Background and Purpose—A reduction in the local availability of nitric oxide (NO) may play a role in the etiology of chronic cerebral vasospasm after subarachnoid hemorrhage (SAH). We investigated the toxicity and efficacy of a locally delivered NO donor from a controlled-release polymer in preventing experimental cerebral vasospasm in rats and rabbits, respectively.

Methods—Diethylenetriamine/NO (DETA/NO) was incorporated into controlled release ethylene-vinyl acetate (EVAc) polymers. Twenty-eight rats were used in a dose-escalation toxicity study to establish a maximally tolerated dose of DETA/NO-EVAc polymer. In the efficacy experiment, 20 rabbits were assigned to 4 experimental groups (n=5 per group): sham operation; SAH only; SAH + empty EVAc polymer; and SAH + DETA/NO-EVAc polymer. Treatment was initiated 30 minutes after blood deposition. Basilar artery lumen patency was assessed 72 hours after hemorrhage to evaluate the efficacy of DETA/NO in preventing cerebral vasospasm.

Results—In the toxicity study, a dose of 3.4 mg/kg was identified as the LD_{20} (dose with 20% mortality during the study period) of this DETA/NO formulation. Brain histology revealed hemorrhage and ischemic changes at the implantation site associated with high concentrations of DETA/NO. In the efficacy study, treatment with DETA/NO-EVAc polymer resulted in a significant decrease in basilar artery vasospasm compared with no treatment (93.0 ± 4.9% versus 71.4 ± 11.9%; P = 0.035) or compared with treatment with blank EVAc polymer (93.0 ± 4.9% versus 73.2 ± 6.4%; P = 0.003).

Conclusions—Local delivery of DETA/NO prevents vasospasm in the rabbit basilar artery. Local delivery of DETA/NO via polymers is a safe and effective strategy for preventing cerebral vasospasm after SAH in this model. (Stroke. 2002; 33:2681-2686.)

Key Words: nitric oxide • polymers • subarachnoid hemorrhage • vasospasm, intracranial • rabbits • rats

An imbalance between endothelin-mediated vasoconstriction and nitric oxide (NO)–mediated vasodilatation has been implicated in the pathogenesis of chronic posthemorrhagic vasospasm after aneurysmal subarachnoid hemorrhage (SAH). Chronic vasospasm develops in approximately 30% of patients who survive the initial rupture of an intracranial aneurysm^1^ and remains a leading cause of mortality and morbidity from aneurysmal SAH.^2,3^ Although the etiology of cerebral vasospasm is uncertain, recent studies indicate that exogenous NO prevents and reverses chronic posthemorrhagic vasospasm.^4–6^ The administration of NO donors or nitric oxide synthase (NOS) substrates ameliorates cerebral vasospasm in experimental and clinical settings.^7–9^ However, the short half-life, side effects, and potential toxicity of NO limit the clinical utility of systemically administered NO for the treatment of cerebral vasospasm.

We recently demonstrated the efficacy of a controlled-release formulation of an NO donor compound—diethylenetriamine/NO (DETA/NO) incorporated in ethylene-vinyl acetate (EVAc) polymer—in preventing and reversing chronic posthemorrhagic vasospasm in the rat femoral artery model. The restricted permeability of the blood-brain barrier to systemic pharmacotherapy limits the spectrum of drugs that can achieve therapeutic levels in the central nervous system (CNS) with acceptable systemic toxicity. Controlled-release polymers for local drug delivery bypass the blood-brain barrier and can deliver drugs safely and effectively into the CNS. As a diazeniumdiolate-class NO donor,
DETA/NO has a relatively long half-life measured in hours,\textsuperscript{7,15} which renders it ideal for targeted and controlled delivery of NO.

In this study we evaluated the efficacy of locally delivered DETA/NO in preventing cerebral vasospasm in the rabbit basilar artery model of vasospasm and determined the toxicity associated with intracranially delivered DETA/NO in the rat brain. Since decreased levels of NO are believed to play a critical role in mediating cerebral vasospasm, we hypothesized that locally delivered exogenous NO from controlled-release polymers would prevent cerebral vasospasm.

