A comparison of the protective effect of dexamethasone to other potential prophylactic agents in a neonatal rat model of cerebral hypoxia-ischemia

PAUL D. CHUMAS, F.R.C.S.(ED), MARC R. DEL BIGIO, M.D., PH.D., JAMES M. DRAKE, F.R.C.S.(C), AND URSULA I. TUOR, PH.D.

Divisions of Neurosurgery, Neuropathology, and Neonatology Research, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

It has recently been reported that pretreatment with a single dose of dexamethasone (0.1 mg/kg) 24 hours before hypoxia in 7-day-old rat pups is protective against an hypoxic-ischemic insult (unilateral carotid artery occlusion followed by 3 hours of hypoxia in 8% O2). The authors now examine whether pretreatment 6 hours before insult is equally effective and compare other agents potentially suitable for prophylaxis in neonatal hypoxia-ischemia, including the calcium antagonists flunarizine (30 mg/kg pretreatment), nimodipine (0.5 mg/kg pretreatment), and the 21-aminosteroid U-74389F (10 mg/kg pre- and posttreatment). For each active agent, there was also a vehicle-treated control group.

Comparison of the mean area of ipsilateral infarction on brain coronal sections showed that there was no statistically significant difference between the various control groups (mean area of infarction 66% ± 4%). Pretreatment with dexamethasone 6 hours prior to hypoxia offered complete protection with no infarction. A beneficial effect was seen following pretreatment with flunarizine (mean area of infarction 33.6% ± 7.8%), although this degree of damage was still significantly different from that seen with dexamethasone pretreatment. Pretreatment with nimodipine or U-74389F offered no protection (mean area of infarction 77.5% ± 4% and 59% ± 10%, respectively). Unlike findings in adult animals and clinical studies, the current studies show that dexamethasone may have a role in the treatment of neonatal hypoxia-ischemia and deserves reappraisal.

KEY WORDS: hypoxia - ischemia - cerebral infarction - dexamethasone - nimodipine - flunarizine - 21-aminosteroid - neonate - rat

PERINATAL hypoxic-ischemic cerebral damage is still a major cause of mortality and morbidity despite the fact that it is often possible to predict neonatal distress. Thus, unlike the situation in the adult population, it may be possible to pretreat the full-term or premature neonate or fetus in the hope of limiting cerebral damage. There are also certain pediatric and adult patients who are subject to planned neurosurgical, vascular, or cardiothoracic intervention in whom it would be feasible to administer a neuroprotective agent.

Recent investigations at this laboratory using a standard model of neonatal hypoxia-ischemia (unilateral carotid artery occlusion followed by 3 hours of hypoxia in 8% O2) have shown that a single dose of dexamethasone (0.1 mg/kg) prevents cerebral infarction providing that it is administered 24 hours prior to the insult. Treatment administered up to 3 hours before hypoxia or after the insult is ineffective. Physiological factors that have been investigated to explain these findings include body temperature, cardiorespiratory function, cerebral blood flow, serum glucose levels and body weight. Only with respect to the latter two parameters is there a difference between dexamethasone-treated and control rat pups, with the dexamethasone-treated group weighing less on the day of hypoxia and having significantly higher serum glucose levels at the end of hypoxia. Further studies have shown that pretreatment with intraperitoneal injections of 10% glucose sufficient to cause hyperglycemia to a similar extent confers some cerebral protection but does not prevent infarction. The neuroprotective effect seen after the administration of dexamethasone in the neonate is surprising as clinical and most experimental studies in adults have shown glucocorticoid therapy to be of no value or detrimental in cerebral ischemia.

In an effort to further characterize the effects of dexamethasone in this model, we reduced the period of pretreatment to 6 hours. As clinical trials in neonates have been proposed to assess the benefits of other agents in preventing neonatal hypoxia-ischemia, we believed
that it would be of value to compare the efficacy of dexamethasone to some of these potential prophylactic agents. As with adult models of cerebral ischemia, calcium channel blockers have been used in the neonate. In particular, pretreatment with the calcium antagonist flunarizine has been shown to confer a degree of protection.\textsuperscript{1,2,4} Because it has been suggested that the protective effect of flunarizine may be complex and involve actions via sodium channels\textsuperscript{51} and free radical scavengers,\textsuperscript{40} we compared the protective capacity of flunarizine with that of the calcium antagonist nimodipine in the same animal model. We also used U-74389F, a 21-aminosteroid or lazaronid, which displays marked inhibition of lipid peroxidation but lacks glucocorticoid activity. Recent studies with this type of drug have shown promising results with regard to prevention of cerebral injury.\textsuperscript{50-52,54} It is believed that the beneficial effect conferred by methylprednisolone administered following spinal cord injury may be mediated via the same mechanism.\textsuperscript{48-50,52}

