Delayed Treatment With Nicotinamide (Vitamin B₃) Improves Neurological Outcome and Reduces Infarct Volume After Transient Focal Cerebral Ischemia in Wistar Rats

Toshihiko Mokudai, MD; Issam A. Ayoub, MSc, MD; Yohtaro Sakakibara, MD; E-Jian Lee, MD; Christopher S. Ogilvy, MD; Kenneth I. Maynard, MSc, PhD

Background and Purpose—We have previously shown that nicotinamide (NAm) acutely reduces brain infarction induced by permanent middle cerebral artery occlusion (MCAo) in rats. In this study, we investigate whether NAm may protect against ischemia/reperfusion injury by improving sensory and motor behavior as well as brain infarction volumes in a model of transient focal cerebral ischemia.

Methods—Forty-eight male Wistar rats were used, and transient focal cerebral ischemia was induced by MCAo for 2 hours, followed by reperfusion for either 3 or 7 days. Animals were treated with either intraperitoneal saline or NAm (500 mg/kg) 2 hours after the onset of MCAo (ie, on reperfusion). Sensory and motor behavior scores and body weight were obtained daily, and brain infarction volumes were measured on euthanasia.

Results—Relative to treatment with saline, treatment with NAm (500 mg/kg IP) 2 hours after the onset of transient focal cerebral ischemia in Wistar rats significantly improved sensory (38%, P < 0.005) and motor (42%, P < 0.05) neurological behavior and weight gain (7%, P < 0.05) up to 7 days after MCAo. The cerebral infarct volumes were also reduced 46% (P < 0.05) at 3 days and 35% (P = 0.09) at 7 days after MCAo.

Conclusions—NAm is a robust neuroprotective agent against ischemia/reperfusion-induced brain injury in rats, even when administered up to 2 hours after the onset of stroke. Delayed NAm treatment improved both anatomic and functional indices of brain damage. Further studies are needed to clarify whether multiple doses of NAm will improve the extent and duration of this neuroprotective effect and to determine the mechanism(s) of action underlying the neuroprotection observed. Because NAm is already used clinically in large doses and has few side effects, these results are encouraging for the further examination of the possible use of NAm as a therapeutic neuroprotective agent in the clinical treatment of acute ischemic stroke. (Stroke. 2000;31:1679-1685.)

Key Words: energy metabolism ■ middle cerebral artery occlusion ■ neuroprotection ■ niacinamide ■ stroke ■ rats

Neuronal ischemia begins as an imbalance between energy supply and demand. The consequence of this energy imbalance is the depletion of ATP, which triggers the onset of numerous ischemia-induced cascades, each of which may lead to irreversible cell injury. Therefore, rectification of the neuronal energy imbalance should lead to neuroprotection of the tissue “at risk” of ischemic injury. This may be achieved by either (1) reducing the neuronal energy demands or (2) increasing the neuronal energy reserve. We have already begun to investigate the first option with some initial success. More recently, we began studies directed at the second option. We showed that a single intraperitoneal injection of nicotinamide (NAm) reduced the infarct volume in a model of permanent middle cerebral artery (MCA) occlusion (MCAo) in Wistar rats. In that study, it was shown that NAm reduced neuronal infarction in a dose-specific manner, even when it was administered up to 2 hours after the onset of the ischemic insult. We used NAm because it prevents the depletion of nicotinamide adenine dinucleotide (NAD⁺), protects against the decreased production of ATP and lactate increases, and has been shown to be neuroprotective against neurochemical toxin-induced lesions in rodent brains. Therefore, NAm can enhance the energetic capacity of neurons and thus has the potential to protect against the initial ischemia-induced energy imbalance induced by cerebral ischemia, via boosting neuronal energy reserves to the tissue at risk.
NAm, a soluble B group vitamin (niacin or vitamin B₃), is an essential precursor of NAD⁺ and a poly-ADP-ribose polymerase (PARP) inhibitor.⁹ In addition to protecting against neurochemical-induced lesions, NAm also protects against trauma and NO exposure in the rat hippocampus.¹⁰

To extend our original findings and in line with the recent recommended standards for the preclinical testing of putative neuroprotective agents,¹¹ the present study was designed to examine whether NAm, administered 2 hours after the onset of transient MCAo, could improve the neurological (behavioral) outcome as well as reduce the infarct volume in a model of transient focal cerebral ischemia in rats after a prolonged recovery, which is more relevant to the clinical scenario.

