The Effect of Isoflurane on Neuronal Necrosis Following Near-complete Forebrain Ischemia in the Rat

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The effect of deep isoflurane anesthesia on ischemically induced neuronal damage was evaluated in the rat. Sixteen mechanically ventilated animals were maintained normocapnic and normothermic while subjected to a near complete forebrain ischemia insult induced with systemic hypotension (MAP = 50 ± mmHg) and bilateral carotid artery occlusion. Prior to ischemia, eight of the rats received isoflurane by inhalation until the EEG demonstrated a burst suppression pattern; the other eight were untreated controls. After 10 min of ischemia, the carotid clamps were removed, blood pressure was restored, and, in the treated group, isoflurane administration discontinued. Following the ischemic insult, the animals were observed over a 7-day period, at which time they were killed and the brains prepared for histologic study. Severity of damage was assessed by a direct count of irreversibly damaged neurons, which appear bright red when stained with cresyl violet-acid fuchsin. Areas of particular interest were those that characteristically display vulnerability to ischemic damage, i.e., hippocampus, caudate nuclei, and neocortex. The control group revealed severe damage in the hippocampal CA1 sector (70% cells acidophilic) with more variability in the caudate nuclei and neocortex. The treated group showed a similar extent of damage with approximately 74% cells acidophilic in hippocampus (CA1). Clinical appearance was indistinguishable between groups. The authors conclude that pretreatment with isoflurane shows no beneficial effects on delayed neuronal necrosis following near-complete forebrain ischemia. (Key words: Anesthetics, volatile; isoflurane. Brain: forebrain ischemia; delayed neuronal necrosis.)

ISOFLURANE, a potent inhalational anesthetic, has found increasing favor in neuroanesthetic practice. Recent evidence has indicated that deep isoflurane anesthesia may provide cerebral protection during ischemic episodes by producing a dose-dependent decrease in canine CMRO₂. This is accompanied by a suppression of electroencephalographic (EEG) activity with maximal reduction of CMRO₂ corresponding to the onset of electrocortical silence.¹ These findings may hold clinical relevance as the human EEG is suppressed by an end-tidal isoflurane concentration of 2.4%,² a dose compatible with maintenance of intraoperative cardiovascular stability.

During episodes of incomplete canine global ischemia, Newberg and Michenfelder³ also have shown that, while cortical tissue energy stores (ATP, ADP, AMP, and PCr) are decreased, cerebral energy charge is better maintained in dogs deeply anesthetized with isoflurane before ischemia. In addition, cerebral lactic acid accumulation in treated animals (5 μmol · g⁻¹) was found to be significantly less than in controls (12 μmol · g⁻¹).

Histopathologic analysis of posts ischemic cerebral outcome has shown that the ultimate degree of brain damage incurred may require hours or days to “mature,” a phenomenon known as delayed neuronal necrosis.⁴-⁶ A model recently developed by Smith et al.⁷ offers the opportunity for evaluation of pharmacologic cerebral protection within this context. With the use of a combination of bilateral common carotid artery occlusion and systemic hypotension in the rat, blood flow to the midbrain and hindbrain remains adequately preserved while a severe, discreet, and quantifiable ischemic insult is delivered to the forebrain.¹-¹¹ From this insult, the animals can be recovered and maintained without special support for subsequent histopathologic analysis. The purpose of this experiment, therefore, was to evaluate the effect of deep isoflurane anesthesia on delayed neuronal necrosis when administered prior to and during near-complete forebrain ischemia insult in the rat.

Methods

Twenty-two fasted male Wistar rats (body weight, 305-345 g) of an SPF strain (Møllegaards Avlaboratorium, Copenhagen, Denmark) were used for this experiment. Following anesthetic induction with 3.0% halothane, the rats were endotracheally intubated and connected to a Starling type respirator (B. Braun, Melsinger AG, West Germany) delivering 0.7% halothane and 30% O₂ in N₂O. The tail artery was catheterized for monitoring of blood pressure and sampling of blood. Via a ventral neck incision, the common carotid arteries were isolated while preserving the vagus nerves and cervical sympathetic plexi. The right jugular vein was cannulated with a silicone catheter, allowing induction of hemorrhagic hypotension during ischemia. Muscle paralysis was provided by a 1-mg iv bolus of suxamethonium chloride (Celocurium, Vitrum AB, Stockholm, Sweden) repeated as necessary. Bipolar EEG recordings were monitored from a pair of nec-
dle electrodes inserted into the temporalis muscle on each side of the head (Mingograf 34®, Elema-Schonander, Stockholm, Sweden).

