Research Paper

Variation in chronic nicotinamide treatment after traumatic brain injury can alter components of functional recovery independent of histological damage

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Abbreviations: TBI, traumatic brain injury; CCI, cortical contusion injury; NAM, nicotinamide; MWM, morris water maze; NA, nicotinic acid; PARP, poly-ADP-ribose polymerase; NAD, nicotinamide adenine dinucleotide; BBB, blood-brain barrier; ROS, reactive oxygen species; ATP, adenosine triphosphate; FPI, fluid percussion injury; ANOVA, analysis of variance; ad lib, ad libitum

Key words: traumatic brain injury, therapy, window of opportunity, vitamin, recovery of function

Previously, we have shown that the window of opportunity for nicotinamide (NAM) therapy (50 mg/kg) following cortical contusion injuries (CCI) extended to 4–8 hrs post-CCI when administered over a six day post-CCI interval. The purpose of the present study was to determine if a more chronic NAM treatment protocol administered following CCI would extend the current window of opportunity for effective treatment onset. Groups of rats received either unilateral CCI’s or sham procedures. Initiation of NAM therapy (50 mg/kg, ip) began at either 15-min, 4-hrs, 8-hrs or 24-hrs post-injury. All groups received daily systemic treatments for 12 days post-CCI at 24 hr intervals. Behavioral assessments were conducted for 28 days post injury and included: vibrissae forelimb placing, bilateral tactile adhesive removal, forelimb asymmetry task and locomotor placing testing. Behavioral analysis on both the tactile removal and locomotor placing tests showed that all NAM-treated groups facilitated recovery of function compared to saline treatment. However, on the vibrissae-forelimb placing and forelimb asymmetry tests only the 4-hr and 8-hr NAM-treated groups were significantly different from the saline-treated group. The lesion analysis showed that treatment with NAM out to 8 hrs post-CCI significantly reduced the size of the injury cavity. The window of opportunity for NAM treatment is task-dependent and in some situations can extend to 24 hrs post-CCI. These results suggest that a long term treatment regimen of 50 mg/kg of NAM starting at the clinically relevant time points may prove efficacious in human TBI.

Introduction

Each year approximately 50,000 people die from traumatic brain injuries (TBI) and another 80,000 to 90,000 become permanently disabled in the US.1-3 Currently, no therapeutics is available to attenuate damage following TBI. A number of novel therapeutics have shown robust behavioral and histological protection in preclinical animal models, but have failed to show beneficial effect in clinical trials. The reason for the lack of translation from animal to human models of TBI is multifaceted, including methodological errors in the clinical setting and errors in preclinical modeling.

Clinically, methodological errors have included patient inclusion criteria and number of patients enrolled to achieve statistical significance.1 Some patients may be too severely injured to show any real improvement (GCS ≤ 8); enrolling these patients may artificially deflate any treatment effect.4 It has been shown that only 8 of the 203 clinical TBI trials evaluating novel therapeutics were sufficiently powered to detect a 10% reduction in mortality rate. Thus, some novel therapeutics, given adequate clinical testing, may have been demonstrated to be effective.5 Preclinically, neglected aspects of testing novel therapeutics have included: determining the window of opportunity for treatment onset and generalizing treatment to two or more models of cerebral insult.1 Treatment duration and dosing parameters should be well defined during the preclinical evaluation of a new therapy. It is unlikely that one or two administrations of a compound will completely arrest its target pathological mechanism and it is possible that acute therapeutic intervention may only delay an inevitable injury cascade; it may take days or weeks of therapeutic intervention to attain efficacy.

Nicotinamide (NAM) is the amide form of niacin (NA or Vitamin B3). NA and NAM are ingested orally in normal dietary intake via yeast, meats, grains and legumes. Although NAM is synthesized endogenously from tryptophan, this reaction does not take place within the central nervous system (CNS) due to the lack of the enzyme quinolinate phosphoribosyl transferase. Thus, any NAM within the CNS has been actively transported across the blood—
for Ca\textsuperscript{2+}, taking up massive amounts. This opens up mitochondrial transition pores, which severely alters ionic homeostasis. High levels of intracellular and mitochondrial Ca\textsuperscript{2+} trigger a massive release of cytochrome C which triggers caspase 9 activation leading to terminal apoptosis. NAM activates protein kinase B and inhibits transcription of proapoptotic genes. A downstream effect of protein kinase B is closing of the mitochondrial transition pores opened by excessive Ca\textsuperscript{2+} influx. The mitochondrion is able to regain ionic homeostasis and bring cytochrome C back to physiological conditions thereby inhibiting caspase 9 mediated cell death.\textsuperscript{8}

