A comparative study of the effects of two nitric oxide synthase inhibitors and two nitric oxide donors on temporary focal cerebral ischemia in the Wistar rat

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Object. A critical review of the literature indicates that the effects of nitric oxide synthase (NOS) inhibitors on focal cerebral ischemia are contradictory. In this experiment the authors methodically examined the dose-dependent effects of two NOS inhibitors and two NO donors on cortical infarction volume in an animal model of temporary focal cerebral ischemia simulating potential ischemia during neurovascular interventions.

Methods. Ninety-two Wistar rats underwent 3 hours of combined left middle cerebral artery and bilateral common carotid artery occlusion after having been anesthetized with 1% halothane. A nonselective NOS inhibitor, NG-nitro-l-arginine-methyl-ester (l-NAME), and two NO donors, 3-morpholinosydnonimine hydrochloride and NOC-18, DETA/NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazene-1,2-diolate, were administered intravenously 30 minutes before ischemia was induced. A selective neuronal NOS inhibitor, 7-nitroindazole (7-NI), was administered intraperitoneally in dimethyl sulfoxide (DMSO) 60 minutes before ischemia was induced. Two ischemic control groups, to which either saline or DMSO was administered, were also included in this study. Seventy-two hours after flow restoration, the animals were perfused with tetrazolium chloride for histological evaluation.

Cortical infarction volume was significantly reduced by 71% in the group treated with 1 mg/kg l-NAME when compared with the saline-treated ischemic control group (27.1 ± 37 mm³ compared with 92.5 ± 26 mm³, p < 0.05). The NOS inhibitor 7-NI significantly reduced cortical infarction volume by 70% and by 92% at doses of 10 and 100 mg/kg: 35.2 ± 32 mm³ (p < 0.05) and 9 ± 13 mm³ (p < 0.005), respectively, when compared with the DMSO-treated ischemic control group (119 ± 43 mm³). There was no significant difference between the saline-treated and DMSO-treated ischemic control groups. Treatment with NO donors did not significantly alter cortical infarction volume.

Conclusions. These results support an important role for NO in ischemic neurotoxicity and indicate that neuronal NOS inhibition may be valuable in reducing cortical injury in patients suffering temporary focal cerebral ischemia during neurovascular procedures.

KEY WORDS • nitric oxide • focal brain ischemia • nitric oxide synthase • NG-nitro-l-arginine-methyl-ester • 7-nitroindazole • rat
Nitric oxide in focal cerebral ischemia

ischemia has raised questions about its similar efficacy in focal cerebral ischemia. In a rat model of focal cerebral ischemia, NO donors were protective if hypotension was avoided. Alternately, in cortical cultures, NO donors were neurotoxic. A comparison of the effects of different NO modulators in vivo between different investigations is complicated by differences in methodology, including animal model, anesthetic agent, occlusion technique, duration of ischemia, and drug dosing. The objective of this study was to examine rigorously the dose-dependent effects of two NOS inhibitors and two NO donors on cortical infarction volume in a model of temporary focal cerebral ischemia that simulates possible ischemia during neurovascular procedures.

Materials and Methods

Following review and approval of our protocol by our institutional Animal Care and Use Committee, 92 adult male Wistar rats were administered halothane in a mixture of oxygen and air through a face mask at 1.5% during the surgical procedure and 1% during the occlusion period. Subcutaneous glycopyrrolate was administered preoperatively at 4 μg/kg to reduce respiratory secretions. Core body temperature was continuously monitored at the beginning of the surgical preparation and throughout the experiment by using a rectal probe. This rectal probe was connected to an infrared heating lamp that maintained both body and head temperature at 37 ± 0.5°C throughout the experiment. Polyethylene catheters (PE-50) were inserted into the right femoral artery and vein to monitor mean arterial blood pressure (MABP) and arterial blood sampling (pH, PaCO₂, PaO₂, and serum glucose). These physiological parameters were measured at the beginning of the surgical preparation, 15 minutes before ischemia was induced, and at the completion of the ischemia experiment.

