Nitrite Infusions to Prevent Delayed Cerebral Vasospasm in a Primate Model of Subarachnoid Hemorrhage

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Context  Delayed cerebral vasospasm causes permanent neurological deficits or death in at least 15% of patients following otherwise successful treatment for ruptured intracranial aneurysm. Decreased bioavailability of nitric oxide has been associated with the development of cerebral vasospasm.

Objective  To determine whether infusions of nitrite will prevent delayed cerebral vasospasm.

Design, Setting, and Subjects  A total of 14 anesthetized cynomolgus monkeys had an autologous blood clot placed around the right middle cerebral artery. Cerebral arteriography was performed before clot placement and on days 7 and 14 to assess vasospasm. The study was conducted from August 2003 to February 2004.

Interventions  A 90-mg sodium nitrite intravenous solution infused over 24 hours plus a 45-mg sodium nitrite bolus daily (n=3); a 180-mg sodium nitrite intravenous solution infused over 24 hours (n=3); or a control saline solution infusion (n=8). Each was infused continuously for 14 days.

Main Outcome Measures  Nitrite, S-nitrosothiol, and methemoglobin levels in blood and cerebrospinal fluid and degree of arteriographic vasospasm.

Results  In control monkeys, mean (SD) cerebrospinal fluid nitrite levels decreased from 3.1 (1.5) µmol/L to 0.4 (0.1) µmol/L at day 7 and to 0.4 (0.4) µmol/L at day 14 (P=.03). All 8 control monkeys developed significant vasospasm of the right middle cerebral artery, which was complicated by stroke and death in 1 animal. Sodium nitrite infusions increased the nitrite and methemoglobin levels (<2.1% of total hemoglobin) in the blood and cerebrospinal fluid without evoking systemic hypotension. Nitrite infusion prevented development of vasospasm (no animals developed significant vasospasm; mean [SD] reduction in right middle cerebral artery area on day 7 after subarachnoid hemorrhage of 8% [9%] in nitrite-treated monkeys vs 47% [5%] in saline-treated controls; P<.001). There was a negative correlation between the concentration of nitrite in cerebrospinal fluid and the degree of cerebral vasospasm (P<.001). Pharmacological effects of nitrite infusion were also associated with the formation of S-nitrosothiol in cerebrospinal fluid. There was no clinical or pathological evidence of nitrite toxicity.

Conclusion  Subacute sodium nitrite infusions prevented delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage.
nism that also produces endothelial dysfunction in hemolytic disease.27

The role of nitric oxide in vasospasm is further confirmed by experimental and clinical studies showing that exogenous delivery of nitric oxide ameliorates or prevents vasospasm.8,20-22,28 A decrease in nitrite levels in cerebrospinal fluid at the time of delayed cerebral vasospasm has been observed.15,23,24 However, a clinical application of these findings has been limited by the following adverse systemic effects of nitric oxide donors: decreased blood pressure, increased intracranial pressure, subsequent decreased cerebral blood flow,20,29,30 and increased risk of ischemic stroke.20 Thus, use of nitric oxide therapy has been restricted to invasive regional intravascular delivery such as intracarotid administration of nitric oxide gas solutions, ultrashort-acting nitric oxide donors,20-21,31,32 and extravascular delivery of nitric oxide donors.20,21,31,32

Endogenous levels of nitrite reflect endothelial NOS activity.33 Recent studies34-37 indicate a new biological role for the nitrite anion as a storage molecule for nitric oxide. Nitrite can be reduced to nitric oxide by mammalian xanthine oxidoreductase38,39 and by nonenzymatic disproportionation,40 although these conversion mechanisms are limited to conditions of hypoxia and tissue acidosis.34

Recent attention has focused on the reaction of deoxyhemoglobin with nitrite to produce nitric oxide under physiological conditions, which may contribute to hypoxic vasodilation.34,36,41,42 Nitrite is converted in the blood to nitric oxide and to potentially vasoactive and biologically active chemical species, including S-nitrosothiols, N-nitrosamines, and iron-nitrosyl complexes.34,35,42,43 Because subarachnoid hemorrhage is associated with the presence of both deoxyhemoglobin and low pH35-47 in the vicinity of arteries surrounded by the subarachnoid clot, we hypothesized that intravenous nitrite infusions would lead to a release of nitric oxide, nitros(yl)ation of cerebrospinal fluid proteins in the vicinity of the affected arteries, and prevent vasospasm. We examined this hypothesis using a well-characterized primate model of subarachnoid hemorrhage.28,48

METHODS
The animal protocol was reviewed by the Animal Care and Use Committee of the National Institute of Neurological Disorders and Stroke and met the National Institutes of Health guidelines for animal care. The study was conducted from August 2003 to February 2004.

