MK-801 Does Not Protect Against Hypoxic-Ischemic Brain Injury in Piglets

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Background and Purpose: The excitatory amino acid inhibitor MK-801 has been shown in many animals species to protect against hypoxic-ischemic brain injury. We sought to determine whether hypoxic-ischemic injury to the newborn pig’s brain could be prevented by the use of MK-801.

Methods: Hypoxic-ischemic injury to the brain was induced in forty 0–3-day-old piglets. They were randomized to receive either 3 mg/kg MK-801 (MK-801 group, n=20) or vehicle (control group, n=19) prior to insult. At time 0, the carotid arteries were ligated and the blood pressure was reduced by one third by hemorrhage. At 15 minutes, inspired oxygen was reduced from 50% to 6%. At 30 minutes, inspired oxygen was changed to 100%, carotid ligatures were released, and the withdrawn blood was reinfused. An additional 14 piglets received 3 mg/kg MK-801 but not hypoxic-ischemic injury (drug-only group), and a final group of 11 piglets were subjected to only a sham operation (sham group).

Results: Neurological examination scores at 24, 48, and 72 hours showed that MK-801 and drug-only piglets were significantly worse than the controls. Pathological examination of the brains at 72 hours showed significantly greater damage in the brains of the MK-801 and control pigs relative to the sham and drug-only groups. No differences were found between the control and the MK-801 groups. No differences were found between the sham and drug-only groups.

Conclusions: MK-801, at a dose of 3 mg/kg, causes neurological dysfunction in piglets lasting at least 72 hours, but neither causes brain damage nor ameliorates the effects of hypoxic-ischemic injury to the brain of the newborn pig. (Stroke 1991;22:1270-1275)

The compound (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, commonly known as MK-801, is a readily diffusible, noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist acting at the associated phencyclidine site. Gill et al have shown that MK-801 is neuroprotective in gerbils for cerebral ischemia when given before ischemia and even when given as late as 48 hours after ischemia. Others have produced similar results. The mechanism of action is thought to be through inhibition of NMDA receptors. Cerebral hypoxia or ischemia triggers release of excitatory amino acids in large quantities, causing calcium entry into the cell and, thus, exacerbating brain injury. We had developed in our laboratory a hypoxic-ischemic brain injury model using the newborn pig in which we chose to test this compound. In the human newborn, unlike the human adult, most clinical injury to the brain is initiated as a hypoxic event, although there is usually some ischemia due to hypoxic dysfunction of the myocardium. However, we found it difficult to generate reproducible hypoxic injury in a purely hypoxic model without an unacceptable rate of death from hypoxic myocardial damage. Thus, an ischemic component, clamping both carotid arteries and reducing the blood pressure by one third, was added to the hypoxic insult to produce a relatively pure brain injury model. Since the circle of Willis connects the vertebral arteries to the carotid arteries in the piglet, this is a partial rather than total ischemia model.

Materials and Methods

These protocols were reviewed by the University Medical Center Animal Use Committee. A total of 65 0–3-day-old Yorkshire Landraise Cross piglets were used in this experiment. They
were divided into four groups. Forty received hypoxic-ischemic injury as described below and were randomly assigned to receive MK-801 (MK-801 group) or vehicle (control group). Fourteen received MK-801 but no hypoxic-ischemic injury (drug-only group), and 11 served as shams (sham group). All piglets were removed from their mothers on the day of the experiment.

The 20 MK-801 and 19 control group piglets received hypoxic-ischemic injury as described previously. They were anesthetized with 1.5% isoflurane and 50% nitrous oxide, intubated, and ventilated with a Harvard Rodent Ventilator (Harvard Apparatus, South Natick, Mass.) adjusted to obtain an initial PCO2 of approximately 5.3 kPa (40 torr). Catheters were placed in the superficial artery of the right rear leg and in the right external jugular vein using sterile technique. A snare was placed around both carotid arteries. After surgery was completed, the isoflurane was reduced to 0.5%, and the animal was paralyzed with an infusion of 0.5 mg/kg/min succinylcholine. The animal’s rectal temperature was maintained at 37.5°C using a servocontrolled infrared lamp.

