increased ratios of "limit" volume/total infarct volume significantly. NAM (500 mg/kg or above) also reduced "limit" volumes significantly.

* P < .05 vs. saline by ANOVA and Newman–Keuls test.

** P < .01 vs. saline by ANOVA and Newman–Keuls test.

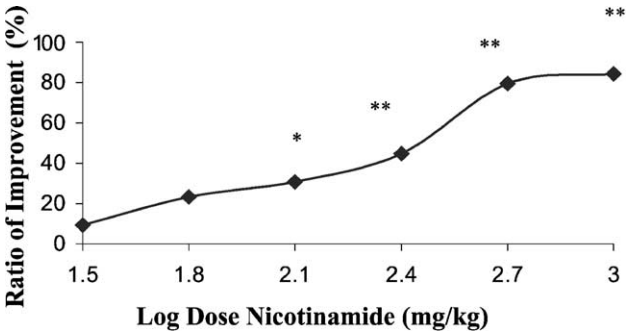

Fig. 3. Improvement in total infarct volumes in nicotinamide-treated MCAO rats compared with saline-treated rats is demonstrated over 24 h of reperfusion after 90 min of ischemia. Values are means. There were six rats in each group. The ratio of improvement was calculated from the total infarct volumes as (control − treated)/control. $*P<.05$ and $**P<.01$ vs. saline by ANOVA and Newman–Keuls test.

48 or 24 h of reperfusion. However, it was found that delayed treatment with nicotinamide could help reduce both the motor neurological scores and sensory scores greatly. The longer the treatment was delayed, the less the neurological deficits were improved. It seemed that early treatment with nicotinamide was more critical to recovery of motor deficits than sensory deficits (Fig. 4).

3.2.2. Infarct volume and time–effect curve

There appeared to be little difference between total infarct volumes in rats in those groups that were treated with nicotinamide within the first 3 h after the onset of reperfusion. Most of the damaged area involved the striatum. However, in those rats that received a very late (>6 h) injection of nicotinamide, the damage could extend to some parts of the cortex. It was found that there existed a significant difference between controls and rats injected with 500 mg/kg of nicotinamide within 6 h after the onset of reperfusion (Fig. 5). In the first 3 h, injection of 500 mg/kg of nicotinamide not only decreased the total

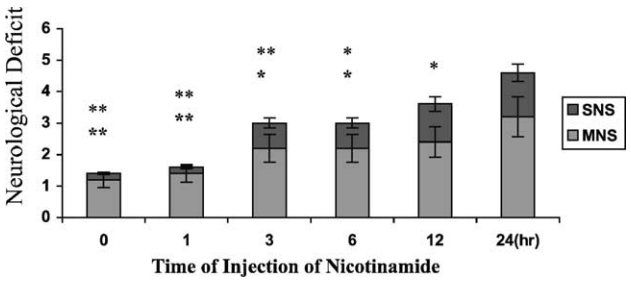

Fig. 4. Effects of delayed nicotinamide treatment on neurological deficits. Values are means ± S.E.M. Control motor neurological scores (MNS) were 3.33 ± 0.47. Control sensory neurological scores (SNS) were 1.67 ± 0.47. There were six rats in each group. Compared with values in the saline-injected (vehicle) group, injection of nicotinamide prior to 24 h of reperfusion reduced both motor and sensory behavior deficits significantly. MNS: $*P<.05$ and $**P<.01$ vs. saline by the Kruskal–Wallis test and the nonparametric multiple comparison test. SNS: $*P<.05$ and $**P<.01$ vs. saline by the Kruskal–Wallis test and the nonparametric multiple comparison test.

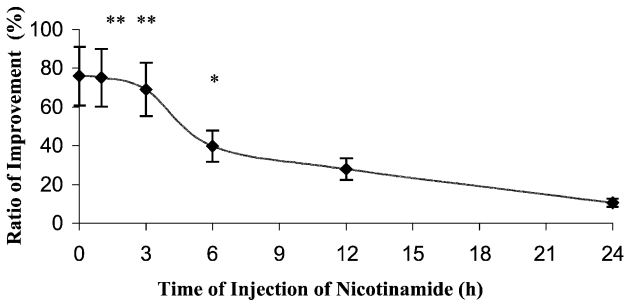

Fig. 5. Improvement in total infarct volumes in nicotinamide-treated MCAO rats compared with saline-treated rats is demonstrated over 48 h of reperfusion after 90 min of ischemia. Nicotinamide was injected at different times after the onset of reperfusion. Values are means ± S.E.M. There were six rats in each group. $*P<.05$ and $**P<.01$ vs. saline by ANOVA and Newman–Keuls test.

