Effect of Mannitol, Nimodipine, and Indomethacin Singly or in Combination on Cerebral Ischemia in Rats

Garnette Sutherland, MD, FRCS(C), Howard Lesiuk, MD, Ranjan Bose, MBBS, MD, and Anders AF Sima, MD, PhD, FRCP(C)

The effects of mannitol, nimodipine, and indomethacin on ischemic neuronal injury were examined in 45 rats divided equally into nine groups subjected to 10 minutes of forebrain ischemia. Of two control groups, one received maintenance fluids while the other received a normal saline bolus. In the remaining seven groups, mannitol, nimodipine, and indomethacin were administered singly or in combination 5 minutes before forebrain ischemia. Seven days after ischemia, the brains were perfusion-fixed, sectioned coronally into 2.8-mm slices, and stained with hematoxylin and eosin. Ischemic neurons were directly counted on predetermined regions of standardized serial sections. Considerable amelioration of ischemic injury (ischemic neurons/total neurons) was observed with mannitol (ischemic injury, 7 ± 5% in the hippocampal CA1/CA2 sectors and 28 ± 17% in the CA3 sector). This is in contrast to control values of 64 ± 11% and 80 ± 6%, respectively, and those obtained in the normal saline group of 70 ± 10% and 59 ± 13%, respectively. The beneficial effect with nimodipine reached significance in only the hippocampal CA3 sector (ischemic injury, 35 ± 21%). Indomethacin showed no significant benefit. Combining the agents resulted in significantly reduced neuronal injury compared with control groups, although the effect was not greater than that achieved with mannitol alone. The degree of ischemic injury was least when all three agents were used in combination (ischemic injury, 12 ± 12% in the hippocampal CA1/CA2 sectors and 4 ± 4% in the CA3 sector). Our data support the concept that successfully blocking the ischemic cascade with a single, diversely acting agent or multiple agents will evoke the best beneficial response. (Stroke 1988; 19:571-578)

Cellular functions are differentially sensitive to increasing cerebral ischemia. Synaptic transmission ceases with a reduction in cerebral blood flow (CBF) to 15–20 ml/100 g cerebral tissue/min. One to two minutes after the onset of severe ischemia (CBF < 6–10 ml/100 g/min), membrane depolarization and potassium efflux occurs, and sodium, water, and calcium enter the cell. These processes represent "membrane failure," they are correlated with "energy failure," and mark the initiation of a complex sequence of metabolic events that, if not corrected, lead to irreversible cellular damage.

Investigators have attempted to modify the effects induced by ischemia by blocking one or more of these metabolic processes. This may be effected by agents that maintain CBF above ischemic thresholds or by agents that alter the metabolic consequences of ischemia.

Given the variety of derangements in ischemia, it is not surprising that a number of investigators have hypothesized that combinations of therapeutic agents may act synergistically to produce a significantly greater beneficial effect than any of the agents used singly. In our study, we further tested this hypothesis using the agents mannitol (M), nimodipine (N), and indomethacin (I) administered singly or in combination in the rat forebrain ischemia model. We chose this model as it induces an incomplete, short-duration ischemic insult that is mild, uniform, and subject to chronic qualitative and quantitative analysis.

Materials and Methods

Experimental Model

Forty-five male Sprague-Dawley rats weighing 400–600 g were used for the experiments. The rats were divided into nine experimental groups. Of the two control groups, one received maintenance fluids while the other received an additional normal saline bolus equal in volume to that of the three agents when used in combination. In the remaining seven groups, M, N, and I were administered singly or in combination before 10 minutes of forebrain ischemia.

All rats were fasted for 2 hours before being anesthetized by i.p injection of 15 mg/kg pentobarbital sodium and 150 mg/kg chloral hydrate. To reduce oral pharyngeal secretions, 0.5 mg/kg atropine was administered i.p. If supplementary anesthesia was necessary before completion of the operative procedures, the rats...
were given a second i.p. injection of 75 mg/kg chloral hydrate. The trachea was intubated under direct vision with a 16-gauge Teflon catheter. Mechanical ventilatory support was maintained throughout the operative procedure (Model 665 ventilator, Harvard, South Natick, Massachusetts), and expired CO₂ was continuously monitored using an infrared gas analyzer (model LB-2, Sensormetics, Anaheim, California). A thermostatically controlled heating unit maintained normothermia (37°C).

