Embolic Stroke in Aged Rats

Nancy Futrell, MD; Julio H. Garcia, MD; Edward Peterson, PhD; and Clark Millikan, MD

Background and Purpose: Although stroke is a disorder associated with aging, experimental studies of stroke are conducted in young adult (2-4-month-old) animals (rat life span, 27-29 months). To determine whether histopathologic changes caused by cerebral infarction would be altered in aged animals, we produced embolic cerebral infarction in 17 aged (23-24-month-old) and 16 young (2-4-month-old) rats.

Methods: The right common carotid artery was irradiated with a laser (632 nm, 200 mW/cm², 15-20 minutes) after the intravenous injection of the photosensitizing dye Photofrin II (12.5 mg/kg). This produces a nonocclusive platelet thrombus that spontaneously embolizes to the brain. Animals were killed 4 days later.

Results: Analysis was done on 142 infarcts, 68 in aged rats and 74 in young rats. Hypercellularity, with infiltration of macrophages, was more common within small infarcts (<1 mm) in young than in aged rats (p=0.002), and hypertrophy of astroglial fibers surrounding the infarcts was more prominent in young rats. Larger infarcts (≥1 mm) were often hypocellular, with a trend toward more macrophages in the periphery of the infarcts in young than in old animals (p=0.170).

Conclusions: The infiltration of macrophages into cerebral infarcts and the hypertrophy of astroglial fibrils surrounding these infarcts are reduced in the aged rat. These age-related differences emphasize the importance of using appropriately aged animals in experimental models of stroke. (Stroke 1991;22:1582-1591)
Futrell et al.  Embolic Stroke in Aged Rats

**TABLE 1. Sizes and Locations of Embolic Infarcts in Rats by Age and Strain**

<table>
<thead>
<tr>
<th>Infarct size (μm)</th>
<th>Location</th>
<th>Aged Fisher rats (23–24 mo old)</th>
<th>Young Fisher rats (2-4 mo old)</th>
<th>Wistar</th>
<th>Total/strain</th>
<th>Total/age</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1,000</td>
<td>Cortex</td>
<td>31</td>
<td>16</td>
<td>9</td>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td>≥1,000</td>
<td></td>
<td></td>
<td>6</td>
<td>9</td>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Hippocampus</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Basal ganglia</td>
<td>9</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Thalamus</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td>49</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

**Materials and Methods**

Seventeen aged (23–24-month-old) female Fisher rats (National Institutes of Aging, Dr. Dewitt Hazard), 12 young (2–4-month-old) male Fisher rats (Charles River Breeders), and four young (2–4-month-old) male Wistar rats (Charles River Breeders) were anesthetized with intraperitoneal chloral hydrate, 4.5 mg%. Initial doses of 8 ml/kg in aged rats and 10 ml/kg in young rats were supplemented as needed. Photofrin II, a mixture of hematoporphyrin ethers, 12.5 mg/kg, was injected into the internal jugular vein. The right carotid artery was surgically exposed; a 5-mm length was irradiated with a red laser (632 nm, 200 mW/cm²), as previously described. The duration of irradiation was 20 minutes in the four young Wistar rats, eight of the young Fisher rats, and six of the aged Fisher rats; duration was 15 minutes in the remaining four young Fisher rats and 11 aged Fisher rats. Core body temperature was maintained between 36.5 and 37.5 during irradiation. Sixty minutes after irradiation, the right carotid artery was ligated distal to the site of irradiation to prevent further embolization.

All rats were killed by transcardiac perfusion under chloral hydrate anesthesia 4 days after the experiment. We infused 300 ml normal saline, followed by 300 ml 10% neutral buffered formalin at a pressure of 100 mm Hg monitored with a blood pressure manometer. Brains were removed from the skull 2 hours or more after perfusion and placed in 10% neutral buffered formalin. Gross coronal sections 3 mm in thickness were made with a razor blade, and the left side of the cortex was marked with the razor blade. Three days later, tissues were processed and embedded in paraffin. Serial cross sections of brain tissue 7 μm in thickness were prepared with a microtome. Twenty serial sections (10 slides) were taken from the face of the block and from the second level, 1 mm deep in the block. One slide from each level was stained with hematoxylin and eosin and another slide with glial fibrillary acidic protein, using the avidin–biotin immunoperoxidase technique. Blank slides were saved for subsequent studies.

Infarcts were located by light microscopy, and two dimensions (the longest dimension and that perpendicular to the longest) were measured with a reticle; the average dimension was calculated. Infarcts were recorded by average dimension (<1 mm, 1–2 mm, >2 mm) and location (Table 1). For cell counting within cerebral infarcts, the central portion of each infarct was photographed (35-mm slides) at x40. The normal tissue in the corresponding anatomic area in the contralateral hemisphere was also photographed at x40 and projected onto a screen. Two blinded observers simultaneously counted all the cells within an area 150x150 μm² in each infarct and the contralateral normal tissue. Large infarcts were counted in two areas, the center and the outer portion, if appropriate.