At time -5 minutes, baseline measurements were taken, then 3 mg/kg MK-801 (Research Biochemicals, Inc., Natick, Mass.) dissolved in 2 cc of the animal’s blood or a placebo (consisting of the animal’s blood stirred as would have been necessary to dissolve the MK-801) was injected. Blood was chosen as the diluent because MK-801 is poorly soluble in saline, and alcohol, in which MK-801 is soluble, has unacceptable neurological effects. Baseline measurements included arterial blood gases, arterial blood pressure, rectal temperature, whole blood lactate, and serum glucose. This set of measurements was repeated at 0, 15, 30, 45, 60, and 90 minutes. At time 0, 500 units/kg heparin was injected, and the carotid arteries were ligated by pulling the snare snug around them. Blood was withdrawn from the arterial catheter into 60-ml syringes to reduce the arterial blood pressure to approximately two thirds of control levels and maintain it at that level. Isoflurane was discontinued at 10 minutes, at which time the animals have been shown to be comatose in pilot studies. Fifteen minutes after the carotid ligation and the reduction of blood pressure to two thirds of normal, the animal was switched from ventilation with 50% nitrous oxide and 50% oxygen to a gas mixture containing 92% nitrous oxide, 2% CO2, and 6% oxygen. This reduced arterial PO2 to approximately 3.3 kPa (25 torr) within 1–2 minutes. Succinylcholine was discontinued at 20 minutes (it is required until this time to prevent gasping). At an experimental time of 30 minutes, after 15 minutes of hypoxia, the animal was reoxygenated by switching the inspired gas from 6% oxygen to 100% oxygen, releasing the carotid ligatures, and reinfusing the blood that had been previously withdrawn. That patency of the carotid arteries is reestablished has been confirmed at autopsy. Ventilation was continued for as much as an additional 48 hours if necessary to maintain the piglets.

Piglets were nursed in cages. Warmth was provided by heat lamp. They were fed 60 cc artificial piglet formula by gavage every 6 hours. Neurological examination was performed by the staff performing the experiment at 2 hours after reoxygenation. In the first 20 piglets (10 MK-801 group, 10 control group), neurological examinations at 1 and 2 days after reoxygenation were performed by the staff performing the experiment; only the 3-day exam was performed by a pediatric neurologist blinded to the experimental assignment of the piglets. In the last 19 piglets (10 MK-801 group, 9 control group), 1-, 2-, and 3-day neurological examinations were performed by a blinded pediatric neurologist. The results were recorded and scored from 5 to 20, with 20 being normal according to a standard scoring system. Three days after the experiment, with the piglet under isoflurane and nitrous oxide anesthesia, the chest was opened, the carotid artery cannulated, and the body perfused with 10% formalin after flushing with 30 cc saline. Formalin was continued until the effluent from the right atrium was clear, thus preserving the brain and killing the animal. The brain was then removed and preserved in formalin for later pathological examination. If the piglet died before completion of the 3 days, its carcass was stored in the refrigerator until morning, and then a gross autopsy was performed and its brain preserved in formalin.

The 14 piglets in the drug-only group received 3 mg/kg MK-801 by ear vein while under general anesthesia with 1.5% isoflurane and 50% nitrous oxide, but they received no hypoxic-ischemic injury. They were then revived and received a 2-hour neurological examination by the experimental staff; 1-, 2-, and 3-day neurological examinations by a blinded pediatric neurologist; and a blinded brain pathological examination, as had the other groups of piglets. One drug-only piglet died between 2 and 24 hours after reoxygenation. Gross autopsy showed only petechiae over the right ventricular epicardium.

The 11 sham-group piglets, after a sham surgical procedure but without hypoxia or ischemia to the brain, received a blinded neurological examination (3-day examination only). They were then killed as previously described, and their brains were submitted for a blinded pathological examination.