infarction volume but also increased the ratio of the "limit" volume/total infarction volume significantly. This means that early treatment with nicotinamide effectively lessened the numbers of necrotic and apoptotic neurons in the core and "limit." Although administration of nicotinamide at 6 h after reperfusion could still control the size of the total infarction, it did not help the ratio of the "limit" volume/total infarction volume. Even though the brain infarctions in late treatment groups were decreased to some extent, there was no statistical difference from the saline group. These results suggest that nicotinamide may play a key role in prohibiting the development of stroke. However, after brain parenchymatous damage is already formed, nicotinamide does not help (Table 2).

3.2.3. Time–effect curve

The neuroprotective effects of nicotinamide were closely related to the time of injection. Delayed administration corresponded with a decreased effect of treatment, in terms of neurological deficit. Since the development of apoptosis in neurons is maximal at about 24–48 h, administration of nicotinamide before this stage should be helpful to save some

Table 2
Effect of delayed treatment with nicotinamide on total infarct volume and limit volume in MCAO rats

Group	Dose (mg/kg)	Total infarct volume (mm^3)	Ratio of limit volume (%)	Limit volume (mm^3)
Saline		323.4 ± 65.2	31.9 ± 14.8	103.3 ± 37.5
NAM (0 h)	500.0	77.7 ± 48.4 **	60.1 ± 14.2 **	46.7 ± 24.2 **
NAM (1 h)	500.0	80.5 ± 48.5 **	62.5 ± 14.9 **	50.3 ± 24.3 **
NAM (3 h)	500.0	100.2 ± 79.5 **	58.8 ± 16.3 **	58.9 ± 17.6 **
NAM (6 h)	500.0	194.6 ± 167.3 *	34.9 ± 21.6	67.9 ± 26.9 *
NAM (12 h)	500.0	233.0 ± 106.5	29.7 ± 10.7	69.3 ± 23.8 *
NAM (24 h)	500.0	288.9 ± 46.2	38.5 ± 15.1	111.3 ± 52.5

Values are means ± S.E.M. There were six rats in each group. Compared with values in the saline-injected (vehicle) group, the delayed injection of nicotinamide (NAM) within 6 h reduced both total infarct volumes and "limit" volumes significantly. Injection of NAM in the first 3 h also increased "limit" volume/total infarct volume ratios significantly.

* $P<.05$ vs. saline by ANOVA and Newman–Keuls test.