Model of Focal Cerebral Ischemia

The original technique, as described by Tamura, et al., was modified for our experiments to increase the severity and reliability of the ischemic model. A ventral midline incision was made for exposure of both common carotid arteries (CCAs). The contralateral right CCA was permanently ligated using a No. 3.0 silk suture. The ipsilateral (left) CCA was temporarily occluded (for 3 hours) using a Mayfield microaneurysm clip.

A skin incision was made between the left outer canthus and the tragus. The temporal muscle was deflected anteriorly and a portion of the left zygomatic arch was removed. Care was taken to avoid damaging the facial nerve. After anterior and downward retraction of the musculature, the mandibular nerve was identified and followed back to the foramen ovale. Using a high-speed air drill, a 3- to 4-mm craniectomy was made just anterior and superior to the foramen ovale. The dura was opened with a sharp needle and the MCA was freed from arachnoid. The portion of the left MCA that crosses the olfactory tract was temporarily occluded (for 3 hours) using a No. 3 Sundt arteriovenous malformation microclip.

Experimental Groups

The animals were divided into 16 groups as follows. The nonspecific NOS inhibitor, N²-nitro-L-arginine-methyl-ester (L-NAME), was administered intravenously 30 minutes before MCA occlusion at doses of 0.1 (six rats), 1 (five rats), 10 (five rats), and 30 (six rats) mg/kg. A selective nNOS inhibitor, 7-nitroindazole (7-NI), was administered intraperitoneally 60 minutes before MCA occlusion at doses of 0.1 (five rats), 1 (six rats), 10 (six rats), and 100 (six rats) mg/kg. Both NO donors, 3-morpholinosydnonimine (SIN-1) and NOC-18, DETA/NO, (Z)-1-[2-(aminoethyl)-N-(2-ammonioethyl)aminodiazene-1-ium-1,2-diolate (DETA NONOate), were administered intravenously 30 minutes before MCA occlusion. The SIN-1 was given at doses of 0.1 (five rats), 1 (six rats), and 10 (six rats) mg/kg, and the DETA NONOate at doses of 0.1, 1, and 10 mg/kg (six rats in each group). The L-NAME, DETA NONOate, and SIN-1 were dissolved in 0.35 ml of 0.9% NaCl. Because of its poor solubility in aqueous solution, the 7-NI was dissolved in 0.35 ml dimethyl sulfoxide (DMSO). Two groups of ischemic control animals (each group consisting of six animals) were administered 0.35 ml of either 0.9% NaCl (intravenously) or DMSO (intraperitoneally) at 30 or 60 minutes before MCA occlusion, respectively. Each solution was prepared directly before administration.

Histological Study

Three days (72 hours) after removal of the left MCA and CCA clips, anesthesia was again induced in the animals by using pentobarbital and the rats were intracardially perfused with a warm (37°C) 2% 2,3,5-triphenyltetrazolium chloride solution. The rat brains were quickly removed, immersed in the 37°C 2,3,5-triphenyltetrazolium chloride solution for 15 minutes to enhance staining, and placed in 10% buffered formaldehyde for 5 days. Twelve serial sections from each brain were cut at 1-mm intervals from the frontal pole and photographed. Photographic slides were analyzed using a computer-assisted image analyzer. Total cortical infarction volume was calculated by integrating the infarcted areas of all twelve sections (area of infarction in square millimeters × thickness of section).

Statistical Analysis

Statistical analysis was performed using analysis of variance with Scheffe’s post hoc test for multiple comparisons. Differences were considered significant if the probability value was less than 0.05. The data are depicted as the mean ± standard deviation.

Sources of Supplies and Equipment

The L-NAME was purchased from Sigma Chemical Co. (St. Louis, MO) and the 7-NI, SIN-1, and DETA NONOate from Alexis Biochemicals Corp. (San Diego, CA). The JAVA image analyzer was obtained from SPSS Inc. (Chicago, IL) and the Sundt No. 3 arteriovenous malformation microclip from Johnson & Johnson Professional, Inc. (Raynham, MA).