Following surgical preparation, the halothane was discontinued and the rat allowed a steady state period of 30 min, with body temperature being maintained between 36.5–37.5°C, arterial PO2 at 90–100 mmHg, and PCO2 at 35–40 mmHg. Before the sampling of blood for the first blood–gas measurement, 50 IU heparin (Vitrum AB, Stockholm, Sweden) was given intravenously. Preischemic plasma glucose levels were measured with a Beckman Glucose Analyzer 2® (Beckman Instruments, Inc., Fullerton, California).

The animals then were randomly assigned to one of two groups. Control rats received no further intervention. The inspired gas mixture was maintained at 30% O2 and 70% N2O, during the ischemia and recovery periods. The treated animals, prior to the onset of ischemia, received a 3–4% inspired concentration of isoflurane (Forene, Abbott Laboratories, Ltd., Queensborough, Kent, England) in 30% O2 and balance N2O, administered to maintain a steady state EEG burst suppression interval of greater than 30 s. To support the arterial blood pressure during isoflurane administration, 4–6 ml blood from a fasted donor rat were infused via the caval catheter to maintain MAP above 100 mmHg.

All rats then underwent a 10-min interval of forebrain ischemia induced by a bolus iv infusion of 3.5 mg trimethaplan camphor sulfonate (Arfonad®, Roche, Basel, Switzerland) to achieve a MAP of 50 ± 5 mmHg, after which both carotid arteries were clamped and a timer was started. Central venous exsanguination to maintain MAP at 50 mmHg throughout the ischemic interval was performed as necessary, usually requiring the withdrawal of 4–6 ml of blood. Onset of ischemia was confirmed by an isoelectric EEG. At the end of the 10-min interval, blood pressure was restored by rapid reinfusion of shed blood, and the carotid clamps were removed. Sodium bicarbonate (0.5 ml 0.6 m) was injected iv to counteract systemic acidosis. In the treated animals, isoflurane was discontinued at the termination of ischemia, and the excess blood administered before ischemia was gradually withdrawn. When the blood pressure was stabilized at preischemic levels, the caval catheter was removed, the jugular vein tied off, and the neck closed with sutures.

Blood pressure was monitored continuously during the early recovery period. At 15-min intervals, the EEG was recorded and arterial PO2, PCO2, and pH measured. The temperature was kept near 37°C. After a recovery of 45–60 min, the animals had regained consciousness, resumed spontaneous respiration, and could be disconnected from the respirator. Intervals required for resumption of spontaneous ventilation and the righting reflex were recorded. The animals subsequently were extubated and the arterial catheter was removed. They were housed then in cages, with access to tap water and pellet food, and maintained for 7 days. During this interval, the animals were observed daily for evidence of hyperexcitability, motor manifestations of seizure activity, feeding behavior, and evidence for neurologic motor deficits.

On day 7 of the experiment, the rats were weighed and anesthetized with 0.7% halothane in 30% O2 and balance N2O, tracheostomized, and connected to the respirator. Via the ascending aorta, the brain was perfusion fixed with 4% formaldehyde buffered to a pH of 7.35, preceded by a 30-s rinse period with saline. Both solutions were prewarmed to 37°C and infused at a pressure of
135 mmHg. The brain was allowed to stabilize in situ until removal the subsequent day for storage in cold fixative. The brains were cut coronally into 2.8-mm-thick slices and dehydrated in graded strengths of ethanol over 2 days. Following clearing in xylol and embedding in paraffin, they were subserially sectioned at 8 μm on a Reichert-Jung® (West Germany) sledge microtome and stained with cresyl violet and acid fuchsin. Sectioning intervals were adapted to obtain specific standard levels of the caudate nucleus, cerebral cortex, and hippocampus for quantification of injury. The damage was assessed as the presence of necrotic acidophilic (pink, red) neurons in the different brain areas. The acidophilic neurons were considered to be irreversibly damaged, since they have been consistently found to undergo cytolysis and removal from brain tissue. The caudate nucleus was quantified in the lateral and dorsal aspects at the level of the septal nuclei at their widest point, the cerebral cortex at the level of subfornical organ, and the hippocampus at three levels throughout its septotemporal extent (Fig. 1). Quantification of damaged neurons was performed by the experimenters, blinded to the condition group, by direct visual counting of acidophilic neurons at a magnification of ×320, using a two-channel laboratory cell counter (Clay Adams, Parsippany, New Jersey).