** $P<.01$ vs. saline by ANOVA and Newman–Keuls test.

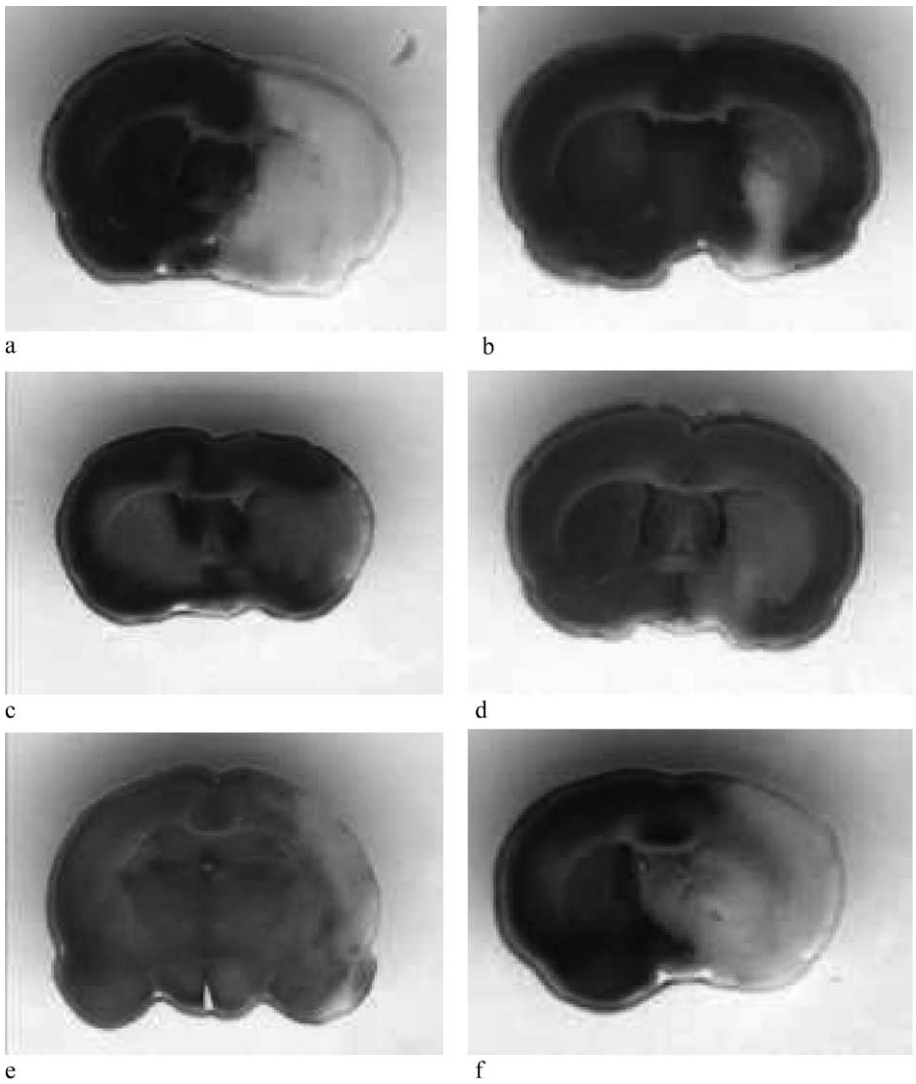

Fig. 6. Nicotinamide injected intraperitoneally at different times after the onset of reperfusion reduced the infarction volume in male Wistar rats induced by MCAO. TTC stained coronal brain sections are from representative animals that received (a) saline, (b) 500 mg/kg NAM at 0 h, (c) 1 h, (d) 3 h, (e) 6 h and (f) 12 h of reperfusion.

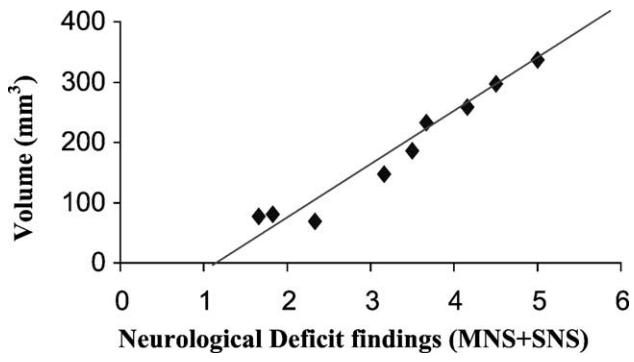

Fig. 7. The correlation between total neurological deficit findings and total infarction volume is shown. Total neurological deficit findings are the sum of motor neurological scores and sensory neurological scores. The linear regression equation was $Y = 83.32X - 88.39$. The correlation coefficient (r) was .98.

brain cells in critical condition (Fig. 6) and thereby improve the neurological deficit. Infarct volume and neurological deficit correlated significantly (Fig. 7, $P < .05$, $r = .98$).

4. Discussion

It is well known that there are at least two different kinds of cell death, necrosis and apoptosis, involved in brain damage. Neurodegeneration associated with stroke is caused by necrosis and apoptosis that can be induced by DNA damage. Nicotinamide decreases DNA damage by inhibiting PARP and increasing NAD levels (Klaidman et al., 1996; Adams et al., 1999). The inhibition of DNA damage prevents necrosis and apoptosis (Adams et al., 1999). The effects of nicotinamide are due to a specific uptake mechanism which allows nicotinamide to penetrate into the brain

rapidly (Spector, 1979). Due to this specific mechanism, nicotinamide can easily reach high levels in the brain, increase brain levels of NAD and ATP and inhibit the use of NAD by PARP by inhibiting the catalytic site of this enzyme.