After preservation in formalin, the brains of all piglets were cut, fixed, and stained. A coronal section was taken at the level of the optic chiasm, and another was taken approximately 3 mm back to demonstrate the cerebral cortex, the hippocampus, and the basal ganglia. Paraffin sections were stained with hematoxylin and eosin and examined by light microscopy. Each section was graded on a scale of 1–10, with 10 being considered normal by a pathologist blinded to the experimental group of the piglets. The scoring system has been published. Cellular changes were classified as hypoxic (considered by the pathologist to be potentially reversible, scores 5–9
depending on size of involved area) or necrotic (thought to be clearly irreversible, scores 1–4 depending on size of involved area). The final score was the sum of the scores of each of the three tissues. Hypoxic changes were largely shrunken hyperchromatic neurons but also included enlarged perivascular spaces and eosinophilic staining neurons with pyknotic nuclei; necrotic changes included loss of neurons with glial and vascular proliferation and increased macrophage activity in the tissue.

Lactate, glucose, and blood gases and pressures were measured by standard techniques. Comparisons were made using a t test (two-tailed) or analysis of variance, depending on the number of groups compared. All results are presented as mean±SEM with nonsignificant (NS) being \( p > 0.05 \).

**Results**

The changes in mean and blood pressure, \( \text{P}O_2 \), and temperature are shown in Figure 1. The planned decrease in blood pressure and \( \text{P}O_2 \) are shown in the upper two graphs. With the administration of MK-801, there was an acute increase in blood pressure of about 20 mm Hg and in heart rate of approximately 50 beats/min as well as an acute increase in electroencephalographic amplitude lasting 1–2 minutes. All these responses returned to near baseline by time 0, although even at time 0, the blood pressure in the MK-801 group (\( n = 19, 68 \pm 2.6 \)) was greater than that in the control group (\( n = 20, 58 \pm 2.0; p<0.01 \)). Aside from the time 0 blood pressure, there were no significant differences between the MK-801 and the control animals in blood pressure, \( \text{P}O_2 \), or rectal temperature. The changes in serum glucose, arterial pH, arterial \( \text{PCO}_2 \), and blood lactate are shown in Figure 2. There were no significant differences between the two groups in any of these four variables.

Ten MK-801 animals and six control animals died before the 3-day examination (\( p=\text{NS} \)). Of the 10 MK-801 animals that died between 2 and 72 hours of age, three were still requiring mechanical ventilation when a ventilator malfunction occurred; two had petechial hemorrhages on the surface of the right ventricle, which suggested right ventricular ischemic damage; one had right upper lobe atelectasis or pneumonia, apparent at autopsy; and four had normal autopsies. Of the six control piglets that died between 2 and 72 hours of age, one died of status epilepticus, two had atelectasis or pneumonia, one had apparent aspiration, and two had a normal gross autopsy.

The results of the neurological exams are shown in Table 1. At 2 hours, the neurologic exam showed the following pattern: MK-801<control=drug only (i.e., the mean score for the MK-801 group was significantly less than that for either the control or the drug-only groups; scores for the drug-only and control groups were not significantly different). Thereafter, a pattern was established: MK-801=drug only<controls. A four-group comparison done at the 72-hour examination showed that MK-801=drug only<control<shams. The results of the pathological examination are shown in Table 2. The upper table shows the results of all pigs. For cortex, basal ganglia, hippocampus, and sum, the results were MK-801<controlsdrug only=shams. In the lower table, data from the animals who survived the full 72 hours and received brain preservation at death is shown. In the cortex, the results were MK-801<drug only=shams, with the results in the controls not significantly different from any of the other groups. In the hippocampus, the results were controls<MK-801=drug only<shams. The difference between the MK-801 and control groups was only at the \( p<0.05 \).
level, and the results for the hippocampus of the MK-801 animals was close to midway between the drug-only scores and the control scores. In the basal ganglion and in the sum of the three tissues, the results were MK-801=controls<drug only=shams.