Results

Physiological Measurements

Physiological parameters measured just before completion of the ischemia experiment for each of the 16 groups are listed in Table 1. These physiological measurements were similar to those obtained at the beginning of the experiment and just prior to induction of ischemia. The MABP decreased momentarily after the intravenous bolus administration of SIN-1 but normalized before left MCA and bilateral CCA occlusion. A slight, nonsignificant reduction in MABP was noted in the DETA NONOate–treated groups. Mean arterial blood pressure was significantly higher in the L-NAME groups when compared with the saline-treated ischemic control group. In the group treated with the highest dose of 7-NI (100 mg/kg administered intraperitoneally), bradycardia occurred with heart rates decreasing to 150 to 200/minute (normal approximately 300/minute).

Ischemic Control Groups

Three hours of left MCA and bilateral CCA occlusion resulted in mean cortical infarction volumes of 92.5 ± 26 mm³ and 119 ± 43 mm³ in the saline- and DMSO-treated ischemic control groups, respectively (Fig. 1). There was
no significant difference (Student’s unpaired t-test) between these two groups. Because the infarcted area was not directly located at the craniotomy site, it was easily distinguishable from possible direct damage caused by the surgical procedure.

To assess the presence and extent of edema, the ischemic hemisphere volume was compared with the hemisphere volume on the contralateral side. In the saline-treated group, the mean hemisphere volume was 648.1 ± 33 mm³ for the ischemic (left) side and 621.2 ± 29 mm³ for the nonischemic (right) side. The right/left ratio (the right hemispheric volume divided by the left hemispheric volume) was 0.96 for this group. The range of the right/left ratio was 0.96 to 1.01 in all groups studied, with no significant differences between groups.

Drug Treatment Groups

The t-NAMe–Treated Groups. The mean cortical infarction volume was reduced by 50 to 71% in all four L-NAMe–treated groups studied when compared with the saline-treated ischemic control group (Fig. 1A). Only in the 1 mg/kg group was this reduction significant (27.1 ± 37 mm³ compared with 92.5 ± 26 mm³; p < 0.05).

The 7-NI–Treated Groups. The mean cortical infarction volume was significantly reduced in the 10 mg/kg–treated group (35.2 ± 32 mm³; p < 0.05) and the 100 mg/kg–treated group (9 ± 13 mm³; p < 0.005) when compared with the DMSO-treated ischemic control group (119 ± 43 mm³ Fig. 1B). The high-dose of 7-NI (100 mg/kg) tended to potentiate anesthesia and delay recovery.

The DETA NONOate–Treated Groups. No significant difference was found between the mean cortical infarction volumes in the DETA NONOate–treated groups and the saline-treated ischemic control group (Fig. 1C).

The SIN-1–Treated Groups. The mean infarction volumes in the SIN-1–treated groups were not significantly different from those in the saline-treated ischemic control group (Fig. 1D).

Discussion

Nitric oxide has been recognized as an important mediator of NMDA and hypoxic neurotoxicity.32 Malinski, et al.,33 conducted the first direct NO measurement study in which a NO-sensitive microsensor was used, and their results confirmed increased levels of NO in focal cerebral ischemia and provided valuable information about the time course of NO production. In that study, a rapid increase in the NO signal was found in the parietal cortex after the onset of focal cerebral ischemia; this increase reached a semiplateau at 6 minutes and the signal decreased to below detectable levels by 60 minutes.33 Using a nitrite assay technique as an indirect method of measuring NO levels, Kader, et al.,27 found a similar pattern with a rise in nitrite levels maximizing at 5 to 10 minutes and normalizing within 60 minutes after onset of ischemia. Although of great importance, absolute values of NO production are very dependent on the local microenvironment and measurement methodology.36

