Clentiazem Reduces Infarct Size in Rabbit Middle Cerebral Artery Occlusion

Leonard Kaminow, MD, and John Bevan, MD

We assessed the value of pretreatment with clentiazem (8-chlorodiltiazem), a diltiazem derivative with cerebroselective properties, on the consequences of surgical occlusion of the middle cerebral artery via a transorbital approach in 38 rabbits. Nineteen rabbits received 1.7 (n=5), 5 (n=8), or 15 (n=6) mg/kg clentiazem orally four times a day for 24 hours before and 48 hours after occlusion. Upon sacrifice, a segment of the right middle cerebral artery distal to the occlusion and a corresponding segment from the nonoccluded left middle cerebral artery were mounted on myographs for in vitro study of their reactivity to histamine, acetylcholine, serotonin, norepinephrine, and electrical stimulation of intramural sympathetic nerves. Morphometric measurements of 2,3,5-triphenyltetrazolium chloride-stained brain slices permitted us to estimate infarct volume. Pretreatment with 1.7, 5, and 15 mg/kg clentiazem significantly reduced infarct volume (p<0.05, p<0.01, and p<0.01, respectively). Mean infarct volume of the 15 mg/kg-treated group was only 4% that of the untreated group. There were no postoperative deaths in any treated group compared with a death rate of 36% in the untreated group. Mean values for vascular smooth muscle contractility to histamine and relaxation to acetylcholine were significantly enhanced in vessels from treated rabbits. These studies indicate that pretreatment with clentiazem offers cerebral protection and significantly reduces infarct volume as well as arterial wall damage beyond the occlusion. (Stroke 1991;22:242-246)

Diltiazem reduces the extent of arterial narrowing and the arterial wall damage in addition to the neurologic deficit related to chronic cerebrovasospasm secondary to subarachnoid hemorrhage in monkeys. Protection is presumably afforded by diltiazem's antagonism of the entry of calcium into vascular smooth muscle or endothelial cells of the cerebral arteries and its intracellular consequences. There is some evidence for a modest selectivity of diltiazem on the mechanisms of calcium entry in cerebral arteries.

The diltiazem derivative clentiazem (8-chlorodiltiazem) has considerable selectivity for the cerebral arterial bed compared with other regional arterial beds and the heart for both receptor- and potential- mediated calcium channel events. By contrast, sympathetic neuronal as well as blood flow and pressure influences on cerebrovascular tone are not interfered with by this calcium channel antagonist.

The spectrum of action of clentiazem and its effectiveness against constrictor mechanisms in the cerebral arterial bed while sparing other cerebral blood flow-regulating mechanisms appear to be potentially advantageous in the treatment of occlusive stroke. This possibility was tested in a rabbit model of middle cerebral artery (MCA) occlusion.

Materials and Methods

The experimental procedure followed National Institutes of Health guidelines and was approved by the Human and Animal Usage Committee of the University of Vermont College of Medicine. Twenty-four hours prior to enucleation, a gastrostomy tube was placed. Thirty-seven adult female and one adult male New Zealand White rabbits weighing 2.1-2.8 kg were anesthetized with 0.1 ml/kg i.m. Innovar (fentanyl citrate/droperidol, Janssen Pharmaceutica Inc., Piscataway, N.J.), followed 5 minutes later by 0.1-0.3 ml/kg i.m. Rompun (xylazine, 3 mg/20 ml; Miles Inc., New Haven, Conn.) then 25-30 mg/kg i.m. ketamine.

The following day the rabbits were anesthetized as before, placed in the left lateral decubitus position, and draped after shaving the right periorbital region. Enucleation was then performed as described by Yamamoto et al. Under a dissecting microscope, a 5x5-mm craniectomy was made above and caudal to and including the right optic foramen using a high-speed drill. After incision of the dura, the intracranial portion of the right internal carotid artery and
The take-off of the right MCA from the circle of Willis were identified. If occlusion was to be carried out, a small opening was made in the arachnoid near the right MCA take-off. Occlusion was accomplished by applying a 3-mm-wide Acland miniature vein clip (Accurate Surgical and Scientific Instruments Corp., Westbury, N.Y.) to the right MCA. No retraction of or contact with brain tissue was necessary. Position of the clip was confirmed by postmortem observation of the crush on the MCA. Effectiveness of the occlusion was assessed by observing the cessation of blood flow directly after placement of the clip. The cranietomy site was covered with absorbable gelatin sponge, and the skin was closed.

