Original Contribution

Effect of Antibiotic Class on Stroke Outcome
Dannielle Zierath, BS; Allison Kunze, BS; Leia Fecteau, BS; Kyra Becker, MD

Background and Purpose—Infections are common after stroke and associated with worse outcome. Clinical trials evaluating the benefit of prophylactic antibiotics have produced mixed results. This study explores the possibility that antibiotics of different classes may differentially affect stroke outcome.

Methods—Lewis rats were subjected to transient cerebral ischemia (2 hours) and survived for 1 month. The day after stroke they were randomized to therapy with ceftiofur (a β-lactam antibiotic), enrofloxacin (a fluoroquinolone antibiotic), or vehicle (as controls) and underwent the equivalent of 7 days of treatment. Behavioral tests were performed weekly until euthanization. In a subset of animals, histology was done.

Results—There were no differences in outcomes at 24 hours or 1 week after stroke among the different groups. At 1 month after stroke, however, performance on the rotarod was worse in enrofloxacin-treated animals when compared with control animals.

Conclusions—Independent of infection, the antibiotic enrofloxacin was associated with worse stroke outcome. These data echo the clinical observations to date and suggest that the secondary effects of antibiotics on stroke outcome should be considered when treating infection in subjects with stroke. The mechanism by which this antibiotic affects outcome needs to be elucidated. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.008663.)

Key Words: antibacterial agents ☼ beta-lactam ☼ ceftiofur ☼ enrofloxacin ☼ fluoroquinolones ☼ stroke

Studies show that patients who become infected in the immediate poststroke period have increased morbidity and mortality in comparison with patients who remain infection free.1 Trials of prophylactic antibiotics to prevent poststroke infection, however, have produced mixed results, which are summarized as follows: levofloxacin did not prevent infection and was associated with worse outcome,2 moxifloxacin did not prevent infection nor affect outcome,3 mezlocillin both prevented infection and improved outcome,4 and minocycline improved outcome (the effect on infection was not assessed).5 All of these studies were relatively small single-center trials, but the disparate findings suggest that the antibiotic class itself may influence stroke outcome. For instance, both minocycline and β-lactam antibiotics have putative neuroprotective properties, whereas fluoroquinolone antibiotics may be neurotoxic.6,7 We thus hypothesized that antibiotics of different classes may differentially affect outcome in experimental stroke. To address this possibility, we compared behavioral and histological outcomes from stroke in animals treated with ceftiofur, a β-lactam antibiotic, enrofloxacin, a fluoroquinolone antibiotic, and vehicle as controls.

Methods

Animals
Male Lewis rats (275–325 g) were purchased from Taconic Farms. All experiments were approved by the Institutional Animal Care and Use Committee.

Middle Cerebral Artery Occlusion
Anesthesia was induced with 5% isoflurane. After midline neck incision, the right common carotid, internal carotid, and pterygopalantine arteries were ligated. A monofilament suture (4.0) was inserted into the common carotid artery and advanced into the internal carotid artery.8 Animals were maintained at normothermia during surgery and reperfused 2 hours after middle cerebral artery occlusion (MCAO). Rectal temperature and body weight were assessed at set time intervals. Animals were euthanized 1 month after MCAO.

Antibiotic Administration
Twenty-four hours after MCAO, animals were randomly treated with ceftiofur or enrofloxacin (according to veterinarian suggested dosing regimens). Briefly, ceftiofur (10 mg/kg) was given subcutaneously daily for 7 days (days 1–8 after MCAO), and enrofloxacin (20 mg/kg) was given subcutaneously on days 1 and 5 after MCAO. Control animals received a matched volume of vehicle at the same time points.

Behavioral Outcomes
The neurological score of animals undergoing MCAO was determined at routine intervals ≤1 month after MCAO.9 Animals were trained on the rotarod before MCAO. After MCAO, rotarod performance was assessed weekly, and the median time they were able to remain on the rotarod >3 trials was determined. Performance of the foot fault test was assessed at these time points, and the results were expressed as a percentage of foot faults per total number of steps taken.10

Received January 12, 2015; final revision received June 1, 2015; accepted June 5, 2015.
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Stroke is available at http://stroke.ahajournals.org
DOI: 10.1161/STROKEAHA.115.008663

Figure 1.
Immunologic Studies
At the time of euthanization, lymphocytes were isolated from both the ischemic and nonischemic hemispheres of the brain. The total number of lymphocytes was determined using a hemocytometer. ELISOPOT assays were done to detect the secretion of interferon (IFN)-γ from the cells (R&D Systems). Briefly, cells were cultured in media alone or in media supplemented with lipopolysaccharide (50 μg/mL; Sigma Aldrich) for 48 hours in 96-well plates (Multiscreen-IP, Millipore). Plates were developed using standard protocols (R&D Systems). After plate development, spots were counted with the aid of a semiautomated system (AID iSPOT).