Nitric Oxide Synthase Inhibitors

Nitric oxide synthase inhibitors have been widely used

<table>
<thead>
<tr>
<th>Groups (no. of rats)</th>
<th>Weight (g)</th>
<th>pH</th>
<th>PaCO₂ (mm Hg)</th>
<th>PaO₂ (mm Hg)</th>
<th>Glucose (mg/dl)</th>
<th>MABP (mm Hg)</th>
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<tr>
<td>t-NAMe</td>
<td></td>
<td>7.35 ± 0.04</td>
<td>48.1 ± 2.3</td>
<td>251 ± 46</td>
<td>194 ± 28</td>
<td>104 ± 8†</td>
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<tr>
<td>0.1 mg/kg (6)</td>
<td>413 ± 42</td>
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<td>1 mg/kg (5)</td>
<td>362 ± 56</td>
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<td>10 mg/kg (5)</td>
<td>390 ± 60</td>
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<td>30 mg/kg (6)</td>
<td>419 ± 8</td>
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<tr>
<td>7-NI</td>
<td></td>
<td>7.35 ± 0.03</td>
<td>47.0 ± 3.4</td>
<td>192 ± 54</td>
<td>174 ± 24</td>
<td>104 ± 8†</td>
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<tr>
<td>0.1 mg/kg (5)</td>
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<td>1 mg/kg (6)</td>
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<td>10 mg/kg (6)</td>
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<td>0.1 mg/kg (5)</td>
<td>371 ± 11</td>
<td>7.40 ± 0.02</td>
<td>40.7 ± 4.0</td>
<td>196 ± 18</td>
<td>159 ± 11</td>
<td>94 ± 6</td>
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<td>1 mg/kg (6)</td>
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<tr>
<td>10 mg/kg (6)</td>
<td>379 ± 48</td>
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<td>DETA NONOate</td>
<td></td>
<td>7.45 ± 0.09</td>
<td>38.6 ± 5.4</td>
<td>239 ± 31</td>
<td>186 ± 32</td>
<td>93 ± 6</td>
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<td>0.1 mg/kg (6)</td>
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<td>1 mg/kg (6)</td>
<td>334 ± 42</td>
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<td>10 mg/kg (6)</td>
<td>419 ± 18</td>
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<tr>
<td>ischemic control</td>
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<td>7.41 ± 0.04</td>
<td>42.9 ± 4.3</td>
<td>246 ± 64</td>
<td>170 ± 22</td>
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<td>0.9% NaCl (6)</td>
<td>372 ± 45</td>
<td>7.44 ± 0.07</td>
<td>38.9 ± 6.8</td>
<td>240 ± 60</td>
<td>192 ± 36</td>
<td>91 ± 6</td>
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<td>DMSO (6)</td>
<td>342 ± 16</td>
<td>7.46 ± 0.04</td>
<td>36.6 ± 4.9</td>
<td>224 ± 24</td>
<td>158 ± 19</td>
<td>90 ± 6</td>
</tr>
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</table>

* Head and body temperature was maintained at 37 ± 0.5°C throughout the experiment. The parameters listed in this table are those obtained at the third measurement just before completion of the ischemia experiment. Physiological parameters obtained in the first two measurement periods at the beginning of the experimental preparation and just prior to ischemia were similar to these values and are not listed. Values are expressed as the mean ± standard deviation.
† p < 0.05 when compared with the ischemic control group (0.9% NaCl).
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Fig. 1. Bar graphs showing infarction volumes in the four drug groups (cross-hatched bars) and their respective saline- or DMSO-treated ischemic control group (slashed bars). A: Groups treated with L-NAME, a nonselective NOS inhibitor, at four different doses. Note a subtle bell-shaped dose response with a significant decrease in infarction volume observed only at the 1-mg/kg dose. B: Groups treated with 7-NI, a selective NOS inhibitor, at four different doses. Note the uniphasic dose response, with the two highest doses resulting in significant reduction in infarction volume, most notably at the 100-mg/kg dose. C: Groups treated with DETA NONOate, a NO donor, at three different doses. There was no significant alteration in infarction volume associated with any dose. D: Groups treated with SIN-1, an NO donor, at three different doses. There was no significant alteration in infarction volume in this group associated with any dose. Values are expressed as the mean ± standard deviation for each group. *p < 0.05; **p < 0.005.

to determine the overall effect of NO on ischemic damage. Selective inhibitors and genetically altered animals have made it possible to estimate contributions of the different isoforms of NOS to neurotoxicity and neuroprotection. Results with nonselective inhibitors such as L-NAME have shown a wide spectrum of results varying from cerebroprotection to augmentation of ischemic damage. Selective nNOS inhibitors and nNOS knockout animal studies have shown consistent protective results.

