Sustained Decrease in Brain Regional Blood Flow After Microsphere Embolism in Rats

Keiko Miyake, MSc; Satoshi Takeo, PhD; and Hiroki Kajihara, MD, PhD

**Background and Purpose:** An experimental model that induces sustained ischemia and infarction may provide useful information relevant to prevention of the development of ischemic brain disease. The purpose of the present study was to elucidate the pathophysiological consequences of cerebral blood flow under sustained cerebral ischemia or oligemia and infarction in rats after microsphere embolism.

**Methods:** We injected 900 microspheres (48 μm in diameter) into the right internal carotid artery of 146 rats and determined the time course of changes in blood flow of the cerebral cortex, striatum, and hippocampus of both hemispheres by the hydrogen clearance method for a period of 28 days after the operation. Infarct area was determined by triphenyltetrazolium chloride staining and hematoxylin and eosin staining methods.

**Results:** Cortical and striatal blood flow of the right hemisphere of microsphere-injected rats was significantly decreased after the embolism, and this was sustained throughout the experiment. Hippocampal blood flow of the microsphere-injected hemisphere was also decreased on days 1 and 3 but tended to return toward control levels thereafter. In the left hemisphere, reduction in regional blood flow was detected in the cortex and hippocampus on day 1 and the striatum on day 3. A triphenyltetrazolium chloride–unstained area had developed by day 3 after the embolism. The extent of the area was similar to that on days 7 and 28. Microscopic examination revealed degenerative areas scattered mainly in the parietotemporal cortex, corpus callosum, hippocampus, thalamus, and lenticular nucleus of the embolized hemisphere, demonstrating the induction of widespread necrosis after embolism.

**Conclusions:** Microsphere embolism resulted in a sustained decrease in regional blood flow and production of cerebral infarction in the brain regions of the microsphere-injected hemisphere. 

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**Key Words** • cerebral blood flow • cerebral infarction • hippocampus • rats

Cerebral embolism with microspheres has been shown to induce biochemical, electrophysiological, neurological, and morphological abnormalities.1–6 Because the diameter of microspheres used as emboli ranged from 15 to 50 μm, the induced embolism has been considered to produce widespread precapillary occlusion, resulting in the development of cerebral ischemia and infarction. In previous studies we have shown that microspheres induced profound changes in cerebral energy metabolites7–9 and neurotransmitter content.10 These included depletion of cerebral high-energy phosphates; accumulation of tissue lactate, glucose, and glycogen; changes in the activity of several enzymes in the tricarboxylic acid cycle; loss of mitochondrial phosphorylation activity; decreases in acetylcholine and neurotransmitter amino acids; and increase in choline of the brain regions of the microsphere-injected hemisphere. Accordingly, microsphere embolism is considered to be capable of inducing severe

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and long-term ischemia or oligemia resulting in disturbances of the energy and neurotransmitter metabolism of the brain. However, the degree of ischemia or oligemia has not been elucidated as yet with respect to cerebral blood flow and morphological structure, which may provide direct evidence for cerebral ischemia and infarction. The purpose of the present study was to determine whether microsphere embolism causes cerebral ischemia or infarction in the hemisphere into which microspheres are introduced.

**Materials and Methods**

We used 242 male Wistar rats weighing 200–220 g (Charles River Japan Inc., Atsugi, Japan) in the present study. The animals were maintained under artificial conditions at 23±1°C, with a constant humidity of 55±5%, a cycle of 12 hours of light and 12 hours of dark, and freely accessed food and tap water, according to the “Guidelines of Experimental Animal Care” issued by the Japanese Prime Minister’s Office. We performed microsphere-induced cerebral embolism by the methods previously described.11 Briefly, 146 rats were anesthetized with 35 mg/kg sodium pentobarbital i.p. After ligation of the right external carotid and pterygopalatine arteries, 900 microspheres (47.5±0.5 μm in diameter; NEN-005, New England Nuclear Inc.,
Boston, Mass.), suspended in 20% dextran solution, were injected into the right internal carotid artery through a polyethylene catheter (3F size, 1.0 mm in diameter, Atom Co., Tokyo) previously inserted into the right common carotid artery. After surgery, the rats were disinfected with povidone-iodine solution. Seventy rats that underwent sham operation were injected with the same volume of vehicle without microspheres, and their right common carotid arteries were ligated. The control group comprised 26 nonoperated rats to examine normal levels of variables of the intact animal. Fifteen hours after the operation, animal behavior was scored on the basis of paucity of movement, truncal curvature, and forced circling during locomotion, which are considered to be typical symptoms of stroke in rats.12,13 Each symptom was ranked from 3 to 0 (3, severe; 2, moderate; 1, light; 0, faint). The sum of these scores was evaluated as a marker of neurological deficits. Rats with >7 points were considered type A; 4–6 points, type B; and <4 points, type C. The type A animals (83 rats) were used for the following experiment.