Model of Cerebral Vasospasm
Fourteen cynomolgus monkeys were studied. After induction of general anesthesia, the monkeys underwent right frontotemporal craniectomy. The proximal 1.4 cm of the right middle cerebral artery (RMCA) (M1 segment) was exposed from a bifurcation of the right internal carotid artery to a trifurcation of the middle cerebral artery. A 5-mL preclotted autologous blood clot was placed around the artery. In this model of subarachnoid hemorrhage, which is widely recognized as the best in vivo model,49 cerebral vasospasm develops in about 95% of the animals and the course of spasm mimics clinical events.49,50 The animals were followed up until day 14 after subarachnoid hemorrhage and were killed at the end of the experiment.

Experimental Design
Initial blood and cerebrospinal fluid samples were collected and all monkeys received cerebral arteriography followed by clot placement around the RMCA on day 0 (baseline) (FIGURE 1). Cerebral arteriography was repeated on days 7 and 14 and cerebrospinal fluid samples were collected. Because no prior data on subacute nitrite dosing in primates or humans were available, we extrapolated the dose from rat31 and human34 studies. The first 3 monkeys received a 90-mg sodium nitrite infusion (Hope Pharmaceuticals, Scottsdale, Ariz) over 24 hours (low-dose infusion group). After we observed no toxic effects during the first day, these animals received an additional 45-mg sodium nitrite bolus daily (1.5 mL over 5 minutes), which was administered at the time the infusion solution was changed, to establish the maximal tolerated dose and to determine pharmacokinetics.

Before each bolus was administered, blood pressure was measured and blood was drawn to determine the nitrite and methemoglobin levels reached during the nitrite infusion. Blood also was drawn 5 minutes after the bolus was delivered to measure nitrite levels. Because the bolus increased blood nitrite levels, produced transient hypotension, and increased methemoglobin levels above normal limits in the first 3 monkeys, the subsequent 3 received a 180-mg sodium nitrite continuous infusion over 24 hours without a bolus (high-dose infusion group). Blood and cerebrospinal fluid samples were collected throughout the study.

The sodium nitrite solutions were delivered at 0.9 µmol/min (low-dose infusion plus bolus group) and 1.8 µmol/min (high-dose infusion group) for 14 days via an ambulatory infusion pump (model 404-SP, Medtronic MiniMed, Northridge, Calif), which was secured in a pocket of the jacket (Lomir Biomedical Inc, Notre-Dame-de-l’Île Perrot, Quebec) fitted to the monkey.

The pump was connected to polyethylene 50 tubing that was passed subcutaneously from the midscapular region and inserted into the external jugular vein under direct visualization. The infusion started 1 hour after craniectomy and clot placement to model the initiation of treatment after craniotomy for control of aneurismatic hemorrhage in human disease. The pump was reloaded daily with sodium nitrite or saline (control group). The incidence and degree of cerebral vasospasm after subarachnoid hemorrhage with nitrite infusion in animals in the low- and high-dose infusion groups were compared with the control animals who received intravenous saline.

Arteriographic Studies
To assess the incidence and degree of vasospasm, cerebral arteriography was performed preoperatively, baseline, and
on days 7 and 14 after subarachnoid hemorrhage, as described previously.28 Each animal underwent at least 2 arteriographies: one before subarachnoid hemorrhage and the other on day 7 after subarachnoid hemorrhage. Arteriographies were performed under general anesthesia induced by a 0.5% mixture of isoflurane and pancuronium. Systemic arterial blood pressure and end expiratory PCO2 of the animals were continuously monitored; both levels remained stable during the procedure.

A femoral cut down to expose the femoral artery was performed under aseptic conditions. An F3 polyethylene catheter (Cook Group, Bloomington, Ind) was advanced under fluoroscopic control to the right internal carotid artery and 0.75 mL of contrast medium (diatrizoate meglumine and diatrizoate sodium) was injected by hand. Subtraction images in the anteroposterior projection were acquired. The area of the proximal 14-mm segment of the RMCA was measured by 3 examiners who were blinded to the study data, using a computerized image analysis system (NIH Image 6.14; FIGURE 2), which has been used to ensure maximal objectivity of measurements by our laboratory since 1992.28,52 Arteriographic vasospasm was quantified relative to each monkey’s baseline arteriogram; it was defined as more than a 25% decrease in the middle cerebral artery area in comparison with the pre-subarachnoid hemorrhage area.