Inhibition of NO’s physiological activity may account for some side effects. Because the head and core body temperature was maintained throughout the experiment at 36.5 to 37.5°C, the effects of the NOS antagonists on infarct size were not related to a hypothermic effect. Vasoconstriction resulting in a rise in MABP was found in studies in which nonspecific NOS inhibitors were used, such as L-NAME, administered intravenously at high dosages, whereas reductions in cerebral blood flow (CBF) were observed in both nNOS-specific (7-NI) and non-specific (for example, L-NAME) inhibitors. Bradycardia and sedation have been reported with the use of L-NAME at higher doses. The 7-NI has potent antinociceptive properties and induces central nervous system depression similar to high doses of narcotic/sedative/hypnotic agents. It may also inhibit eNOS and, therefore, may not be a completely selective nNOS inhibitor. In our present study, bradycardia and sedation were seen in the group treated with an intraperitoneal injection of 100 mg/kg of 7-NI. In the L-NAME groups studied, the MABP increased after intravenous administration of L-NAME.
Nitric Oxide Donors

The antiischemic effects of nitrovasodilators in coronary artery disease have been recognized for more than 100 years and are still used to treat acute coronary syndromes. In focal cerebral ischemia NO donors appeared to reduce ischemic brain damage if hypotension was pharmacologically avoided by coadministration of an inotrope. Cyclic guanosine monophosphate–mediated vasodilation and reduction in platelet aggregation, as well as direct downregulation of the NMDA receptor by reacting with the redox modulary site, have been proposed to contribute to the cerebroprotective effect of NO. No protective effect was found in vitro, however. The NO donor sodium nitroprusside showed concentration-dependent cell death curves similar to those of NMDA. A dual role for NO was proposed in which neuronal NO overproduction played an important role in the development of ischemic damage, whereas endothelial and perivascular NO could protect against ischemia by increasing regional CBF and preventing platelet aggregation.

One of the two NO donors used in this study, SIN-1, is routinely used in interventional cardiology as a coronary artery bolus injection. In a study by Shukla, et al., SIN-1 was shown to cross the blood-brain barrier in rats. Nitric oxide formation from SIN-1 occurs spontaneously and does not require the presence of cysteine. Two factors were identified as influencing NO release: PO2 and pH. During cerebral ischemia, oxidative capacity appeared to be high enough to guarantee NO release. With the exception of NO, superoxide was shown to be a product of the SIN-1 oxidation. Superoxide generation was also described with purified nNOS at suboptimal L-arginine concentrations. By preventing superoxide formation, L-arginine infusion could provide additional protection to the previously discovered beneficial effects on regional CBF. Together superoxide and NO form peroxynitrite, which is the major contributor to NO and superoxide toxicity.