Cerebral blood flow was measured in seven control, 44 microsphere-injected, and 38 sham-operated rats. The animals were anesthetized with intraperitoneal administration of 35 mg/kg sodium pentobarbital. Arterial blood pressure was monitored via a cannula inserted into the right femoral artery and measured by means of a pressure transducer (model AP-601G, Nihonkohden Co., Tokyo). The heart rate was triggered through the electrocardiogram (lead II) and counted by means of a heart rate counter (model AT-601G, Nihonkohden) throughout the experiment. Rectal temperature of the rat was maintained at 36.5±0.5°C by warming the animal with an electronic panel heater placed underneath it. Tissue blood flow of brain regions was measured according to the method of hydrogen clearance by means of a hydrogen clearance flow meter (model UPS-400, Unique Medical Inc., Tokyo). The head of the rat was fixed in a head holder (model SR-5, Narishige Instrument Co., Tokyo). Three small bar holes were made with an electric mini-drill (model 28400, Proxxton, FRG) in the skull of each side. Two Teflon-coated platinum electrodes, 100 μm in diameter with a 1-mm uncoated portion at their tips and plated with platinum black, were positioned at a depth of 2 mm, 2 mm posterior and 2 mm lateral to the bregma for measurement of blood flow of the cortex, at a depth of 4 mm, 2 mm anterior and 3 mm lateral to the bregma for that of the striatum, and at a depth of 5 mm, 6 mm posterior and 5 mm lateral to the bregma for that of the hippocampus of each hemisphere according to the guide of an atlas of anatomy of rat brain,14 using a stereotactic apparatus (model SR-5, Narishige). The reference Ag–AgCl electrode was inserted under the skin of the neck. The animals inhaled a gas mixture of 90 ml/min air and 10 ml/min hydrogen through a mask loosely fitted to the nose. After ensuring the equilibration of the brain tissue with hydrogen gas, the cerebral blood flow was measured. At approximately 30-minute intervals, the blood flow of the other brain regions was similarly determined.

In a separate set of experiments, we performed blood gas analysis of 10 control rats and four sham-operated and four microsphere-injected rats on day 3 after embolism under the present experimental conditions to ensure the validity of the experimental conditions used. The rats were anesthetized with 35 mg/kg of sodium pentobarbital as described above. A cannula was inserted into the right femoral artery for sampling the blood. Then, the animals inhaled air containing 10% hydrogen gas as described above. Blood (0.2 ml) was sampled before inhalation (0 minutes), three times after equilibration of hydrogen gas in the tissue (30, 60, and 90 minutes), and at the end of the experiment (120 minutes). Then, the blood was immediately analyzed with a blood gas analyzer (model 288, Ciba-Corning Japan, Tokyo).

For determination of the infarct area, 21 microsphere-injected and 23 sham-operated rats were lightly anesthetized with ether and decapitated at different time intervals (days 1, 3, 7, and 28) after the operation. Five rats were also decapitated without any operation (control). The brains were rapidly isolated and photographed. After cooling the brain in a stainless-steel container surrounded with ice, the brain was positioned on a brain holder and coronally sectioned 3, 5, and 7 mm from the frontal pole with razors. The sectioned brain tissue was incubated at 37°C for 30 minutes with 2% of 2,3,5-triphenyltetrazolium chloride (TTC) in physiological saline.15 The slices were transiently immersed into a 10% formalin solution, and then their photographs were taken. The sum of TTC-stained and TTC-unstained (including weakly stained) areas of three brain slices was estimated by a planimetric method.