The rabbits were assigned randomly to one of five treatment groups. Group 1 (eight rabbits) underwent gastrostomy and enucleation without occlusion; group 2 (11 rabbits) underwent gastrostomy, enucleation, and occlusion; groups 3, 4, and 5 (five, eight, and six rabbits, respectively) underwent treatment via the gastrostomy tube with 1.7, 5.0, or 15.0 mg/kg clentiazem in 5 ml of 5% dextrose in normal saline q.i.d., respectively, for 24 hours before and 48 hours after enucleation and occlusion.

All rabbits were killed 48 hours after surgery (approximately 1 hour after the final clentiazem dose in groups 3, 4, and 5) following intracardiac injection of 2 ml heparin sodium sulfate by a stunning blow to the cervical spine or by intraperitoneal injection of sodium pentobarbital and exsanguination. Immediately after sacrifice, the brains were removed and placed in Krebs' bicarbonate solution. After arterial segments had been removed for assessment of MCA function using in vitro techniques (see below), the brains were sliced into 4-mm coronal sections. The cerebral hemispheres and brain stem to the fifth cranial nerve were included. The slices were incubated for 30 minutes at 37°C in 2,3,5-triphenyltetrazolium chloride (TTC) and then photographed. Infarct volume was quantified as the sum of the percentage areas of the slices that did not stain with TTC. Areas were based on quantitative morphology using a Nikon Optiphot microscope (Garden City, N.Y.) and a Sigmascan software area program (Jandel Scientific, Sausalito, Calif.).

We studied vessels from five rabbits in group 1, five rabbits in group 2, 10 rabbits in group 4, and two rabbits in group 5 for responses to various agents and procedures assessing cerebral artery function. A 2-mm portion of the right MCA 5 mm distal to the occlusion and a corresponding portion of the left MCA were mounted in the conventional manner in a tissue bath containing Krebs' bicarbonate solution. After 1 hour's equilibration 10^{-6} M cimetidine was added to the bath, and 10 minutes later the contraction to 10^{-5} M histamine was recorded without washing. The relaxations to acetylcholine in logarithmic increments between 10^{-8} and 10^{-3} M were then determined. Vessels were then washed for 30 minutes. Contraction to 10^{-7} to 10^{-5} M histamine in logarithmic increments was recorded, and then the vessels were washed for 30 minutes. Electrical stimulation of the intramural nerves was carried out using a just-supramaximal voltage for 25 seconds at 8 Hz with a pulse duration of 0.03 seconds. This was repeated twice after 5-minute intervals and after a 15-minute incubation in 3x10^{-7} M tetrodotoxin. The vessels were again washed for 30 minutes, and then serotonin was added cumulatively in half-logarithmic steps from 10^{-7} to 10^{-6} M. Again the vessels were washed for 30 minutes, and 10^{-7} M propranolol and 10^{-6} M deoxytocopherol acetate were added. Ten minutes later, a dose-response curve was generated for norepinephrine by adding it in half-logarithmic steps from 10^{-7} to 10^{-6} M. At the culmination of the response to norepinephrine, 10^{-4} M histamine and 89 mM potassium chloride were added to the bath.

Infarct volume was compared between groups by using the nonparametric Mann-Whitney U test. This method allowed for inclusion of deaths in the study. In vitro responses of the vessels were compared by using repeated-measures design and ranked comparison techniques. Repeated-measures design evaluates individuals (vessels) at each of several evenly spaced levels (concentrations). The set of measurements is therefore an observed dose-response curve. The treatments (sham occlusion, occlusion without clentiazem, and occlusion with clentiazem pretreatment) can be combined as grouping variables. Using repeated-measures analysis of variance, dose-response curves can be compared.

