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Intranasal administration with NAD^+ profoundly decreases brain injury in a rat model of transient focal ischemia

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Methods and Materials
- *3.1. Materials*
- *3.2. Focal brain ischemia*
- *3.3. Intranasal drug delivery*
- *3.4. Quantifications of infarct size*
- *3.5. Neurological assessments*
- *3.6. Measurements of NAD^+ levels in rat brains*
- *3.7. Statistical analyses*

4. Results
5. Discussion
6. Acknowledgments
7. References

1. ABSTRACT

Excessive poly(ADP-ribose) polymerase-1 (PARP-1) activation plays a significant role in ischemic brain damage. Increasing evidence has supported the hypothesis that PARP-1 induces cell death by depleting intracellular NAD^+. Based on our *in vitro* finding that NAD^+ treatment can abolish PARP-1-mediated cell death, we hypothesized that NAD^+ administration may decrease ischemic brain injury. In this study, we used a rat model of transient focal ischemia to test this hypothesis. We observed that intranasal NAD^+ delivery significantly increased NAD^+ contents in the brains. Intranasal delivery with 10 mg / kg NAD^+ at 2 hours after ischemic onset profoundly decreased infarct formation when assessed either at 24 or 72 hours after ischemia. The NAD^+ administration also significantly attenuated ischemia-induced neurological deficits. In contrast, intranasal administration with 10 mg / kg nicotinamide did not decrease ischemic brain damage. These results provide the first *in vivo* evidence that NAD^+ metabolism is a new target for treating brain ischemia, and that NAD^+ administration may be a novel strategy for decreasing brain damage in cerebral ischemia and possibly other PARP-1-associated neurological diseases.

2. INTRODUCTION

Accumulating evidence has indicated a crucial role of oxidative stress in ischemic brain damage (1). Therefore, in order to obtain novel insights for treating brain ischemia and several other neurological diseases, it is of significance to search for new strategies for blocking oxidative cell injury. Excessive poly(ADP-ribose) polymerase-1 (PARP-1) activation has been indicated as one of the mediating factors in cell death induced by oxidative stress and oxygen-glucose deprivation (2, 3). Animal studies have also indicated that PARP-1 is a key pathological factor in cerebral ischemia: Both PARP inhibitors and genetic inhibition of PARP-1 can greatly decrease ischemic brain damage (2, 4). Many studies have further suggested that PARP-1 may be a therapeutic target for multiple neurological diseases including Parkinson's diseases, traumatic brain injury and hypoglycemic brain injury (3, 5).

NAD^+ plays critical roles not only in energy metabolism and mitochondrial activities, but also in calcium homeostasis, gene expression, aging and cell death (5, 6). While it has long been hypothesized that NAD^+ depletion mediates PARP-1 cytotoxicity, recent studies have provided direct evidence supporting this hypothesis (7-9):

Delayed treatment with NAD^+ can significantly decrease PARP-1-mediated cell death, which was profound even when NAD^+ was added at 3 - 4 hours after PARP-1 activation. These studies suggest that NAD^+ is one of the most effective drugs with the longest window of opportunity for decreasing PARP-1 toxicity *in vitro*. It has also been found that NAD^+ treatment can block various PARP-1-mediated pathological changes, including glycolytic inhibition (7, 10), mitochondrial permeability transition, mitochondrial depolarization, and nuclear translocation of apoptosis-inducing factor (9). However, there has been no *in vivo* study determining the therapeutic potential of NAD^+ for neurological diseases. The goal of our current study is to use a rat model of transient focal ischemia to test our hypothesis that NAD^+ administration can decrease ischemic brain injury *in vivo*.

Drug delivery into the brains is commonly limited by the blood-brain barriers. A number of studies have shown that the drug delivery by intranasal approach can produce neuroprotective effects in several models of neurological diseases (11). Our latest studies have also used the poly(ADP-ribose) glycohydrolase inhibitor gallotannin as a model drug to indicate that intranasal drug delivery is markedly more effective than intravenous drug injection in decreasing ischemic brain injury (12). Thus, in this study we conducted intranasal NAD^+ delivery to determine the effects of NAD^+ administration on ischemic brain damage.