Materials and Methods

Experimental Design

This report consists of 2 independent experiments. In the first experiment, a dose-escalation study was performed to determine the toxicity and a maximum tolerated dose of DETA/NO delivered from polymers implanted in the rat brain. The animals were randomized to 5 experimental groups and implanted with 0% (0 mg/kg of DETA/NO; n=5), 10% (3.4±0.1 mg/kg; [mean±SEM]; n=5), 20% (6.1±0.1 mg/kg; n=5), 30% (11.5±0.7 mg/kg; n=5), and 40% (13.0±0.3 mg/kg; n=8) DETA/NO-EVAc polymers. Animal survival and brain histology were assessed over 6 weeks. In the second experiment, an efficacy study was performed to determine whether intracranial delivery of DETA/NO from polymer implanted in the rabbit subarachnoid space prevents posthemorrhagic cerebral vasospasm. With the use of the rabbit basilar artery model of posthemorrhagic cerebral vasospasm, control or 0.48±0.01 mg/kg DETA/NO in the form of DETA/NO-EVAc polymers was implanted in the cisterna magna after injection of autologous blood in the subarachnoid space. The animals were randomized to 4 groups: (1) sham operation; (2) injection of nonheparinized autologous blood into the cisterna magna (SAH only group); (3) blood injection and implantation of empty EVAc polymer 30 minutes after SAH (SAH+empty EVAc polymer group); and (4) blood injection and implantation of DETA/NO-EVAc polymer 30 minutes after SAH (SAH+DETA/NO-EVAc polymer group). The basilar artery lumen patency was assessed 72 hours after hemorrhage to evaluate the efficacy of locally delivered DETA/NO in preventing vasospasm.

Animals

For the toxicity study, 28 adult, female Fischer 344 rats with a mean weight of 150 g (range, 110 to 165 g) were used in a dose-escalation study. In the efficacy experiment, 20 male New Zealand White rabbits with a mean weight of 2.7 kg (range, 1.9 to 4.0 kg) were assigned to 4 experimental groups as outlined above. The animals were kept in standard animal facilities with free access to Baltimore city water and rodent chow; all experimental protocols were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Polymer Preparation

We have previously described the technique for incorporating DETA/NO into controlled-release EVAc polymer.\textsuperscript{6,12} Briefly, 4 different weight ratios of the NO donor compound (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate (DETA/NO) (Alexsis Biochemicals) and EVAc polymer (40% vinyl acetate by weight; DuPont) were dissolved in methylene chloride (Fischer Chemicals) to yield EVAc polymers loaded with 10%, 20%, 30%, and 40% (wt/wt) DETA/NO. Suspensions were poured into cylindrical glass molds at −70°C, and after uniform freezing for 1 hour, the resulting polymer cylinders were transferred to glass plates at −80°C for 2 days and then to −21°C for 1 week. The polymer rods were then transferred to a vacuum desiccator at room temperature for 2 days to extract the remaining methylene chloride. Empty EVAc polymer implants containing 0% DETA/NO (wt/wt) were synthesized in an analogous fashion while omitting DETA/NO. The resulting dry empty or DETA/NO-EVAc polymer rods were cut into 5-mg cylindrical fragments (height=6 mm; diameter=1 mm) and stored at −20°C in sterile glass containers before implantation.

Toxicity Study in Rats

Animal Preparation

The animals were anesthetized with a 3-mL/kg intraperitoneal injection of a solution of ketamine (25 mg/mL), xylazine (2.5 mg/mL), and 14.25% ethanol in normal saline. We have previously described the procedure for polymer implantation in the rat brain.\textsuperscript{12} Briefly, the heads of anesthetized rats were shaved and aseptically prepared with povidone/iodine. Via a midline incision, a 3-mm burr hole was drilled through the skull 5 mm posterior to the bregma and 3 mm lateral to the sagittal suture. The dura was incised, and the polymer was inserted into the brain parenchyma. The incision was closed with surgical staples after wound irrigation. The animals were weighed at the beginning and end of the study; during daily examination for 6 weeks, animal survival, neurological deficits, and changes in grooming behavior were assessed. The animals that died before the end of the study underwent necropsies. Those animals that survived to the end of the study underwent brain examination after euthanasia.