**Materials and Methods**

**Animal Model of Hypoxia-Ischemia**

Animals were drawn from multiple litters for all treatment groups and concurrent control groups. A well-established and widely used model involving a modification in the Levine preparation was employed to produce cerebral infarction.\textsuperscript{4,6,14,50} In brief, 7-day-old Sprague-Dawley rats were anesthetized with 4% halothane (with a 1% mixture for maintenance), the neck incision was infiltrated with 2% lidocaine, and the right carotid artery was isolated and ligated. The pups were returned to the dam during a 3-hour recovery period, then placed in 8% oxygen with 92% nitrogen in a plastic chamber inside a neonatal incubator with an air temperature of 37°C. Blood glucose was measured before and at the end of hypoxia with glucose oxidase reagent strips using a drop of blood taken from the tail vein in some of the rat pups treated with dexamethasone, vehicle, or flunarizine. The experimental protocol was approved by the Animal Care Committee of the Hospital for Sick Children.

**Drug Administration**

All of the drugs were protected from daylight and stored as suggested by the manufacturer. Drug doses used were in accordance with previous investigations or as suggested by the manufacturer. For each agent, the concentration was adjusted to give a similar volume (0.1 ml/10 gm), and the vehicle for each agent was administered at the same volume and over the same time schedule. Dexamethasone sodium phosphate (0.1 mg/kg)\textsuperscript{*} was injected intraperitoneally 6 hours prior to hypoxia in nine rats. A dose of U-74389F\textsuperscript{†} (10 mg/kg) was administered intraperitoneally at 30 minutes prior to hypoxia and also at the end of hypoxia in 12 animals.

Flunarizine\textsuperscript{‡} was administered in 14 rats at the same dose and over the same time schedule as has been previously shown to be neuroprotective\textsuperscript{7,41} (30 mg/kg intraperitoneally, split dose with one-half administered at the end of carotid artery occlusion and one-half administered 30 minutes prior to hypoxia). Although other investigators have shown that the most efficacious method of administration of nimodipine is via a continuous infusion, this was not feasible in the current model; nimodipine\textsuperscript{§} was therefore administered intraperitoneally at a dose of 0.5 mg/kg 1 hour prior to hypoxia in 12 animals. Nimodipine was also administered in five rats at a dose of 2 mg/kg in a similar fashion to flunarizine administration, with one-half of the dose given after carotid artery occlusion and the remainder given 30 minutes prior to hypoxia. To avoid potential hypotension, a lower dose of nimodipine (70 μg/kg) was administered to an additional group of five rat pups. The number of rat pups in the control groups were as follows: dexamethasone vehicle, 10; flunarizine vehicle, 10; nimodipine vehicle, 10; and U-74389 vehicle, eight.

**Assessment of Pathological Changes**

Five to 8 days after hypoxia-ischemia, with the animals under deep anesthesia (50 mg/kg pentobarbital), the brains were perfusion-fixed via the heart with buffered 10% formalin. The brains were removed, sectioned coronally, and embedded in paraffin. Sections 4-μm thick were cut and stained with hematoxylin and eosin. Three coronal levels were examined histologically: immediately anterior to the corpus callosum, at the level of the third ventricle and posterior striatum, and at the level of the rostral midbrain and posterior hippocampus. All sections were reviewed by a single observer (M.R.D.) who was unaware of the treatment regimens. The areas of necrosis, infarction, and ischemic neurons were marked on prepared schematic diagrams, and the total area of infarction and/or necrosis for each hemisphere was measured using a microcomputer image analysis system.\textsuperscript{\textdagger}

**Statistical Analysis**

Analysis of variance followed by Student's t-test with Bonferroni correction for multiple comparison of means was used to compare the area of infarction, serum glucose, and body weight between the various treatment regimens and control groups. Mortality was compared by Fisher's exact test.