Drug-only piglets were consistently lethargic and difficult to arouse. Most were jittery, and many exhibited rigidity similar to that seen in some animals with ketamine anesthesia. Even at 72 hours, the drug-only piglets (n=13) were significantly abnormal (by neurological examination<sub>16</sub>, 4=normal; differences, p<0.01) when scored for mental status (mean±SEM, 2.7±0.2), pupillary reflex (2.5±0.4), suck reflex (2.6±0.4), stepping reflex (2.6±0.4), righting reflex (1.7±0.4), motor (1.2±0.2), and coordination (1.4±0.2).

Discussion

The excitatory amino acid inhibitor MK-801 has a profound sedative effect on the newborn pig that is apparent even 3 days after drug administration. This resulted in neurological examination scores worse than those seen in the hypoxic-ischemic control pigs throughout most of the experiment. Histopathologic examination of the brain, however, showed that the amount of brain damage seen in the controls and in the MK-801-treated animals was very similar, whereas the drug-only and sham groups showed little damage. There was no difference seen between the MK-801-treated animals and the controls except in the hippocampus, and then only if all animals dying before brain preservation are excluded. In theory, this could be due to a preservative effect of MK-801 in hypoxic-ischemic injury that occurs only in the hippocampus. Indeed, the hippocampus is the tissue in which the neuroprotective effect of excitatory amino acid antagonists have been most frequently reported<sup>2-3,12,13</sup>. This was balanced by somewhat worse, although not statistically significantly worse, outcomes in the cortex of the MK-801-treated animals that survived to brain preservation. Thus, no significant difference between the controls and the MK-801 animals in overall brain outcome was seen.

Why, then, have so many investigators reported evidence of MK-801 protection against CNS injury? There have been numerous reports showing its effectiveness<sup>13-15</sup> however, most have looked only at hippocampal injury,<sup>2,3,12,13,16,17</sup> and most have been in small animals without adequate temperature control during the experiment<sup>2-3,16,18-20</sup>. As was shown by Buchan and Pulsinelli<sup>21</sup> in adult gerbils and by other authors<sup>12,17</sup> in infant rats, and others<sup>14,15,17</sup> in larger adult animal models, if the body temperature is maintained throughout the experiment, the protective effect of MK-801 is not seen. However, Gill and Woodruff<sup>12</sup> in adult gerbils, and others,<sup>14,15,17</sup> found a neuroprotective effect even when body temperatures were kept constant. In addition, at least four studies show in vitro effects of MK-801 demonstrating brain preservation in neuronal slices or neuronal cultures.<sup>27-30</sup>

It is possible, of course, that differences in species and age may also contribute to the observed differences. The neuroprotective effect of MK-801 in immature animals has, up to this point, been tested...
only in rats. As far as we are aware, the protective effect of MK-801 in the brain has not been previously tested in the newborn or adult pig. Receptors for MK-801 are broadly present within different species,31 including the pig,32 and at different ages.33-34 Our data support the presence of MK-801 receptors within the neonatal piglet brain. Indeed, the profound and long-lasting neurological deficit produced by MK-801 suggests a greatly enhanced sensitivity to MK-801 in this model. It is possible, however, that the anesthetic and neuroprotective effects may be mediated by different receptors.35 Anesthesia may markedly alter the effects of MK-801.36 Thus, different protocols for anesthetic use may in part explain the differences noted between our results and those of others. Is 3 days long enough to see the delayed neuronal injury prevented by MK-801? In the studies cited in this paper that evaluate the effects of MK-801, there is no pattern of efficacy related to length of time between injury and pathological examination. The median time of examination was 3 days. Acidosis may reduce the effect of MK-801.37-39 A relatively extreme degree of blood lactic acidosis was induced in this particular experiment. It has been suggested that differences in results with MK-801 may relate to differences in the degree of ischemia3940 with focal ischemia, where blood flow is reduced but not eliminated in part of the specimen, producing positive results,4,5,15-17,20 and global ischemia, where blood flow is totally eliminated, producing negative results.21-26 However, examples of efficacy in global12,13 and partial global ischemia exist.18,19 This experiment shows that with partial global ischemia in the piglet, MK-801 is ineffective. Our speculation is that the effectiveness of MK-801 will be very species-, age-, and circumstance-specific. We feel that reports on MK-801 have not included an array of experimental circumstances broad enough to delineate the patterns in its effectiveness.