**Results**

The number of rabbits that died and the infarct volume in each group are illustrated in Figure 1 and listed in Table 1. A basal ganglia infarct was defined as one that occupied <3% of the brain volume and was confined to periventricular structures. All rabbits in group 2 developed cerebral infarcts, while none in group 1 did. The mean±SEM infarct volume of the seven (64%) group 2 rabbits that survived for 48 hours was 9.9±2.11%; only one (9%) group 2 rabbit had a basal ganglia infarct. In group 5 no rabbit died, and of the four (67%) with infarcts all had basal
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TABLE 1. Outcome of Middle Cerebral Artery Occlusion in Rabbits

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham-occluded (n=8)</th>
<th>Untreated (n=11)</th>
<th>1.7 mg Clentiazem (n=5)</th>
<th>5 mg Clentiazem (n=8)</th>
<th>15 mg Clentiazem (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>No infarct</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Infarct</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Basal ganglia infarct</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Death</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>Mean infarct volume (%)</td>
<td>0*</td>
<td>9.93</td>
<td>5.98†</td>
<td>5.20*</td>
<td>0.38*</td>
</tr>
<tr>
<td>Percent reduction from untreated group</td>
<td>...</td>
<td>...</td>
<td>39.8</td>
<td>47.6</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Basal ganglia infarct, infarct in characteristic location occupying <3% of brain volume.
*tp<0.01, 0.05, respectively, different from untreated group by Mann-Whitney U test.

ganglia infarcts. Results intermediate between these two extremes were seen in groups 3 and 4.

All brains were divided into six 4-mm-thick slices except one in which only five slices were made. Infarct volume differed significantly between group 2 and group 1 (p<0.01), group 3 (p<0.05), and groups 4 and 5 (p<0.01 each). There were no significant differences among groups 1, 3, 4, and 5. Whereas group 2 had an overall death rate of 36%, no rabbit died in group 1, 3, 4, or 5.

Necropsies showed extensive cerebral decomposition, and it is presumed that postoperative deaths occurred secondary to cerebral edema and transtentorial herniation. Each rabbit was arousable and ambulatory postoperatively, and no animal had evidence of infection. Gross subarachnoid hemorrhage was seen in three rabbits in group 1 and in two rabbits in group 4; in one the in vitro reactions were different from those expected.

Data from the right MCA segments of rabbits in groups 4 and 5 were pooled and compared with data from the right MCA segments of rabbits in groups 1 and 2. Four vessels were excluded from analysis because they did not respond to histamine, acetylcholine, serotonin, and potassium chloride. In each case, the vessel was damaged during mounting on the myograph. The histamine and norepinephrine dose-response curves are shown in Figure 2. In both instances, sensitivity to these agents (defined as the ED50) did not change in group 1. Mean values for contractility (wall force development) were depressed at all agonist dosages in group 2. Overall responses to histamine were significantly greater for groups 4 and 5 combined than for group 2 (p=0.019) by ranked comparison. The norepinephrine dose-response curve of groups 4 and 5 combined approached but did not differ significantly from that of group 2 (p=0.060). There was no correlation between reduction in the maximum response to histamine and infarct volume within groups or within the entire series. Sham operation reduced the maximum histamine contractility of the right MCA segment by guest on July 14, 2017 http://stroke.ahajournals.org/ Downloaded from
compared with that of the left MCA segment by an average of 27.0%, a change that was not significant. Relaxation to $10^{-8}$ to $10^{-4}$ M acetylcholine in these small arteries is endothelium dependent. In group 2 (Figure 2) the dose-dependency of relaxation to acetylcholine was lost, and the response to a threshold dose was significantly reduced compared with group 1 (26.6% relaxation in group 2 versus 52.9% in group 1). Mean values for relaxation to acetylcholine for groups 4 and 5 combined were greater than those for group 2, and clentiazem-treated segments relaxed significantly ($p = 0.038$).

Contraction to serotonin was unaffected by MCA occlusion (Figure 2). Mean values in groups 4 and 5 combined were greater than those in group 1 by 23.7%; the increase, however, was not significant.