A 24-gauge Teflon catheter was inserted through the tail artery into the descending aorta for blood pressure monitoring. Both carotid arteries were exposed through a neck incision, and a 22-gauge Teflon catheter was inserted into the external jugular vein and connected to an infusion pump. This provided the route for the infusion of maintenance fluids (1.5 ml Ringer's lactate/kg/hr) and pharmacologic agents. After 20 minutes of stabilization, the agents were administered singly or in combination as follows: 0.25 g/kg M over 60 seconds, 4 mg/kg I over 60 seconds, and N at staged intervals, the first 10 μg/kg N 5 minutes before ischemia and two additional 5-μg/kg injections at 10-minute intervals after the first injection. Three separate boluses were given so that adequate peri-ischemic blood levels would be obtained. Due to the light sensitivity of N, it was prepared, handled, and administered under red light. Five minutes after the infusion of normal saline or agent(s), forebrain ischemia was induced by bilateral carotid occlusion using temporary vascular clips coincident with a reduction in systemic blood pressure to a mean of 50 torr by aspiration of blood from the aortic cannula. Ten minutes after producing a mean blood pressure of 50 torr, the vascular clips were removed and the aspirated blood was rapidly reinfused. Both before and after the ischemic insult, blood gas analyses were obtained (Model 1032 blood gas analyzer, Instrumentation Laboratories, Dayton, Ohio). Hematocrit was determined before and after agent administration and ischemia. Postischemia ventilatory support was continued until the rat was breathing well and moving its extremities. The oropharynx was suctioned and the endotracheal tube was removed. The rat was returned to its cage and allowed tap water and food pellets at will.

On Day 7 after ischemia, the rats were anesthetized with 35 mg/kg pentobarbital sodium. The left ventricle of the heart was cannulated with a 20-gauge Teflon cannula, and 50 ml normal saline at 37°C was used to flush the intravascular compartment. Each rat was perfusion-fixed with 1 l of 2.5% glutaraldehyde in cacodylate buffer (pH 7.25). After fixation, the brain was removed and placed in the same fixative for 2 weeks before sectioning. The brains were cut coronally into 2.8-mm slices, dehydrated in graded concentrations of ethanol, and embedded in paraffin. Eight-micrometer serial sections were cut and stained with hematoxylin and eosin. All sections were examined to determine the qualitative and topographic extent of ischemic brain damage. For quantification of ischemic neuronal injury, a standard section including the cerebral cortex and hippocampus was used (Figure 1). Predetermined regions of this section consisting of frontal cortex (FC), inferior frontal cortex (IFC), and hippocampus including the dentate gyrus were photographed, and ischemic neuronal injury was quantitatively determined by direct visual counting of all neurons. The FC region consisted of an area of approximately 4 mm², while the IFC region was approximately 2 mm². The frequency of ischemic neurons was calculated by dividing the number of acidophilic and/or pyknotic neurons by the total number of neurons. The examiner was unaware of the identity of sections examined.

Statistical Analysis

All results are presented as mean±SEM. Groups were compared using analysis of variance (ANOVA) followed by Duncan’s intergroup comparison test.