For microscopic examination, five microsphere-injected, five sham-operated, and four control rats were decapitated on day 3 after the operation, and the brain was quickly isolated. The specimens were fixed for 1 week in 10% formalin solution buffered with phosphates (pH 7.4). Thereafter, serial 2-mm sections were cut from the frontal area of each brain and embedded in paraffin. The paraffin sections were stained with hematoxylin and eosin and observed by light microscopy. The microscopic observation of the samples was performed by a person unaware of the study group.

The results are expressed as mean±SEM. Statistical significance for comparison of values in time course studies was evaluated using analysis of variance, followed by Dunnett’s t test. A confidence value of >95% was considered significant (p<0.05).

Results

The operated rats were inspected 15 hours after surgery. Thirty rats (21%) died within 24 hours of the embolism. Ninety-seven embolized rats (66%) showed type A symptoms, 13 rats (9%) type B, and six rats (4%) type C, which was similar to the previous results.9,11 Among the type A rats, 14 rats (10%) died within 3 days of the embolism. The initial score of microsphere-injected rats used in the present study was 8.6±0.1 (n=74). The scores of rats on days 3, 5, 7, 14, and 28 after the operation were 6.6±0.6, 5.3±0.7, 4.4±1.3, 2.4±1.0, and 1.1±0.5 (n=13–74), respectively, which are similar to earlier findings.11 No neurological deficits were seen in sham-operated rats throughout the experiment.

In the first set of experiments, we performed blood gas analysis to ascertain the physiological profile of anesthetized rats under the same conditions as those undergoing determination of cerebral blood flow ac-
were observed and monitored crosphere-injected of the flow.

As shown in Table 1, there were no differences in pH, $P_{O_2}$, and $P_{CO_2}$ values of control animals that persisted after the operation, whereas the level of $P_{O_2}$ decreased after microsphere injection.

The brains of microsphere-injected and sham-operated rats were isolated on days 1, 3, 7, and 28 after the operation and used for gross observation and TTC staining. On day 3, cerebral vasodilation, edema, and hemorrhage were seen in the right hemisphere of microsphere-injected rats. There was a marked degeneration and/or atrophy of the right hemisphere of

<table>
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<th>Blood pressure (mm Hg)</th>
<th>Time after operation (day)</th>
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<th>3</th>
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<tr>
<td>Sham operated</td>
<td>102±2</td>
<td>100±4</td>
<td>98±3</td>
<td>109±4</td>
<td>100±4</td>
<td>109±1</td>
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<tr>
<td>Microsphere injected</td>
<td>102±2</td>
<td>112±5</td>
<td>110±2</td>
<td>108±3</td>
<td>119±7</td>
<td>113±1</td>
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Heart rate (bpm)

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<th>Blood pressure (mm Hg)</th>
<th>Time after operation (day)</th>
<th>1</th>
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<tr>
<td>Sham operated</td>
<td>423±10</td>
<td>412±17</td>
<td>426±15</td>
<td>449±10</td>
<td>413±10</td>
<td>416±19</td>
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<tr>
<td>Microsphere injected</td>
<td>418±18</td>
<td>411±13</td>
<td>415±14</td>
<td>403±8</td>
<td>411±15</td>
<td>404±7</td>
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Arterial blood pressure and heart rate of control rats were 103±2 mm Hg and 421±17 beats per minute (bpm) ($n=10$, respectively). Each value represents mean±SEM of 7–14 experiments (for details of experiments, see “Materials and Methods”). No significant difference from control was seen at any time examined.