When cerebral ischemia and reperfusion occur, necrosis follows rapidly, within a few minutes or hours. The core of the lesion in stroke is mainly constituted of necrotic cells. However, patients suffering from stroke typically exhibit a progressive neurodegeneration over the first day. This may be due to a delayed increase in the lesion in their brains. Although necrosis develops in a rapid process within hours after ischemia and reperfusion, apoptosis is a delayed, but critical, process that may require days. The progression of the lesion in ischemia–reperfusion may involve an apoptotic process (Du et al., 1996; Adams et al., 1996; Mukherjee et al., 1997). It has been found that a mildly ischemic area, the penumbra area, borders the core ischemic area (Du et al., 1996; Qin, 1998). Penumbral cells undergo an insult that is not as severe as the insult suffered by the core cells. Most core cells probably die within minutes or hours through a necrotic process. The inner part of the penumbra may become part of the core. The outer part of the penumbra may become what has been designated the ''limit.'' Cells in the ''limit'' may die through necrosis and a delayed process that involves apoptosis.

Previous reports have shown that nicotinamide reduced brain infarctions in rats 24 h after permanent or transient MCAO (Mokudai et al., 2000; Ayoub et al., 1999; Sakabibara et al., 2000). However, the optimal dose and time of administration are not known. In this study, we designed two different experiments to look for the most suitable dosage of nicotinamide and the most effective administration period. Published studies have found that nicotinamide can be used safely in rats at doses below 600 mg/kg daily for several weeks. However, daily doses of 600 mg/kg of nicotinamide cause kidney and liver hypertrophy and increase hepatic lipid content within 2–5 weeks (Kang-Lee et al., 1983). Doses of 900 mg/kg daily for 3 weeks cause lipid peroxidation in the liver (Henning et al., 1989). The LD_{50} of oral nicotinamide has been reported to be about 2.7 g/kg (Unna, 1939). The LD_{50} of intraperitoneal nicotinamide or subcutaneous nicotinamide have been reported to be about 1.5 and 1.7 g/kg, respectively (Ellinger et al., 1947; Brazda and Coulson, 1946). The present study did not examine the toxicity of nicotinamide in detail.

In order to rescue the still viable but injured nerve cells within the ischemic area, effective therapy should be started at the earliest possible time. Measures to halt or reverse necrosis or programmed cell death, to enhance the intrinsic autoprotective and repair mechanisms, involve supplying enough energy in time to neurons in the critical area. In contrast to the saline-treated group, it is very clear that nicotinamide treatment, at the right dose and time of administration, decreased the amount of necrosis in the core. The ''limit'' in some of the nicotinamide-treated groups decreased implying that the number of apoptotic cells decreased. The prevention of apoptosis by nicotinamide in neuronal and endothelial cell cultures has been found by others (Lin et al., 2000; Maiese et al., 2001).

Damaged cells in the core respond to nicotinamide more than do damaged cells in the ''limit.'' This can be seen in the dose–response curve where the total infarction volume decreased significantly at doses as low as 125 mg/kg. The volume of the ''limit'' decreased significantly only after 500 mg/kg of nicotinamide. In fact, the percentage of the ''limit'' volume in the total infarct volume increased after administration of some doses of nicotinamide. However, rescue of damaged cells in the core must be accomplished within 6 h, whereas damaged cells in the ''limit'' can still be rescued as late as 12 h after reperfusion. This strongly implies that damaged cells in the core tend to die rapidly by mostly necrotic mechanisms, whereas ''limit'' cells die by delayed mechanisms probably involving apoptosis.

Other work has also shown that 500 mg/kg of nicotinamide is the optimal dose for prevention of infarction (Mokudai et al., 2000; Ayoub and Maynard, 2002). However, this previous work found that doses higher than 500 mg/kg were not protective. The current study found protection by nicotinamide up to 1000 mg/kg. The difference between the current and previous studies is that the current study used ketamine in conjuction with nicotinamide, whereas the previous study did not use ketamine.

Motor and sensory neurological findings and anatomic lesions of the brain were simultaneously affected by nicotinamide with increasing dosage. In a time-dependent manner, we have confirmed a significant correlation between the effects on neurological deficits and brain damage, as total infarct volume (Fig. 7). Although delayed treatment with nicotinamide, 12 h after reperfusion, could not reduce the total infarction volumes effectively, the neurobehavioral scores in MCAO rats were still obviously improved at that time. This implies that a small reduction in total infarction volume can significantly improve behavioral outcome. Concurrent work has confirmed that nicotinamide administered as long as 6 h after ischemia onset can protect against infarction assessed 7 days later (Ayoub and Maynard, 2002).

