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Reduction of Perinatal Hypoxic-Ischemic Brain Damage with Allopurinol

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ABSTRACT. Cytotoxic free radicals are generated during cerebral hypoxia-ischemia and reperfusion. We studied the efficacy of allopurinol, a xanthine oxidase inhibitor and free radical scavenger, in reducing posthypoxic-ischemic damage in the developing brain of 7-d-old rat pups. Hypoxic-ischemic injury to the right cerebral hemisphere was produced by ligation of the right common carotid artery followed by 3 h of hypoxia with 8% oxygen. Thirty to 45 min before the hypoxia, the rats received either allopurinol (dose = 130–138 mg/kg) or an equal vol of saline (0.2 mL). Some pups were killed at 42 h of recovery for measurement of cerebral hemispheric water content, whereas others were killed at 30 or more d for neuropathologic examination. A total of 18 allopurinol treated rats had significantly less water content in the right hemisphere (89.07 ± 0.32%) than 23 saline-treated animals (91.64 ± 0.25%, mean ± SEM, $p < 0.0001$). Rank scoring of neuropathologic alterations revealed that the allopurinol treated rats were less damaged ($p = 0.001$). Only two of 13 brains from the allopurinol group suffered infarction compared to 10 of the 14 saline-treated animals. The results indicate that allopurinol reduces both cerebral edema and the extent of perinatal hypoxic-ischemic brain damage. (*Pediatr Res* 27: 332–336, 1990)

Free radicals contribute to the pathogenesis of hypoxic-ischemic brain damage (1–5), and successful management of traumatic or ischemic brain injury includes prevention of free radical damage (6–10).

During cerebral hypoxia-ischemia, tissue stores of ATP are degraded sequentially to ADP, AMP, adenosine, inosine, and hypoxanthine (11, 12). Hypoxanthine levels accumulate as further metabolism is impeded by tissue hypoxia (13, 14). During reperfusion and reoxygenation, there is a transient rise in xanthine with a concomitant fall in hypoxanthine, reflecting the conversion of hypoxanthine to xanthine by the enzyme xanthine oxidase (15). This reaction generates superoxide and secondarily derived cytotoxic species including hydroxyl radicals (2). Free radicals are highly reactive molecules capable of damaging cells by peroxidation of membrane phospholipids (16) and by oxidation of cellular proteins and nucleic acids (17).

Allopurinol is both an inhibitor of xanthine oxidase (18) and a scavenger of free radicals (19, 20). It has been used successfully to reduce ischemic injury of the heart (21), kidney (22), small intestine (23), and adult rat brain (24, 25). Accordingly, we aimed to establish if allopurinol could reduce hypoxic-ischemic injury to the developing brain of the 7-d-old rat pup.

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MATERIALS AND METHODS

To evaluate the potential neuroprotective effect of allopurinol, we studied 7-d postnatal rat pups in which we induced a hypoxic-ischemic insult to the right cerebral hemisphere (see below). Animals were killed after either a short (42 h) or long (30 d) period of recovery to evaluate the extent of the lesion.

Animals in the short recovery study were evaluated for brain water content, whereas those in the long recovery study were killed after 30 d of age to evaluate long-term neuropathologic alterations. The rat pups were treated alternately with either saline or allopurinol (see below).

Animal model. Seven-d-old Wistar (Charles River, Wilmington, MA) rat pups of either sex, weighing between 12–18 g were anesthetized with a mixture of halothane (4% halothane, 1–1.5% for maintenance), 30% oxygen, and balance nitrous oxide. The right common carotid artery of each pup was ligated with 4–0 surgical silk. The wound was then sutured and the animal allowed to recover. The duration of anesthesia was about 5 min. After surgery, the rat pups were returned to their dams for 2½ h. Pups from mixed litters were then randomly divided into two equal treatment groups. One group received an injection of normal saline (0.2 mL) given s.c. into the back. The other group received 2 mg (0.2 mL) of allopurinol (Zyloprim sodium, Burroughs Wellcome Co., Research Triangle Park, NC). Zyloprim, the sodium salt of allopurinol, after dilution to 10 mg/mL with sterile water, had a pH of 11.5. To permit absorption and distribution of allopurinol, the animals were allowed to recover for another 30 min after injection before being exposed to cerebral hypoxia-ischemia (see below).