Histology
In a subset of animals, brains were removed at the time of euthanization, postfixed in 4% paraformaldehyde, 24 hours, saturated in 30% sucrose for 48 hours, placed in optimal cutting temperature compound, flash frozen in dry ice cooled isopentane, and stored at −80°C until sectioning. Coronal sections (20 μm) were taken from Bregma +1.70 mm, −0.40 mm, and −1.80 mm. The area of the ischemic and nonischemic hemispheres at each level was determined using Image J and cresyl violet stained sections. The degree of atrophy is expressed as the percent of the ischemic hemisphere lost relative to the nonischemic hemisphere. Sections at −0.40 mm were stained with Fluoro-Jade-B (Histo-Chem Inc) and OX-42 (abcam), and labeled cells were counted as previously described.11

Statistics
Parametric data are displayed as mean±SD and compared using the t test or ANOVA with post hoc Dunnett test. Nonparametric data are displayed as median (interquartile range) and compared using the Kruskall–Wallis H test. Significance was set at P<0.05.

Results
Behavioral Outcomes
Before and immediately after the week of antibiotic treatment, physiological variables and the neurological score were similar in all groups (Table 1). Figure 2 shows the rectal temperature (A) and changes in weight (B) over the month after stroke. At week 4 after ABX therapy, enrofloxacin-treated animals had lower body temperatures. Changes in bodyweight were similar among groups. There were no differences in performance on the foot fault test among the 3 groups. By 3 weeks after stroke, there were significant differences in the median performance of animals (14 [5–100] versus 78 [21–100]; P=0.04). Individual animal performances are depicted in Figure 3. At week 4, 14 of 31 (45%) control animals, 15 of 22 (68%) ceftiofur-treated animals, and 8 of 25 (32%) enrofloxacin-treated animals had returned to baseline performance (P=0.04).

Immunologic Assays
There were no differences in the number of lymphocytes or the IFN-γ secretion of the lymphocytes isolated from the ischemic hemisphere 1 month after stroke (Table 2). In the nonischemic hemisphere, however, ceftiofur-treated rats had fewer lymphocytes, but the number of lymphocytes secreting IFN-γ was significantly augmented in response to lipopolysaccharide stimulation.

Histological Analyses
Histology showed no difference in the degree of hemispheric atrophy among ceftiofur-treated, enrofloxacin-treated, and control animals. Similarly, there were no differences in the number of microglia stained with OX-42 or the number of degenerating neurons stained with Fluoro-Jade-Bs among antibiotic-treated and control animals.

Discussion
Given the disparate results of the trials of antibiotic prophylaxis in patients with acute stroke, this study was undertaken to determine whether the class of antibiotics may affect stroke outcome independent of infection. That antibiotics may have off-target effects has been appreciated for decades, and the potential for antibiotics to affect the brain is demonstrated by the propensity of many antibiotics to lower the seizure threshold. In this study, we used ceftiofur as a representative of the β-lactam class of antibiotics and enrofloxacin as representative of the fluoroquinolone class of antibiotics. Both ceftiofur and enrofloxacin were developed for veterinary use and are used widely throughout the animal kingdom, especially in food animals.12,13

As a class, fluoroquinolones inhibit γ-aminobutyric acid-A receptor binding; at least some of these drugs may also act at the N-methyl-D-aspartate receptor and can alter K+ currents.14–18 In the initial clinical trials aimed at assessing the antibacterial properties of the fluoroquinolones, neurological symptoms, such as dizziness, headache, and sleep disturbances, were relatively common; hallucinations, depression, and seizures were also reported.19 Multiple reports of fluoroquinolone induced delirium, and nonconvulsive status epilepticus can also be found in the literature.20–25 Enrofloxacin, the fluoroquinolone used in this study, has been linked to
retinal cell death in felines. Under normal circumstances, the accumulation of fluoroquinolones in the brain is limited via P-glycoprotein efflux mechanism. In the setting of stroke, however, it is likely that this efflux mechanism becomes dysfunctional, which means that the concentration of these drugs, especially in the peri-infarct region, would rise, thus increasing the potential for central nervous system toxicity.

The β-lactam ABXs are well known for their ability to induce seizures. In most experimental studies, these drugs are applied directly to the brain to precipitate seizures; in excessive doses, systemic administration may also cause seizures. Similar to the fluoroquinolones, the β-lactams are thought to cause seizures through inhibition of γ-aminobutyric acid-A receptors. More recent data, however, suggest that β-lactam antibiotics may also be neuroprotective by virtue of their ability to induce glutamate transporter expression. Ceftriaxone, a clinically used β-lactam antibiotic, has been shown to improve outcome in experimental models of stroke.

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<tr>
<th>Table 1. Physiological Variables Before and After Antibiotic Therapy</th>
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<tr>
<td>Temperature, °C</td>
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<tr>
<td>Change in body weight, %</td>
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<td>Neurological score</td>
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MCAO indicates middle cerebral artery occlusion; and NS, not significant (P>0.10).