NAM also exerts protective effects by inhibiting poly-ADP-ribose polymerase (PARP). Due to oxidative damage caused by the failure of the electron transport chain and propagation of reactive oxygen species (ROS), DNA becomes damaged.\textsuperscript{9} Following breaks in DNA strands caused by oxidative damage, PARP binds to single or double strand breaks and cleaves NAD to catalyze the transfer of ADP-ribose, which binds to acceptor proteins and to PARP itself reducing amount of free NAD for normal energy metabolism.\textsuperscript{10} Chronic PARP activation quickly depletes cellular NAD stores; administration of NAM has been shown to inhibit PARP, increase levels of NAD in cortical areas affected by ischemic events, and restore ATP levels.\textsuperscript{11,13}

Recent research has shown that while NAM administration increases brain concentrations of NAD, the effect of this increase may only serve to inhibit PARP and decrease oxidative stress and not restore metabolic functioning.\textsuperscript{11} Peak plasma concentrations of NAM have been demonstrated within 45 min after systemic injection in the rat.\textsuperscript{14} The increase of cortical NAD and how long this elevation might last after a 50 mg/kg dose of NAM is not known. However, if this effect is short lived after a single bolus injection, then treatment beginning at 8 hrs may not have spiked NAD levels at a therapeutically relevant time or delayed pathological mechanisms. It has been reported that PARP demonstrates two peaks following injury, one at 30 min and at 24 hrs.\textsuperscript{15} It is possible that single administrations of NAM at later time points (i.e., 6–8 hrs) may temporarily attenuate pathological mechanisms upstream of PARP activation, thus pushing the second peak of PARP somewhere outside of the treatment range. In contrast, if the effects of PARP activation were apparent at 24 hrs, spiking the concentration of NAD at this time would have produced the therapeutic effect observed following treatment onset at 24 hrs. Thus, the initial protection observed would be a result of acute prevention of apoptosis and not mainly due to restoration of metabolic function. Therefore, it is of interest to determine the effect of repeated dosing of NAM for the treatment of TBI because by increasing the duration of therapy it is likely to increase the beneficial effects on recovery of function.

NAM was first investigated in experimental models of ischemia. Administration of NAM following permanent middle cerebral artery occlusion was effective at reducing infarction volumes in male rats.\textsuperscript{16} This study began treatment 1 hr prior to ischemia onset and tested the effectiveness of three doses: 50 mg/kg, 500 mg/kg and 1000 mg/kg. Histological data revealed an inverted U-shaped distribution with the 50 and 1000 mg/kg groups failing to show neuroprotection as assessed by infarction volume. The 500 mg/kg group showed a significant reduction in infarction volume relative to saline 24 hrs post-stroke. A single dose of 500 mg/kg of NAM was found to have a window of opportunity that extended to 2 hrs, but not 3 or 4 hrs post-stroke using a measure of infarction volume.\textsuperscript{16} In another study, a 500 mg/kg dose was the most effective at reducing infarction volume; the window of opportunity remained at 2 hrs.\textsuperscript{17} It was later demonstrated that the window of opportunity extended to 4 hrs post-stroke with the 500 mg/kg dose as measured by infarction and neuroscore at 7 days post-stroke.\textsuperscript{18}

Recently, NAM has been demonstrated to be effective in models of TBI. It has been shown that NAM was effective at improving behavioral and histological outcome following experimental TBI.\textsuperscript{19} Animals received a bilateral CCI to the medial prefrontal cortex and were administered NAM (500 mg/kg) at 15 min with a booster at 24 hrs post injury. Behavioral evaluation revealed that NAM-treated animals were significantly less impaired than saline-treated animals on sensorimotor (e.g., and cognitive measures) but not on a skilled forelimb use task. NAM was also shown to decrease the size of the lesion cavity and downregulate the glial response.\textsuperscript{19} It has also been recently shown that NAM administration acutely reduced apoptosis, BBB breach and neuronal death following CCI.\textsuperscript{20,21}