A previous study (24), supported by limited experiments in our laboratory, suggested a dose in excess of 100 mg/kg was needed to demonstrate a neuroprotective effect. The dose used in this study was limited by injection volume and by the occurrence of subcutaneous hemorrhages if higher concentrations of allopurinol were used.

As each pup received the same amount of allopurinol (2 mg), variations in animal wt would have caused minor differences in the mg/kg dose per animal. The average amount of allopurinol received by the 63 pups in the short recovery and 35 pups in the long recovery study was 138 and 130 mg/kg, respectively.

Inasmuch as allopurinol has an alkaline pH, the acid base status of eight allopurinol-treated pups was compared with eight saline-treated pups. Blood was collected from severed neck vessels 1 h after s.c. allopurinol (135 mg/kg) or an equivalent vol of saline.

Allopurinol did not cause a significant alteration in acid base status. The allopurinol group had a pH of 7.31 ± 0.09 (mean ± SEM) *versus* 7.26 ± 0.04 in the saline-treated group ($p = 0.19$).

Bicarbonate levels were 21.2 ± 4.9 and 18.9 ± 2.9 in the allopurinol and saline groups, respectively ($p = 0.27$).

Four rat pups (two from each treatment group) were placed in each of four 500-mL airtight jars and exposed to an 8% oxygen-92% nitrogen mixture (26). The jars were partially submerged in a 37°C water bath to maintain a constant thermal environment. Air temperature in the jar was measured at 32°C. After 3 h of hypoxia, the jars were opened to room air and the surviving pups returned to their dams. Survival was defined as spontaneous recovery in air after 3 h of hypoxia. This insult is known to produce selective neuronal necrosis or infarction restricted to the cerebral hemisphere ipsilateral to the common carotid artery ligation (26).

Brain swelling: water content. After 42 h of recovery with their dams, the pups were decapitated and their brains removed. A portion (150–200 mg) from the posterior half of each cerebral hemisphere was placed in a preweighed 5-mL glass vial and then reweighed on a microanalytical balance. The posterior half of the right hemisphere (ipsilateral to common carotid artery ligation) represented the area most severely injured, whereas the corresponding area in the left hemisphere served as control. Subsequently, the specimen was dessicated at 70°C for 48–72 h. Reweighing ascertained the dry wt of the tissue, and by subtraction from the wet wt the water content of the hemisphere was obtained. Water content was determined as a percentage of wet wt according to the formula:

$$\text{water content (WC\%)} = \frac{\text{wet wt} - \text{dry wt}}{\text{wet wt}} \times 100$$

In a separate experiment, twelve 9-d-old rat pups, which were neither ligated nor rendered hypoxic, served as controls. Water content was measured when the pups were the same age as the experimental animals at 42 h of recovery. Water content for each hemisphere was determined as above.

Neuropathologic procedures. To examine long-term neuropathologic outcome, 35 rat pups were carotid artery ligated and rendered hypoxic in the manner described above. However, for this study, allopurinol was given 45 min before hypoxia in an effort to achieve better drug distribution. At the end of the hypoxic exposure, surviving animals were returned to their dams. Treatment groups were identified by clipping the tip of the tail or cutting a small notch in the right ear. These procedures incurred minimal bleeding.

At 30–44 d of recovery the animals were killed with a lethal dose of pentobarbital (150 mg). The brains were carefully removed from the skull and immediately placed in formaldehyde, acetic acid, and methanol, 1:1:8.

Individual brains were later grouped into one of four categories based on the gross morphologic appearance of the right cerebral hemisphere (normal, mild, moderate, severe) (Fig. 1). (Recall that it is the right common carotid artery which was occluded.) Normal referred to no difference in size between the two hemispheres; mild referred to some discrepancy in size between hemispheres with right side smaller than left without any visible infarct; moderate indicated marked discrepancy in size due to a large infarct in the right hemisphere with some preservation of the posterior aspect; and severe related to extensive infarction of the right hemisphere with almost total destruction of its posterior aspect. The anteromedial aspect of the right cerebral hemisphere was always spared even in the severe group.

Two examiners (R.C.V. and J.T.) allocated the brains into one of the four pathologic categories. Examiners were blinded to treatment and were unaware of each other's findings. After grouping, the brains were ranked by each examiner in ascending order of severity and the rankings compared.