**Results**

Histological assessment of vehicle-treated rat pups revealed ipsilateral infarction of the frontal, parietal, and temporal cortex with occasional sparing of the...
piriform and medial frontal cortex and amygdala. In addition, the caudate-putamen, hippocampus, and dorsolateral nuclei of the thalamus were affected (Fig. 1). In areas of infarction, the tissues had lost their physical integrity and exhibited a mild infiltration by macrophages. In the less severely affected boundary regions, there was individual cell necrosis with astrogial reaction. Usually there were scattered eosinophilic neurons in the contralateral amygdala and piriform cortex, but rarely in the hippocampus and deep cerebral cortical layers, especially the parasagittal region. Occasionally an area of contralateral infarction was observed in the parasagittal region.

The mean area (± standard error of the mean) of infarction did not differ significantly between the various vehicles used in this study; dexamethasone vehicle, 66% ± 9%; nimodipine vehicle, 77% ± 5%; flunarizine vehicle, 50% ± 10%; and U-74389F vehicle, 80% ± 3%; therefore, the vehicle groups were combined for further analysis. Pretreatment with dexamethasone at 6 hours before hypoxia prevented infarction in all nine brains examined (area of infarction 0%), and this difference was statistically significant in comparison to the combined vehicle group and the groups treated with other treatment agents (p < 0.0001). Microscopic examination of these brains revealed either no pathological change or at most rare eosinophilic neurons in the hippocampus and piriform cortex. Pretreatment with flunarizine offered some protection, with a mean area of ipsilateral infarction of 33.6% ± 7.8% (p < 0.05). In contrast, pretreatment with U-74389F or nimodipine (0.5 mg/kg) did not confer any protection (mean area of infarction 59% ± 10% and 77.5% ± 59%, respectively) (Fig. 2). Nimodipine at the higher dose of 2 mg/kg was associated with a prohibitively high mortality rate (four of five rats) and was therefore abandoned.

Nimodipine at the lowest dose (70 µg/kg) failed to show any protective effect (mean area of infarction 74% ± 6%).

There was no significant difference in the rates of mortality during hypoxia except for that seen with the high-dose nimodipine (Table 1). Although no formal behavioral testing was conducted, there were no obvious differences between animals with and without cerebral infarcts. There was a statistically significant increase in the blood glucose level prior to hypoxia in the group treated with dexamethasone 6 hours prior to hypoxia (p < 0.01). In the flunarizine pretreatment group there was a trend toward hyperglycemia but this did not reach statistical significance when compared to the vehicle group. Likewise, by the end of hypoxia, the vehicle- and flunarizine-treated groups had both become hyperglycemic while the serum glucose level in the dexamethasone-treated rat pups had not altered significantly (Table 1).

Discussion

The results from the present study show that pretreatment with low-dose dexamethasone 6 hours prior to hypoxia is capable of completely preventing infarction in this hypoxia-ischemia model. Under identical conditions, neither nimodipine nor U-74389F offered protection; however, the calcium antagonist flunarizine conferred moderate protection against infarction, as has been previously shown. 

**Calcium Channel Blockers**

Cerebral palsy, epilepsy, learning disabilities, and mental retardation remain common sequelae to perinatal hypoxic-ischemic insult, despite the fact that the neonatal brain is relatively resistant to anoxia compared

**Fig. 1.** Photographs of brain sections from a vehicle-treated (control) rat pup (A, B, and C) and brain sections of a rat pup pretreated with dexamethasone 6 hours prior to an hypoxic-ischemic insult (D, E, and F). There is obvious damage in the right hemisphere of the control animal but no infarction in the dexamethasone-treated animal. H & E.
Effect of dexamethasone in rat cerebral hypoxia-ischemia

to the adult brain. This resistance is thought to reflect the lower metabolic rate that is a consequence of lower neuronal activity. However, once a sufficiently severe insult has occurred, a similar pathophysiological cascade is believed to take place in both the neonatal and the adult brain, with calcium influx playing a pivotal role.

Although the relative importance of agonist-operated calcium channels, such as the glutamate N-methyl-D-aspartate (NMDA) receptor channels and voltage-sensitive calcium channels, probably varies with cell type, there is mounting evidence that the glutamate-mediated calcium flux is one of the most important mediators of neuronal damage. Evidence for excitotoxic injury exists in neonatal animals, but the possible long-term complications of manipulating glutamate receptors at this crucial stage of development limit the clinical application of NMDA antagonists.