NAM was also effective in attenuating behavioral and histological measures at two different doses in a diffuse model of TBI: fluid percussion injury (FPI).\textsuperscript{22} Animals received FPI injuries and were treated with either 500 mg/kg or 50 mg/kg NAM at 15 mins post injury with a single 24 hr booster. Behavioral evaluation over 35 testing days showed significant performance improvement relative to saline-treated animals on measures of sensorimotor functioning, sensorimotor integration, and motor functioning. The 500 mg/kg treatment condition was also effective in attenuating cognitive dysfunction, but the 50 mg/kg was not. Histologically, both treatment conditions significantly reduced cavity size and glial cell proliferation relative to saline-treated animals.\textsuperscript{22} Acute neuroprotection and a time-dependent modulation of reactive gliosis at 24 hrs and 7 days post-FPI has also been recently shown.\textsuperscript{23}

A recent study has evaluated the window of opportunity for treatment onset using a 50 mg/kg dose following CCI. Animals received injection regimens starting at 15-min, 4-hr or 8-hr post injury with 50 mg/kg boosters at 24 hr intervals for five days following bilateral frontal CCI. Testing revealed that the 15-min, 4-hr and 8-hr treatment groups were significantly less impaired in the sensorimotor and sensory tasks. However, only the 15-min and 4-hr treatment groups demonstrated a significant reduction in cognitive deficits. Histologically, the 15-min and 4-hr treatment groups had significantly smaller cavities than saline-treated animals.\textsuperscript{24}

The objective of the present study was to extend the window of opportunity of NAM administration from 4 hrs to 24 hrs post injury. Animals were administered NAM for 12 days using the clinically relevant dose of 50 mg/kg. Administration regimens overlapped the duration of time that metabolic dysfunction has been proposed to occur.\textsuperscript{25,26}
Results

Lesion analysis. Examination of the extent of injury measured by the percent reduction of lesion volume in the ipsilateral hemisphere compared to the intact contralateral hemisphere was analyzed in a one way ANOVA (4 hr-NAM 8 hr-NAM, 24 hr-NAM, Saline and Sham). A significant group effect was observed, \( F(4,32) = 6.70, p < 0.001 \). Post hoc analysis with Tukey’s HSD demonstrated the 4 hr-NAM \( [HSD(13) = 8.10, p < 0.05] \) and 8 hr-NAM groups \( [HSD(14) = 9.06, p < 0.02] \) were significantly different from the saline-treated groups but the 24 hr-NAM group was not \( (p > 0.10) \) (see Fig. 1). However, shams were not significantly different from any of the NAM-treated groups in respect to cortical volume reduction \( (p > 0.05) \). Representative histological images through the site of injury are presented in Figure 2.

Bilateral tactile adhesive removal task. The latency to remove the adhesive patch from the right forelimb was examined using a 5 x 8 ANOVA with repeated measures. Group (4 hr-NAM, 8 hr-NAM, 24 hr-NAM, Saline and Sham) and day (2, 4, 6, 8, 10, 12, 21 and 28 post-CCI) were included as the between and within group factors, respectively. All animals showed improved latencies to remove the adhesive patch across testing days, the main effect for day was significant \( [F(7,224) = 33.16, p < 0.001] \). A significant group main effect was observed in the latency to remove the adhesive patches from affected forelimb \( [F(4,32) = 11.04, p < 0.001] \). The group x day interaction was also significant, suggesting differential recovery between groups across time \( [F(28,224) = 3.00, p < 0.001] \) (See Fig. 3). Post hoc comparisons with planned t-tests revealed that the 4 hr-NAM group was significantly different from the saline-treated group on days 2 \([t(13) = 3.50, p < 0.008]\), 4 \([t(13) = 3.82, p < 0.004]\) and 6 \([t(13) = 2.47, p < 0.04]\). Comparisons of the 8 hr-NAM group to saline were also significantly different on days 4 \([t(14) = 2.21, p < 0.05]\), 6 \([t(14) = 3.63, p < 0.003]\) and 8 \([t(14) = 2.17, p < 0.05]\). The comparisons between the 24 hr-NAM and saline-treated group were not significantly different on days 2 \([t(14) = 2.59, p < 0.02]\), 4 \([t(14) = 4.09, p < 0.002]\) and 6 \([t(14) = 3.20, p < 0.008]\), and 8 \([t(14) = 2.87, p < 0.02]\). Comparisons between the NAM-treated groups were not significantly different \( (p > 0.05) \).