Results

Physiologic Observations

Table 1 shows the mean blood pressure and hematocrit before and after agent administration and ischemia. In all groups, the infusion of normal saline or therapeutic agent(s) resulted in a transient decrease in hematocrit, returning to preocclusion values 20 minutes after forebrain ischemia. In keeping with the pharmacologic action of N, its first infusion (10 μg/kg) singly or in combination with other agents resulted in a significant decrease in mean blood pressure (p<0.01). This decrease was transient, and blood pressure returned to preinfusion values in 2-3 minutes. As the second N infusion (5 μg/kg) took place during the induction of ischemia, fluctuations in blood pressure were controlled by either the administration or withdrawal of additional blood. The third infusion of N (5 μg/kg) produced a decrease in blood pressure that was not significantly different from its preinfusion value (p>0.05).
Following the ischemic insult, mean blood pressure increased in all groups except M + N + I. This increase reached significance in the control and normal saline groups compared with preischemic values (p<0.05). In all groups, a mild metabolic acidosis followed the ischemic insult, which was reflected by a small increase in \( P_{co2} \) (3.2 ± 0.5 torr), a decrease in \( \text{HCO}_3^- \) (2.2 ± 0.2 mmol/l), and a negative base excess (-2.8 ± 0.2 mmol/l). These changes were not significantly different between groups.

*Histopathology — Qualitative Findings*

The distribution of ischemic changes was similar in all groups, varying only in severity. Ischemic neurons were characterized by retraction of the cell body, eosinophilia of the cytoplasm, disappearance of Nissl bodies, and pyknosis and hyperchromasia of the nucleus (Figure 2). Neocortical damage was restricted to cortical layers III—VI and was most severe in the anterior and middle cerebral arteries, tapering posteriorly as the territory of the posterior cerebral artery was approached. Infarction with cavitation was not observed. Striatal damage was noted in only a few of the affected rats (3 control, 3 normal saline, and 2 I rats). The damage was restricted to the dorsal lateral portion of the caudoputamen.

Damage to the thalamus was not observed. In the hindbrain, ischemic injury was restricted to the superior vermis and was observed only in those rats in which striatal damage was also observed.

Of all the brain regions, ischemic injury was most severe in the hippocampus. In this area, the CA1/CA2 and CA3 sectors sustained the greatest injury, followed by the CA4 sector and inner dentate blade. Ischemic injury to the outer dentate blade and the subiculum was restricted to rats that had sustained extensive injury. In all but three rats, the hippocampal injury was bilateral and of equal severity.

*Histopathology — Quantitative Findings*

Total number of cells for the regions examined are presented in Table 2. Minimal intergroup differences were observed, and therefore, it is unlikely that significant cellular phagocytosis occurred during the experiments. In the subiculum, all groups had significantly fewer neurons than M. This likely reflects difficulty in defining this region as well as the relative

### Table 1. Mean Blood Pressure and Hematocrit Before and After Agent or Ischemia in Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Before agent</th>
<th>After agent (20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood pressure</td>
<td>Hematocrit</td>
</tr>
<tr>
<td>C</td>
<td>112.0 ± 5.6</td>
<td>45.0 ± 1.1</td>
</tr>
<tr>
<td>NS</td>
<td>108.8 ± 5.0</td>
<td>54.0 ± 1.1</td>
</tr>
<tr>
<td>M</td>
<td>133.0 ± 5.2</td>
<td>48.8 ± 1.2</td>
</tr>
<tr>
<td>N</td>
<td>113.4 ± 3.2</td>
<td>47.3 ± 1.4</td>
</tr>
<tr>
<td>I</td>
<td>112.0 ± 5.8</td>
<td>45.6 ± 1.0</td>
</tr>
<tr>
<td>M + N</td>
<td>116.4 ± 7.2</td>
<td>45.8 ± 1.7</td>
</tr>
<tr>
<td>M + I</td>
<td>116.2 ± 7.1</td>
<td>45.1 ± 2.2</td>
</tr>
<tr>
<td>N + I</td>
<td>111.8 ± 7.2</td>
<td>48.7 ± 0.6</td>
</tr>
<tr>
<td>M + N + I</td>
<td>115.0 ± 8.0</td>
<td>47.6 ± 1.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n = 5 for each group. Analysis of variance followed by Duncan’s test for multiple comparisons. C, control; NS, normal saline; M, 0.25 g/kg mannitol; N, 20 μg/kg nimodipine; I, 4 mg/kg indomethacin.

*p<0.05 compared with before agent.