Figure 2. Changes in temperature (A) and weight (B) over time. Data are presented as the mean and SD. Statistics are by ANOVA; **P<0.01 in comparison with control. MCAO indicates middle cerebral artery occlusion.
Stroke  August 2015

reports on potential neuroprotective effects of ceftiofur, the β-lactam used in this study. Antibiotics in other classes, such as minocycline, have also been found to affect neurological outcome after stroke, but these antibiotics do not have broad spectrum activity against hospital-acquired bacterial pathogens and are rarely used in the clinical setting.

In addition to a direct effect on brain, antibiotics might also affect the development of the immune response, which can indirectly affect stroke outcome. For instance, both in vitro and in vivo studies show that fluoroquinolones suppress the release of inflammatory cytokines (particularly tumor necrosis factor-α) from lymphocytes and monocytes while increasing interleukin-10. Both β-lactam antibiotics similarly modulate the production of inflammatory cytokines. Both ceftiofur and enrofloxacin seem to decrease B-cell maturation. In our study, the number of lymphocytes in the ischemic hemisphere and their ability to respond to lipopolysaccharide were similar in all 3 groups. In the noninfarcted hemisphere, however, the number of lymphocytes was relatively decreased in ceftiofur-treated animals, but their ability to respond to lipopolysaccharide relatively increased compared with enrofloxacin-treated and control animals. Whether these immunologic effects of ABXs are responsible for modulating stroke outcome or the effects are related to a more direct effect of the drugs on neural cells is unclear.

In this study, neurological outcomes were similar among animals randomized to treatment with ceftiofur or enrofloxacin and control animals at 24 hours and 8 days after MCAO (and 7 days of antibiotics), suggesting that baseline stroke severities were also similar. By 4 weeks after MCAO (and 3 weeks after cessation of ABX), however, those animals that received the fluoroquinolone antibiotic enrofloxacin performed worse than control animals on the rotarod. We previously showed that in Lewis rats, the rotarod is the most sensitive measure of long-term stroke outcome. These data suggest that antibiotic treatment with enrofloxacin put in motion a process that affected stroke recovery. Gross histological analysis showed no difference in the degree of brain atrophy among antibiotic-treated and control animals at 1 month. Furthermore, in the relatively small subset of animals in which we performed immunocytochemistry, there was no difference in the number of cells labeled with OX-42 (microglia) or Fluoro-Jade B. Of note, studies suggest

Table 2. Characteristics of Lymphocytes Isolated From the Brain in Antibiotic-Treated Rats

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<tr>
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<th>Infarcted Hemisphere</th>
<th>Noninfarcted Hemisphere</th>
<th>P Value</th>
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<tbody>
<tr>
<td></td>
<td>Saline, n=19 Ceftiofur, n=13</td>
<td>Enrofloxacin, n=20</td>
<td></td>
</tr>
<tr>
<td>Total number of lymphocytes (×10⁶)</td>
<td>7.2±2.2 6.3±2.6</td>
<td>6.6±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Relative increase in number of lymphocytes secreting IFN-γ in response to LPS</td>
<td>2.2±2.0 1.7±1.2</td>
<td>1.9±1.4</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>Saline, n=19 Ceftiofur, n=13</td>
<td>Enrofloxacin, n=20</td>
<td>P Value</td>
</tr>
<tr>
<td>Total number of lymphocytes (×10⁶)</td>
<td>7.7±2.9 5.2±2.1</td>
<td>6.8±2.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Relative increase in number of lymphocytes secreting IFN-γ in response to LPS</td>
<td>1.5±0.7 2.8±1.5</td>
<td>1.8±1.3</td>
<td>0.02</td>
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IFN indicates interferon; LPS, lipopolysaccharide; and NS, not significant (P>0.10).
that fluoroquinolones have the capacity to impair both differentiation and proliferation of embryonic brain cells in culture,50 the β-lactam antibiotic ceftriaxone, on the other hand, has been shown to upregulate neurotrophins in the peri-infarct zone.33

Whereas more detailed histological analyses in larger animal cohorts would reveal differences between groups is not known. The antibiotics used in this study were drugs developed for veterinary use, but they are representative of the major antibiotic classes (fluoroquinolones and β-lactams) used in patients with poststroke infections. Furthermore, a veterinary study showed that enrofloxacin, but not ceftiofur, reduced equine bone marrow–derived mesenchymal stromal cell viability in vitro,51 again suggesting that the off-target effects of these drugs may be significant.

In summary, our data show that the fluoroquinolone antibiotic enrofloxacin started 1 day after MCAO can profoundly affect stroke outcome in uninfected animals, at least as measured by rotorad performance. The mechanisms by which this antibiotic affects outcome is unclear but warrants further investigation because these drugs are commonly used in clinical practice.

Sources of Funding
This work was funded by National Institute of Neurological Disorders and Stroke (5R01NS056457).

Disclosures
None.

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Stroke. published online July 2, 2015;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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/early/2015/07/02/STROKEAHA.115.008663