After the brains were categorized and ranked according to gross pathology, two brains from each category (one from each treatment group) were examined histologically. This was done to evaluate the microscopic characteristics of each category and to establish if the nature of damage was the same in the two treatment groups. Seven brains were selected (as the brains in the moderate category were all from the saline group, only one was chosen from that category). The brains were coronally cut into 1- to 2-mm slices and then embedded entirely in paraffin for sectioning and staining with hematoxylin and eosin.

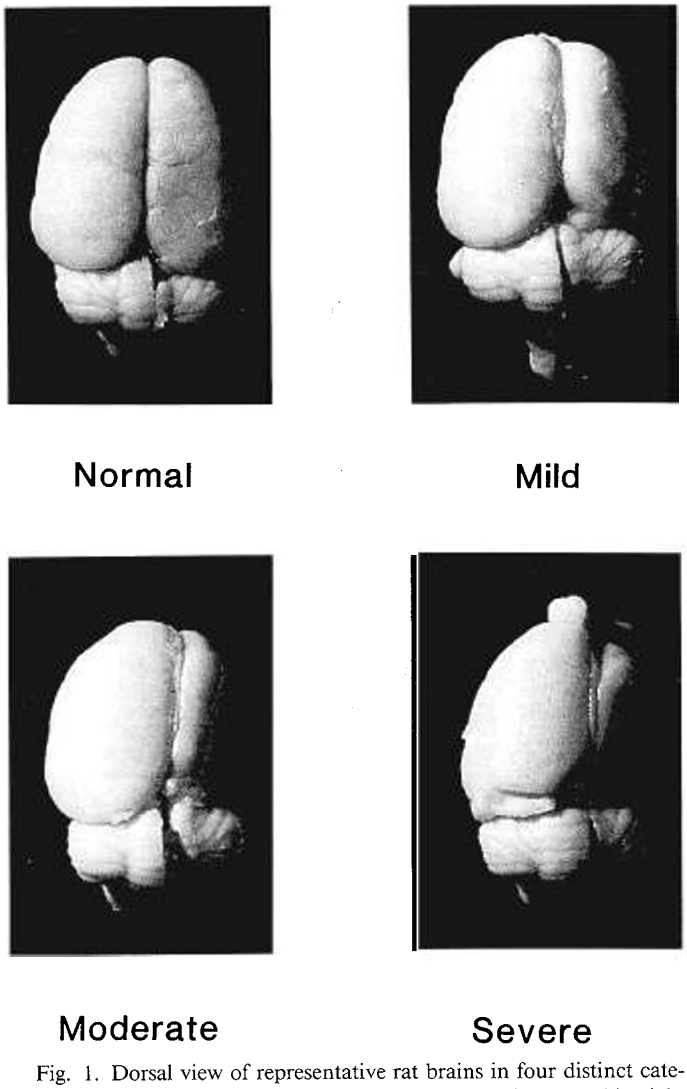

Fig. 1. Dorsal view of representative rat brains in four distinct categories. Normal: symmetric cerebral hemispheres; mild: atrophic right hemisphere; moderate: large infarct with relative preservation of medial aspect of right hemisphere; severe: extensive infarct of right hemisphere including the entire posterior medial aspect.

Statistical analyses. We used the two-tailed Student's t test, Fischer exact test or Mann-Whitney U test where appropriate. $p < 0.05$ was considered significant. The survival studies were analyzed with the Cochran-Mantel-Haenszel statistic and the Breslow-Day test.

RESULTS

Survival studies. In the short-term recovery study, 13/31 (41%) of the allopurinol-treated pups died. The 18 survivors generated an odds for dying ratio of 13/18 (0.72). In the same study, 9/32 (28%) of the saline treated pups died. The 23 survivors generated an odds for dying ratio of 9/23 (0.39). The survival odds for the allopurinol- and saline-treated pups were not significantly different ($p = 0.25$, Cochran-Mantel-Haenszel statistic).

In the long-term recovery study 6/19 (32%) of the allopurinol-treated pups died. The 13 allopurinol survivors made the odds for dying 6/13 (0.46). In the saline treated, 1/16 (1%) died. The 15 survivors made the odds for dying 1/15 (0.03). The difference

in survival odds for the two treatment groups in this study was not statistically significant ($p = 0.07$, Cochran-Mantel-Haenszel statistic).

As more allopurinol-treated pups died in the long-term study compared to the short-term study, we compared the survival data between studies to find that they were not statistically different ($p = 0.28$ Breslow-Day test).