Clinical and Laboratory Observations

The monkeys were observed daily for changes in weight, eating behavior, and general physical and neurological status. Daily blood samples and blood pressure were collected when the infusion solution pump was changed (under ketamine anesthesia). The blood was collected to assess methemoglobin and nitrite levels. Cerebrospinal fluid samples were collected via suboccipital puncture and were collected at the time of arteriography (days 0, 7, and 14). Blood pressure was measured using a new-Arterial clots were placed and arteriography was performed on days 0, 7, and 14. In the low-dose group, blood was drawn prior to bolus. Asterisk indicates missing data in the low-dose nitrite group on day 3. Dashed line in panel D indicates the 2% level, which is the upper limit of normal.
Figure 2. Characteristic Cerebral Arteriograms Before and After Subarachnoid Hemorrhage

Cerebral arteriograms on day 7 of a control monkey treated with intravenous saline for 14 days demonstrate severe vasospasm of the M1 segment of the right middle cerebral artery compared with a monkey treated with nitrite.

born’s cuff (Zimmer Inc, Dover, Ohio). The remaining plasma and cerebrospinal fluid were stored at –80°C.

Measurement of Nitrite and Nitric Oxide–Modified Protein Levels

For blood nitrite measurements, 100 µL of whole blood were mixed with 900 µL of a nitrite-stabilizing solution containing 80 mM of potassium hexacyanoferrate (III), 20 mM of N-ethylmaleimide, 200 µL of diethylenetriaminepentaacetic acid, and 0.2% of Nonidet P-40 (Abbott Laboratories, North Chicago, Ill). The nitrite in whole blood was then measured using triiodide-based reductive chemiluminescence using a nitric oxide analyzer (model 280, Seivers, Boulder, Colo) as previously described and validated.35–38 Cerebrospinal fluid was used to measure nitrite and S-nitrosothiol via a stabilization solution containing 10 mM of N-ethylmaleimide and 2 mM of diethylene triamine pentaacetic acid. To determine the levels of specific nitric oxide adducts (N-nitrosamines, iron-nitrosylhemoglobin, and S-nitrosothiols), samples were reacted with and without 5 mM of mercuric chloride. Nitric oxide–modified protein is converted to nitrite in the presence of mercuric chloride and N-nitrosamines when iron-nitrosyl complexes are stable. Both blood and cerebrospinal fluid samples were treated with a 0.5% solution of acid sulfanilamide, which eliminates nitrite.53,54

Statistical Analysis

Data are presented as mean (SDs). Degree of vasospasm, levels of nitrite and methemoglobin, and blood pressure were assessed using 2-tailed t tests and analysis of variance followed by the Tukey test for post hoc comparisons of mean values. Statview statistical software (version 5.0, SAS Institute Inc, Cary, NC) was used; statistical significance was defined as P < .05.

RESULTS

Clinical Status

Of 14 monkeys, 13 survived the study without complication. One monkey from the control group with severe vasospasm of the RMCA became somnolent and died on day 8 after subarachnoid hemorrhage (24 hours after cerebral arteriography). Autopsy confirmed the presence of ischemic stroke in the territory of the RMCA. None of the monkeys in the low- or high-dose infusion groups developed symptoms of stroke or nitrite toxicity due to methemoglobinemia (Figure 1). Transient decreases in blood pressure (lasting for 15 to 25 minutes) were observed immediately after the nitrite boluses were delivered in the low-dose infusion group (mean [SD], 97 [3] mm Hg to 80 [3] mm Hg; P < .001), but no changes in blood pressure were observed with continuous infusions (prior to receiving the bolus in the low-dose infusion group and at all measured times in the high-dose infusion group; Figure 1).

Degree of Vasospasm

All of the control monkeys who had subarachnoid hemorrhage (n = 8) developed vasospasm (Figure 2 and Figure 3). The degree of vasospasm peaked on day 7 (mean [SD], 97 [3] mm Hg; range, 47%–54%; P < .001) and significantly decreased on day 14 (–1.9% [7.5%]; range, –7% to 3%; P < .001 vs day 7). None of the monkeys treated with nitrite developed significant vasospasm on day 7 after subarachnoid hemorrhage (mean [SD] reduction from baseline, 47% [5%] on day 0; range, 41%–54%; P < .001) and significantly decreased on day 14 (–0.90 [95% confidence interval, –1.24 to –0.73]; P < .001; Figure 4).

