Reduction of Central Nervous System Reperfusion Injury in Rabbits Using Doxycycline Treatment

Wayne M. Clark, MD; Frank A. Calcagno; Walter L. Gabler, DDS, PhD; John R. Smith, PhD; Bruce M. Coull, MD

**Background and Purpose** Activated leukocytes appear to potentiate central nervous system reperfusion injury, and agents that block leukocyte adhesion have shown neuroprotective efficacy in experimental models. Doxycycline, a tetracycline antibiotic, inhibits leukocyte function in vitro, presumably through divalent cation binding. We used a model of focal central nervous system reperfusion injury to determine the efficacy of doxycycline treatment in preserving neurological function.

**Methods** Rabbits randomly received 10 mg/kg IV doxycycline 30 minutes before ischemia (pretreatment group) or 45 minutes after ischemia (posttreatment group) or received phosphate-buffered saline vehicle (control group) followed by 10 mg/kg q 8 hours times two. The average length of reversible spinal cord ischemia required to produce paraplegia (P50) at 18 hours was calculated for each group.

**Results** For the control group (n=13), the P50 was 22.8±2.2 minutes; for the pretreatment group (n=14), 35.5±2.4 minutes (P<.01; t=3.8); and for the posttreatment group (n=13), 31.4±4.2 minutes (not significant; t=1.6). Doxycycline also attenuated postischemic decreases in in vivo leukocyte counts and inhibited in vitro leukocyte adhesion. Therapeutic doxycycline levels at 24 hours were confirmed in the plasma and spinal cord.

**Conclusions** This significant protective effect suggests that doxycycline, a safe and readily available agent, may play a role in reducing clinical central nervous system reperfusion injury.

In the present study, we used a selective model of CNS ischemia in rabbits to test whether treatment with doxycycline would improve functional outcome. We also assessed the effects of doxycycline on rabbit leukocyte function to determine a potential neuroprotective mechanism.

**Materials and Methods** Male New Zealand White rabbits weighing 2 to 3 kg were used. The rabbit spinal cord ischemia model has been previously described in detail.12 Under halothane anesthesia, rabbits have a snare ligature occluding device placed around the abdominal aorta just below the left renal artery. The end of the occluder is left accessible through the skin. The rabbit is allowed to recover for a minimum of 2 hours. To induce ischemia, the occluder is tightened and clamped. All rabbits are completely paraplegic within 2 minutes. The animals show no evidence of discomfort, and the procedure appears to be painless, based on lack of previously measured changes in heart rate, blood pressure, or circulating catecholamine levels. At the end of a variable predetermined occlusion period, the device is unclamped and removed, and the skin is closed. Animals are exposed to varying durations of ischemia. Animals with shorter occlusion times tend to regain function, whereas those with longer occlusion durations remain permanently paraplegic.

**See Editorial Comment, page 1416**

*Leukocytes* • cerebral ischemia • reperfusion • doxycycline

**Key Words** — doxycycline — cerebral ischemia — reperfusion — leukocytes

Received October 26, 1993; final revision received January 11, 1994; accepted February 28, 1994.

From the Oregon Stroke Center, Department of Neurology (W.M.C., F.A.C., B.M.C.), and Departments of Biologic Structure and Function and Oral Molecular Biology, School of Dentistry (W.L.G., J.R.S.), Oregon Health Sciences University, and the Department of Neurology (B.M.C.), Veterans Administration Medical Center, Portland, Ore.

Correspondence to Dr Wayne M. Clark, Department of Neurology L226, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd, Portland, OR 97201.

© 1994 American Heart Association, Inc.
Each animal was evaluated 18 hours later for degree of neurological impairment by an observer who was blinded to the treatment or ischemic duration. Each animal was scored as 0, paraplegic (no hindlimb movement), or 1, functional (normal/paretic). Animals were then killed at 24 hours. In the present study, a 10-cc blood sample was obtained after surgery (baseline) and at 24 hours after ischemia for complete blood count and doxycycline level determinations measured by spectrophotofluorometer. Spinal cords were removed at 24 hours for measurement of doxycycline levels.

After full recovery from surgery, animals were randomly assigned to one of three groups: the pretreatment group, which received 10 mg/kg IV doxycycline (Sigma Chemical Co) 2 mg/mL dissolved in phosphate-buffered saline (PBS; Sigma) 30 minutes before ischemia, followed by 10 mg/kg BID every 8 hours; the posttreatment group, which received 10 mg/kg IV doxycycline 45 minutes after the onset of ischemia, followed by 10 mg/kg q 8 hours twice; or the control (untreated ischemic) group, which received PBS equivalent to that in the pretreatment group. Preliminary studies found that 10 mg/kg doxycycline produced therapeutic levels with an approximate 12-hour half-life in rabbits. The animals were observed continuously and evaluated at 18 hours by an investigator blinded to group. A 30-minute pretreatment period was chosen because it was effective in previous antiadhesion antibody leukocyte studies and it allowed time for maximal leukocyte uptake. A 45-minute posttreatment period was chosen to determine the effects of doxycycline treatment during reperfusion because the aorta of all animals is unclamped by 45 minutes.