Hypercellular infarcts were defined as those containing 30 cells more than the corresponding normal tissue in an area 150x150 μm². Hypocellular infarcts contain 10 cells less than the corresponding normal tissue in an area 150x150 μm². A reactive periphery refers to a hypercellular reaction in the outer portion of a large infarct with a hypocellular center. Normal cell counts generally ranged between 25 and 55 in an area 150x150 μm², except in the molecular layer of the hippocampus, where the normal count ranged from 2 to 15 cells.

For the comparison of number of infarcts, four groups were defined. The four consisted of aged Fisher, young Fisher irradiated 15 minutes, young Fisher irradiated 20 minutes, and young Wistar. Because the number of infarcts is a count variable and is not normally distributed, the nonparametric two-sample Mann-Whitney test was used. A total of six comparisons were considered. The level of rejection was lowered to 0.01 to adjust for multiple testing.
FIGURE 1. Photomicrographs of cortical infarcts in young and aged rats. Panel A: Hypercellular cortical infarct in young rat at 4 days after infarction. Panel B: Glial fibrillary acidic protein (GFAP) of infarct in panel A. Note the long glial processes extending toward infarcted tissue and intense astroglial hypertrophy surrounding the infarct.

Scale markers = 100 μm.
Panel C: Hypocellular cortical infarct in aged rat at 4 days after infarction, similar in size and location to the infarct in panel A at 4 days. Panel D: GFAP of infarct in panel C. Astroglial cells are hypertrophied, but processes are much shorter than in young rat (panel B). Scale marker=100 μm.
To compare young versus aged rats for differences in the percentage of small infarcts displaying hypercellularity or hypocellularity, a ratio estimate approach from sampling theory was used. For each group the number of infarcts varies. It would be inappropriate to assume that the hypercellularity status of infarcts was independent across all animals. Hence, we used a technique that adjusts for the dependence within animals. This approach assumes that each animal is a cluster and computes estimates of the percentages and variances across clusters. For testing the two groups, an estimate of the covariance was also computed. This approach was also used to analyze the relative size of infarcts for different comparison groups of interest. All tests reported are two-sided, and \( p < 0.05 \) was considered significant except where noted.

### Results

All six of the aged Fisher rats irradiated for 20 minutes either died or were killed because they appeared near death from 2 to 48 hours after irradiation. Three of the 11 aged rats irradiated for 15 minutes were eliminated from the series; one had adhesive arachnoiditis, and two died before the scheduled sacrifice time (within 8 hours). The remaining eight aged rats irradiated for 15 minutes survived to the protocol sacrifice time. All 16 young rats, 12 irradiated for 20 minutes and four irradiated for 15 minutes, survived 4 days. There were 142 cerebral infarcts in the 24 rats, 68 infarcts in eight aged rats and 74 infarcts in 16 young rats. The numbers, sizes, and distributions of these lesions are listed in Table 1. The mean number of infarcts was 5.9 per rat, with a range of 0–16 per rat. The distribution of infarct number was not normal, with a range of 0–16 per rat. The distribution of infarct number was not normal, with a range of 0–16 per rat.

Infarcts <1 mm (average dimension) in size in young rats were more often hypercellular compared with surrounding normal tissue (Figure 1A). Astrocytes were absent within the infarcts, but there was a striking hypertrophy of astrocytic processes around the infarct with elongated fibrils projecting toward the infarct in young rats (Figure 1B). In the aged rats, infarcts <1 mm in size were often hypocellular (Figure 1C), compared with contiguous tissue and the contralateral hemisphere. Of the infarcts <1 mm in young rats, 33.3% were hypercellular, compared with 6.1% for the aged rats \( (p=0.002) \). Hypocellularity was present in 18.4% of the infarcts in aged rats but in only 2.1% of the infarcts in young rats \( (p=0.001) \). These statistics are summarized in Table 2. There was much less glial hypertrophy around the infarcts in old rats, and the astrocytic processes were shorter (Figure 1D). There were no astrocytes with eosinophilic cytoplasm in young or aged rats; “reactive” astrocytes were only identifiable using glial fibrillary acidic protein. The cells within the infarcts were mainly macrophages in young rats (Figure 2A). Only a few macrophages were present within the infarcts in the aged rats (Figure 2B). Occasional mitoses that appeared to be in macrophages occurred in both old and young rats.