3. MATERIALS AND METHODS

3.1. Materials

Reagents were purchased from Sigma Chemical Co (St. Louis, MO, USA) except where noted.

3.2. Focal brain ischemia

All of the procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Research Subcommittee of San Francisco VA Medical Center. Adult male Sprague Dawley rats (300-340 grams) were subjected to transient focal ischemia by intraluminal middle cerebral artery occlusion (MCAo) with a nylon suture, as described previously (13). The rats were anesthetized with 2.0% isoflurane in 30% oxygen and 70% nitrous oxide using a face mask. The rectal temperature was controlled at 37.0 ± 0.5°C during surgery with a feedback-regulated heating pad. Cannulation of a femoral artery allowed monitoring of blood pressure and arterial blood gases. Samples for analysis were taken immediately after cannulation, 60 minutes after occlusion, and 60 minutes after reperfusion. After a midline skin incision, the left external carotid artery was exposed, and its branches were electrocoagulated. A 20-mm 3-0 surgical monofilament nylon suture, blunted at the end, was introduced into the left internal carotid artery through the external carotid artery stump. After 120 minutes of MCAo, blood flow was restored by the withdrawal of the nylon suture.

3.3. Intranasal drug delivery

Six μl of NAD^+ or nicotinamide, which was dissolved in phosphate-buffered saline (PBS), was applied in one side of the nose of rats each time, with the nose at another side blocked for 5 seconds to enhance the influx of the solutions through the nostrial tract. This procedure was repeated every 2 minutes alternatively on each side of the nose, totally for 10 times.

3.4. Quantifications of infarct size

Rats were killed at either 24 or 72 hours after ischemia, and the brains were rapidly removed. All brains were carefully evaluated for macroscopic hemorrhagic changes before 2,3,5-triphenyltetrazolium chloride (TTC) staining. TTC staining is widely used for determining infarct volume either at 24 hrs or at later time points after ischemia (14, 15). The tissue was sliced into 2-mm-thick coronal sections by a rodent matrix, and the slices were stained in TTC for 20 min. When the stain had developed, the brain sections were immersed in 4% paraformaldehyde overnight. Six coronal sections per animal were scanned. Striatal and cortical areas of infarction were measured and analyzed using Image J (NIH) version 1.63.

3.5. Neurological assessments

Neurological deficits were assessed at either 24 or 72 hrs after ischemia according to the method of Belayev et al. (16). The following categories were scored to evaluate motor neurological deficits: 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), and Grade 4 (forelimb flexion and difficulty or inability to ambulate). The maximal score of neurological deficits is 4.

3.6. Measurements of NAD^+ levels in rat brains

The method of Zeng et al. (17), with modifications, was used for the NAD^+ measurements. In brief, rats were sacrificed and their brains were obtained, which were immediately frozen in dry ice. Frozen brain slices were ground into fine powder in a mortar that was cooled on dry ice. The powder was then transferred into 1 ml of ice-cold, 5% perchloric acid. After three times of 2-sec sonication in ice bath, the samples were centrifuged at 14,000 g for 10 min. The supernatant was used for NAD^+ measurements according to our previously described NAD^+ cycling assay by a plate reader (18).

3.7. Statistical analyses

All data are presented as means ± standard errors (SE). Data were assessed by analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. p values less than 0.05 were considered statistically significant.