Statistical analysis was performed on the counts obtained from the caudate nucleus and CA1 sector of the hippocampus using the nonparametric Wilcoxon rank sum test, pooling per cent dead cells from the left and right hemispheres. Cortical injury was graded on a scale of 0-3: 0 = no acidophilic cells; 1 < 10%, 2 = 10-75%, and 3 > 75% acidophilic cells. The Student’s t test was used to analyze physiologic variables.

**Results**

Physiologic values are given in Table 1. The MAP profile was similar between groups with one exception. Mean preischemic MAP in the isoflurane group was 20 mmHg less than in the controls (P < 0.01). However, in all treated animals the preischemic MAP was maintained above 100 mmHg by infusion of donor blood. Otherwise, hemodynamic conditions were identical. Figure 2 depicts a typical MAP profile from the isoflurane-treated group. The animals were normothermic during ischemia. Arterial blood gases and pH measurements at various intervals throughout the experimental procedure showed no statistical difference between groups. The duration of recirculation elapsed before onset of spontaneous ventilation, and resumption of the righting reflex was also consistent between groups.

Recordings from the bipolar EEG electrodes demonstrated a progressive slowing of activity as the isoflurane concentration was steadily increased. A burst suppression

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**TABLE 1. Values (mean ± SD) for Physiologic Variables Measured at Various Intervals During Experimental Procedure**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Isoflurane</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>321 ± 15</td>
<td>316 ± 10</td>
</tr>
<tr>
<td>Preischemia</td>
<td>321 ± 22</td>
<td>309 ± 23</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>7.4 ± 0.6</td>
<td>6.9 ± 0.11</td>
</tr>
<tr>
<td>Body temperature</td>
<td>37.2 ± 0.3</td>
<td>37.0 ± 0.3</td>
</tr>
<tr>
<td>Ischemia (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>124 ± 10</td>
<td>104 ± 4*</td>
</tr>
<tr>
<td>R = 1 min</td>
<td>105 ± 4</td>
<td>105 ± 7</td>
</tr>
<tr>
<td>R = 15 min</td>
<td>128 ± 12</td>
<td>126 ± 14</td>
</tr>
<tr>
<td>R = 30 min</td>
<td>122 ± 10</td>
<td>120 ± 10</td>
</tr>
<tr>
<td>R = 45 min</td>
<td>121 ± 12</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>Onset spontaneous</td>
<td>44 ± 8</td>
<td>40 ± 10</td>
</tr>
<tr>
<td>Respiration (min postischemia)</td>
<td>50 ± 8</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>Righting reflex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(min postischemia)</td>
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R = recirculation interval. * Statistical significance, P = <0.01.
Table 2. Number of Acidophilic Cells in the Evaluated Regions of the Rat Brain Following 10 Min Ischemia and 7 Days Recovery

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>Isoflurane</th>
</tr>
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<tbody>
<tr>
<td>Hippocampal CA1</td>
<td>1,100 ± 88</td>
<td>1,174 ± 67</td>
</tr>
<tr>
<td>Caudate</td>
<td>12 ± 5</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>Cortex</td>
<td>20 ± 11</td>
<td>55 ± 15</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Mean total cell count for CA1 level: 2–4 = 1,578 (Smith M-I, Wieloch T, Auer RN, unpublished data).

A pattern appeared at an inspired concentration of approximately 3.5%. When the isoelectric interval between burst activity was greater than 30 s, ischemia was induced, which produced onset of isoelectricity within 20 s. This ischemia-induced pattern of isoelectricity was then indistinguishable between groups. In both groups of animals, the EEG remained isoelectric for approximately 20 min into the recirculation period, when a low-voltage continuous EEG recording gradually resumed.

Of the initial 22 animals, six (three treated and three control) were eliminated from the study. Four rats had postextubation airway obstruction, identified as glottic edema secondary to intubation trauma. Two rats (one isoflurane and one control) failed to establish MAP > 100 mmHg at the termination of ischemia.

Subsequent to the acute recovery the animals were neurologically indistinguishable. Rats in both groups were typically docile on days 1 and 2 but by day 3 showed signs of hyperexcitability to both sound and handling. No overt seizure activity was noted in any animal, although the hyperexcitability persisted through day 7. All rats had resumed ambulation within 6 h, with no evidence of residual paresis or plegia throughout the remainder of the observation period. The animals typically resumed feeding on day 2 to overcome a transient postischemic body weight loss (range of 12–55 g) and returned to normal weight by day 7 without a difference between groups (table 1).