Materials and Methods
All procedures performed on the animals in the present study were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, whose standards meet that of the Federal and State reviewing organizations.

Animal Preparation and Monitoring
Forty-eight male Wistar rats weighing 300 to 330 g (Charles River Laboratories, Wilmington, Mass) were allowed free access to food and water before and after surgery. Halothane anesthesia (1% to 2% in 50% N₂O/50% O₂) was used in free-breathing animals whose body temperatures were kept stable at 36.5 ± 0.5°C by use of a heating pad and rectal probe (Yellow Springs Instruments) from the beginning of the surgical procedure through recovery from anesthesia. The right femoral artery was cannulated for measurement of arterial blood gases, glucose, hematocrit, mean arterial blood pressure, and heart rate. These physiological parameters were monitored before, during, and after MCAo. After the procedure, anesthesia was withdrawn, and once the animals recovered from the mild stimulus, the rectal probe was removed, and the animal was returned to its cage and given water and food ad libitum.

Experimental Model
All rats were subjected to 2 hours of right MCAo. Regional cerebral blood flow measurements were not monitored. Transient focal cerebral ischemia was induced by use of a well-established and modified procedure.¹² ¹³ Under the operating microscope, the right common carotid artery was exposed through a midline incision in the neck. A 4-0 nylon suture with its tip rounded by heating over a flame and subsequently coated with poly-L-lysine (Sigma Chemical Co) was introduced into the external carotid artery and then advanced into the internal carotid artery for a length of 19 to 20 mm from the bifurcation. This method placed the tip of the suture at the origin of the anterior cerebral artery, thereby occluding the MCA. The suture was left in place for 2 hours, and the animals were allowed to awaken from the anesthesia after closure of the operation sites. During another brief period of anesthesia, the suture was gently removed at 2 hours after MCAo.

Drug Administration and Follow-Up Periods
NAm (500 mg/kg) or saline that served as the vehicle-control was administered intraperitoneally at the time of reperfusion. The rats were assigned to 1 of 2 follow-up groups, which were euthanized after either 3 or 7 days. Animals were assigned treatment in a random fashion, and the investigators performing the MCAo procedure, administering the drugs, measuring the infarction volumes, conducting the neurobehavioral test, and recording the weight of the animals were blinded as to the experimental protocol.

Quantification of Ischemic Damage

Neurobehavioral Testing
After surgery, each animal’s neurological function was evaluated on a daily basis. A modification of previously published methods was used to evaluate the sensory and motor disturbance.¹³ ¹⁵ Accordingly, 5 categories of motor neurological findings were scored: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push, and unilateral circling; and 4, forelimb flexion and being unable or difficult to ambulate. To score the sensory neurological findings, the affected forelimb received forward and sideways visual tests, which were scored as follows: 0, complete immediate placing; 1, incomplete and/or delayed placing (<2 seconds); and 2, absence of placing. In addition, each animal’s body weight was measured concurrently with the neurobehavioral testing.

Infarct Assessment
The animals were euthanized under ketamine (44 mg/kg IP) and xylazine (13 mg/kg IP) anesthesia, followed by decapitation on either day 3 or day 7. The brain was then rapidly removed, cut into seven 2-mm-thick coronal sections by use of a rat brain matrix (RBM 4000C, ASI Instruments), stained with 2,3,5-triphenyltetrazolium chloride at room temperature for 30 minutes, and then fixed in 10% buffered formalin.¹⁴ After 48 to 72 hours, the infarct area on each slice was determined by using a computerized image analyzer (Bioquant, R and M Biometrics), and the infarct areas were calculated to obtain the infarct volumes per brain (in mm³).¹⁵ Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using an “indirect method” (area of intact contralateral [left] hemisphere minus area of intact regions of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere.¹⁶

Statistical Analysis
Physiological data obtained before, during, and after ischemia and infarct volume were analyzed by ANOVA, followed by the Fisher least significant difference (protected t) post hoc tests where necessary. Body weights, which were collected daily, were analyzed by repeated measures ANOVA, followed by the Fisher least significant difference post hoc tests, and neurobehavioral scores were analyzed by a nonparametric test for independent groups, ie, the Wilcoxon rank sum/Mann-Whitney U test. The data were expressed as the mean±SEM, and the differences were considered to be statistically significant at the P≤0.05 level.