4. RESULTS

To assess the capacity of intranasal delivery approach to deliver NAD^+ into rat brains, we determined the effects of intranasal administration with 20 mg / kg NAD^+ on the NAD^+ levels in normal rat brains. The results showed that the NAD^+ administration can significantly

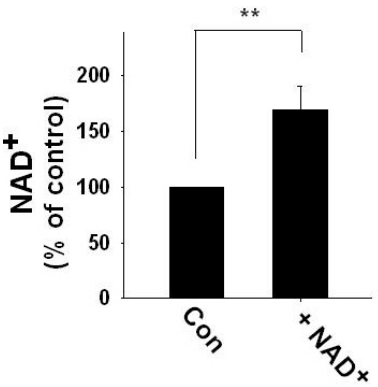

Figure 1. Intranasal NAD^+ administration significantly increased NAD^+ levels in rat brains. Rats were administered with 20 mg / kg NAD^+. Thirty minutes after the initiation of the administration the rat brains were collected and the NAD^+ levels in the brains were assessed by the NAD^+ cycling assay. N = 5; data are Mean ± SE; ** $p < 0.01$.

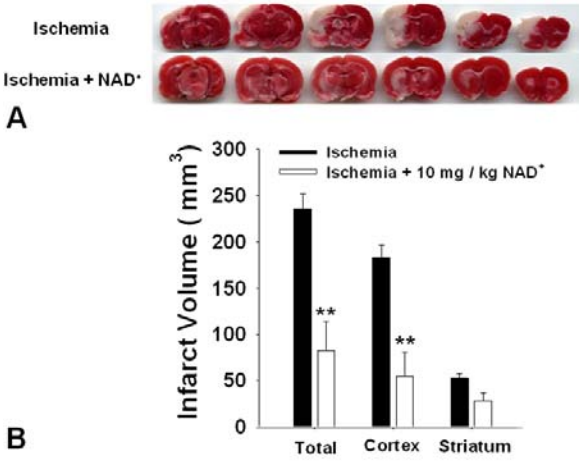

Figure 2. Intranasal NAD^+ administration profoundly decreased infarct formation assessed at 24 hrs after ischemia. Rats were subject to 2-hr MCAo, which were administered with 10 mg / kg NAD^+ immediately after reperfusion. Twenty four hours after ischemia, the rats were sacrificed and the infarct volume was determined. Massive infarct formation was found in the brains of rats subject to ischemia-reperfusion, which was decreased by intranasal administration with 10 mg / kg NAD^+ (Figure 2A). Figure 2B shows the quantifications of the infarct volume of rats subject to ischemia or ischemia with NAD^+ administration. N = 8 - 10; data are Mean ± SE; ** $p < 0.01$.

increase the NAD^+ levels in the brains (Figure 1), suggesting that NAD^+ can be delivered into the brains by this approach.

We used a rat brain ischemia model with 2-hr MCAo to determine the therapeutic potential of NAD^+ administration on ischemic brain injury. Since our pilot studies suggested that intranasal administration with NAD^+ at 10 mg / kg may be most effective for decreasing ischemic brain injury, we determined the effects of the intranasal administration with 10 mg / kg NAD^+ at 2 hrs after ischemia (i.e., immediately after reperfusion) on various major physiological parameters of rats, including body temperature, blood pressure, CO_2 and pH. There was no any statistically significant difference in these parameters between the rats subject to ischemia only and the rats subject to ischemia plus the NAD^+ administration, assessed at 1 hr prior to ischemia, 1 hr after ischemia and 1 hr after reperfusion (not shown).

We determined the effects of intranasal NAD^+ administration on ischemic brain injury by assessing infarct formation and neurological deficits at 24 hrs after ischemia. Ischemia / reperfusion led to massive infarct formation (Figure 2A), which was profoundly decreased by the intranasal administration with 10 mg / kg NAD^+ (Figures 2A and 2B). The NAD^+ administration also significantly attenuated ischemia / reperfusion-induced neurological deficits (Figures 3).