To determine whether doxycycline modifies rabbit leukocyte function, in vitro leukocyte adhesion was assessed in two separate experiments. To determine whether in vitro concentrations of doxycycline affect both leukoaggregation and platelet aggregation, a mononuclear cell (MNC) function, an 80-cc blood sample was obtained from a donor rabbit during euthanasia. Rabbit neutrophils and MNC were isolated by using a modified dextran sedimentation and Percoll density gradient centrifugation method. The final purity of the two isolated cell populations was 97% neutrophils and 95% MNC respectively by morphological and nonspecific esterase staining evaluation. After hypotonic red cell lysis, cells were resuspended in PBS and 100 μL of a known cell concentration was added to each well. The wells of microtiter plates were coated by adding 200 μL of a known cell concentration to each well and incubating for 2 hours at 37°C followed by three rinses with distilled water and air drying. Test systems consisted of 5×10⁵ cells per well, 0 to 25 μg/mL doxycycline, and 2×10⁴ mol/L formyl-methionyleucylphenylalanine, 100 ng/mL phorbol 12-myristate 13-acetate, or buffer alone (PBS). To account for nonspecific leukocyte-substrata binding, one half of the wells of each test system contained 5 mmol/L iodoacetamide. The optical density values of iodoacetamide-inhibited adherent cells system were subtracted from those of adherent cells of noninhibited systems to give specific adhesion values.

Cells, test agents, and buffer were placed in wells, and the plates were incubated at 37°C for 30 minutes before the addition to the activator. The plates were then incubated for an additional 3 hours, at which time the media and nonadherent cells were removed using a standardized technique. One hundred microliters of a PBS solution containing 0.25% rose bengal was added to each well and allowed to incubate for 5 minutes. The stain was removed, and the wells were washed three times with 200 μL of saline. Finally, 200 μL of a 1:1 PBS (1:1) solution was added to each well to release the cell-bound stain, and the plates were incubated at 37°C for at least 60 minutes. The optical density values of the released stain were measured at a 550-nm wavelength. The results were expressed as a percentage of the total number of cells initially added (percent adhesion). The data are presented as the arithmetic mean of at least eight wells.

In a second, separate experiment, we investigated whether the in vivo doxycycline dose used in our therapeutic study was sufficient to inhibit neutrophil adhesion. A 20-cc blood sample was obtained at baseline and 1 hour after administration of a 10-mg/kg dose of doxycycline in five rabbits. Neutrophils were isolated as previously described. Neutrophil adherence to laminin, a basement membrane protein, was determined by a modification of the method used by Bohnsack et al that we have previously described. After a standardized washing of nonadherent cells, a myeloperoxidase activity assay was used to determine cell adherence. The myeloperoxidase activity in the cell adhesion well was compared with a standard curve generated by known cell concentrations derived from each rabbit. The number of adherent cells present in the coated plates was calculated by comparing optical density readings with those of the known cell concentration values. The results were expressed as a percentage of the total number of cells initially added (percent adhesion).

**Statistical Analysis**

We constructed quantal dose-response curves from the neurological function ratings at 18 hours. This analysis involves iterative fitting of a logistic function. The derivation of this type of analysis has been described in detail, and its utility as a pharmacological screen has been demonstrated. At each point on the abscissa the percentage of paraplegic animals in the group is calculated and plotted as the ordinate. The total increases from 0% at the shortest duration of occlusion to 100% at the longest. The resulting sigmoid curve represents the effect of a range of durations on neurological outcome. The point at which 50% of the animals are paraplegic is calculated. This point, termed P50, is the average length of ischemia that produces impairment in 50% of the animals. An agent that is effective in reducing neurological damage will shift the curve (and P50) to the right; ie, the animals will on average tolerate a longer period of ischemia. To determine significance, t tests were performed (P<.05).

For the adhesion and hematologic values, differences among groups were assessed by ANOVA; when there was a significant difference (P<.05) among groups, the probability value was determined by Bonferroni-corrected post hoc t tests.

**Results**

The results of doxycycline treatment in the spinal cord ischemia model are presented in Table 1. The average length of ischemia that produced impairment (P50) in the control group (n=13) was 22.8±2.2 minutes (mean±SE); in the pretreatment group (n=14), 35.5±2.4 minutes (P<.01; t=3.8); and in the posttreatment group (n=13), 31.4±4.2 minutes (not significant; t=1.6). Thus, pretreatment with doxycycline but not delayed treatment produced a significant reduction in ischemic injury in this model. The Figure shows the quantal dose-response curves for these groups. Doxycycline treatment showed no effects on respiration or rectal temperature. We did not assess blood pressure or pulse in this study.