There were no significant differences among groups to transmural electrical stimulation (data not shown).

Discussion

We examined the effect of a cerebroselective dil-tiazem derivative, clentiazem, on the cerebral and cerebrovascular consequences of MCA occlusion in rabbits. Occlusion via the transorbital approach, described initially by Sundt and Waltz in cats and recently by Yamamoto et al in rabbits, gave a reliable and reproducible infarct. Morphometric examination of TTC-stained brain slices revealed a significant reduction in brain pathologic changes at all three doses of clentiazem used, with the 15 mg/kg dose showing the greatest protection; specifically, there was a 96% reduction in infarct volume. Protection was in fact even more substantial since no rabbits treated with this dose died. After MCA occlusion, sensitivity to histamine (defined by the ED$_{50}$) was unchanged. On the other hand, mean contractility to $10^{-4}$ M histamine, which causes maximum smooth muscle contraction in the presence of an H$_3$ antagonist, was reduced less in the clentiazem-treated groups. We conclude that the reduction in contractility reflects vascular smooth muscle damage. The response to norepinephrine was similarly influenced, presumably due to the same change in vascular smooth muscle. Relaxation to the threshold concentration of acetylcholine ($10^{-7}$ M) was depressed only in MCA segments from group 2 rabbits. Relaxation to acetylcholine is mediated by a factor released from the intima that acts on the vascular smooth muscle cells. Thus, this reduction may represent endothelial damage. However, the relative contributions of endothelial and vascular smooth muscle cell damage to the overall change in the acetylcholine effect cannot be distinguished.

Ipsilateral MCA segments from sham-operated rabbits showed depressed responses compared with contralateral MCA segments although the dura was not opened. Operative trauma, local bleeding, and the residual bony opening may have compromised normal vascular and other functions. This observation clearly emphasizes one limitation of this model of occlusive stroke. However, based on the mean maximum contractility values, changes associated with the experimental procedures per se are much less than those associated with occlusion.

Comparisons of our results with those of other reports emphasize the protective efficacy of clentiazem. Models of focal cerebral ischemia in baboons and rats in which nimodipine was administered after MCA occlusion showed no benefit, although when given as a pretreatment nimodipine was of some value. Roy et al concluded that pretreatment as well as posttreatment with intravenous verapamil and diltiazem in cats provided no benefit and in fact might have been detrimental by causing a steal of blood flow from the ischemic areas.

A number of properties of clentiazem may account for its efficacy in this study. First, clentiazem exhibits a high degree of cerebrovascular selectivity against agonist- and potassium-induced contractions. During and after the occlusive episode, part of the distal changes must result from the vasoconstriction brought about by ischemic products released from damaged tissues that act through these mechanisms. Second, the drug does not seem to interfere with a number of processes responsible for the autoregulation of pial artery diameter. Thus, some of the vascular homeostatic mechanisms are retained during clentiazem administration, allowing some matching of blood flow to local need. There is some difficulty with this explanation since once the clip was applied, no blood flow was seen. However, at necropsy no established clot was seen; thus, the blood vessel changes may be due not simply to occlusion, but rather to anoxia. Therefore, increased vessel reactivity and decreased infarct volume may be due to retrograde blood flow or to increased flow via a collateral circulation such as the lenticulostriate vessels or vessels in the watershed zones connecting the MCA with other cerebral vessels.

Third, the concentration of clentiazem required to affect the cerebral vasculature is very much lower than that depressing the action of the heart and is achieved at doses that do not significantly lower arterial blood pressure in rabbits. Finally, clentiazem has been reported to penetrate the blood-brain barrier more effectively than its parent compound. Clentiazem's value can also probably be ascribed to its ability to antagonize the vasoconstriction that must occur in the ischemic area and adjacent vascular systems. The depression of vascular contractility and the relaxation of MCA segments from beyond the occlusion were significantly reduced by pretreatment. These represent predominant functions of the vascular smooth muscle and endothelial cell, respectively. Whether clentiazem has an additional cellular protective function has not been investigated.

References


**KEY WORDS** • cerebral infarction • neuroprotection • rabbits
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