Histological Assessment

Six weeks after implantation, all surviving animals were killed by CO2 inhalation, and the brains were removed and fixed in 10% formalin solution. The specimens were paraffin-embedded, and 1 coronal section centered through the polymer implant was taken from each rat and stained with hematoxylin and eosin. These tissue sections were examined microscopically by one of the authors (C.G.E.) for signs of hemorrhage, ischemic damage, or other pathological changes. Hemorrhage in and around the implantation site was graded according to the following system: 0, no significant macrophages and/or red blood cells; 1, scattered macrophages and/or red blood cells; 2, numerous macrophages and/or red blood cells; and 3, confluent macrophages and/or red blood cells. Ischemic damage around the implantation site was graded as follows: 0, no signs of ischemia; 1, single ischemic/necrotic focus; 2, multiple ischemic/necrotic foci; and 3, confluent ischemic/necrotic foci.

Efficacy Study in Rabbits

Animal Preparation

A modified rabbit basilar artery model of cerebral vasospasm was used to study the efficacy of intracranial DETA/NO-EVAc polymers in preventing experimental cerebral vasospasm (Figure 1). The animals were anesthetized with a 0.6-mL/kg intramuscular injection of a 5:1 mixture of ketamine (25 mg/mL) and xylazine (2.5 mg/mL). A 20-mg/kg intramuscular injection of ceftriaxone was administered prophylactically to all animals. The animals were placed prone on the operating table with the neck flexed, and the suboccipital and cervical regions were shaved and prepared in sterile fashion with 70% ethanol and povidone/iodine. A midline suboccipital incision extending from the inion was made, and the ligamentum nuchae was identified. With the use of blunt dissection, the underlying tissue was mobilized and removed from the opisthion, revealing the atlanto-occipital membrane. To further anesthetize the dural nerve endings in the underlying atlanto-occipital membrane, 1% lidocaine (1 mL) was applied topically onto the atlanto-occipital membrane for 1 minute and soaked up. In the sham operation animals, which did not receive an injection of blood in the cisterna magna, the wound was irrigated and closed in a layered fashion by interrupted sutures and surgical staples. To minimize postoperative pain, a 0.04-mg/kg IM injection of buprenorphine was given.

The animals in the 3 other experimental groups received injection of blood into the cisterna magna. A 23-gauge butterfly needle was used to penetrate the cisterna magna and 0.7 to 1.0 mL of cerebrospinal fluid was aspirated over a 30-second period. Three milliliters of autologous nonheparinized blood was collected from the central ear artery. A SAH was induced by injecting 1.5 to 2 mL of autologous nonheparinized blood into the cisterna magna over 30 to
by guest on October 14, 2017 http://stroke.ahajournals.org/ Downloaded from

The incision was closed as 0.48/H11006 inserted into the subarachnoid space. The DETA/NO-EVAc polymer 5-mg DETA/NO-EVAc or empty EVAc polymer fragment was allowed to recover, while the SAH animals that received polymer in the head-down position, the animals in the SAH only group were to confine the blood to the intracranial cisterns. After 30 minutes injection of blood into the cisterna magna.16,17 Therefore, the animals described above, and the animals were allowed to recover before being returned to their housing. A 0.04-mg/kg IM injection of buprenorphine was given to minimize pain. Postoperatively, all animals were monitored daily for signs of neurological deficits or behavioral changes.