### Table 2. Total Number of Neurons in Rat Brain Regions

<table>
<thead>
<tr>
<th>Region</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>Subiculum</td>
<td>181 ± 22*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
</tr>
<tr>
<td>CA1/CA2</td>
<td>679 ± 51</td>
</tr>
<tr>
<td>CA3</td>
<td>616 ± 63</td>
</tr>
<tr>
<td>CA4</td>
<td>192 ± 23</td>
</tr>
<tr>
<td>Inner dentate blade</td>
<td>1105 ± 51</td>
</tr>
<tr>
<td>Outer dentate blade</td>
<td>702 ± 79</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>1260 ± 202</td>
</tr>
<tr>
<td>Inferior frontal cortex</td>
<td>951 ± 196</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, analysis of variance followed by Duncan’s test for multiple comparisons. C, control; NS, normal saline; M, 0.25 g/kg mannitol; N, 20 μg/kg nimodipine; I, 4 mg/kg indomethacin.

*p<0.05 compared with M.
paucity of neurons rather than cellular phagocytosis.

**Hippocampus.** Neuronal injury within the subiculum was relatively mild and variable, although it reached 38 ± 17% in the normal saline group. Significantly ($p<0.05$) less neuronal injury (≤9%) was found in all groups except the control (17%) or N (12%) groups compared with the normal saline group. The extent of ischemic damage in the CA1/CA2 sector for the various groups is presented in Figure 3. In this region, ischemic neuronal injury exceeded 60% in both the control (64 ± 10%) and normal saline (70 ± 10%) groups. Only M used singly (7 ± 5%) showed a significant reduction in ischemic neuronal injury compared with the control ($p<0.05$) and normal saline ($p<0.01$) groups. M also showed a significant reduction in neuronal injury when compared with the I (51 ± 14%) group ($p<0.05$). Although N appeared to be beneficial, it failed to show any significant effect. When compared with either the
control or normal saline groups, pairing the agents resulted in a significant decrease in neuronal injury in both the M + N (19 ± 17%) and the N + I (21 ± 15%) groups (p < 0.05). M + N + I also resulted in significantly less (12 ± 12%) neuronal injury compared with the control (p < 0.05) or normal saline (p < 0.01) groups (Figure 3). A similar effect was observed in the CA3 sector (Figure 4), which differed from the CA1/CA2 sector in that N used singly showed significantly less neuronal injury than the control group, as did all paired drug combinations (p < 0.05). Due to the great variability in ischemic injury within the CA4 sector, no significant intergroup differences were obtained.

**Dentate gyrus.** Ischemic injury was mild within the outer dentate blade, and in no group was a significant reduction in ischemic neuronal injury observed. In the inner dentate blade (Figure 5), however, ischemic injury exceeded 50% in the control group (54 ± 11%). Compared with the control group, ischemic neuronal injury was significantly less in the M, the M + I, and the M + N + I groups (p < 0.05).
Average number of total neurons in examined sections was not explicable by a variable loss of neurons per group, as the total number of neurons did not differ between groups; this possibility is negated.

Experimental Model

would not be explicable by a variable loss of neurons per group, as the total number of neurons did not differ between groups; this possibility is negated.

Discussion

Forebrain ischemia was induced by a method similar to that used by Smith et al. This model produces an incomplete ischemic insult of short duration that is uniformly reproducible, affecting mainly watershed territories. Histopathologic changes become more pronounced as the duration of ischemia increases. In the neocortex, neuronal injury is usually confined to layers III–VI. Pyramidal neurons of the hippocampus (sectors CA1–CA4) and the inner dentate blade are also selectively injured.

Selective neuronal vulnerability may reflect a multitude of variables such as variations in collateral flow, neuronal hyperexcitability, the release of excitotoxins, intracellular calcium overload, and alterations in protein synthesis. Pathophysiologic changes of ischemia occur as a function of time in this model. This maturation process, therefore, suggests that although dissipative electrolyte shifts may mark the threshold of irreversible ischemic injury, intrinsic cellular mechanisms are operant in its final expression.