Larger infarcts were often hypocellular, with coagulation necrosis in young and aged rats (Figures 2C and 2D). For aged rats, 47% of the large (≥1 mm) infarcts were hypocellular, compared with only 18% of the small infarcts \( (p=0.032) \). For young rats, 39% of large infarcts were hypocellular, compared with 2% of the small infarcts \( (p=0.001) \). Macrophages were often present in a reactive periphery in young rats (Figure 2C); this reactive periphery was less prominent in aged rats (Figure 2D; \( p=0.170 \)). There was mild astrocytic hypertrophy around larger infarcts, but it was less intense than that around small infarcts and was more evident near infarcts with a reactive periphery.

Infarcts with a longest dimension >1,600 \( \mu \)m (the longest dimension seen in an earlier series of 80 photochemically induced embolic cerebral infarcts in 18 young Wistar rats) were tabulated. There were

### Table 2. Summary of Test Significance Levels

<table>
<thead>
<tr>
<th>Cellularity</th>
<th>Infarct size</th>
<th>Age</th>
<th>Hypothesis</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypercellar</td>
<td>Small</td>
<td>...</td>
<td>Young (33%) vs. aged (6%)</td>
<td>0.002</td>
</tr>
<tr>
<td>Hypercellar</td>
<td>Large</td>
<td>...</td>
<td>Young (23%) vs. aged (5%)</td>
<td>0.148</td>
</tr>
<tr>
<td>Hypercellar</td>
<td>...</td>
<td>Young</td>
<td>Small (33%) vs. large (23%)</td>
<td>0.441</td>
</tr>
<tr>
<td>Hypercellar</td>
<td>...</td>
<td>Aged</td>
<td>Small (6%) vs. large (5%)</td>
<td>0.841</td>
</tr>
<tr>
<td>Hypocellular</td>
<td>Small</td>
<td>...</td>
<td>Young (2%) vs. aged (18%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypocellular</td>
<td>Large</td>
<td>...</td>
<td>Young (30%) vs. aged (47%)</td>
<td>0.599</td>
</tr>
<tr>
<td>Hypocellular</td>
<td>...</td>
<td>Young</td>
<td>Small (2%) vs. large (39%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypocellular</td>
<td>...</td>
<td>Aged</td>
<td>Small (18%) vs. large (47%)</td>
<td>0.032</td>
</tr>
<tr>
<td>Periphery of infarct</td>
<td>Hypercellar</td>
<td>Large</td>
<td>Young (23%) vs. aged (5%)</td>
<td>0.170</td>
</tr>
</tbody>
</table>

Small, infarct <1 mm average dimension (see text for details); Large, infarct ≥1 mm average dimension; Young, rat 2–4 mo old; Aged, rat 23–24 mo old.
10 in aged Fisher rats, four in young Fisher rats, and four in young Wistar rats. Ten of these were >3 mm (three in old rats, seven in young rats), including three involving much of the middle cerebral artery territory (one in an old rat and two in young rats). The largest infarcts were in an aged rat that was found dead just before the scheduled 4-day sacrifice and was excluded from the statistical analysis. This rat had infarcts of most of the middle and posterior cerebral artery territories, with massive swelling and midline shift.

The mortality from anesthesia (death before the photochemical experiment) in our entire experience with aged animals is approximately 10%. The mortality often occurred in animals that were wasted (weighing <250 g) and in those with large pituitary adenomas compressing the hypothalamus and the thalamus. There was no early experimental mortality (within 4 hours of the experiment) with 15 minutes of laser irradiation in this series of aged animals, but two of 11 animals died before 4 days.

Discussion

The mortality rate in aged rats is greater than that in young rats, both from adequate anesthesia and from the effects of the photochemical experiment. This is probably related to intercurrent diseases, frequent in aged rats,39 some of which could produce the general wasting that was associated with increased mortality. The high mortality in animals with pituitary adenomas may be secondary to autonomic instability during anesthesia, caused by compression of the hypothalamus and thalamus. The aged rats did not survive following 20 minutes of laser irradiation, but altering the laser irradiation to 15 minutes improved survival in the aged rats while still producing adequate numbers of infarcts in young rats.

The most striking finding in this study was the marked reduction in macrophage activity within cerebral infarcts of aged rats. Most brain macrophages are derived from circulating mononuclear leukocytes,30 with convincing evidence emanating from studies using tritiated thymidine labeling of peripheral blood elements.31 The triggering mechanism for monocyte and macrophage attraction to cerebral infarcts is unknown, but glial cells may play a role in modulation of the immune response in the brain.32 The mechanism for the entry of leukocytes into infarcts is likely adhesion molecules, which are present on the surfaces of these cells, enabling them to bind to the vascular endothelium.33 Although the response of the immune system is known to be decreased in aged animals, the immune alterations are complex, and it is uncertain whether the defect resides in the signaling mechanisms or in the ability of leukocytes to respond to signals. Leukocytes in aging animals may be unable to bind to the endothelium or to use certain modulating substances.34 Conflicting data on active suppression of the immune system with aging21,35 and decreased function of immunologic stem cells36,37 may reflect both the complexity of the regulation of the immune response and the variability in model systems. The variability in the inflammatory response in our study does not appear to be sex related; four 24-month-old male rats have been subsequently studied 2 and 4 days after embolic cerebral infarction, and the infarcts are predominantly hypocellular (N. Futrell, J.H. Garcia, unpublished observations). Aged female rats were initially used because they were readily available.