Clinical trials using voltage-sensitive calcium channel blockers in adult patients are in progress. Three types of channels have been identified: long-lasting, transient, and neither long-lasting nor transient. The dihydropyridine class of calcium antagonists, of which nimodipine is a member, acts only on the long-lasting type. These calcium antagonists inhibit cerebrovascular contraction but may also have a direct protective effect on neurons and glia. Flunarizine is a piperazine derivative that acts on all three channel types as well as inhibits veratridine-sensitive sodium channels. Flunarizine is also distinct from the dihydropyridines in having no antihypertensive effect and no inhibition of cardiac inotropism.

Our results show that neither flunarizine (30 mg/kg) nor nimodipine (0.5 mg/kg and 70 μg/kg) pretreatment is effective in preventing infarction in this neonatal hypoxic-ischemic model. However, as has been previously shown, flunarizine pretreatment significantly decreases the area of infarction, possibly by a combination of vasoactive and neuroprotective effects. It should be noted that, at a lower dose (10 mg/kg intravenously), flunarizine has no beneficial effect and that the dose of nimodipine (30 mg/kg) used in this and other neonatal studies far exceeds that usually suggested (1.5 to 5 mg/kg intravenously).

Inhibition of Lipid Peroxidation

Lipid peroxidation with free radical formation has been proposed as one of the lethal processes in cells subjected to hypoxia-ischemia. There is mounting evidence that the beneficial effects seen after high-dose methylprednisolone treatment for spinal cord trauma are due to inhibition of this process. The 21-aminosteroids (lazaroids) have an increased capacity to inhibit lipid peroxidation but induce no action on the steroid receptor and hence have no steroid side effects. Although the dose of dexamethasone used in our study is of a much lower magnitude than the levels required for lipid peroxidation inhibition by methylprednisolone, we believed that it would be informative to try U-74389F treatment in this model. Despite previous success in reducing cerebral and spinal damage in animal models of trauma and ischemia with this class of drug, we observed no beneficial effect. Likewise, U-74006F, which is similar to U-74689F, failed to prevent cerebral damage in a model of forebrain ischemia, and U-74389F failed to ameliorate purely hypoxic brain damage in neonatal rats. It should be noted, however, that there are no data concerning bioavailability of 21-aminosteroids after intraperitoneal administration.

Steroid Actions

In contrast to the actions of nimodipine, flunarizine, and U-74389F, a single low dose of dexamethasone is capable of completely preventing infarction in the current model when given 6 hours prior to hypoxia. Steroids have a myriad of effects and the mechanism by which dexamethasone prevents hypoxic-ischemic damage remains unknown. A possible mode of action is growth retardation which has been shown to be partially protective against hypoxia-ischemia in the immature brain. Although a significant difference in weight was seen in the animals pretreated with dexamethasone 24

![Fig. 2. Bar graph showing the area of infarction ipsilateral (solid bars) and contralateral (hatched bars) to the occluded carotid artery in 7-day-old rats subjected to 3 hours of hypoxia in 8% O2. The ipsilateral area of infarction differs significantly (p < 0.05) between the dexamethasone (Dex) and flunarizine (Flu)-treated groups, and both are significantly less than the combined vehicle group. The infarct area in the U-74389F- and nimodipine (Nimod)-treated groups is not significantly different from that in the control group. Data are presented as the mean ± standard error of the mean. Statistical significance: * = p < 0.05, compared to vehicle-treated rats; + = p < 0.05, compared to vehicle- and dexamethasone-treated animals.]

### TABLE I

Mortality rates and serum glucose levels in neonatal rats subjected to hypoxia-ischemia

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of Rats</th>
<th>No. (%) Deaths During Hypoxia</th>
<th>Serum Glucose (mM/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prehypoxia</td>
<td>Post-Hypoxia</td>
<td></td>
</tr>
<tr>
<td>combined vehicle</td>
<td>38</td>
<td>8 (21%)</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>dexamethasone</td>
<td>9</td>
<td>0 (0%)</td>
<td>7.4 ± 0.6†</td>
</tr>
<tr>
<td>flunarizine</td>
<td>14</td>
<td>1 (7.1%)</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>nimodipine</td>
<td>12</td>
<td>5 (42%)†</td>
<td>—</td>
</tr>
<tr>
<td>U-74389F</td>
<td>12</td>
<td>1 (8.3%)</td>
<td>—</td>
</tr>
</tbody>
</table>

*Means are expressed ± standard error of the mean. Statistical significance: † = p < 0.01, ‡ = p < 0.05, compared to combined vehicle group. — = not measured.

J. Neurosurg. / Volume 79 / September, 1993
hours before hypoxia, there was no difference in weight after pretreatment at 6 hours in this study.

