Ischemic Neurons in Rat Brains After 6, 8, or 10 Minutes of Transient Hypoxic Ischemia*

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ABSTRACT

The incidence and distribution of ischemic (necrotic) neurons in the brains of rats 72 hr after hypoxic ischemia induced via asphyxiation is described and scored. Anesthetized Sprague-Dawley rats (10/group) were endotracheally intubated and had their airways occluded for 6, 8, or 10 min, which resulted, respectively, in approximately 3, 5, or 7 min of pulselessness (MABP < 10 mm Hg). Survival was 10/10, 9/10, and 6/10 in the 6-, 8-, and 10-min groups; deaths occurred within 1 hr after resuscitation. At 72 hr, rats were reanesthetized and their brains were perfusion-fixed with 3% buffered paraformaldehyde. Paraffin-embedded, 5-μm-thick, H&E-stained sections at 5 coronal levels of the brain had shrunken, hypereosinophilic ischemic neurons in 12 anatomic regions. Ischemic neurons were most consistently found in the lateral reticular thalamic nucleus; lateral caudoputamen; CA1 region of the hippocampus; subiculum; and, with longer asphyxia times, among cerebellar Purkinje neurons. Categorical histologic damage scores were assigned to affected regions on the basis of manual counts of ischemic neurons and summed for the whole brain. Brain histologic damage scores were significantly (p < 0.01) different for the 6-, 8-, and 10-min groups (means of 8 ± 2; 14 ± 4; and 22 ± 4). Brain regions where both the number of rats affected and ranked categorical scores for ischemic neurons increased with asphyxia time were the lateral caudoputamen and cerebellar Purkinje neurons.

Keywords. Neuropathology; animal model; asphyxiation; clinical deficit; cardiac arrest

INTRODUCTION

Previous clinical observations and experimental studies have demonstrated 2 fundamental properties of neuronal death after global ischemic brain damage: the phenomenon of delayed neuronal death and the existence of selective vulnerability (14). Because neuronal death is delayed, sufficient time must elapse after the insult to allow development of the full spectrum of neuronal death prior to histologic examination. Since different brain regions have different vulnerabilities, all regions of the brain must be assessed.

Our research group has described and scored the prevalence of necrotic neurons throughout the postischemic brain to screen therapeutic interventions in monkeys (9) and dogs (13, 18). The present paper describes the abundance and distribution of ischemic (necrotic) neurons in rat brains 72 hr after 6, 8, or 10 min of hypoxic ischemia and subsequent pulselessness (transient cardiac arrest) induced by asphyxiation. A method of scoring this histologic brain damage and the correlation of histologic brain damage scores with duration of asphyxia is presented. An abstract of this material has been previously published (12).

MATERIALS AND METHODS

These studies were performed with the approval of the Institutional Animal Care and Use Committee of the University of Pittsburgh. Thirty male outbred Sprague-Dawley rats (Zivic Miller, Zelienople, PA) weighing 350–450 g were randomly assigned to 3 groups of 10 rats each. One group was subjected to 6 min of asphyxia; the second group had 8 min of asphyxia; and the third group had 10 min of asphyxia.

Experimental Insult

Details of this asphyxial hypoxic ischemia cardiac arrest outcome model have been published (7). Briefly, rats were anesthetized with halothane, oxygen, and nitrous oxide, endotracheally intubated, and maintained on a piston ventilator. They were kept at a tympanic membrane temperature of 37.5 ± 0.2°C with the aid of a heating pad and heat lamp. The left femoral artery and vein were cannulated, and mean arterial blood pressure and ECG were continually recorded. Blood gases, pH, glucose, and hematocrit were measured before and after asphyxia. Vecuronium, 2 mg/kg, was administered iv to eliminate respiratory efforts, the ventilator was disconnected, and the tracheal tube was clamped during the period of asphyxia. Asphyxia time was measured beginning with the occlusion of the endotracheal tube. Pulselessness (mean arterial blood pressure < 10 mm Hg without arterial pressure fluctuations) started at about 3–4 min of apnea. After the measured period of asphyxia (which included cardiac arrest), resuscitation was accomplished by unclamping the tracheal tube and ventilating with 100% oxygen, giving epinephrine (0.01 mg/kg iv) and sodium bicarbonate (1 mmol/kg iv), and applying sternal compressions at a rate of 200/min. Resuscitation with a return to spontaneous circulation (ROSC) was accomplished within 2 min (± 45 sec). Thirty minutes after ROSC, a 1-ml bolus of isotonic saline was given (iv) to each rat. Sixty minutes after ROSC, the catheters were removed and the ventilator disconnected. Spontaneous respirations on a T-tube with supplemental oxygen were continued until a gag reflex was present, and the rat was then extubated. All rats that...
died in this study did so within an hour after resuscitation. Subcutaneous isotonic saline, 20 ml/kg daily, was given after recovery to all rats that were judged to be dehydrated by clinical observation and skin turgor.