The profound protective effects of the intranasal NAD^+ administration were also observed at 72 hrs after ischemia. Quantifications of the infarct volumes indicate that administration with 10 mg / kg NAD^+ decreased cortical, striatal and total infarct volume by 95%, 60% and 86%, respectively (Figures 4A). The extent of this protection is not statistically different from the 10 mg / kg NAD^+-produced protection assessed at 24 hrs after ischemia (Figure 2B). In contrast, neither intranasal administration with 5 mg / kg NAD^+ (Figure 4A) nor administration with 20 mg / kg NAD^+ (not shown) produced significant protection against ischemic brain injury. Consistent with their effects on infarct formation, the intranasal administration with 10 mg / kg NAD^+, but not with 5 mg / kg NAD^+, significantly attenuated the ischemia / reperfusion-produced neurological deficits (Figure 4B).

Nicotinamide --- a catabolic end product of NAD^+ --- is a relatively weak inhibitor of PARP. It has been reported that intravenous or intraperitoneal administration with nicotinamide at relatively high concentrations can decrease ischemic brain damage (19, 20). We conducted studies to compare the effect of intranasal administration with nicotinamide with that of intranasal NAD^+ administration. In contrast to the profound protective effects of 10 mg / kg NAD^+, intranasal administration with 10 mg / kg nicotinamide did not decrease infarct formation (Figures 5) or neurological deficits (not shown).

5. DISCUSSION

Our results provide the first *in vivo* evidence that NAD^+ administration can profoundly decrease brain damage under certain pathological conditions. It is noteworthy that NAD^+ can reduce infarct formation by up to 86 % even when administered at 2 hrs after ischemic onset. Compared with other studies that apply drugs during post-ischemia phases in order for decreasing ischemic brain

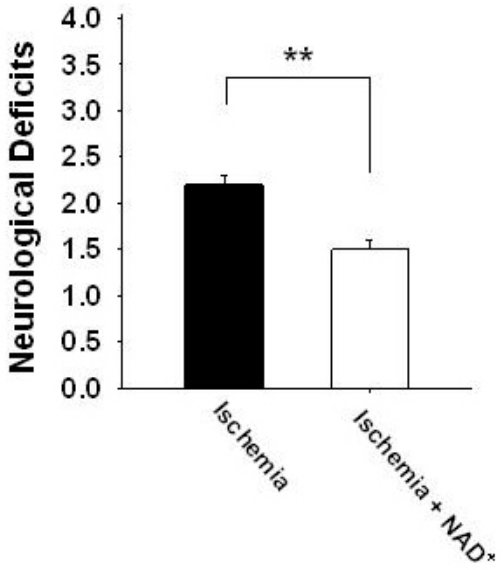

Figure 3. Intranasal administration with 10 mg / kg NAD$^+$ decreased neurological deficits assessed at 24 hrs after ischemia. Rats were subject to 2-hr MCAo, which were administered with 10 mg / kg NAD$^+$ immediately after reperfusion. Neurological deficits were assessed at 24 hrs after ischemic onset. N = 8-10; data are Mean ± SE; ** $p < 0.01$.