The time courses of changes in blood flow of the cerebral cortex, striatum, and hippocampus of microsphere-injected, sham-operated, and control (without any operation) rats are shown in Figure 1. The initial values of the cortical, striatal, and hippocampal blood flow of the right hemisphere were 42.9±4.7, 36.4±1.3, and 51.0±3.5 ml/100 g tissue per minute, respectively. Microsphere embolism induced a significant reduction in the cortical blood flow of both hemispheres on day 1 after the operation. This low level of cortical blood flow persisted in the right hemisphere throughout the experiment, whereas the cortical blood flow of the left hemisphere returned to normal levels on day 3 but tended to decrease after day 7. The striatal blood flow of the right hemisphere was decreased on day 3 after the operation. This low level was maintained up to 14 days after the operation. A significant decrease in the striatal blood flow of the left hemisphere was also seen on day 3 after the operation, followed by a gradual return to normal levels thereafter. The hippocampal blood flow of the right hemisphere was similar to that of the left hemisphere; however, the blood flow of either hemisphere of sham-operated groups throughout the experiment.

**Figure 1.** Line graphs show time course of changes in blood flow of cerebral cortex, striatum, and hippocampus of right and left hemispheres of microsphere-injected (● and ■), sham-operated (○ and □), and control (○ and □ at day 0; $n=10$) rats, respectively. Each value represents mean±SEM of the following numbers of experiments: microsphere-injected rats, $n=7$, 8, 9, 8, and 6, and sham-operated rats, $n=7$, 6, 7, 5, and 7 on days 1, 3, 5, 7, and 14 after the operation, respectively. *p<0.05 different from control group.
microsphere-injected rats on day 28 after the operation. No such abnormalities were seen in the left hemisphere of microsphere-injected rats and either hemisphere of sham-operated rats throughout the experiment.

TTC staining of brain slices was performed to determine cerebral infarct area (Figure 2). The unstained areas, which included weakly stained areas of the right hemisphere of microsphere-injected rats, was constant by day 3 after the operation. The TTC-unstained area 3 days after the operation was approximately 79% that of the corresponding sham-operated animals. The TTC-stained areas of the right hemisphere on days 7 and 28 were almost the same as those on day 3. No TTC-unstained area was seen in the brain slices of either hemisphere of sham-operated rats, even though the right common carotid artery was permanently ligated.

Brain sections of rats on day 3 after the operation were stained by hematoxylin and eosin. In the brains of sham-operated rats on day 3, bilateral cortices, corpus callosum, hippocampi, thalami, hypothalami, lenticular nuclei, and internal capsules showed no microscopic or histological changes (Figure 3, upper panel). In contrast, the right hemispheres of microsphere-embolized rats were seen to be somewhat swollen by gross observation. By light microscopy, the affected right hemisphere was seen to have undergone degeneration and stained palely compared with the left unaffected hemisphere (Figure 3, lower panel). Scattered necrotic areas, variable in size and shape, were seen mainly in the parietotemporal cortex, corpus callosum, hippocampus, thalamus, and lenticular nucleus of the affected hemisphere. Small hemorrhagic foci were frequently observed in the necrotic areas.

Discussion

The purpose of the present study was to provide direct evidence of cerebral ischemia and/or infarction after microsphere embolism, which may result in severe and sustained damage to brain function and metabolism.

First, we determined cerebral blood flow of microsphere-embolized rats for 28 days after induction of the embolism. A sustained and marked decrease in the blood flow of the cerebral cortex and striatum of the right hemisphere was seen in microsphere-injected rats throughout the experiment. This indicates that microsphere embolism induced sustained ischemia or oligemia in the brain region of the cerebral hemisphere where microspheres were introduced.

The blood flow of the left hemisphere was decreased only in the cerebral cortex, striatum, and/or hippocampus 1 to 3 days after the operation. Several possible explanations can be considered for this phenomenon. These include an attenuation of microspheres to the contralateral hemisphere via Willis’ circle and/or a change in distribution of blood flow normally circulating in the left hemisphere by a mechanism to compensate for a decrease in blood flow of the ipsilateral hemisphere. Since the diameter of the posterior communicating artery of the rat brain, a vessel consisting of Willis’ circle, is $>50 \, \mu m^{10}$, the former is a plausible
mechanism underlying the decrease in blood flow of the contralateral hemisphere.

