Sustained Damage to Energy Metabolism of Brain Regions After Microsphere Embolism in Rats

Satoshi Takeo, PhD; Taku Taguchi, MSc; Kouichi Tanonaka, PhD; Keiko Miyake, MSc; Tomoko Horiguchi, BSc; Norio Takagi, BSc; and Kannosuke Fujimori, PhD

Background and Purpose: Information on sustained damage to cerebral function and metabolism after cerebral ischemia is useful for prophylaxis and therapeutics of cerebral infarction. The purpose of the present study was to induce sustained damage to brain regions after cerebral ischemia in experimental animals. For this purpose, we examined animal behavior and cerebral energy metabolism following microsphere embolism in rats.

Methods: We injected 900 microspheres (48 µm in diameter) into the right internal carotid artery of 110 rats and determined the time course of changes in the rats' behavior and the energy metabolism of the cortex, striatum, and hippocampus of both hemispheres. We injected the same volume of vehicle, without microspheres, into 28 sham-operated rats; there were 14 nonoperated control rats.

Results: Peak increase in lactate content and decrease in adenosine triphosphate and creatine phosphate of these brain regions of the right hemisphere were seen on the first day after microsphere embolism, whereas peak increases in glucose and glycogen contents of these regions were observed on the third day. Most of the metabolic alterations in all these regions continued for up to 28 days after operation, although they recovered toward control levels with time after the operation. The extent and trend of metabolite changes of the right hemisphere after microsphere embolism were similar in the three brain regions. In the left hemisphere, similar metabolic changes were observed, but to a lesser degree. The time course of changes in behavioral scores following microsphere embolism revealed marked stroke-like symptoms on the first day and relatively rapid disappearance of the symptoms with time after embolism.

Conclusions: Microsphere embolism is capable of inducing widespread, sustained damage to energy metabolism of brain regions. (Stroke 1992;23:62–68)

Shortage or lack of cerebral blood flow induces cerebral oligemia or ischemia, which, even if short, may lead to serious damage to brain function and metabolism. Numerous experimental studies have been attempted, particularly in small animals such as rats and mice, to mimic cerebral oligemia or ischemia and to elucidate its pathophysiological consequences. These included decapitation ischemia,1 compression ischemia,2 and ischemia induced by ligation of middle cerebral artery3 or both carotid and vertebral arteries.4 Embolization with blood clots,5 microspheres,6 or silicon rubber7 has also been shown to induce focal ischemia in the brain regions. Most of these experimental observations, however, concerned pathophysiology of short-term ischemia and thus do not provide information concerning long-lasting cerebral ischemia or damage to cerebral function and metabolism. The purpose of the present study was to induce sustained cerebral damage to brain regions, which may mimic pathophysiological alteration of long-term cerebral ischemia. For this purpose, we produced irreversible embolism with microsphere injection and examined the time course of changes in animal behavior and energy metabolism in brain regions. As markers of energy metabolism in the present study, we measured the level of metabolites relating to energy production in the cerebral cortex, striatum, and hippocampus, which are recognized to be extremely sensitive to oxygen deficiency.8–10

From the Department of Pharmacology (S.T., T.T., K.T., K.M., T.H., N.T.), Tokyo College of Pharmacy, and the Division of Pharmacology (K.F.), Biological Safety Research Center, National Institute of Hygienic Sciences, Tokyo, Japan.

Address for correspondence: Satoshi Takeo, PhD, Department of Pharmacology, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachiouji, Tokyo 192-03, Japan.

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Materials and Methods

We used 177 male Wistar rats weighing 180–220 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. Briefly, 110 rats were anesthetized with 30 mg/kg sodium pentobarbital i.p. After the right external carotid and the right pterygopalatine arteries were ligated with strings, 900 microspheres (47.5±0.5 μm diameter; NEN-005, New England Nuclear Inc., Boston, Mass.), suspended in 20% dextran solution, were injected into the right internal carotid artery through a 2 Fr. polyethylene catheter (Atom Co., Tokyo, Japan) previously inserted into the right common carotid artery. In the present study, we administered a slightly larger number of microspheres than before (approximately a 32% increase) to induce a more definite change in the cerebral energy metabolism. Twenty-eight rats that underwent sham operation were injected with the same volume of vehicle without microspheres. The control group comprised 14 nonoperated rats.

Fifteen hours after the operation, the behavior of the rat was scored on the basis of paucity of movement, truncal curvature, and force circling during locomotion, which are considered to be typical symptoms of stroke. The score of each item was ranked from 3 to 0 (3, very severe; 2, severe; and 1, moderate). Rats with >7 points were considered type A; 4–6, type B; and <4, type C. In the present study, we used only type A animals for the studies on brain energy metabolites and behavioral symptoms.