Complete blood count and plasma doxycycline levels were obtained at baseline and at 24 hours. Doxycycline levels at 24 hours were as follows: pretreatment group (n=10), plasma 0.35±0.08 μg/mL and spinal cord 1.16±0.27 μg/mg; and posttreatment group (n=9), plasma 0.20±0.03 μg/mL and spinal cord 1.76±0.43 μg/mg (not significant between groups). In addition, a peak 1-hour plasma doxycycline level of 1.7 μg/mL was found in two animals. Table 2 shows hematologic variables. There was a significant hematocrit drop from baseline in all three groups due to surgical blood loss.
TABLE 1. Efficacy of Doxycycline Treatment in the Rabbit Spinal Cord Ischemia Model

<table>
<thead>
<tr>
<th>Ischemic Duration, min</th>
<th>Neurological Status</th>
<th>Control group</th>
<th>Doxycycline 30-minute pretreatment group</th>
<th>Doxycycline 45-minute posttreatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal or Paralytic, n</td>
<td>Paraplegic, n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>14</td>
<td>0</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>1</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1</td>
<td>28</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>0</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>0</td>
<td>40</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>0</td>
<td>42</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0</td>
<td>44</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>46</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>52</td>
<td>40</td>
</tr>
</tbody>
</table>

TABLE 2. Hematologic Profile at Baseline and 24 Hours by Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit, %</th>
<th>Leukocyte, 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>40.4±2.1</td>
<td>7.6±1.9</td>
</tr>
<tr>
<td>24 hours</td>
<td>35.0±2.4*</td>
<td>5.8±2.0*</td>
</tr>
<tr>
<td>Pretreatment (n=12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>41.9±3.1</td>
<td>8.1±1.4</td>
</tr>
<tr>
<td>24 hours</td>
<td>37.8±4.4*</td>
<td>8.7±3.3</td>
</tr>
<tr>
<td>Posttreatment (n=11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>38.5±1.8</td>
<td>8.1±1.9</td>
</tr>
<tr>
<td>24 hours</td>
<td>33.3±2.3*</td>
<td>5.3±2.0*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

*P<.05 different from corresponding baseline values.
importantly inhibited both neutrophil and mononuclear cell in vitro adhesion and prevented the postischemic decrease in total leukocyte count that is seen in this model. The results of this study may seem paradoxical in that an antibiotic is being given to block leukocyte function. The explanation is that although all tetracyclines appear to have anti-inflammatory properties, they have a far greater ability to inhibit bacterial replication. Tetracyclines produce bacteriostatic effects through inhibition of protein synthesis. However, the ability of tetracyclines to inhibit leukocyte function appears to be unrelated to their antimicrobial effects, because studies using a modified nonantibacterial tetracycline still found anti-inflammatory effects. The degree of neurological protection seen with doxycycline pretreatment is similar to that which we have previously observed in this model using specific antiadhesion molecule monoclonal antibodies. Although there was a strong trend toward neuroprotection when doxycycline treatment was delayed for 45 minutes, it did not reach statistical significance. The possibility that we did not detect a real treatment effect because of inadequate sample must be considered (type II error). Based on the assumption that the observed difference in P values between the delayed treatment and control groups is real, if we had chosen to design the experiment to have a power of 90% and set the α error at the usual 5%, we would have needed 17 animals in each group to find a statistically significant difference. The 45-minute delay in treatment was chosen so that all animals would be treated during the reperfusion period. Consequently, any leukocyte adhesion during the initial reflow period would have occurred before the start of doxycycline treatment. In our previous studies demonstrating neuroprotection in this model, the antiadhesion monoclonal antibodies were given only pretreatment. In a similar spinal cord model, Lindsberg et al found beneficial effects when antiadhesion treatments were given 30 minutes after ischemia. In our study, it is possible that a shorter posttreatment period might have produced neuroprotection. The peak plasma and spinal cord levels of doxycycline found in this experimental study are within the peak doxycycline plasma levels (1 to 3 μg/mL) recommended in clinical treatment. However, since doxycycline appears to produce a dose-dependent inhibition of leukocyte function, higher treatment doses may have produced greater therapeutic effects.