Total number of neurons illustrates that the results would not be explicable by a variable loss of neurons between groups as a result of phagocytosis. Because the data were presented as the ratio of ischemic neurons to total neurons, a reduced ratio could have occurred if the agent or combination of agents accelerated the process of ischemic cell damage, such that the cells appearing eosinophilic in control brains were no longer visible in the treated groups. As the total number of neurons did not differ between groups, this possibility is negated.

Pharmacologic Modification of Ischemic Neuronal Injury—Agents Used Singly

Our study has demonstrated considerable amelioration of ischemic injury with M. Other reports have attributed similar observations to improvement in local CBF secondary to decreases in blood viscosity and hemoglobin content and direct vasodilatation. In addition, M favorably affects ischemic edema and functions as a hydrophilic free radical scavenger. Therefore, M has multiple, potentially beneficial effects on the ischemic cascade.

The beneficial effect of N on neuronal ischemia was not as pronounced although it reached significance in the hippocampal CA3 sector. Calcium antagonists have two potentially beneficial effects. Through vasodilatory mechanisms, they may enhance CBF during and following ischemia, although evidence for such a mechanism remains controversial. They also inhibit calcium fluxes into ischemic cells, thereby preventing the secondary effects of cytosolic free calcium. Free calcium activates calcium-dependent phospholipases, resulting in production of free fatty acids and lysophospholipids that are cytotoxic to the cell. In addition, calcium alters various enzyme systems including proteases, nuclease, adenylate cyclase, Na,K-ATPase, and glycogen phosphorylase. Any potential effect observed with N may reflect one or more of the above mechanisms. The failure of N to equal the beneficial effect of M suggests that calcium may not have a central role in ischemic neuronal injury, a finding that has been alluded to in previous reports.

Treatment with I failed to produce a significant beneficial effect. Accumulation of prostaglandins during incomplete ischemia is inhibited through pretreatment with I via inhibition of cyclooxygenase. Reperfusion is associated with an imbalance between the production of thromboxane A2 and prostacyclin. Preferential synthesis of thromboxane A2 may result in vasoconstriction. This may explain, in part, the posts ischemic hypoperfusion syndrome. Several studies have shown inhibition of posts ischemic hypoperfusion with I. The failure of I to prevent ischemic neuronal injury in our study suggests that posts ischemic hypoperfusion may not be a major factor in the initiation or propagation of ischemic neuronal injury.

Pharmacologic Modification of Ischemic Neuronal Injury—Combinations of Agents

M + N or M + I produced a favorable response; however, this benefit is not greater than that achieved with M alone. These observations are further evidence for the multifaceted effects of M that override any further benefit that may be achieved through the...
addition of N or I. N + I resulted in qualitatively less ischemic injury than was observed with either agent alone, suggesting a synergistic effect between the two agents. The degree of ischemic injury was least when all three agents were used in combination. In the CA3 sector (Figure 4), M + N + I resulted in significantly less neuronal injury than in either the control or normal saline groups. Its protective effect appeared to be greater than that of M, which was significantly different only from the control group. Similarly, for IFC, only the M + N + I group showed significantly less neuronal damage than the control group.

Our data support the concept that successful blockage of the ischemic cascade with a multifaceted single agent or multiple agents will produce the most beneficial response. Combinations of agents may elevate CBF above ischemic thresholds, thereby preventing energy failure and its resulting dissipative ion fluxes. In addition, M would favorably affect ischemic cerebral edema, thus maintaining the optimal diffusion distance for both oxygen and substrate. Preventing calcium influx into ischemic cells may inhibit the accumulation of free fatty acids and their metabolites, prostaglandins, leukotrienes, and endoperoxides. In addition, it would prevent the activation of several calcium-dependent enzyme systems and the uncoupling of oxidative phosphorylation by mitochondrial sequestration of calcium. The addition of I, which inhibits ischemia-induced imbalance between thromboxane A 2 and prostacyclin, could favorably affect the postischemic hyperperfusion state.

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nimodipine
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