Vibrissae-forelimb placing. The percentage of unsuccessful placing attempts with the contralateral forelimb was examined using a 5 x 9 ANOVA with repeated measures. Group (4 hr-NAM, 8 hr-NAM, 24 hr-NAM, Saline and Sham) and day (2, 4, 6, 8, 10, 12, 14, 21 and 28 post-CCI) were included as the between and within group factors, respectively. There was a general improvement in recovery of function across testing days, the main effect for day was significant \( [F(8,256) = 16.89, p < 0.001] \). A significant group main effect was observed in placing performance in the affected contralateral forelimb \( [F(4,32) = 12.34, p < 0.001] \). The group x day interaction was also significant, suggesting differential improvement between groups \( [F(32,256) = 2.59, p < 0.003] \) (See Fig. 4). Post hoc comparisons with planned t-tests revealed that the 4 hr-NAM group was significantly different from the saline-treated group on days 12 \([t(13) = 2.85, p < 0.01]\), 14 \([t(13) = 3.79, p < 0.002]\), 21 \([t(13) = 3.69, p < 0.003]\) and 28 \([t(13) = 3.45, p < 0.004]\). Comparisons of the 8 hr-NAM group to saline were also significantly different on days 8 \([t(14) = 2.55, p < 0.02]\), 10 \([t(14) = 2.65, p < 0.02]\), 12 \([t(14) = 2.60, p < 0.02]\), 14 \([t(14) = 2.67, p < 0.02]\), 21 \([t(14) = 2.88, p < 0.01]\) and 28 \([t(14) = 2.68, p < 0.02]\). The comparisons between the 24 hr-NAM and saline-treated group were not significantly different \( (p > 0.05) \). Comparisons between the NAM-treated groups were not significantly different \( (p > 0.05) \).
differential recovery between groups across time \( [F(8,64) = 0.92, p > 0.50] \). With the non-significant interaction effect, post hoc comparisons with planned T ukey HSD tests were performed between groups. This comparison revealed that the 4 hr-NAM group was significantly different from the saline-treated group \( [HSD(13) = 19.67, p < 0.001] \) (See Fig. 6). Comparisons of the 8 hr-NAM group to saline were also significantly different \( [HSD(14) = 3.75, p < 0.002] \). The 24 hr-NAM group was not significantly different compared to the saline group \( [HSD(14) = 3.75, p < 0.002] \). Comparisons between the 4 hr-NAM group were only significantly different from the 24 hr-NAM on the first test day \( (p < 0.05) \).

### Discussion

This study sought to extend the window of opportunity for NAM therapy by prolonging administration to include time points covering metabolic dysfunction post-TBI. Animals within the present study received NAM at the clinically relevant dose of 50 mg/kg and at clinically relevant administration times; 4, 8 and 24 hrs post-injury with 12 days of daily boosters. The injury model utilized produced enduring deficits in forelimb functioning as assessed in locomotor placing, forelimb asymmetry, bilateral tactile adhesive removal, and vibrissae forelimb placing over the course of 28 days. Histological evaluation was performed to evaluate the treatment regimen's ability to reduce injury extent.

The results of this study have shown that administration of NAM following CCI had a task dependent effect on the window of opportunity for recovery of function. In general, the 13-dose regimen of NAM significantly lessened the behavioral impairments observed following injury and led to a more rapid and sustained improvement in functional recovery. On 2 of the 4 behavioral tests examined the window of opportunity for this dosing regimen of NAM extended
out to 24 hrs post-CCI. On the bilateral tactile removal test it was found that administration of NAM at the 4-hr, 8-hr and 24-hr post-CCI time points significantly improved recovery of function on this test. On post-CCI day 2 it was found that both the 4-hr and 8-hr groups showed a significantly reduced initial impairment on this test; and the 24-hr group showed a greatly reduced impairment, compared to the saline-treated group. Over the first week of testing all of the NAM groups showed a significantly improved level of performance compared to the saline-treated group. In general, the data from the bilateral tactile removal test demonstrates that there were no significant differences between any of the NAM treatment groups. Thus, on this test a very wide window of opportunity was present. A similar level of performance was also shown on the locomotor placing test. The 4-hr NAM group demonstrated no behavioral impairments on this test for the extent of the testing period post-CCI; the 8-hr NAM group was significantly reduced on all test days compared to the saline-treated group and the 24-hr group showed a strong reduction on the first test day and significant reductions on all subsequent test days. Comparisons between the NAM-treated groups showed that the 4-hr group was significantly improved compared to the 8 and 24-hr groups. Inspection of the graph in figure 5 reveals a classic window of opportunity effect for NAM therapy post-CCI, with the earlier dosing points providing superior behavioral performance compared to later time points; however, in this case all of the NAM treatment groups significantly outperformed the vehicle control group. Thus, the earlier the treatment can be initiated post-injury the better the expected recovery outcome. In general, on these 2 behavioral tests (bilateral tactile removal and locomotor placing) the window of opportunity extends out to 24 hrs post-CCI.