Results

Mortality
Mortality was 20.0%. Twelve animals died and were therefore not included in the 48 animals used for data analysis after MCAo in the saline- and NAm-treated groups. Ten animals died spontaneously before completing the recovery protocol: 7 were saline-injected (of which 4 were from the 3-day group), and 3 were treated with NAm (of which 1 was from the 3-day group). Two animals died on the operating table because of the occluding suture could not be advanced and thus create the MCAo (therefore, death occurred before either saline or NAm treatment).

Physiological Variables
Table 1 shows the data of physiological parameters obtained in the 48 rats that completed the present study. All data were kept within normal physiological limits before, during, and after ischemia.

Functional Outcome and Weight Gain
Compared with saline-treated rats, NAm-treated rats had significantly improved sensory and motor neurological scores at 3 and 7 days after MCAo (Table 2). NAm-treated rats also...
showed improved (P<0.05) weight gain up to 7 days after MCAo (Figure 1).

Infarct Volume
Two hours of MCAo caused lesions that were reproducible but variable in size (3-day control group, 147±71 mm³; 3-day NAm-treated group, 87±83 mm³; 1-week control group, 113±49 mm³; and 1-week NAm-treated group, 87±68 mm³). To compensate for edema formation in the ipsilateral hemisphere as well as for brain size variation from rat to rat, the infarct volume was analyzed as a percentage change relative to the contralateral (unaffected) hemisphere. Infarction lesions were reduced by 46% and 35% in the NAm-treated groups euthanized at 3 and 7 days, respectively. Only in rats euthanized at 3 days after MCAo was the reduction statistically (P<0.05) significant (Figure 2).

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<th>TABLE 1. Physiological Parameters in All Treatment Groups</th>
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Values are mean±SEM. Hct indicates hematocrit; Gluc, glucose; MABP, mean arterial blood pressure; HR, heart rate; and n, number of animals. All animals were maintained at 36.5±0.5°C rectal temperature. Physiological parameters were stable and normal in all treatment groups.

Physiological parameters are for vehicle (saline)-injected (control) animals and nicotinamide-treated (treated) animals before, during, or after ischemia by transient MCAos.

*P<0.05 vs saline.

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<th>TABLE 2. NAm Improves Sensory and Motor Behavioral Scores Throughout 3- or 7-Day Periods After Onset of MCAo</th>
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Values are mean±SEM. There were 12 animals per group. Intraperitoneal injection of NAm (500 mg/kg) 2 hours after the onset of MCAo (ie, upon reperfusion) in Wistar rats significantly reduced sensory and motor ischemia-induced behavioral deficits compared with saline-injected control values. *P<0.005 and †P<0.05 vs saline.

Figure 1. Improvement in weight gain in NAm-treated rats compared with saline-injected control rats is demonstrated over a 7-day period after transient MCAo. Data are represented as mean±SEM. There were 12 animals per group. *P<0.05 vs saline-injected rats.
Discussion

Although our previous data showed that NAm reduced brain infarction in rats 24 hours after permanent MCAo, the present experiments were not designed to address the possible mechanism(s) of the neuroprotective action of NAm. Instead, it was felt that, initially, we needed to determine whether NAm could not only reduce brain infarction caused by ischemia but also improve the neurological behavior in these animals and also to determine whether this protection would be long-lasting in a model that included not only ischemia but reperfusion as well. We used only 1 dose of NAm in the present study because it was the only dose that proved to be protective. Similarly, we used 2 hours as the treatment delay because we previously showed neuroprotection at this time point but not at 3 or 4 hours after MCAo. The present results indicate that NAm (500 mg/kg) given at 2 hours after the onset of the ischemic insult (ie, at the time of reperfusion) not only reduced the infarction volume after MCAo in Wistar rats but also improved the sensory and motor neurological outcome and improved weight gain.

There appears to be a discrepancy between the significant improvement of the sensory and motor deficits and the lack of anatomic protection (as measured by infarction volume) after NAm treatment compared with saline injection at 1 week after MCAo. The reduction in infarct volume lost significance ($P=0.09$) at 1 week after MCAo because of the smaller lesions observed in the control group at 1 week after MCAo. In fact, the effect of NAm on the infarction volume in these animals was almost identical at both 3 and 7 days after MCAo (see Figure 3), and we believe that this is confirmed by the beneficial effect of NAm observed in the sensory and motor scores.