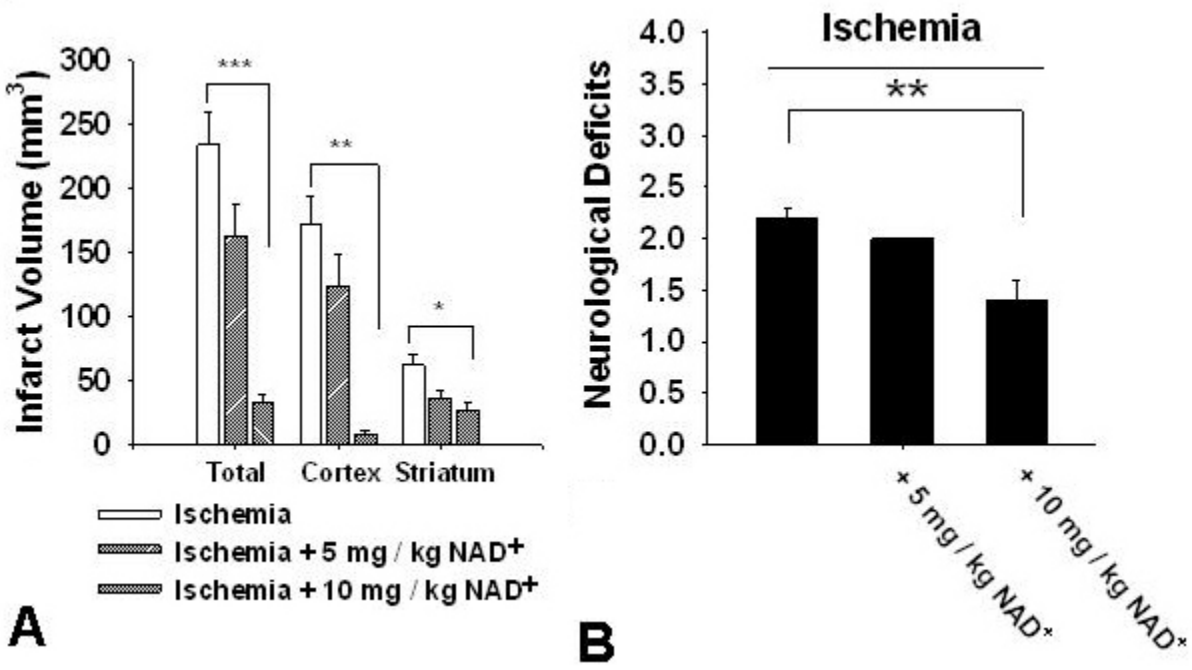

Figure 4. Intranasal NAD$^+$ administration profoundly decreased infarct formation assessed at 72 hrs after ischemia. Rats were subject to 2-hr MCAo, which were administered with 5 or 10 mg / kg NAD$^+$ immediately after reperfusion. After 3 days the rats were sacrificed and the infarct volume was determined. Figure 4A shows the quantifications of the infarct volume of rats subject to ischemia, ischemia + 5 mg / kg NAD$^+$ or ischemia + 10 mg / kg NAD$^+$. Intranasal NAD$^+$ administration also significantly attenuated ischemia / reperfusion-produced neurological deficits (Figure 4B). N = 5 - 8; data are Mean ± SE; *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

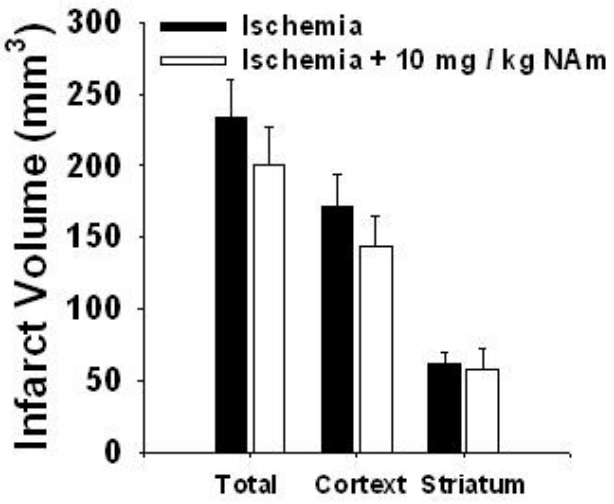

Figure 5. Intranasal administration with 10 mg / kg nicotinamide did not decrease ischemic brain damage. Rats were subject to 2-hr MCAo, which were administered with 10 mg /kg nicotinamide (Nam) immediately after reperfusion. After 3 days the rats were sacrificed and the infarct volume assessed. N = 5 - 6; data are Mean $\pm$ SE.

injury, the protective effect of NAD$^+$ could be one of the most profound effects ever reported. In this study we also provided evidence that by the intranasal delivery approach NAD$^+$ can be delivered into the brains. Collectively, our studies suggest that NAD$^+$ metabolism may be a new target for treating cerebral ischemia, and intranasal NAD$^+$ administration may become a novel therapeutic strategy for cerebral ischemia and other PARP-1-related diseases.