At an appropriate point in the experimental sequence, the microsphere-injected rats, sham-operated rats, and nonoperated control rats were killed with focal irradiation of microwave to the head for 0.85 seconds by a microwave applicator at 5.0 kW (model TMW-6402c, Muromachi Kikai Co., Tokyo, Japan). After the rats were decapitated, their heads were immersed in liquid nitrogen and left for 10 seconds (near-freezing). The cerebral hemispheres were isolated and separated into three regions: cortex, striatum, and hippocampus. Each region was homogenized in 0.2 M HClO₄ and 0.01% ethylenediaminetetraacetic acid with a Polytron homogenizer (model PT-10, Kinematica, Switzerland) for 15 seconds at maximum speed. After being left for 10 minutes at 0°C, the homogenate was centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was neutralized with 2.5 M K₂CO₃. The resultant solution was then centrifuged at 10,000g for 15 minutes at 4°C. The supernatant was filtered through a membrane filter (0.45 μm), and tissue lactate, adenosine 5'-triphosphate (ATP), creatine phosphate, glucose, and glycogen of the filtrate were determined. Numbers of determinants in each experimental sequence were eight for the microsphere-injected and sham-operated rats and fourteen for the control rats.

For determination of plasma glucose concentrations, 25 rats were anesthetized with diethylether, and their femoral veins were exposed. Blood sampling was performed by puncturing the vein with a needle, with syringe, containing a trace of heparin. After centrifugation at 1,000g for 15 minutes at 4°C, the plasma was separated. Plasma glucose concentrations were determined by the o-toluidine–boric acid method.

In the present study, tissue metabolite contents are expressed in micromoles per gram of frozen tissue. In a preliminary study, we examined the water content of wet and frozen tissues of the cortex, striatum, and hippocampus of microsphere-injected and sham-operated rats. Water content values of the wet cortex, striatum, and hippocampus of the right hemisphere were 77.8±0.3, 75.0±0.7, and 77.1±0.7%, and those of the frozen ones 77.9±0.3, 75.4±0.8, and 77.4±0.7%, respectively (n=6 each). This indicates that there is no difference in water content between wet and frozen tissues. This trend in water content of wet and frozen tissues of the regional brain was similar to that in microsphere-injected rats. Microsphere injection did not elicit significant changes in water content of the cortex and hippocampus, but slightly increased water content of the striatum. Water content values of the left hemisphere of microsphere-injected rats and of both hemispheres of sham-operated rats were almost the same as those of controls at periods after the operation.

The results are expressed as mean±SEM. Statistical significance for comparison of values in time-
Results

Of the 110 microsphere-injected rats, twenty-one (19%) died within 24 hours of operation. After observation of animal behavior, we found that 69 rats (63%) showed typical type A symptoms, of which 11 rats (10%) died within 3 days of the operation. Twelve rats (11%) showed type B symptoms and 8 rats (7%) type C symptoms. The sham-operated rats (28 animals), which showed no stroke-like symptoms, survived throughout the experiments.

Behavioral scores of the operated rats (examined 15 hours after operation, then at 10 AM each day), shown in Figure 1, decreased with time after the operation. A marked decline in behavioral scores was seen 5 days after operation, which indicated a significant recovery from stroke-like symptoms when estimated by the methods of Friedman's test, followed by Dunnett's t test (p < 0.05).

Figures 2–6 show the time courses of changes in cerebral metabolite contents (lactate, ATP, creatine phosphate, glucose, and glycogen) of microsphere-injected and sham-operated rats after microsphere embolism are examined, and the results are shown in Figures 2 to 6, respectively. It should be noted that, in sham-operated rats, there were no appreciable changes in metabolites examined except for slight decreases in high-energy phosphates of the three brain regions on the first day after embolism.

In microsphere-injected rats, marked increases in lactate content of the three regions of the right hemisphere were seen 1 day after the operation. These increases gradually attenuated up to 7 days after the operation, but lactate levels in these three regions remained high compared with those in control rats. Changes in lactate contents of the cortex, striatum, and hippocampus of the left hemisphere revealed a trend similar to those of the right hemisphere, but to a lesser degree. A marked reduction in the ATP and creatine phosphate content of the brain regions of the right hemispheres of the microsphere-
FIGURE 4. Time course of changes in creatine phosphate (CP) levels in the cerebral cortex (panel a), striatum (panel b), and hippocampus (panel c) in the right (●) and left (■) hemispheres of microsphere-injected rats and in the right (○) and left (♀) hemispheres of sham-operated rats. Each value represents the mean±SEM of eight values from days 1–28 after embolism for microsphere-injected and sham-operated rats and 14 values for control (nonoperated) rats.

injected rats was seen 1 day after the operation. The decreases were slightly attenuated with time after the operation, but ATP and creatine phosphate levels in these three regions were consistently low when compared with those in control rats. Changes in the ATP and creatine phosphate levels of the cortex, striatum, and hippocampus of the left hemisphere revealed a trend similar to those of the right hemisphere, but to a lesser degree.