It is important to point out that the rats in this study were pretreated with ketamine before induction of ischemia. Ketamine may have neuroprotective actions (Spandou et al., 1999; Pfenninger and Himmelseher, 1997). Ketamine blocks NMDA receptors (Orser et al., 1997), decreases intracranial pressure (Albanese et al., 1997) and may have radical scavenging activity. The present study was not designed to investigate the neuroprotective actions of ketamine.

Ketamine was used in this study because our previous work has found that ketamine synergized the activity of nicotinamide in the inhibition of infarct formation. Without

ketamine, nicotinamide has a modest effect. Neither nicotinamide nor ketamine alters blood gases, pH or other physiological parameters (Mokudai et al., 2000; Ayoub et al., 1999; Sakabibara et al., 2000, Albanese et al., 1997). Ketamine may decrease body temperature. However, ketamine by itself does not decrease infarct formation (Chang et al., 2002; Ridenour et al., 1991). Therefore, the ketamine-induced decrease in body temperature and intracranial pressure by themselves is not enough to prevent infarct formation.

Other agents used in this study were nitrous oxide and isoflurane. Neither nitrous oxide nor isoflurane appears to alter the neuroprotective activities of nicotinamide or ketamine in the current stroke model (Chang et al., 2002). Isoflurane has been found to be neuroprotective in a stroke model (Soonthon-Brant et al., 1999). This suggests that isoflurane could contribute to the neuroprotection produced by the combination of nicotinamide and ketamine. However, previous work from the current laboratory has shown that nicotinamide and ketamine, without isoflurane, produced a substantial neuroprotection in another stroke model (Klaidman et al., 1996).

Our laboratory has investigated and confirmed that the neuroprotective effects of nicotinamide are due to several mechanisms (Klaidman et al., 1996; Adams et al., 1996, 1999; Mukherjee et al., 1997). Nicotinamide was found to increase NAD levels dramatically, which leads to less DNA fragmentation and may allow rapid DNA repair. The inhibition of DNA fragmentation was shown to prevent apoptosis in the brain. Nicotinamide was shown to prevent necrosis when administered prior to the induction of oxidative stress. In addition, nicotinamide was shown to inhibit brain PARP, when brain nicotinamide levels are high. Inhibition of PARP is likely to be involved in the prevention of NAD and ATP depletion. Nicotinamide was found to inhibit xanthine oxidase and was capable of scavenging oxygen free radicals (Adams et al., 1999; Mukherjee et al., 1997). Thus, nicotinamide has antioxidant effects as well as DNA protective effects. Recent research has indicated that administration of nicotinamide resulted in higher brain NAD^+, NADH, $NADP^+$ and NADPH levels in some regions. The synthesis of these pyridine nucleotides was up-regulated during oxidative stress in animals treated with nicotinamide (Klaidman et al., 2000).

There are currently several strategies for the development of therapeutic agents for stroke. Tissue plasminogen activator (tPA) and similar agents are somewhat effective in a small percentage of patients, where they may serve to dissolve blood clots and help restore blood flow. Another strategy is to use neuroprotective agents which can decrease the progression of neurodegeneration by inhibiting DNA fragmentation caused by oxygen free radical production associated with ischemia and reperfusion. It is unlikely that the neuroprotective effects of nicotinamide are due to an increase in regional cerebral blood flow and cerebral metabolic rate (Huang and Chao, 1960). In fact, it has been reported that administration of 500 mg/kg of nicotinamide decreases regional cerebral blood flow in normal rats (Brown et al., 1999). Hence, It is clear that nicotinamide plays its role through preservation of energy and inhibition of PARP. Nicotinamide, or NAD, has been used for many years to treat neurodegenerative disorders such as dementia associated with pellagra, alcoholism-associated dementia, Parkinson's disease and Alzheimer's disease (Adams et al., 1999; Birkmayer, 1996; Birkmayer et al., 1993). Nicotinamide may possess other effects, such as anticonvulsant activity, anticoagulant activity and inhibition of lipid peroxidation (Kang-Lee et al., 1983; Kryzhanovskii et al., 1980; Braslavskii et al., 1982; Chumakov and Starchik, 1991). Nicotinamide exhibits very little toxicity in patients even at high doses (Adams et al., 1999; Horsman et al., 1993).

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