Animal wt. The wt of the pups at 7 d of age were compared for treatment group and survival outcome. In the short-term recovery study, the average wt was 14.47 ± 0.19 g (mean ± SEM). Wt were the same for treatment groups and for survival outcome ($p = 0.37$ analysis of variance). In the long-term recovery study the average rat pup weighed 15.39 ± 0.25 g (mean ± SEM). The 13 allopurinol survivors and six nonsurvivors weighed the same as the 15 saline survivors ($p = 0.39$ analysis of variance).

Water content. The cerebral hemisphere ipsilateral to the carotid artery ligation in the allopurinol treated pups had a lower water content (89.07 ± 0.32%, mean ± SEM) than the saline-treated pups where water content was 91.64 ± 0.25% ($p < 0.0001$) (Table 1). The water content of the left hemisphere (contralateral to the ligation) of both the allopurinol and saline-treated pups was unchanged from the control values of nonligated, nonhypoxic rat pups.

Gross neuropathology. From among the 13 surviving allopurinol-treated and 15 surviving saline-treated pups, one saline-treated pup died during the long-term recovery period. Two examiners (R.C.V. or J.T.), blinded to treatment, grouped each of the fixed brains of 27 surviving pups into one of four categories: normal, mild, moderate, severe. The results of the first examiner are illustrated in Table 2. Significantly less damage was seen in the allopurinol treated pups (Fisher exact $p = 0.005$). Only two of 13 allopurinol-treated pups had more than mild damage, whereas 10 of 14 saline-treated animals fell into the moderate or severe categories characterized by marked atrophic and cystic damage. The second examiner confirmed that the allopurinol treated were less damaged ($p = 0.012$). This categorization differed from the first examiner only slightly (see legend to Table 2).

The brains were also ranked in order of severity and the sum of the ranks compared. The examiners concurred that the allopurinol treated animals were significantly less damaged ($p = 0.001$, Mann-Whitney U).

Histopathology. The normal category showed no abnormalities. Alterations of the right cerebral cortex in the mild category included thinning of the cerebral cortex. The cortical thickness was reduced to two-thirds to three-fourths normal size in the middle cerebral artery territory due to neuronal loss and gliosis in a roughly columnar fashion with marked shrinkage of the hippocampus due to severe neuronal loss and gliosis with only parts of the fascia dentata remaining (Fig. 2). There were also patchy areas of neuronal loss and gliosis present in the dorsolateral and ventroposterior thalamic nuclei. The moderate category had extensive cystic infarction of the right hemisphere involving cerebral cortex and white matter (anterior, superior, and medial aspects of the frontal lobe was relatively spared), the entire hippocampus, amygdaloid nucleus, basal ganglia, and the dorsolateral thalamus. Septal nuclei had only patchy loss of neurons and gliosis. The right superior colliculus was mildly atrophic, and the right pyramidal tract in the brainstem was small. In the severe category, the right cerebral hemisphere was virtually totally destroyed and replaced by a cystic infarct with the exception of a small rim of cortex medially and anteriorly as well as the medial hypothalamus. There was a small strip of neuronal loss and gliosis in the supracallosal cortex bilaterally, with the lesion on the right side being larger. There was also moderate to marked atrophy of the right superior colliculus and pyramidal tract.

Table 1. *Water content as percentage of cerebral hemisphere wt in control and experimental (hypoxic-ischemic) rat pups pretreated with allopurinol or saline**

Group	Cerebral hemispheric water content %	
	Ipsilateral (R)	Contralateral (L)
Controls ($n = 12$)	87.72 ± 0.06	87.71 ± 0.04
Experimental		
Allopurinol ($n = 18$)	89.07 ± 0.32†‡	87.66 ± 0.16
Saline ($n = 23$)	91.64 ± 0.25	87.85 ± 0.09

* Values are mean ± SEM for the numbers of animals in parentheses. Controls were subjected to neither ligation nor hypoxia. Experimental rats were subjected to right (ipsilateral) carotid ligation and 3 h of hypoxia with 8% oxygen.

† Significantly different from saline treated at $p < 0.0001$ (two-tailed, unpaired Student's t test).

‡ Significantly different from controls at $p < 0.01$.