In summary, MK-801 does not protect against hypoxic-ischemic damage to the brain of the newborn piglet. It does, however, exert a profound and prolonged sedative effect that is evident for at least 3 days following drug administration.

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References

Table 1. Piglet Neurological Exam Scores

<table>
<thead>
<tr>
<th>Time of examination</th>
<th>Groups</th>
<th>MK-801 Score</th>
<th>Control Score</th>
<th>Drug only Score</th>
<th>Sham Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Score</td>
<td>n</td>
<td>Score</td>
<td>n</td>
</tr>
<tr>
<td>2 hr</td>
<td>20</td>
<td>6.5±0.3*†</td>
<td>19</td>
<td>8.5±0.6</td>
<td>14</td>
</tr>
<tr>
<td>1 day</td>
<td>12</td>
<td>7.5±0.5*</td>
<td>17</td>
<td>14.7±0.8†</td>
<td>14</td>
</tr>
<tr>
<td>2 days</td>
<td>10</td>
<td>8.1±0.9*</td>
<td>15</td>
<td>16.2±0.9†</td>
<td>13</td>
</tr>
<tr>
<td>3 days</td>
<td>10</td>
<td>9.7±1.0*‡</td>
<td>14</td>
<td>15.6±1.5†‡</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*<p<0.01 vs. control.
†<p<0.01 vs. drug only.
‡<p<0.01 vs. sham.

Table 2. Piglet Pathological Examination Results

<table>
<thead>
<tr>
<th>Area</th>
<th>Groups</th>
<th>MK-801 Result</th>
<th>Control Result</th>
<th>Drug only Result</th>
<th>Sham Result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Result</td>
<td>n</td>
<td>Result</td>
<td>n</td>
</tr>
<tr>
<td>All piglets</td>
<td>20</td>
<td>6.6±0.6*†</td>
<td>19</td>
<td>6.9±0.7*†</td>
<td>14</td>
</tr>
<tr>
<td>Cortex</td>
<td>20</td>
<td>7.6±0.6*‡</td>
<td>19</td>
<td>7.3±0.6‡</td>
<td>14</td>
</tr>
<tr>
<td>Basal ganglion</td>
<td>20</td>
<td>7.4±0.6‡</td>
<td>19</td>
<td>6.5±0.7‡</td>
<td>14</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>20</td>
<td>21.6±1.7‡</td>
<td>19</td>
<td>20.2±1.8‡</td>
<td>14</td>
</tr>
<tr>
<td>Sum</td>
<td>20</td>
<td>21.6±1.7‡</td>
<td>19</td>
<td>20.2±1.8‡</td>
<td>14</td>
</tr>
<tr>
<td>Piglets with brain preservation at death</td>
<td>10</td>
<td>6.5±0.9*†</td>
<td>14</td>
<td>7.6±0.7</td>
<td>13</td>
</tr>
<tr>
<td>Cortex</td>
<td>10</td>
<td>7.6±0.8*‡</td>
<td>14</td>
<td>7.6±0.5‡</td>
<td>13</td>
</tr>
<tr>
<td>Basal ganglion</td>
<td>10</td>
<td>8.2±0.7‡</td>
<td>14</td>
<td>6.2±0.8‡</td>
<td>13</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>10</td>
<td>22.6±1.6‡</td>
<td>14</td>
<td>21.4±2.0‡</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
*<p<0.05, †<p<0.01 vs. sham group; *<p<0.05, †<p<0.01 vs. drug-only group; †<p<0.05 vs. control group.


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