We found a significant decline in total leukocyte count in both untreated animals and in animals whose treatment was delayed until after reperfusion was initiated. One possible explanation for these findings is that during initial reperfusion a population of leukocytes is sequestered in the microcirculation so that the leukocytes are no longer present in the 24-hour sample. This finding would fit with the theory that leukocytes adhere to endothelium and are trapped in the microcirculation early during the reperfusion period, contributing to the “no-reflow phenomenon.” A similar drop in leukocyte counts was not seen in the group pretreated with doxycycline, suggesting that pretreatment with doxycycline may be preventing in vivo leukocyte adherence and sequestration.

The results of our in vitro studies provide further evidence that doxycycline may be inhibiting leukocyte adherence. We found that doxycycline, in concentrations similar to that used in our treatment study, produces inhibition of specific monocyte and neutrophil adhesion in rabbits. We have previously found that doxycycline, along with other tetracyclines, produces specific inhibition of human mononuclear cell and neutrophil adhesion. This inhibition is believed to be due to the ability of the tetracyclines to bind the divalent cations Ca$^{2+}$ and Mg$^{2+}$, because the addition of these cations prevents this inhibition. Specific leukocyte adhesion to the endothelium is predominantly mediated by a leukocyte membrane glycoprotein receptor complex termed CD-18. This receptor comprises three beta subunits that require Ca$^{2+}$ and Mg$^{2+}$ to associate. By preventing CD-18 reception function, doxycycline may also be inhibiting adhesion in a similar fashion to specific monoclonal antibodies that are directed against these adhesion receptors. We have previously found that in vitro tetracycline treatment also inhibits other neutrophil functions, including oxygen free radical generation, degranulation, and collagenase activity. Leukocyte granule contents, reactive oxygen metabolites, and elastase release have been found to injure endothelium and potentiate ischemic injury. The ability of

### Table 4. Effect of In Vivo Doxycycline Administration on Neutrophil Adhesion to Laminstin

<table>
<thead>
<tr>
<th>Group</th>
<th>Unstimulated</th>
<th>FMLP†</th>
<th>Unstimulated</th>
<th>FMLP†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (n=5)</td>
<td>4.7±1.1</td>
<td>18.3±3.5</td>
<td>4.7±1.1</td>
<td>18.3±3.5</td>
</tr>
<tr>
<td>Posttreatment (n=5)</td>
<td>1.5±1.5</td>
<td>9.3±2.9*</td>
<td>1.5±1.5</td>
<td>9.3±2.9*</td>
</tr>
</tbody>
</table>

Values are mean±SE percent. FMLP indicates formylmethionylleucylphenylalanine. *P<.05 different from FMLP-stimulated samples from animals that did not receive doxycycline treatment by Bonferroni-corrected post hoc comparison.
doxycycline treatment to suppress these functions may also have been important in producing the neuroprotective effects seen in our study. We found that the dose of doxycycline used in this study produced plasma levels of doxycycline that were sufficient to inhibit in vitro leukocyte function. Doxycycline appears to be sequestered in the CNS, based on our finding of relatively higher levels in the spinal cord. Because doxycycline binds Ca$^{2+}$ and Mg$^{2+}$, an alternative explanation for the observed neuroprotective effects is that doxycycline is decreasing the locally available Ca$^{2+}$ in the ischemic CNS tissue. This could directly inhibit the excitatory ischemic cascade that is felt to be detrimental to CNS tissue during ischemia. Further work with in vitro neuronal or microglial cultures is needed to investigate this theory.

To our knowledge, this is the first investigation of doxycycline treatment in CNS ischemia. We have recently demonstrated that doxycycline treatment is also protective in a rat model of hepatic reperfusion injury. In this study, both pretreatment (1 hour) and posttreatment (1 hour into reperfusion) with 10 mg/kg doxycycline reduced hepatic injury as assessed by serum alanine aminotransferase levels.

We conclude that treatment with doxycycline reduces CNS ischemic injury in this reperfusion model, with inhibition of leukocyte adhesion a potential mechanism. These findings support the role of leukocytes as active participants in CNS injury. Doxycycline is a safe and readily available clinical therapeutic agent. In our model it appears to have therapeutic potential equal to and would be much less expensive than specific monoclonal antiadhesion antibodies. Finding an agent that could safely reduce CNS reperfusion injury would have widespread clinical benefit when used after thrombolysis or as a pretreatment in clinical situations in which the risk of stroke is high.

Acknowledgments

Supported in part by a National Stroke Association/Allied Signal Inc. Award (W.M.C.), a National Institutes of Health Clinician Investigator Development Award (W.M.C.), NINDS 2P01 NS17493-08 (B.M.C.), and the Kettering Foundation (W.L.G.).

References

Reduction of central nervous system reperfusion injury in rabbits using doxycycline treatment.

W M Clark, F A Calcagno, W L Gabler, J R Smith and B M Coull

Stroke. 1994;25:1411-1415
doi: 10.1161/01.STR.25.7.1411

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/25/7/1411

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/