We observed two interesting phenomena in the time course study of regional blood flow. One is a relatively minor decrease in cerebral blood flow of the striatum on day 1 after the embolism, followed by a sustained decrease in the blood flow thereafter. This suggests that the striatal area is indirectly occluded by the microsphere embolism. The other is a gradual reversal of the hippocampal blood flow with time after the embolism. There are a number of possible explanations for the findings, such as an increase in reactive hyperemia, development of collateral blood flow, and/or changes in blood flow distribution with time after the operation. The exact mechanisms underlying such changes in cerebral blood flow cannot be addressed at present. In spite of that, it is possible to consider that sustained and almost irreversible cerebral ischemia is induced, at least, in the cortical and striatal regions after microsphere embolism, which may lead to functional, morphological, and metabolic impairments in these or adjacent brain areas.

Reduction in blood flow of brain regions was predicted from several findings in our previous studies. These include decreases in tissue high-energy phosphate and acetylcholine and increases in tissue lactate and choline. These changes are considered to be biochemical markers of an ischemic state. We have now demonstrated that microsphere embolism decreases cerebral blood flow, which results in profound metabolic disturbance in the brain.

The TTC-staining method is a convenient method of determining cerebral infarct area, and a close correlation with evaluation of infarct size by hematoxylin and eosin has been described in the literature. Our results show the formation of widespread TTC-unstained or weakly stained areas in the brain slices of the right hemisphere from microsphere-injected animals on day 3 after the operation. The TTC-unstained areas on day 28 were similar to those on day 3, suggesting that cerebral infarction develops within 3 days after the embolism, and the infarct area does not expand further during the period studied in these experiments.

In accordance with this, we observed in the microscopic study that there was widespread degeneration of the microsphere-injected hemisphere, as evidenced by the existence of areas sparsely stained with hematoxylin and eosin. The necrotic areas, variable in size and shape, with small hemorrhagic foci were scattered widely in various brain regions, which is indicative of multifocal necrosis.

Numerous studies have attempted to measure blood flow of brain regions after induction of ischemia and to correlate the reduction in regional blood flow with histological, biochemical, and neurological alterations during the acute phase of ischemia or a short period after ischemia. These include correlation of severe and uniform reduction of blood flow of all brain regions after global ischemia with changes in extracellular amino acid levels in rats and correlation of near zero blood flow in the striatum after four-vessel occlusion with altered monoamine neurotransmitter release in rats. There are also reports of tissue high-energy phosphate depletion in rats treated with a combination of elevation of intracranial pressure and unilateral common carotid artery occlusion and histological changes recorded after occlusion of the proximal middle cerebral artery. Although these results demonstrated the reduction in cerebral blood flow resulting in metabolic and morphological impairments, the close relation between these variables with respect to the extent and period of long-term ischemia or infarction has not yet been established. The previous and present studies in our ischemic model showed a dissociation of change, particularly recovery, of cerebral blood flow and neurological deficit or metabolic disturbance to some extent.

In summary, the present study provides direct evidence of reduction in blood flow of brain regions after microsphere embolism. The induced ischemia resulted in a sustained and severe cerebral infarction within 3 days after the embolism. This model may be useful for the study of functional, biochemical, pathophysiological, and morphological alterations of long-term cerebral ischemia and infarction.

References

Cerebral embolism with microspheres has been shown to induce abnormalities that resemble the development of cerebral ischemia and infarction. By using this approach in the rat, the authors have published a number of papers on the changes of cerebral energy metabolites and neurotransmitter content. In this manuscript the authors continued their effort in examining the changes of brain blood flow and morphological structure.

Their experimental procedure and results were clearly documented. About two thirds of their rats developed strokelike symptoms after microsphere injection through the right internal carotid artery. Generally, the reduction of regional blood flows and degenerative areas were confined to the right hemisphere. The morphological damage appeared to be extensive and irreversible. The surprise finding in their study was that the strokelike symptoms in these microsphere-injected rats disappeared spontaneously in about 2 weeks. The reason for the dissociation between the morphological damage and the neurological deficits remained unclear.

Microsphere-induced embolism in the rat is a useful model in the sense that it can uncover the sequence of events and the extent of damage after sustained cerebral ischemia or oligemia. However, its usefulness as a stroke model may be limited. Since the microsphere-injected rats can recover fully from the strokelike symptoms without any treatment, this may complicate evaluation of potential therapeutic intervention in this animal model.

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