While it has been long hypothesized that PARP-1 induces cell death by depleting NAD$^+$, only recently there has been direct evidence supporting this hypothesis: NAD$^+$ repletion can abolish PARP-1 cytotoxicity even when applied at 3 – 4 hours after PARP-1 activation (7, 9). This observation, together with the reports that liposomal NAD$^+$ delivery can partially prevent peroxynitrite-induced mitochondrial depolarization (8), indicates a key role of NAD$^+$ depletion in PARP-1 cytotoxicity. Recent studies have also indicated that mitochondrial permeability transition (MPT) and apoptosis inducing factor (AIF) translocation are important steps leading from PARP-1 activation to cell death (9, 21).

While it has been long thought that the major cellular functions of NAD$^+$ and NADH are modulating cellular energy metabolism, increasing evidence has suggested that NAD$^+$ and NADH also play key roles in calcium homeostasis, gene expression, cell death and aging process (5, 6). Considering this information as well as the significant lack of information regarding these molecules under pathological conditions, future studies into the roles of NAD$^+$ and NADH under various pathological conditions may expose novel mechanisms of various diseases.

Cumulative evidence has suggested that NAD$^+$ may mediate cell death via multiple mechanisms (5). For examples, NADH / NAD$^+$ ratio is a major index of cellular reducing potential, which can modulate MPT --- a mediator of both apoptosis and necrosis under many conditions; and both NAD$^+$ and NADH mediate energy metabolism that could determine cell death modes. Therefore, while our current study has shown the profound protective effects of NAD$^+$ administration against ischemic insults, much future investigation is needed to elucidate the mechanisms underlying the protective effects of NAD$^+$.

NAD$^+$ decreases have been found in ischemic brains in which PARP-1 plays a key pathological role (4). These results, combined with the *in vitro* studies suggesting that NAD$^+$ depletion mediates PARP-1 cytotoxicity, suggest that the NAD$^+$ decreases may also contribute to the PARP-1 toxicity *in vivo*. Several animal studies have further suggested that NAD$^+$ loss is a significant factor determining the relationships between PARP and ischemic brain damage. In a mild ischemia model in which there was no NAD$^+$ decrease, PARP activation could be beneficial by promoting DNA repair (22). Recent studies have also indicated that PARP-1 plays either detrimental or beneficial effects in male or female rats subject to ischemic insults (23, 24). A role of NAD$^+$ in this intriguing gender effect has been implicated by a study using a perinatal brain injury model (24). Our current observation that NAD$^+$ administration can prevent ischemic brain injury could provide essential basis for further establishing the roles of altered NAD$^+$ metabolism in ischemic brain injury.

Intranasal drug delivery approach could have multiple merits over traditional drug delivery approaches: First, it may deliver drugs into the brains by bypassing the blood-brain barriers, which is one of the major obstacles for treating central nervous system (CNS) diseases (11); second, it may decrease the amount of drugs needed to affect CNS, which could significantly decrease the cost of treatments; and third, it could reduce the probability that the protective effects of certain drugs on CNS may result from the drug effects on peripheral systems. Our current

study has further indicated the effectiveness of this drug delivery approach.

Multiple studies have suggested that nicotinamide can decease ischemic brain injury, but at doses higher than 125 mg / kg (19). Our study shows that intranasal administration with nicotinamide at 10 mg / kg can not affect ischemic brain damage, in contrast to the profound protective effects of 10 mg / kg NAD^+. While future studies are still needed to compare the capacity of NAD^+ and nicotinamide to enter into neurons and glial cells, our results highlight the therapeutic potential of NAD^+ for cerebral ischemia. Since there has been little treatment approach for stroke patients at several hours after stroke occurs, new strategies for reducing ischemic brain injury are critically needed. Based on the information provided by previous reports and our current study, future investigation into the biological properties of NAD^+ and NADH under ischemic conditions may significantly deepen our understanding about the mechanisms of ischemic brain damage and suggest new therapeutic approaches for this debilitating illness.

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