The results of the vibrissae-forelimb placing test showed that all of the NAM-treated animals showed some degree of recovery compared to the saline-treated animals, with most of the significant recovery occurring after 10 days of testing which is typical for this particular behavioral test. However, on this test only the 4-hr and 8-hr NAM groups were significantly improved compared to saline treatment. Treatment with NAM at the 4-hr or 8-hr NAM groups significantly improved performance compared to the saline-treated group. Symbols indicate significant differences (p < 0.05) between comparisons of NAM-treated groups and the saline-treated group.

Figure 5. The effects of a 13 day regimen of NAM (50 mg/kg, ip) or saline administered following CCI or sham surgery on the locomotor placing test. The graph shows the plotted mean ±SEM forelimb foot-faults impairment scores. Treatment with the 4-hr, 8-hr and 24-hr NAM groups significantly improved performance compared to the saline-treated group. Symbols indicate significant differences (p < 0.05) between comparisons of NAM-treated groups and the saline-treated group.

Figure 6. The effects of a 13 day regimen of NAM or vehicle administered following frontal CCI or sham surgery on the forelimb asymmetry test. The graph shows the plotted mean ±SEM percentage of forelimb asymmetry scores. The no bias line at 50% demonstrates a level of performance where both forelimbs are being used equally on this test. Treatment with the 4-hr and 8-hr NAM groups significantly improved performance compared to the saline-treated group. Symbols indicate significant differences (p < 0.05) between comparisons of NAM-treated groups and the saline-treated group.
therapy was initiated at 8 hrs post-CCI which clearly represents a clinically relevant treatment window.

The results of the anatomical analysis also showed a time-dependent effect for NAM treatment. Both the 4-hr and 8-hr treatment groups showed a preservation of cortical tissue loss following CCI compared to the saline-treated group. The 24-hr group was not significantly different compared to the saline-treated group; however, the cortical loss was reduced. In a previous window of opportunity study for NAM therapy it was found that 6 daily doses of low dose NAM did not provide significant cortical protection following CCI. Thus, it appears that by increasing the length of NAM therapy post-CCI that greater beneficial effects can be observed. This finding suggests that by providing NAM therapy during the time of metabolic crisis following CCI that a significant gain in tissue sparing and behavioral recovery can be achieved.

The results of this study indicate that a 13-dose regimen of NAM significantly improved performance on all behavioral measures of sensorimotor function when administered as late as 8 hrs post-CCI. Furthermore, significant improvements in sensorimotor performance were observed when administered as late as 24 hrs post-CCI on certain tests. NAM administration also significantly reduced tissue loss in the injured cortex when administered 8 hrs following injury. In conclusion, by increasing the length of time of treatment duration the window of opportunity can be extended for NAM therapy in the traumatically injured rodent brain.

**Methods**

**Subjects.** Forty male Sprague-Dawley rats, 3–5 months of age were included in this experiment. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee. The study was conducted in a facility certified by the American Association for the Accreditation of Laboratory Animal Care. Rats were maintained on a standard 12-hr light/dark cycle with food and water available ad lib.

**Surgery.** The surgical procedure was performed using aseptic techniques and conditions. The CCI model utilized in the proposed study is based on previous studies. Animals were anesthetized using a mixture of isoflurane (2–4%) and oxygen (0.8 L/min). When the animal became unresponsive (no ocular or pedal reflexes) the head was shaved and scrubbed with 70% alcohol and placed into a stereotaxic device. A midline incision was made in the skin and underlying fascia was reflected. A circular craniotomy (4.0 mm) was performed by scoring forelimb placing reaction. Each rat was held by the trunk ensuring the forelimbs were free to move. One side of the rat was oriented parallel to a Plexiglas surface and was slowly moved until the vibrissae on one side touched the surface. A reliable lateralized placing response was elicited in intact rats each time the vibrissae made contact with the surface. A successful forelimb placing response was recorded if the animal raised its forelimb and placed it on the surface in response to stimulation of the vibrissae ipsilateral to the forelimb. Each rat was given 10 trials for each forelimb. If a placing response was not elicited within 5 sec of vibrissae stimulation, the trial was recorded as unsuccessful. Baseline performance was recorded prior to injury. The animals were tested on post-operative days 2, 4, 6, 8, 10, 12, 14, 21 and 28. Refer to Table 1 for complete testing schedules.