Table 2. *Neuropathologic grading**†

Gross pathologic category	Treatment group	
	Allopurinol (no. of animals)	Saline
Normal	7	1
Mild	4	3
Moderate	0	5
Severe	2	5

* $p = 0.005$ (two-tailed Fisher's exact).

† Neuropathologic grading performed by first examiner. Second examiner differed from above in that one allopurinol animal in the mild category was called normal and one saline in the severe group was called moderate. $p = 0.012$ Fisher's exact.

DISCUSSION

The results of our study show that allopurinol substantially reduces hypoxic-ischemic brain damage in 7-d-old rat pups. In this perinatal model, brain swelling and infarction are produced by a combination of hypoxia and ischemia. These dual insults are necessary as carotid artery ligation or hypoxia alone is unable to cause injury (26).

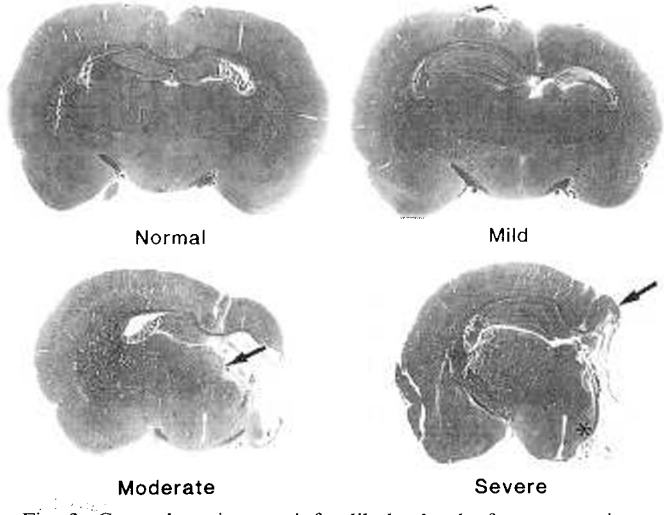

Fig. 2. Coronal sections at infundibular level of representative rat brains from the four categories (compare with Fig. 1). Normal; mild: atrophy of the right hemisphere; moderate: large cystic infarct extending deep and involving dorsolateral thalamus (*arrow*); severe: extensive infarct with almost total destruction of the right hemisphere with preservation of a small rim of cortex dorsomedially (*arrow*) and medial hypothalamus (*asterisk*). (Hemotoxylin and eosin stain, magnification 4×.)

Allopurinol, an analog of hypoxanthine, inhibits xanthine oxidase by virtue of its own substrate activity with this enzyme. The product of the reaction, oxypurinol, is a noncompetitive inhibitor of xanthine oxidase and produces a stable complex with the enzyme. Allopurinol and oxypurinol are distributed in total body water, with the exception of brain, in which their concentrations are approximately one-third those of other tissues. Neither compound is bound to plasma proteins (27).

We chose to measure cerebral water content at 42 h of recovery from hypoxia-ischemia as previous work in our laboratory had shown that edema is well-established at this interval (28). The vasogenic component of cerebral edema requires injury to the capillary endothelial cell, which then allows macromolecules, *e.g.* albumin, and water to pass through the blood-brain barrier (29). Cerebral capillary endothelial cells may be particularly susceptible to free radical attack because xanthine oxidase is concentrated 3.7 times within these cells compared to a homogenate of rat cerebral cortex (30). Zweier *et al.* (31) showed that endothelial cells are potent sources of cytotoxic superoxide and hydroxyl free radicals. They demonstrated that xanthine oxidase was the source of free radicals and that free radical generation could be inhibited with allopurinol and oxypurinol. Chan *et al.* (32) showed that intracerebral injection of the free radical generating system (hypoxanthine and xanthine oxidase) in rats resulted in structural damage to neurons, increased permeability of the blood-brain barrier, and increased brain water content.

Free radicals stimulate membrane phospholipase activity during ischemia causing the release of polyunsaturated fatty acids. The oxidation of these fatty acids via the cyclooxygenase and lipooxygenase pathways forms thromboxanes, prostaglandins, and leukotrienes that induce chemotaxis, inflammation and more oxygen free radicals (33–36). Free radicals have other sources during ischemia including mitochondrial oxidation (37), lactic acidosis (38), and activated white cells (39). Patt *et al.* (40) measured hydrogen peroxide in the brains of gerbil exposed to unilateral carotid artery occlusion and reperfusion. Gerbils were fed a tungsten-rich diet to inactivate xanthine oxidase and those who received this diet for 4, 5, and 6 wk developed progressive decreases in brain xanthine oxidase activity. This correlated with decreased cerebral edema and brain hydrogen peroxide levels, suggesting that hydrogen peroxide was derived from xanthine oxidase activity, and that it contributes to reperfusion-induced cerebral edema.