Care should be taken in handling sydnonimines such as SIN-1 because they are highly susceptible to oxygen and light. For this reason all our SIN-1 solutions were prepared directly before use and administered as an intravenous bolus. The half-life of NO in air-saturated buffer was calculated to be 6 seconds, indicating that NO formation is the main determinant of NO levels. Noack and Feilisch studied time-dependent formation of various metabolites of SIN-1 and their velocity of NO liberation, revealing the half-life of SIN-1 to be approximately 150 minutes. The SIN-1 metabolite SIN-1A reached a peak concentration at approximately 75 minutes with a half-life lasting approximately 300 minutes. The initial NO liberation velocity for SIN-1A was measured to be four times higher than that for SIN-1. Accounting for each relative NO liberating capacity, the overall half-life for NO donation is approximately 230 minutes and the maximum NO liberation is at approximately 30 minutes. In our study a temporary reduction in MAP was seen after intravenous administration. Before MCA and bilateral CCA occlusion, the MABP had returned to baseline levels without the use of vasopressor agents. The protective effect of a NO donor may be difficult to determine when a vasopressor is used simultaneously to maintain blood pressure by some investigators. Using a permanent MCA occlusion model with phenylephrine-induced hypertension, Drummond and associates showed a reduction in brain regions in which local CBF is equal to or lower than levels that may result in neuronal death. Using permanent MCA occlusion in spontaneously hypertensive rats, Maiese, et al., found no significant difference in infarction volume when the MAP was elevated using phenylephrine. One possible explanation for this discrepancy may be that the protective effect of phenylephrine depends on improvement in collateral flow. Collateral circulation is less developed in spontaneously hypertensive rats, which may explain the lack of efficacy of phenylephrine. In this present study, SIN-1 without vasopressor did not significantly alter mean cortical infarction volume.

The other NO donor used in this study, DETA NONOate, is a zwitterionic polyamine/NO adduct that releases two molecules of NO per molecule of DETA NONOate. The half-life for DETA NONOate was found to be 3400 minutes; therefore, it is more stable and longer acting than SIN-1. Although it is longer acting and releases higher concentrations of NO than SIN-1, DETA NONOate also did not significantly affect mean cortical infarction volume in our study.

In our model of focal cerebral ischemia in the Wistar rat, NO donors resulted in unchanged mean cortical infarction volumes throughout the concentration range used. However, both a nonspecific (L-NAME) and an nNOS-specific (7-NI) inhibitor reduced infarction volume, which suggests that excessive NO production is detrimental. However, the effect of NO inhibitors on other physiological roles of NO should not be overlooked. Pajewski and associates studied the effect of L-NAME and 7-NI on anesthesia, discovering that inhibition of the NO pathway decreased levels of consciousness, augmenting sedation.
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analgesia, and anesthesia. Potencies for 7-NI were found to vary between mice and rats in a study by Moore, et al. In that study, the authors showed that there was inhibition of nNOS without alterations in MAPB in a dose range of 10 to 80 mg/kg. The lack of effect on MAPB would indicate that there would be minimal or no effect on eNOS. Traystman and colleagues showed that a 20-mg/kg intravenously injected L-NAME produced the same amount of NOS enzyme inhibition (> 70%) but the half-lives varied widely among cats, dogs, and pigs. These observations suggest that extrapolations between species can lead to inaccurate dosing. The most effective dose for 7-NI found in our study was 100 mg/kg administered intraperitoneally, although a significant reduction in infarction volume was also demonstrated at 10 and 100 mg/kg. Dalkara, et al., found 25- and 50-mg/kg doses to be effective in reducing infarction volume by 25 to 27%.

The efficacy of different therapies appears to depend on the severity of the ischemic insult, time of onset, dosing of therapy, and the patient’s general vascular condition. Margail, et al., described the time course of glutamate concentrations during temporary MCA and bilateral CCA occlusion. Because the glutamate surge occurred minutes after occlusion and lasted for only approximately 80 minutes, the therapeutic window for intervention was found to be very short. Direct measurement of NO production showed a similar pattern. Atherosclerotic lesions tended to modify the response of human (coronary) arteries to vasoactive substances that curtailed the efficacy of the therapy. Dosing appeared to be a major determinant of the overall effect, especially in the case of L-NAME, a low dose of which (0.1 mg/kg) has been shown to be protective with a minimum effect on MAPB and a high dose (10 mg/kg) was more often detrimental.

Before extrapolations can be made to human pathophysiological states, more information should be obtained about the activation of pathophysiological events in humans. The therapeutic window for NOS inhibition appears to be short, limiting its efficacy in cases of stroke. In temporary arterial occlusion during vascular surgery, drug therapy could be instituted before occlusion to prevent ischemic brain damage. The results of this experiment indicate that NOS inhibition may be a valuable neuroprotective intervention during neurovascular or endovascular procedures.

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