**Histopathology Techniques**

Preliminary studies were done in which rats after 8 min of asphyxia were killed at intervals of up to 96 hr postinsult and the entire brain step-sectioned at 200-μm intervals. From these preliminary studies, it was determined that the numbers of ischemic neurons did not increase after 72 hr of asphyxia (i.e., that delayed neuronal death was complete), and that 5 coronal sections would include a portion of each neuronal population having ischemic neurons. These 5 coronal levels corresponded to a standard rat brain atlas (10) as follows: Level I was between plates 16 and 22; Level II was between plates 29–31; Level III was between plates 36–38; Level IV was between plates 40–46; and Level V was between plates 59–65. The number of ischemic neurons at all levels in pertinent anatomic regions (bilaterally) were summed for the regional count of ischemic neurons.

Seventy-two hours after the hypoxic ischemic insult, rats were reanesthetized with halothane, oxygen, and nitrous oxide and endotracheally intubated. Rats were perfused with 150 ml of 3% buffered paraformaldehyde from a drip bag suspended 100 cm above the table administered via sternal thoracotomy and cannulation of the left ventricle with insertion of the cannula tip into the ascending aorta. Brains were hardened in situ for at least 24 hr at 4°C, and after removal from the skull, were stored in 3% paraformaldehyde. A rat brain matrix (ASI Instruments, Inc., Warren, MI) was used to obtain uniform sections of the brain. Single sections were taken from: the level of the optic chiasm; just anterior to the pons; and at the widest part of the cerebellum. Due to the relative paucity of surface landmarks for levels II and III, the middle third of the brain was cut into 2-mm-thick sections and then step-sectioned at 300-μm intervals. Sections corresponding to the desired levels were then chosen from the resulting slides. Routine paraffin-embedding was followed by sectioning to 5-μm thickness and staining with hematoxylin and eosin. Transmission electron microscopy was done on the caudoputamen of a separate rat subjected to 8 min of asphyxia to further characterize the neuronal alterations. For electron microscopy, initial perfusion-fixation and subsequent immersion in 3% paraformaldehyde was done as described above; however, postfixation was in 1% osmium tetroxide. Dehydration through graded ethanol was followed by embedding in methacrylate and sectioning at 90-nm thickness on a Sorvall MT 5000 microtome. Examination was with a Phillips 400 transmission electron microscope.

**Histopathology Evaluation**

All sections were systematically examined at 200× total magnification, and all regions with ischemic (necrotic) neurons were noted. Necrotic neurons in affected neuronal populations (bilaterally) were manually counted by a veterinary pathologist (AR), without regard for the total number of neurons in the region, and put into 5 ranked categories as follows: 0 necrotic neurons = 0; 1–15 = (+1); 16–30 = (+2); 31–50 = (+3); and >50 = (+4). Ranked category assignments for all regions were summed to obtain the overall brain histologic damage score. Karyorrhectic nuclei were counted by visually reconstructing each nucleus from the fragments.

**Statistics**

Overall brain histologic damage scores were compared using the Mann–Whitney U-test. Regional trends in increased incidence with asphyxia time were calculated using a Cochran–Armitage trend test, and regional trends in increased categorical severity were calculated using Jonckheere’s test (5).

**RESULTS**

Time from the onset of asphyxia (clamping of the endotracheal tube) to pulselessness (mean arterial blood pressure < 10 mm Hg) was 195 ± 30 sec. Return to spontaneous circulation (ROSC) was accomplished within 60 sec. One of the 10 rats in the 8-min, and 4 of 10 in the 10-min asphyxia groups died within an hour after resuscitation and were excluded from histologic evaluation.

When examined 72 hr after the hypoxic ischemic insult, ischemic (necrotic) neurons in the brain were shrunken; had pyknotic or karyorrhectic nuclei; and their cytoplasm was brightly eosinophilic and lacked visible Nissl substance. Karyorrhexis was only prominent in the dead neurons in the subiculum. In previous studies “instrumented” controls have had brains without ischemic neurons (7).