Although results of our study show allopurinol markedly reduced brain water content in the right hemisphere (ipsilateral to the ligation), it had no effect on the left hemisphere. This suggests that the drug does not exert an osmotic influence.

Our results are consistent with the work of Itoh *et al.* (24) who treated adult rats with large doses of allopurinol (200 mg/kg) 24 and again 1 h before 4 h of reversible cerebral ischemia. The study showed that cerebral water content 1 h after reperfusion was reduced with allopurinol. The findings were accompanied by significantly improved survival rates. Pigott *et al.* (41) demonstrated that allopurinol treatment prevented death in all six adult rats exposed to 15 min of global cerebral ischemia, whereas no rats treated with normal saline survived. They also showed that the arachidonic acid content of ischemic brain was reduced by allopurinol pretreatment. Recently, Martz *et al.* (25) reduced cerebral infarct volume in adult rats by 32–35% with allopurinol treatment, which was given in doses of 100 mg/kg 48, 24, and 1 h before permanent middle cerebral artery occlusion.

The model of perinatal hypoxic-ischemic brain damage used in this study includes ligation of the right common carotid artery that reduces blood flow in individual structures of the right hemisphere to 16–41% of normal (42). Although incomplete, this degree of ischemia combined with hypoxia, is severe enough to cause a loss of ATP (43, 44) and an accumulation of hypoxanthine (Palmer C, unpublished data). This incomplete ischemia might also have supplied enough oxygen to tissues at the edge of the ischemic zone to permit xanthine oxidase activity or to enable the oxygen requiring cycloxygenase and lipoxygenase enzyme systems to generate eicosanoids and more free radicals (34). Furthermore, oxygen free radicals cause inhibition of mitochondrial respiration (45). In this regard, studies in adult animals have shown a near complete recovery of the cerebral energy state upon recirculation after complete ischemia but not after incomplete ischemia (46, 47).

Although much of allopurinol's success in reducing ischemic-reperfusion injury has been attributed to its ability to inhibit xanthine oxidase, other mechanisms warrant consideration. The reaction catalyzed by xanthine oxidase is the first irreversible step in the catabolic breakdown of ATP. Inhibition of xanthine oxidase activity by allopurinol might have reduced the depletion of purine bases and enhanced their reconversion to nucleotides. Lasley *et al.* (48) using a Langendorff isolated heart apparatus, showed that hearts treated with allopurinol exhibit greater ATP levels and improved function during reperfusion following ischemia than controls. Cunningham *et al.* (49) reported a conservation of ATP and total adenine nucleotides in ischemic kidneys after allopurinol treatment. Relevant to the brain, the key enzyme in the purine salvage pathway, hypoxanthine-guanine-phosphoribosyltransferase, which catalyzes the one step formation of nucleotides from hypoxanthine, is found homogeneously in the rat CNS. Moreover, there is a 3-fold increase in its activity in the first 15 to 20 d of postnatal life (50). The brain even more than other tissues recycles hypoxanthine and converts it into purine nucleotides (51). In addition, allopurinol ribonucleotide, a metabolite of allopurinol, inhibits 5′ nucleotidase, the enzyme that catalyzes the dephosphorylation of AMP (52). These findings suggest that allopurinol may have enhanced the preservation of adenine cerebral nucleotides, including ATP, during both hypoxia-ischemia and recovery in addition to its inhibition of xanthine oxidase activity.

Of equal or greater interest is the fact that allopurinol and its active metabolite, oxypurinol, are scavengers of free radicals (19, 20). Furthermore, allopurinol has a dose-related inhibitory effect on polymorphonuclear leukocyte lysosomal enzyme release (53). These two dose-related actions are important because of the large doses of allopurinol used in this and other studies that have shown a neuroprotective effect.

In conclusion, we have shown that high dose allopurinol treatment substantially reduces hypoxic-ischemic brain damage in 7-d-old rats. Further studies are required to define its neuroprotective mechanism and its potential for preventing human hypoxic-ischemic brain injury.

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