Significant increases in tissue glucose and glycogen contents in the three brain regions of the right hemisphere were seen in microsphere-injected rats throughout the experiment, with the exception of glucose levels in the cortex on days 7–28 after operation. The peak increases of both metabolites were seen 3 days after operation. Thereafter, the increases were slightly attenuated up to 7 days after the operation. The glucose and glycogen levels in the cortex, striatum, and hippocampus of the right hemisphere 28 days after the operation still remained at a high level as compared with controls. Changes in glucose and glycogen levels in the cortex, striatum, and hippocampus of the left hemisphere revealed a trend similar to those of the right hemisphere, but to a lesser degree.

Plasma glucose concentrations of microsphere-injected rats on days 3, 7, and 28 after operation were 118±10, 117±5, and 121±10 mg/dl (n=5, 6, and 4), respectively, which are almost the same as control values (121±10 mg/dl, n=10).

Discussion

In this study, we have shown that approximately 50% of the animals that underwent operation and had typical symptoms of stroke survived throughout the experiment. As described earlier, several investigators have employed microsphere-induced cerebral embolism to induce focal cerebral ischemia. However, there are very few reports in which the rate of success of the operation was described in detail. Because the microsphere-induced cerebral embolism is, in our experience, greatly influenced by animal species, strains, and body weight, as well as numbers of microspheres used, information concerning the
The rate of success of the operation is relevant to pharmacological and therapeutic studies of embolism-induced cerebral ischemia and infarction. The time course of changes in behavioral scores of animals undergoing operation showed reduction of the score with time after the operation. Fourteen days after operation, very faint stroke-like symptoms were observed, indicating that animal behavior is greatly improved with time after microsphere embolism.

We determined cerebral metabolites after fixation of the brain by microwave irradiation and near-freezing method. It should be noted that the lactate levels in the brain cortex in the present study were similar to those in the studies of other investigators and to those obtained by funnel-freezing. Furthermore, the values of total cerebral high-energy phosphate (ATP+creatine phosphate) determined by this method were also similar to, or slightly higher than, those obtained by the funnel-freezing method and the freeze-blowing method, which are widely accepted methods using liquid nitrogen for determination of cerebral labile metabolites. This suggests that microwave irradiation followed by near-freezing is the preferable method for determination of labile cerebral metabolites. On the contrary, our values of tissue glucose and glycogen contents are lower than those reported by others. It should be noted that the tissue glucose and glycogen contents obtained in the present study are referred to as HClO₄-extractable glucose and glycogen levels because we found in a previous study that cerebral glucose and glycogen levels were dependent on properties of the extracting and the reacting medium, that is, whether the media for extraction and reaction are alkaline, HCl-acidic, or HClO₄-acidic. The glycogen content measured is also dependent on the methods used for cleaving glucose moieties from glycogen molecules, that is, whether the moieties are cleaved by enzymes or chemicals.

Using the above method, we examined the time course of changes in labile metabolites of three regions of the cerebral hemispheres, the cortex, striatum, and hippocampus. Significant increases in tissue lactate content and decreases in ATP and creatine phosphate content of these three regions of the right hemisphere were seen after microsphere embolism and lasted throughout the experiment (28 days). Since both increase in tissue lactate and decrease in tissue high-energy phosphates are indicative of ischemia, the findings suggest that microsphere embolism manipulated by the current methods induces long-term cerebral ischemia. We also observed a severe decrease in blood flow of the brain regions in microsphere-injected rats. Pulsinelli et al. demonstrated severe nerve-cell death associated with a decrease in cerebral high-energy phosphates and an increase in tissue lactate after induction of cerebral ischemia, suggesting altered energy metabolism either as a result of, or as a cause of, delayed neuronal death.

Gurdjian and Duffy have shown that exposure of brain to hypoxia elicited flattening of electroencephalogram and cessation of respiration before a marked reduction of tissue ATP. Likewise, it has been shown that a profound fall in creatine phosphate and ATP occurs after brain function has been depressed in hypoxic or asphyxial animals. These observations suggest that a reduction of cerebral high-energy phosphates is associated with, or preceded by, a functional failure of cerebral nerve cells. High-energy phosphates of the regional brain in the present study was decreased by >50% 1 day after and by approximately 20% 28 days after the operation. This suggests that microsphere-induced cerebral embolism causes severe functional and metabolic damage to the cortex, striatum, and hippocampus of the microsphere-injected hemisphere.