The mechanism for the decreased astroglial hypertrophy surrounding cerebral infarcts in aged rats compared with young rats is unknown. Because macrophages produce cytokines38 and some cytokines stimulate astrocytes,39,40 the decreased macrophage response in old animals could result in decreased astrocytic hypertrophy. The intensity of macrophage infiltration and astrocytic hypertrophy could be determined by some other factor that is altered with aging.

Although there was a tendency toward hypocellularity in the center of large infarcts in both young and aged rats, there was a macrophage response in the outer portion of these infarcts that was, again, most prominent in young animals. Since most brain macrophages come from the circulation,30 it is not surprising to find them mainly in the periphery of large infarcts (near normally perfused tissue) as early as 4 days after infarction.

The occurrence of large infarcts in this series is different from that observed in our previous series of young animals with photochemical irradiation but no ligation of the right common carotid artery. The carotid was ligated to limit the period of active embolism, thus providing a more precise timing of the embolic infarcts. Ligation of the right common carotid artery may have decreased perfusion pressure, thus decreasing collateral flow to the infarcted area and increasing infarct size. This is consistent with our results in another series of rats with left common carotid artery occlusion and photochemical irradiation of the right common carotid artery.41 Not only were infarcts and emboli evenly distributed between the right and left hemispheres, but large infarcts were often present. Because both hemispheres were likely perfused in large part by the right common carotid artery (left common carotid artery ligated), decreased perfusion pressure to both hemispheres could have reduced collateral circulation. It seems that the size of an infarct is determined not only by the size of the embolus but also by local perfusion characteristics and that platelet emboli can be associated with surprisingly large infarcts in certain settings.

One aged rat survived more than 3 days with infarcts in the territory supplied by much of the middle and posterior cerebral arteries. Two aged rats survived 4 days with much of the middle cerebral artery territory infarcted. These results contrast with death within 5 hours3 after middle cerebral artery occlusion in mature adult (12-month-old) rats. There are two major differences between this experiment.
FIGURE 2. Panel A: High-power photomicrograph of the infarct in Figure 1A, a young rat at 4 days after infarction. The infarcted tissue is packed with lipid-laden macrophages. Scale marker=50 µm. Panel B: High-power photomicrograph of the infarct in Figure 1C, an aged rat at 4 days after infarction. Occasional macrophages can be detected (arrow). Scale marker=50 µm.
Panel C: The edge of a large cortical infarct in a young rat at 4 days after infarction. Most of the infarct is hypocellular (right). Note two occluded vessels within this severely infarcted tissue (arrows). Entire infarct is surrounded by a reactive periphery (left) with lipid-laden macrophages. This macrophage reaction is less than that seen within smaller infarcts (Figure 1A). Scale marker=100 μm. Panel D: The edge of a large cortical infarct in an aged rat at 4 days after infarction. Most of the infarct is hypocellular and is associated with an occluded vessel (arrow), as in the young rat in panel C. There is no appreciable reactive periphery on left. Scale marker=100 μm.
and the failed attempt to keep mature adult (12-month-old) rats alive after middle cerebral artery occlusion. First, our technique is relatively noninvasive, whereas the MCA occlusion required craniotomy.3 Second, we used chloral hydrate anesthesia, which has an acceptable mortality in our series of aged rats.

In conclusion, by decreasing the duration of laser radiation to 15 minutes, we have produced the first nonlethal stroke model in aged rats, and we have demonstrated that macrophage infiltration into cerebral infarcts in aged rats is decreased in comparison with young rats. Because macrophages produce numerous cytokines, some of which have trophic properties, this decreased macrophage response could be a factor in a worsened recovery from stroke with aging. These findings underline the importance of using aged animals in stroke research to avoid age-related distortions in the study of a disorder related to aging in humans.

Acknowledgments

We gratefully acknowledge the technical assistance of Nancy Allar, Alexandra Halverson, and Cessalyn Y. Harvey in the laboratory; George Potts, BA, for assistance with the manuscript; and K.M.A. Welch, MD, for review of the manuscript.

References


Key Words • aging • blood platelets • embolism • rats
The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://stroke.ahajournals.org/content/22/12/1582