Nitric Oxide Metabolites and Methemoglobin Levels

Nitrite levels were measured in blood samples daily and in cerebrospinal fluid on days 0, 7, and 14 in 3 monkeys in the control group (Figure 1). These
monkeys developed moderate to severe vasospasm after subarachnoid hemorrhage. Blood nitrite levels remained unchanged compared with pre-subarachnoid hemorrhage values; however, cerebrospinal fluid nitrite levels were significantly lower on days 7 and 14 compared with day 0 (P = .03). Cerebrospinal fluid nitric oxide–modified protein levels, plasma methemoglobin levels, and mean arterial blood pressure remained unchanged.

In the 3 monkeys in the low-dose infusion plus bolus group, the levels of nitrite in blood and cerebrospinal fluid and levels of nitric oxide–modified protein in cerebrospinal fluid significantly increased compared with levels on day 0 and compared with controls (P = .02). Blood methemoglobin levels also increased significantly on days 4, 5, and 6 (P = .01) compared with the levels in controls. This low-dose infusion group received a bolus of nitrite of 45 mg/d for 14 days. Following these boluses, the mean (SD) level of whole blood nitrite increased from 24.7 (13.1) µmol/L to 328.7 (61) µmol/L (P = .009); level of blood methemoglobin increased from 0.9% (0.4%) to 10.8% (5.1%) (P = .02); and systemic blood pressure decreased from 97 (3) mm Hg to 80 (3) mm Hg (P < .001). The cumulative nitrite dose in the low-dose infusion with bolus group was lower than in the high-dose infusion group. The cumulative nitrite dose in the low-dose infusion plus bolus group produced transiently higher blood nitrite levels (>150 µmol/L) resulting in the higher cerebrospinal fluid nitrite levels, which appeared to exert more potent antivasospastic effects.

The 3 monkeys in the high-dose infusion group had significantly increased nitrite levels in the blood (P < .001) and cerebrospinal fluid (P = .01; Figure 1). This group also had significantly increased levels of nitric oxide–modified protein in their cerebrospinal fluid compared with levels on day 0 and compared with controls (P = .04). Levels of methemoglobin were significantly increased above the baseline level (day 0), but remained within clinically acceptable limits (<2%) throughout the study.

**COMMENT**

This study shows that continuous intravenous infusions of nitrite for 14 days prevent delayed cerebral vasospasm after subarachnoid hemorrhage in a primate model. Moreover, the results suggest that nitrite therapy is safe and is associated with limited adverse effects. This study suggests that lower nitrite levels in cerebrospinal fluid correlate with the development of vasospasm and that nitrite administration repletes these levels and generates cerebrospinal fluid nitric oxide and S-nitrosothiols, which are potent vasodilating molecules. Widely used treatments such as nimodipine and hypertension-hypervolemia-hemodilution (triple-H) therapy do not influence either the incidence or severity of vasospasm despite possibly improving outcome. Angioplasty and papaverine alone transiently reverse vasospasm, without changing overall morbidity and mortality. Thus, these results suggest a new, safe, inexpensive, and rationally designed therapy for a disease for which no current preventative therapy exists.

There have been several experimental and clinical trials using nitric oxide donors delivered intra-arterially, intravenously, intrathecally, and/or intraventricularly in an attempt to restore regional nitric oxide bioavailability and prevent or reverse vasospasm. However, using nitric oxide donors in animals and humans has been limited by systemic hypotensive effects, nondiscriminative dilation of the cerebral vasculature resulting in cerebral blood flow “steal syndrome,” increased intracranial pressure, and decreased cerebral perfusion pressure. Intracarotid and local delivery of nitric oxide donor (isolating the nitric oxide effects to the brain vasculature) eliminate hypotension, increase intracranial pressure, decrease cerebral perfusion pressure, and demonstrate that local delivery of nitric oxide can prevent vasospasm after subarachnoid hemorrhage.

There is little doubt that oxyhemoglobin and deoxyhemoglobin are slowly released from erythrocytes after aneurysmal bleeding and are directly and/or indirectly responsible for vasospasm. We and others have reported a significant concentration of deoxyhemoglobin in a subarachnoid clot in the direct vicinity of vessels in spasm. Deoxyhemoglobin levels peak in the vicinity of cerebral vessels in spasm around day 7 after subarachnoid hemorrhage. At the same time, dysfunction with preserved expression of endothelial NOS has occurred and decreased nitrite levels in cerebrospinal fluid are observed. The latter is closely associated with the degree of vasospasm. While some researchers have challenged this observation, our study demonstrated a similar and marked reduction of nitrite in cerebrospinal fluid