Histological Assessment
In the rabbit basilar artery model of cerebral vasospasm, angiographic evidence of vasoconstriction is evident 72 hours after injection of blood into the cisterna magna.16,17 Therefore, the animals were killed 72 hours after the surgeries and underwent in situ perfusion-fixation. Anesthesia was induced as described above, and a 200-mg/kg intraperitoneal injection of sodium pentobarbital was administered. A thoracotomy was performed, and the left ventricle was cannulated with polyethylene tubing. The descending aorta was clamped, and the right atrium was pierced for drainage. Transcardiac perfusion was performed with 300 mL of normal saline solution followed by 500 mL of ice-cold freshly depolymerized 4% paraformaldehyde in 0.1 mol/L phosphate buffer at a standard height of 100 cm from the chest. The basilar arteries and the brain stem were harvested en bloc and cryoprotected in 20% sucrose in 0.1 mol/L phosphate buffer for 3 days at 4°C. After snap-freezing in dry ice–equilibrated isopentane cooled to −60°C, the specimens were stored at −80°C. The specimens were mounted in tissue freezing compound (Triangle Biomedical Sciences) and sectioned transversely into 10-μm slices with a microtome cryostat (Microm GmbH) at 200-μm intervals beginning at the basilar termination. The tissue slices were mounted on Superfrost Plus slides (Fisher Scientific) and stained with standard hematoxylin and eosin followed by coverslip mounting with Permount medium (Fischer Chemicals).

Morphometric Analysis
Vessel patency was quantified by measuring the basilar artery circumference with the use of a computerized image analysis system (MCID; Imaging Research). To correct for vessel deformation and off-transverse sections, the internal circumferences of 5 different sections of each vessel separated by 200 μm were measured and averaged. The luminal cross-sectional area of each vessel was estimated with the use of the calculated radius (r value obtained from the measured circumference (r=measured circumference/2π, area of circle=πr²). Then the estimated mean cross-sectional area of each basilar artery was converted into a mean percent lumen patency, defined as the ratio of the blood-exposed artery area to the average of control basilar artery area in the sham operation group.

Statistical Analyses
For the toxicity experiment in rats, animal weights are expressed as mean±SEM. Animal survival was plotted as a Kaplan-Meier curve, and statistical significance was determined by Kruskal-Wallis 1-way ANOVA.

For the DETA/NO efficacy experiments in rabbits, basilar artery percent lumen patencies for the experimental groups were expressed as mean±SEM. The mean percent lumen patencies of the treatment groups were compared by a Kruskal-Wallis 1-way ANOVA followed by the Newman-Keuls post hoc test (SigmaStat 2.03; SPSS). A probability value of <0.05 was considered significant.

Results
Toxicity of Controlled-Release DETA/NO in Rat Brain
There was a trend toward increasing toxicity with escalating doses of DETA/NO administered to the rat brain (Figure 2); the 13.0-mg/kg dose was associated with 50% mortality at the end of 6 weeks (P=0.07). The 11.5- and 3.4-mg/kg dose groups had 20% mortality during the study period. Therefore, we defined a dose of 3.4 mg/kg as the LD₅₀ of this DETA/NO formulation. There were no deaths associated with the implantation of 6.1- or 0-mg/kg doses of DETA/NO. Animal mortality in the 13.0-, 11.5-, and 3.4-mg/kg groups occurred by postoperative day 2. Necropsies performed on these animals revealed large intraparenchymal and incisional hemorrhages. With the exclusion of these 6 early deaths, the remaining rats in the DETA/NO groups and all animals receiving empty EVAc implants survived to the completion of the experiment. Weight gain, grooming behavior, and motor activity of rats in all DETA/NO groups were indistinguishable from those of the control rats.