These function-improving and infarct-reducing effects of NAm could not be accounted for by changes in mean arterial blood pressure, heart rate, or hemodilution (as measured by blood hematocrit), because these parameters were not significantly different when saline-injected control and NAm-treated animals were compared at any time before, during, or after MCAo. The only significant change found in the blood gases was seen in the NAm-treated group, but this change is unlikely to be of importance. The actual values for $PO_2$ and pH in the 3- and 7-day groups (Table 1), although significantly different, were within the normal ranges for these parameters. In addition, the differences were observed only before the onset of MCAo and NAm treatment.

NAm has been shown to protect against necrosis and/or apoptosis in oxidative stress–induced injury in the mouse brain. Given that both these types of cell death are likely to contribute to the immediate and delayed brain infarction observed at both 3 and 7 days after the onset of MCAo, our

Figure 2. NAm treatment 2 hours after onset of MCAo reduced infarction in male Wistar rats. 2,3,5-Triphenyltetrazolium chloride–stained coronal brain sections are from representative animals that received a saline (I) or 500 mg/kg IP NAm (II) injection and were euthanized 3 days after MCAo. Infarcts are observed (pale region) involving the cerebral cortex and underlying striatum, representative of the MCA perfusion region.

Figure 3. Infarct volumes were significantly reduced with NAm treatment in the 3-day but not 7-day groups. Data are expressed as percentage of contralateral (control) hemisphere, and data are represented as a superimposed scatterplot showing the infarction volume for each animal in the group as well as the mean±SEM. Each group consisted of 12 animals. $^P<0.05$ vs saline-treated rats.
results suggest that NAm treatment may also be protecting against apoptosis and/or necrosis induced by focal cerebral ischemia/reperfusion. However, this suggestion remains to be proven, inasmuch as we have not directly measured apoptosis with the appropriate techniques, such as DNA laddering, staining by terminal deoxynucleotidyl transferase–mediated dUTP nick-end-labeling, or electron microscopy.

Neuroprotection obtained with the transient model of focal cerebral ischemia at 3 and 7 days also suggests that NAm may protect against reperfusion injury as well, because the animals were reperfused 2 hours after MCAo. Ischemia/reperfusion results in an inflammatory response mediated by cytokines (eg, tissue necrosis factor and interleukin-1β), chemokines, and adhesion molecules (eg, intercellular adhesion molecule and selectins).18 In addition, there may be injury due to ischemia-induced hyperemia. Therefore, the significant reduction in infarction volume in the ischemia/reperfusion model is good indirect evidence that NAm may also protect against reperfusion injury.

NAm has the potential to be neuroprotective via various mechanisms. We initially tested it because it is reported to prevent the injury-induced depletion of neuronal ATP and boost the amount of ATP in the tissue because it is a precursor of NAD⁺.1–9 Therefore, we reasoned that it should protect the brain at risk of infarction by rectifying the ischemia-induced energy imbalance caused by focal cerebral ischemia.

NAm is also a PARP inhibitor, and, it is known that PARP activation contributes to neuronal damage after focal ischemia because ischemic infarction is reduced in PARP-null mice. Moreover, wild-type mice and various species of rats treated with PARP inhibitors, such as 3-aminobenzamide, 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl-1(2H)-isoquinolinolone and GPI-6150, also exhibited reduced infarction volumes.20–25 Interestingly, the injurious effects of excessive PARP activation, like ischemia, may be due to the depletion of ATP, the augmentation of excitotoxicity mediated by NO and glutamate,26–28 and/or free radical damage.29 However, it has been argued that the beneficial effects of NAm in the brain may be attributed more to the elevation of NAD⁺ levels and the sparing of ATP levels rather than to NAm-induced PARP inhibition.6

It is unlikely that the neuroprotective effect of NAm is due to an increase in regional cerebral blood flow (rCBF) and cerebral metabolic rate of oxygen.30 The neuroprotective dose of NAm (500 mg/kg) administered intraperitoneally in Wistar rats has recently been reported to decrease rCBF in normal animals and not change rCBF in rat brain tumors.31 Hence, it is unlikely that in the present studies, the identical dose and route of administration of NAm improved collateral rCBF and thus reduced the injury induced by temporary MCAo.

NAm is also reported to be an anticonvulsant,32,33 anticoagulant,34 and angiogenic35 agent and an inhibitor of lipid peroxidation.33 Thus, there are numerous ways in which NAm could potentially act to protect against injury due to focal cerebral ischemia. Experiments are presently under way to examine which action and to what extent each action contributes to the overall neuroprotective effect of NAm against focal cerebral ischemia. However, we propose that it may be precisely due to the many possible protective actions, directed at both neurons and glia, short and long term, that NAm has such a robust effect in both permanent and temporary models of focal cerebral ischemia that involve cerebral ischemia and reperfusion.