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ies that have shown nitrite infusions at pharmacological and near-physiological concentrations producing vasodilation and increasing blood flow. This bioactivity was associated with a reaction of nitrite with deoxyhemoglobin to produce nitric oxide, iron nitrosylhemoglobin, and to a lesser extent S-nitrosothiols. These results are consistent with a deoxyhemoglobin-mediated nitrite reaction that ultimately produces methemoglobin and nitric oxide.13,41,42

The same conditions (ie, the presence of deoxyhemoglobin) and reduced pH45,47,76-78 exist in the subarachnoid after subarachnoid hemorrhage.77,79 Lower levels of nitrite in cerebrospinal fluid after subarachnoid hemorrhage and during the development of vasospasm can be evoked by decreased nitric oxide production by both neuronal17 and endothelial NOS,15 and by increased nitrite consumption. The potent vasodilating effects of nitrite34,37,43 are consistent with reactions between nitrite and hemoglobin to form nitric oxide, S-nitrosothiols, and methemoglobin—chemistries that not only vasodilate, but also inhibit further nitric oxide scavenging by hemoglobin.

We demonstrate herein that intravenously administered nitrite in a primate model increases nitrite levels in the blood and in the cerebrospinal fluid, reacts in cerebrospinal fluid to produce S-nitrosothiol, and potently and completely inhibits middle cerebral artery vasospasm after subarachnoid hemorrhage. This observation provides additional evidence for the recently appreciated potent bioactivity of nitrite.34,37,43,51,75,80 Considering the lack of currently available therapies that prevent this devastating complication of subarachnoid hemorrhage and the safety and efficacy associated with nitrite therapy in a primate model, these results support the careful implementation of phase 1 and 2 trials in humans.

However, this study has limitations. The results we observed with relatively healthy monkeys may not extrapolate to patients who have subarachnoid hemorrhage and who are cardiologically and neurologically unstable. Due to possible cardiovascular effects that might result in decreased cerebral perfusion pressure and the unknown effects of prolonged low levels of methemoglobinemia, careful studies are needed of dosing and adverse effects of sodium nitrite in healthy volunteers and in at-risk patients. Such studies should elucidate the pharmacokinetics of sodium nitrite in humans, establish a proper dosage and safety profile, and offer a new therapeutic modality for patients surviving subarachnoid hemorrhage.

Author Contributions: Drs Pluta, Gladwin, and Oldfield had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Drs Gladwin and Oldfield share senior authorship equally.

Study concept and design: Pluta, Dejam, Gladwin, Oldfield.

Acquisition of data: Pluta, Dejam, Grimes, Gladwin, Oldfield.

Analysis and interpretation of data: Pluta, Dejam, Grimes, Gladwin, Oldfield.

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Figure 4. Relationship Between Degree of Vasospasm and Nitrite Levels in Cerebrospinal Fluid at Day 7

The low-dose infusion group received a 90-mg sodium nitrite intravenous solution infused over 24 hours with a 45-mg sodium nitrite bolus daily. The high-dose infusion group received a 180-mg sodium nitrite intravenous solution infused over 24 hours. The control group received a saline solution infusion. The nitrite levels in cerebrospinal fluid inversely correlated with degree of right middle cerebral artery vasospasm (R2 = -0.50; 95% confidence interval, -1.24 to -0.73, P < .001). Despite the lower dose of nitrite in the low-dose infusion group, the actual whole blood nitrite levels were transiently (about 150 min/d) significantly higher than the peak levels in the high-dose group. This finding explains the strong correlation between nitrite levels and cerebrospinal fluid and degree of vasospasm for baseline (day 0) and days 7 and 14 for all animals. The low-dose group had less vasospasm and higher methemoglobin levels, which confirm a dose-dependent effect of nitrite in cerebrospinal fluid. The solid line indicates linear regression and the dashed lines indicate the upper and lower 95% confidence intervals.

in untreated primates—a result consistent with the hypothesis that decreased nitric oxide availability contributes to development of cerebral vasospasm after subarachnoid hemorrhage17 and nitrite in cerebrospinal fluid can be consumed after subarachnoid hemorrhage.

Gradients of nitrite are present in human circulation from artery to vein, with increased nitrite consumption occurring with increased metabolic stress.30 Recently, we reported that intravenous sodium nitrite infusion selectively opens the blood-tumor barrier via a nitric oxide–related mechanism, suggesting that nitrite may be a naturally circulating nitric oxide donor.51,75 Bioactivity of nitrite has been confirmed in human study.
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Nothing in life is to be feared. It is only to be understood.
—Marie Curie (1867-1934)