Figures 1 and 2 show the brain regions containing neuronal populations with ischemic neurons, and dots indicate general areas where ischemic neurons were concentrated. For all 3 asphyxia times, the scores for the cerebral cortex (the total for the 4 levels that included cerebrum) were +1 (less than 15 ischemic neurons total), while the incidence (number of rats with ischemic neurons in the cortex) increased with asphyxia time (Table I).

At the most rostral level (Level I), the induseum griseum, a small collection of neurons just above the medial corpus callosum, sometimes had ischemic neurons, but neither their incidence in the group of rats nor their rank category corresponded with asphyxia time. Ischemic neurons were most frequent in the lateral caudoputamen. Both the ranked category and the incidence (number of rats affected) in the caudoputamen increased (p < 0.05 with trend test) with asphyxia time (Table I).

The lateral reticular thalamic nucleus (Level II) had a 100% incidence (all surviving rats affected) of ischemic neurons, and ranked category increased with asphyxia time (Table I). The rostral hippocampal formation had ischemic neurons in 3 different regions: CA1 pyramidal neurons (100% incidence but variable ranked categories); CA4 polymorph neurons (high incidence but low categorical rank); and subiculum (high incidence and categorical rank that increased with asphyxia time) (Fig. 2 and Table I). The selective vulnerability of the CA1 region of hippocampal pyramidal cells was striking (Fig. 3). The ischemic neurons of the subiculum at 72 hr after...
Asphyxia had little visible cytoplasm; their presence was marked by karyorrhectic nuclear debris (Fig. 4).

The hippocampal formation was also present on levels III and IV. Ischemic neurons on Level III were included with those at Level II for determining regional categorical ranks and incidence, but hippocampal neurons at Level IV were not found to be ischemic. At Level IV, ischemic neurons in the substantia nigra had low incidence and categorical rank (Table I). The substantia nigra was present also at Level III, but ischemic neurons were not found at this level. Purkinje neurons in the cerebellum (Level V) were not affected after 6 min of asphyxia, but their category ranks increased with asphyxia time in all surviving rats in the 8- and 10-min asphyxia groups (Table I). The appropriate trend tests showed significance ($p < 0.05$) in increases in both the incidence and the mean categorical scores for cerebellar Purkinje neurons. Thus, brain regions that had significantly ($p < 0.05$) increased incidence and/or ranked category of ischemic neurons with increased asphyxia time included: cortex (increased incidence); caudoputamen (increased incidence and ranked category); subiculum (increased ranked category); thalamus (increased ranked category); and cerebellum (increased incidence and ranked category) (Table I).

Transmission electron microscopy confirmed that ischemic neurons in the caudoputamen were shrunken, and electron dense (Fig. 5).

The mean brain histologic damage scores for the rats that survived to 72 hr in the 6-, 8-, and 10-min asphyxia time groups were 8 ($\pm 2$); 14 ($\pm 4$); and 22 ($\pm 4$), respectively (Fig. 6). The Mann–Whitney $U$-test comparing each pair of time groups gave values of $p < 0.01$. Brain histologic damage scores increased (worsened) with increased asphyxia time.

## DISCUSSION

Waiting for a long period after an ischemic insult might result in neurons being resorbed and invisible to manual light microscopic counting methods, while evaluation after too short an interval might not allow appreciation of the full extent of damage due to the delayed expression of neuronal death. Seventy-two hours was chosen as the interval between the hypoxic ischemic insult and histological examination, based on preliminary studies with this model indicating that visible neuronal death increased up to 72 hr but was not increased further at 96 hr. In a 4-vessel occlusion model of transient brain isch-
chemia in the rat, a significant increase in ischemic neurons was noted in the neocortex and hippocampus between 24 and 72 hr postischemia (11), supporting the conclusion that delayed neuronal death continues until at least 72 hr.

In this asphyxia model of brain damage in the rat, the paucity of damage at all levels of the cortex, and the 100% incidence of ischemic neurons in the lateral reticular nucleus of the thalamus, were both striking. In studies with transient brain ischemia in dogs and monkeys, there were many ischemic neurons in the cortex but few in the thalamus (9, 13). Even among experiments using rats, distribution of ischemic neurons in the brain appears to vary with the techniques used to create transient ischemia. In a model of rat brain ischemia based on occlusion of the common carotid arteries, neuronal damage was reported to be concentrated in the septal two-thirds of the hippocampus, and scoring at 16–18 wk postischemia was based on a comparison of remaining viable neurons in the affected hippocampus in comparison to neuron numbers in control rats (11). For the present asphyxia model, there were ischemic neurons in so many
regions that determining baseline neuronal numbers by manual counting in controls in all the regions was not feasible. Since the absolute number of ischemic neurons depends on the total number of neurons present in a particular region, the present study assumed that evaluation of consistent levels of the brain would produce consistency in the number of neurons across animals. A possible disadvantage of the present method is that dead neurons that disappeared from the plane of section could not be evaluated. A digitizer method for assessing damaged neurons has been studied (3). Theoretically, automated methods of cell discrimination, which could count the enormous numbers of neurons throughout the brain, could enable comparisons between normal numbers of neurons and neurons remaining “intact” after the ischemic insult—the difference would then be the number of neurons destroyed by the insult.