Pretreatment with dexamethasone 3 hours before hypoxia has been previously shown to be ineffective.\(^4\) The 3- to 6-hour time delay required for dexamethasone treatment to show a neuroprotective effect suggests that protein synthesis may be involved. Conversely, because chronic administration of dexamethasone results in diminished brain weight, deoxyribonucleic acid content, and gene expression of glial fibrillary acidic protein and myelin basic protein in the developing rat,\(^6\) it is possible that dexamethasone acts via protein synthesis inhibition. Pretreatment with dexamethasone is ineffective in preventing kainic acid-induced seizures in neonatal rats\(^1\) and glucocorticoids have been shown to enhance kainic acid-induced brain damage;\(^1\) therefore, it is likely that dexamethasone acts before the Ca\(^{2+}\) influx is initiated.

One potential mode by which glucocorticoids might ameliorate hypoxic-ischemic damage is through an effect on the blood-brain barrier (BBB). Several studies indicate that glucocorticoids not only reduce the BBB disruption association with tumors, cerebral infarction, and kainic acid-induced seizures\(^2,25,31,42\) but that they also decrease the permeability of the intact adult BBB.\(^2,36,53\) In the neonate or premature infant in particular, the BBB may be relatively more permeable than in the adult,\(^47\) and thus dexamethasone treatment could provide some protection during hypoxia/ischemia by altering BBB function prior to the insult.

Another mode of action in the neonate might be alteration of cerebral energy stores. In the neonatal period, it is known that the brain is not solely reliant on glucose but may also utilize other metabolites such as ketones and lactate.\(^2,23,50,52,53\) Chronic administration of hydrocortisone to neonatal mice results in increased brain glucose, glycogen, \(\beta\)-hydroxybutyrate, and cerebral energy reserve with prolonged maintenance of brain adenine triphosphate (ATP) levels after decapitation.\(^43\) Even in adult animals there is some evidence that prior treatment with dexamethasone maintains ATP levels and electrical activity during cerebral ischemia, but is associated with seizures in the reperfusion period and increased cerebral damage.\(^47\) It is conceivable that the secondary insult from the seizures masks the beneficial effect of dexamethasone treatment. If so, then combining glucocorticoid pretreatment with an anticonvulsant agent may prove to be prophylactic even in adult models of hypoxia-ischemia.

Glucocorticoids cause marked gluconeogenesis. Indeed, pretreatment with dexamethasone 6 hours prior to hypoxia results in mild hyperglycemia throughout the period of hypoxia. In contrast, the vehicle-treated rat pups became hypoglycemic during hypoxia. Contrary to what is observed in the adult, hyperglycemia induced by glucose supplementation during or at the end of hypoxia reduces the size of infarction in this neonatal model\(^2,48\) and is associated with increased survival after anoxia.\(^2,24\) Furthermore, it has recently been reported that extreme hyperglycemia (serum glucose levels of 35 to 40 mM) offer complete protection from infarction in this model.\(^35\) Flunarizine in high doses (> 10 mg/kg intravenously) has also been shown to cause hyperglycemia in adult rats.\(^3\) Although a statistically significant difference in serum glucose levels between the vehicle- and flunarizine-treated rat pups was not shown in the present study (perhaps reflecting the timing of the samples and the mode of drug administration), it is possible that some of the protective effect seen with flunarizine is mediated via metabolic changes. While it is well recognized that glucose metabolism in the immature brain differs from that in the adult brain, the protective role during hypoxia-ischemia remains poorly understood.\(^50,53\)

**Conclusions**

The striking ability of pretreatment with low-dose dexamethasone 6 hours before hypoxia to prevent cerebral infarction in the neonatal rat model of hypoxia-ischemia is encouraging and requires further elucidation. In contrast, neither the calcium antagonist nimodipine nor the 21-aminosteroid U-74389F showed any beneficial effect when administered prophylactically. High-dose flunarizine only reduced the size of the infarct. The mechanism of action for these drugs remains obscure. In view of these findings, we recommend that the role of dexamethasone and hyperglycemia in cerebral and spinal insults be studied further to determine whether they may be used to benefit the pediatric population.

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Effect of dexamethasone in rat cerebral hypoxia-ischemia

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Address for Dr. Chumas: Department of Neurosurgery, Institute of Neurological Sciences, Southern General Hospital, Glasgow, Scotland.

Address reprint requests to: Ursula I. Tuor, Ph.D., Division of Neonatology, McMaster Building, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.