The data in the present study provide very encouraging and favorable conditions that could eventually lead to the testing of NAm in stroke patients in a clinical safety trial. First, previous putative neuroprotective agents that have been subjected to clinical trials did not always show the protection in both transient and permanent models of stroke that we have illustrated for NAm. Second, the therapeutic window in both these models is at least 2 hours after the onset of stroke. This window was obtained by using a permanent MCAo model and may therefore improve with reperfusion. In addition, multiple dosing with NAm may also enhance the therapeutic window, because the treatment paradigm used in the present experiments involved only a single intraperitoneal injection of NAm. Third, NAm is already used clinically, primarily in the treatment of pellagra.36 Moreover, the pharmacokinetics of NAm have been reported in healthy adults, and NAm in large doses (ie, up to 6 g) in normal humans is reported to have very mild side effects.37

In conclusion, delayed treatment with NAm protects against cerebral ischemia/reperfusion by improving behavior and weight loss for up to 7 days after MCAo and by reducing the brain infarction in Wistar rats for up to 3 days after the onset of stroke. Further studies are needed to examine the many ways by which NAm is achieving this profound neuroprotective effect and to decipher optimal conditions in which it may be used to lengthen the duration and improve the degree of neuroprotection.

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References

The cellular and molecular mechanisms that ultimately determine ischemic neuronal insults are broad in nature, but offer the potential to serve as selective molecular targets against neuronal injury. For example, neuroprotective agents that focus on “downstream” cellular pathways such as trophic factors, 1 metabolic glutamate receptor agonists, 2 and cys-teine protease inhibitors 3 can prevent the induction of both necrotic and apoptotic ischemic cellular injury. With the proper therapeutic intervention, clinical neurodegenerative disease may not only be preventable but also reversible.

Yet, the ability to effectively translate therapeutic intervention into the clinical spectrum requires an initial understanding of the predominant cellular mechanisms that may mediate neuronal injury during stroke. In this respect, the present study by Mokudai and colleagues examines the agent nicotinamide, an essential precursor of nicotinamide adenine dinucleotide, in a focal rat model of cerebral ischemia with reperfusion. To extrapolate a possible clinical utility for their work, the investigators use both anatomic and behavioral analyses in a posttreatment experimental paradigm.

The investigators use a concentration of nicotinamide intraperitoneally that is within a nontoxic clinical range. 4 They demonstrate that a significant reduction in infarct size following nicotinamide application occurs at 3 days but not 7 days post-treatment with an inhibitor of poly(ADP-ribose) polymerase attenuates cerebral damage in focal ischemia. Brain Res. 1999;829:46–54.


Editorial Comment

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The investigators use a concentration of nicotinamide intraperitoneally that is within a nontoxic clinical range. 4 They demonstrate that a significant reduction in infarct size following nicotinamide application occurs at 3 days but not 7 days after the transient focal cerebral ischemic insult. Interestingly, the behavior analysis suggests that motor and sensory deficits are significantly reduced with nicotinamide application at both 3 days and 7 days after cerebral infarction. These results are important and serve to highlight some of the difficulties with clinical outcome trials that demonstrate a
limited correlation between anatomic injury observed on imaging studies and functional deficits documented on clinical examination.5,6 Such observations should motivate rather than dissuade both basic and clinical investigators to further elucidate the cellular mechanisms that contribute to ischemic neuronal injury.

In this regard, future work that is directed to investigate the character of the injury, such as necrotic versus apoptotic disease, neuronal versus vascular injury, and the underlying cellular and molecular pathways that can contribute to the injury, such as the activity of specific cysteine proteases, is necessary to formulate a better understanding of the ability of a particular neuronal insult to influence both clinical plasticity and functional outcome in the nervous system. Thus, neuroprotective agents such as nicotinamide should be viewed as possessing 2 distinct utilities that function not only as agents to prevent or reverse clinical neuronal injury but also as investigational tools to elucidate the cellular pathways that modulate subsequent neuronal function. Yet, in all aspects, these separate functions for a neuroprotective agent should be considered parallel in nature to successfully lay the foundation for the development of more safe and efficacious future neuroprotective strategies for the treatment of stroke.

Kenneth Maiese, MD, Guest Editor
Departments of Neurology and Anatomy & Cell Biology
Centers for Molecular Medicine and Molecular Toxicology
Wayne State University School of Medicine
Detroit, Michigan

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