**Vibrissae-forelimb placing.** Sensorimotor function was evaluated by scoring forelimb placing reaction. Each rat was held by

**Experimental Design**

<table>
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<th>Condition</th>
<th>Treatment Initiation</th>
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<td>CCI + NAM</td>
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<tr>
<td>Group 2</td>
<td>CCI + NAM</td>
<td>8 hrs post-CCI</td>
<td>8</td>
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<tr>
<td>Group 3</td>
<td>CCI + NAM</td>
<td>24 hr post-CCI</td>
<td>8</td>
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<tr>
<td>Group 4</td>
<td>CCI + Saline</td>
<td>4 hrs post-CCI</td>
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<tr>
<td>Group 5</td>
<td>Sham + Saline</td>
<td>4 hrs post-CCI</td>
<td>6</td>
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</tbody>
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**Table 1**

<table>
<thead>
<tr>
<th>Timeline</th>
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<th>Surgery</th>
<th>NAM treatment protocol</th>
<th>Bilateral adhesive removal test</th>
<th>Vibrissae-forelimb placing test</th>
<th>Locomotor placing test</th>
<th>Forelimb asymmetry test</th>
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<tr>
<td></td>
<td>Days -7 to 0</td>
<td>Day 0</td>
<td>Day 0-12</td>
<td>Days 2,4,6,8,10,12,21,28</td>
<td>Days 2,4,6,8,10,12,14,21,28</td>
<td>Days 2,8,14</td>
<td>Days 2,6,12</td>
<td>Day 30</td>
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on an elevated grid floor (56.5 x 54 cm) and allowed to explore for 3 min while being videotaped. The openings in the grid were 3.5 cm². While moving around the grid an intact animal occasionally made a “foot-fault”; this occurred when a rat inaccurately placed a limb and it fell through a hole in the grid floor. Over the course of testing, intact animals made few foot-faults. A rat with a lesion to the sensorimotor cortex will make forelimb movements with the forelimb contralateral to the injury. To weight the frequency of foot-faults relative to amount of movement the number of steps was recorded. The deficit assessment was calculated using the equation:

\[\frac{\text{ipsilateral} + \frac{1}{2}\text{both}}{\text{ ipsilateral} + \text{contralateral} + \text{both}} \times 100\].

The animals were tested on post-operative days 2, 8 and 14.

**Forelimb asymmetry task.** Following unilateral injury to the sensorimotor cortex animals display a preference for the unimpaired forelimb,²⁴,³⁰ To assess this bias, animals were placed in a 20 x 20 x 30 cm clear cage and videotaped for 5 min while exploring the walls of the cage. During rearing behavior, intact animals used both forelimbs equally during vertical and horizontal movements. An impaired animal reared and supported its weight against the wall with the unaffected forelimb and during horizontal movements. While scoring the taped session a movement in which both forelimbs made contact with the wall of the cage either at the same time or in short succession was scored as a “both”. Rears in which the animal made either a left or right placement singly was scored as left or right. Weight bearing movements were considered those forelimb places in which the palm of the forepaw made contact with the glass and/or remained stable until the animal moved horizontally or vertically. A score of forelimb asymmetry bias was calculated by the following equation:

\[\frac{\text{ipsilateral} + \frac{1}{2}\text{both}}{\text{ ipsilateral} + \text{contralateral} + \text{both}} \times 100\].

The animals were tested on post-operative days 2, 6 and 12.

**Histology.** At 30 days post-injury, rats were anesthetized with urethane (3.0 g/kg, 0.5 g/ml i.p.) and transcardially perfused with 0.9% phosphate buffered saline (PBS) followed by 10% phosphate buffered formalin (PBF). Brains were removed from the cranium and serially sectioned at 40 μm. Sections and the section thickness (40 μm) was multiplied by the reference to changes in nicotinamide and NAD+ levels in ischemic core and penumbra. Fujishima M. Nicotinamide attenuates focal ischemic brain injury in rats: with special reference to changes in nicotinamide and NAD+ levels in ischemic core and penumbra. Neuroreport 2002; 11:213-6.

**References**


31. Hoane MR, Raad C, Barth TM. Non-competitive NMDA antagonists and anti-oxidant drugs reduce striatal atrophy and facilitate recovery of function following lesions of the rat cortex. Restor Neurol Neurosci 1997; 11:71-82.