Histopathological evaluation performed 6 weeks after implantation revealed increasing hemorrhage and ischemic damage at the implantation site associated with increasing concentration of DETA/NO (Figure 3). Hemosiderin-laden macrophages and extravasated red blood cells were mostly scattered in the 0- to 11.5-mg/kg DETA/NO groups but were numerous to confluent in the rats receiving 13.0-mg/kg DETA/NO implants (Figure 3D). Ischemic damage ranged from focal neuronal loss to frank necrosis. Dystrophic calci-
Effect of Controlled-Release DETA/NO on Basilar Artery Vasospasm After SAH

Treatment with DETA/NO-EVAc polymer resulted in a significant increase in basilar artery lumen patency compared with no treatment (93.0±4.9% versus 71.4±11.9%; P=0.035) or compared with treatment with blank EVAc polymer (93.0±4.9% versus 73.2±6.4%; P=0.003). In comparison to the control group, there was a statistically significant reduction in the mean basilar artery cross-sectional areas in the SAH only (100±5.4% versus 71.4±11.9%; P=0.006) and SAH+empty EVAc groups (100±5.4% versus 73.2±6.4%; P=0.006) (Figure 4). Therefore, the presence of a polymer does not affect lumen patency.

There were no adverse events in rabbits receiving blank or DETA/NO-EVAc polymer implants with a dose of 0.48 mg/kg. At the time of brain removal, there was a visible layer of subarachnoid clot that surrounded the ventral brain stem and enveloped the basilar artery. There was no evidence of vascular toxicity during the examination of basilar artery cross-sections under the light microscope (Figure 5).

Discussion

In this investigation we demonstrate the safety and efficacy of controlled-release DETA/NO for the prevention of cerebral vasospasm in the rabbit basilar artery model. Our results indicate that local administration of 0.48 mg/kg DETA/NO from EVAc controlled-release polymers initiated 30 minutes after hemorrhage prevents the onset of delayed posthemorrhagic cerebral vasospasm in rabbits. The interstitial implantation of DETA/NO-EVAc polymer in the brain of rats is tolerated by the animals up to a DETA/NO dose of 3.4 mg/kg, suggesting that the DETA/NO-EVAc polymer formulation may be used safely for local drug delivery to the CNS.

The pathogenesis of cerebral vasospasm in the setting of SAH entails an alteration in cerebral vessel autoregulation possibly driven by decreased local availability of NO.5,18–21 As the most notable endothelium-derived relaxation factor, NO is a ubiquitous effector molecule with extensive and diverse roles in neurotransmission, inflammation, and vascular autoregulation.22 In response to a variety of intracellular and extracellular stimuli, NO is synthesized from L-arginine by the NOS enzyme, of which, 3 isoforms—endothelial, neuronal, and inducible NOS—have been isolated in the brain.23 The dynamic release of NO from the endothelium is necessary for autoregulation of cerebral vascular tone.24 As a result, it has been postulated that a prolonged reduction in the local availability of NO after SAH may be pivotal to the pathogenesis of cerebral vasospasm.5,18,21 Investigators have demonstrated significant decrease in neuronal NOS activity after SAH in a primate model of cerebral vasospasm,19,20 while inactivation of NOS by exogenous inhibitors after induction of SAH leads to delayed vasospasm.25

A substantial body of experimental evidence indicates that exogenous repletion of NO counteracts vasospasm after SAH. Investigators have succeeded in treating experimental and clinical cerebral vasospasm by administration of various NO or NO donor compounds and NOS substrates through intravascular, intrathecal, and periadventitial routes.4,9–20 At the same time, the complications associated with systemic ad-
administration of NO-related agents and the requirement for repeated treatments due to transient bioavailability of NO have prompted the search for more practical NO donors and delivery strategies.

Local CNS pharmacotherapy with NO donor compounds with the use of controlled-release polymers may offer efficacy and safety advantages over conventional systemic, intrathecal, or intraventricular administration. Controlled-release polymers allow for local, sustained, and tightly regulated drug delivery to the CNS with reproducible release kinetics. The development and clinical application of carmustine-loaded polymers have demonstrated the effectiveness of local chemotherapy against malignant gliomas in experimental models and ongoing clinical trials. The use of polymer matrices to deliver pharmacotherapy interstitially to the CNS has also been accomplished in experimental models of neurodegenerative disorders, brain edema, and chronic posthemorrhagic vasospasm.