The scoring system presented was developed because a regional counting approach helped to organize the absolute number of ischemic neurons according to distribution. Because there were differing numbers of total neurons in each region, ranked categories based on the absolute number of ischemic neurons did not reflect the proportion of that region affected, but rather the number that contributed to the overall number of ischemic neurons in the entire brain. Summing the regional histologic damage scores in each rat resulted in a score for the entire brain that correlated with asphyxia time. Manual counting rons in the entire brain. Summing the regional histologic proportion of that region affected, but rather the number of neurons in each region, ranked categories based on the distribution. Because there were differing numbers of total neurons destroyed by the insult.

It is intuitive that the number of ischemic neurons should increase with an increase in asphyxia time. However, this correlation was not consistently found at 96 hr after more than 12.5 min of cardiac ventricular fibrillation in dogs (13). In the present asphyxia model in rats, an increase in asphyxia time resulted in an increase in both incidence (number of affected rats) and ranked categories of ischemic neurons in the caudoputamen and cerebellum. A previous report using a rat model of carotid arterial occlusion and hypotension suggested that neuronal damage in the caudoputamen was an all-or-none phenomenon (17). However, in the present model, neuronal death in the caudoputamen clearly increased with asphyxia time. Incidence alone increased in the cerebral cortex because none of the groups ever had more than a few ischemic neurons, even when all levels of the cortex were considered. Ranked category of ischemic neurons alone increased with asphyxia time in the lateral reticular thalamic nucleus because there was a 100% incidence for all 3 asphyxia times in this region. The vulnerability of the thalamus to ischemia has previously been noted in both rodents (17) and humans (8).

The selective vulnerability of the CA1 region of hippocampal pyramidal neurons to ischemia is well recognized (16). In the present model, all surviving rats had CA1 damage. However, ranked category of ischemic hippocampal CA1 pyramidal neurons was not useful in distinguishing between durations of asphyxia since there were fewer ischemic neurons noted after 10 vs 8 min of asphyxia. In a gerbil carotid artery occlusion model of brain ischemia, hippocampal neurons showed damage more quickly following prolonged ischemia than after shorter periods of ischemia (2). If hippocampal neurons die more quickly after 10 vs 8 min of asphyxia in the present model, they may have been more rapidly absorbed, resulting in fewer visible ischemic neurons for the 10- vs the 8-min insult.

There was some variation among animals within the same asphyxia time groups. This variation might have been due to the small (less than 1 min) difference in the length of pulseless in rats in the same asphyxia time group, but was more likely due to individual biologic variation. Similar variability was seen in dogs made pulseless by cardiac ventricular fibrillation and resuscitated with cardiopulmonary bypass, techniques that enabled very precise, reproducible durations of brain ischemia (15).

A recent study (4) suggests that astrocytes and microglia in addition to necrotic neurons can be evaluated in neurotoxicity safety studies, and that special stains can contribute to an understanding of the processes involved in brain lesions. While such an approach certainly gives a more thorough understanding of neurotoxicants, there is also a need for a relatively low-cost, yet accurate, system of evaluating relative brain damage in initial efficacy screens of agents designed to ameliorate brain damage after injury. Because neurons are recognized as the functional units of the brain (6), and since neurons have no

![Graph](https://example.com/graph.png)
postnatal ability to multiply (1), the death of neurons can be considered an important manifestation of brain damage. Knowledge of the distribution and regional numbers of ischemic neurons with increasing duration of asphyxia in this model will facilitate its use in screening therapeutic interventions for transient brain ischemia. Despite some regions not showing an increase of ischemic neurons with an increase in asphyxia time in the present study, the group total ranked brain histologic damage scores did show significant increases with asphyxia time. Brain regions where both the number of rats affected and the categorical scores for ischemic neurons increased with asphyxia time in this model were the lateral caudoputamen and cerebellar Purkinje neurons.

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REFERENCES