Tissue glucose and glycogen contents of the three regions of the right hemisphere were significantly increased after microsphere embolism. Peak increases seen 3 days after the operation appeared to be different from peak alterations in tissue lactate and high-energy phosphates. The increase, although attenuated with time after the operation, lasted for up to 28 days after the operation, which suggests that
severe impairment of glucose metabolism continued throughout the experiment. Several reports have shown that brain glucose content\textsuperscript{30} or glucose utilization\textsuperscript{31} decreased shortly after acute cerebral ischemia, which was associated with an increase in plasma glucose levels.\textsuperscript{31,32} However, little information concerning brain glucose content in long-term cerebral ischemia is available. In contrast, there are several reports that describe abnormal changes in brain glycogen content a few days after cerebral ischemia. For example, histological examination of ischemic brain revealed an accumulation of glycogen granules in various areas of the brain,\textsuperscript{33} particularly in glial cells of white and gray matters, which disappeared several days after induction of ischemia.\textsuperscript{34} The accumulation of cerebral glycogen is attributed to enhancement of uridine-diphosphoglucosetransferase activity during the early phase of hypoxia.\textsuperscript{35} Our findings of an increase in tissue glycogen after the cerebral embolism is in good agreement with these observations. Kogure et al\textsuperscript{36} have demonstrated increases in brain glucose and glycogen contents 24 hours after microsphere embolism, although this study did not include measurements at later times. Thus, sustained high levels of glucose and glycogen in the brain regions may be characteristic of microsphere embolism.

The increase in tissue glucose content may result from an imbalance between glucose uptake from blood and glucose utilization through the glycolytic pathway in the brain. Uptake of glucose from the circulating blood might be enhanced in the oligemic brain owing to a compensatory mechanism for shortage of glucose in brain cells. It has been demonstrated that glucose uptake occurs by facilitated diffusion which fits to a theory of Michaelis-Menten with a $K_m$ value of 7.2 mM; therefore, brain glucose uptake depends on the concentration gradient at physiological concentrations of plasma glucose (approximately 6 mM) under normal state. In the present study, we observed no significant differences in plasma glucose levels on days 3, 7, and 28 after embolism. Consequently, sustained increase in brain glucose uptake from plasma is unlikely to occur under the present experimental conditions.

In previous studies in which similar experimental conditions with a smaller number of microspheres were employed,\textsuperscript{24,37} we observed similar, but relatively milder, damage; that is, a significant increase in tissue glucose was seen up to 5 days after microsphere embolism. This increase was found to be associated with increased or unchanged activities of Embden-Meyerhof enzymes such as phosphofructokinase, hexokinase, and pyruvate kinase, and with critical impairment of succinate dehydrogenase activity and mitochondrial oxidative phosphorylation. This suggests severe impairment in the mitochondrial function. Thus, the observed long-lasting increase in tissue glucose may be due to an inhibition of mitochondrial energy production after microsphere embolism.

Several regional differences in the metabolite content of the brain were seen; that is, lactate content of the striatum was found to be increased after microsphere embolism to a greater extent than that of the cortex or hippocampus. The peak increase in glucose content of the hippocampus 3 days after operation was slightly less than that of the cortex or striatum. The peak increase in the glycogen content of the cortex at 3 days was more marked than that of other brain regions. However, the trend of metabolite changes induced by microsphere embolism was found to be similar in these three regions. This suggests that damage to energy metabolism is uniformly developed in all these regions of the brain by the current microsphere embolism.

In our study, we observed significant alterations in tissue high-energy phosphates, lactate, glucose, and glycogen contents of the cortex, striatum, and hippocampus of the left hemisphere after microsphere-induced cerebral embolism that were similar to, but less severe than, those of the right hemisphere. Yamada et al\textsuperscript{38} induced embolization of the internal carotid artery with silicon rubber (500 \textmu m in diameter, 1 mm in length) and found an appreciable reduction of tissue ATP content of the embolized cerebral hemisphere and a similar trend in the contralateral hemisphere. They postulated that the latter was caused either by extension of brain edema or by diachisis. This might account, in part, for our findings of the reduction of energy metabolism in the brain regions of the contralateral hemisphere. However, the extent of impairment of brain energy metabolism in the contralateral hemisphere in the present study was more severe than in the study of Yamada et al. It is commonly recognized that the circle of Willis in the brain is well developed in rats; therefore plausible that considerable amounts of microspheres injected through the right carotid artery might be distributed to the contralateral cerebral hemisphere. This is a possible explanation for the appreciable changes in brain energy metabolites of the left hemisphere observed in the present study.

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**References**


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