We have previously described the release kinetics of the DETA/NO-EVAc polymer used in this investigation and demonstrated the favorable pharmacokinetics of this polymer construct for intracranial delivery of exogenous NO. Apart from the inherent stability of the DETA/NO compound, the 20%-loaded DETA/NO-EVAc polymer construct releases approximately 13% of its initial loading dose during the 3-day treatment period used in the efficacy study. This controlled and sustained release of DETA/NO may prove valuable since a sustained increase in the local availability of NO in the subarachnoid space would obviate the need for repeated drug infusion or ventricular access. Furthermore, surgical placement of controlled-release DETA/NO polymer in the vicinity of aneurysmal rupture could provide targeted therapy to arteries at risk in the subarachnoid space. However, the extent of diffusion for the DETA/NO compound in the subarachnoid space from a fixed DETA/NO-EVAc source is unclear and remains the focus of additional investigation.

There is conflicting evidence concerning the potential cytotoxic or cytoprotective roles of NO in the CNS. Because of the multiple biological effects of NO in the CNS, a distinction in the pathophysiological effects of endothelial and neuronal NO is warranted. Endothelial NO plays a neuroprotective role in experimental cerebral ischemia by augmenting cerebral blood flow in the pial microcirculation. In contrast, excessive neuronal NO production after ischemia/reperfusion induces neurotoxicity by interacting with reactive oxygen species and by participating in the glutamate excitotoxicity cascade contingent on the redox state of the local microenvironment. However, a neuroprotective role for NO in the setting of glutamate excitotoxicity has been demonstrated that involves downregulation of the N-methyl-D-aspartate receptor at a redox modulatory site. Therefore, the outcome of the pleiotropic neuroprotective or neurotoxic effects of NO in the CNS is determined by the redox microenvironment, presence of reactive molecular species, or amount of isoform-specific NO.

In the present study histopathological sequelae of NO-related toxicity after DETA/NO-EVAc implantation in the rat brain consisted of dose-dependent evidence of hemorrhage and ischemic changes. However, the LD₅₀ dose, 3.4 mg/kg, of the DETA/NO-EVAc intraparenchymal implant was 7-fold greater than the 0.48-mg/kg dose of intracisternally implanted DETA/NO used in the efficacy study, implying that this novel...
formulation of DETA/NO-EVAc polymer possesses a favorable therapeutic index. Indeed, a decrease in ischemic injury was reported in a canine model of cerebral vasospasm after intrathecal infusion of DETA/NO,40 and no serious complications were identified in patients intrathecal administration of sodium nitroprusside—both prophylactically and as adjuvant treatment—for post-SAH cerebral vasospasm.9,41

In summary, we describe the safe and effective intracranial administration of an exogenous NO donor compound from controlled-release polymers. Intrathecal administration of 0.48 mg/kg DETA/NO-EVAc polymer after SAH prevents the onset of cerebral vasospasm in the rabbit basilar artery model. Moreover, the intraparenchymal implantation of DETA/NO-EVAc polymer in the rat brain is safe up to a DETA/NO dose of 3.4 mg/kg. We conclude that the DETA/NO-EVAc polymer and its intracranial delivery hold therapeutic potential in pathological states that respond to increased concentration of NO.

Acknowledgments

This work was supported in part by a Student Scholarship in Cardiovascular Disease and Stroke from the American Heart Association (P.G.), the Dr Harold Lamport Student Research Award (P.G.), and the John W. Brantigan Clinical Research Fund (T.S.T.).

References

Prevention of Experimental Cerebral Vasospasm by Intracranial Delivery of a Nitric Oxide Donor From a Controlled-Release Polymer: Toxicity and Efficacy Studies in Rabbits and Rats

Patrik Gabikian, Richard E. Clatterbuck, Charles G. Eberhart, Betty M. Tyler, Travis S. Tierney and Rafael J. Tamargo

Stroke. 2002;33:2681-2686
doi: 10.1161/01.STR.0000033931.62992.B1

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/33/11/2681

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/