Twelve to 24 h after perfusion fixation the brains were removed and observed grossly. Flattening of gyri or other signs of edema were not present. Quantitative assessment of neuronal necrosis did not show a statistically significant difference between the isoflurane-treated and control groups in the extent of damage incurred within the caudate nuclei or CA1 sector of the hippocampus (table 2). Dense hippocampal CA1 injury was seen in both groups, with 70% of the control and 74% of the treated neurons staining acidophilic, frequently with condensed or absent nuclei, and stained dendritic processes. Both groups contained a large number of macrophages in this region, as well as in the caudate nuclei, associated with the acidophilic neurons. CA3, CA4, and the dentate nuclei, although not directly quantitated, showed infrequent acidophilic neurons often surrounded by macrophages but with no discernible difference between groups.

Isoflurane also had no effect on the histologic outcome in the cerebral cortex. All rats fell into the injury rank of 1 on a scale of 0–3. In layers 3–5, the range of acidophilic neurons varied between 17–303 (bilateral sum), but the groups were not statistically compared because of the enormous background field of normal neurons (estimated to be 8,000 per section). Infarcts were not noted, but again macrophages were frequently seen grouped in association with the acidophilic cortical neurons.

Discussion

The present experiment did not demonstrate a difference between isoflurane-treated and control rats with respect to outcome in selectively vulnerable cerebral structures following near-complete forebrain ischemia. Within the forebrain, the ischemia technique employed severely reduces rCBF as determined autoradiographically (2 ml·100 g⁻¹·min⁻¹ in frontal cortex; 2 ml·100 g⁻¹·min⁻¹ in caudoputamen and 1 ml·100 g⁻¹·min⁻¹ in hippocampus).11 Values in this range produce uniform EEG quiescence within 20 s, making this model similar to one of cardiac arrest with respect to neuronal electrical activity and energy requirements within the regions studied.

Experiments evaluating the protective effects of thio- pental have shown that drugs that function solely through suppression of electrical activity can provide protection only when the extent of hypoxia is insufficient to abolish such activity.12 For this reason, our results are compatible with those of Newberg and Michenfelder,3 which would predict a lack of protection by isoflurane under conditions of ischemia severe enough to produce EEG isoelectricity. Further investigations into potential benefits of isoflurane treatment on histopathologic outcome under conditions of less severe ischemia are indicated.

Previous investigation has demonstrated a relationship between the duration of ischemia and the density and distribution of delayed neuronal necrosis.8 Within the hippocampus (CA1) and cortex, the density is graded, i.e., increasing numbers of dead cells parallel the duration of ischemia. Within the caudate nucleus virtually no acidophilic neurons appear until 10 min of ischemia. Therefore, if isoflurane-induced preservation of energy charge and reduction of lactate accumulation are produced by mechanisms other than suppression of electrical activity, treated animals would be expected to show less neuronal necrosis. The present study failed to show evidence for an alternative mechanism of action for isoflurane-induced cerebral protection.

Biochemical processes other than depletion of the cerebral energy state also may be responsible for mediating...
ischemic brain damage. Glucose-induced intracellular acidosis, disturbed calcium ion homeostasis, and a related intracellular accumulation of calcium have been advanced as factors necessary for the production of irreversible neuronal damage. Recent evidence suggests that the phenomenon of delayed neuronal necrosis may be transsynaptically mediated by release of excitatory neurotransmitters (glutamate and aspartate), which are neurotoxic in high concentrations. The effect of isoflurane on cerebral energy state is not expected to reverse these mechanisms, and this also would account for the lack of beneficial effect in our study. Contrarily, flunarizine, a calcium channel blocker, has been shown to ameliorate ischemic brain damage in this model.

The significance of the intermittent EEG burst activities seen in the dog as well as in this rat model has not been characterized. The possibility exists that this activity represents unsuppressed subcortical electrical function, which if located in the hippocampus would in part also explain the lack of effect within the CA1 sector.

In conclusion, rats receiving 10 min of near-complete forebrain ischemia showed no difference between control animals and those receiving isoflurane administered to produce a burst suppression EEG pattern. No deleterious effect from isoflurane was seen. However, the results of this study indicate that isoflurane will not provide cerebral protection under conditions of severe ischemia.

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References