Cerebral Ischemia Model Using Mongolian Gerbils—Comparison Between Unilateral and Bilateral Carotid Occlusion Models

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Abstract We permanently occluded unilaterally and/or bilaterally the carotid arteries of anesthetized Mongolian gerbils (60–80 g) and compared the two models. In the former, stroke-positive animals were selected by calculating the stroke index score of the conscious animals. Selection was not made in the latter. We measured the rCBF of the cerebral cortex, hippocampus, and diencephalon using the ^{3}H-nicotine scintillation method; analyzed the EEG using the wave-form recognition method (Fujimori); measured ATP, PCr (phosphocreatine), lactate, and glucose content in the cerebral hemisphere using the Lowry method; and measured infarct size on HE-stained coronal sections. All parameter values were uniform in the gerbils of the unilateral model, whereas great variation was observed in the right and left cerebral cortex, hippocampus, and diencephalon in the bilateral occlusion model. Therefore, we have discarded the bilateral model and used the stroke-positive unilateral model only.

By changing the length of time of the unilateral carotid occlusions and intervals, we found that two 10-min unilateral carotid occlusions with a 5-h interval between them achieved a threshold ischemic insult in gerbils, which produced uniform cortical focal infarctions that evolved in the maturing DSNN on the coronal surface sectioned at the chiasmatic level (Face A). This model showed a marked reduction in the occurrence of ischemic epilepsy and death.

Keywords Unilateral and bilateral carotid occlusion • Stroke index score • Stroke-positive Mongolian gerbil

Introduction

Studies on human pathological anatomy have not been able to elucidate the pathological changes that occur in the cerebrum in the acute phase after stroke onset. Until the early 1970s, although there was a different vulnerability depending on the neuron, neurons had been thought to die soon, even after a short period of ischemia. Except in the case of the Levine preparation using ischemia and hypoxia on gerbils [9], investigations into the acute phase of neuronal changes and/or evolution of an infarction after ischemic stroke have mainly used large animals such as cats and rhesus monkeys for focal ischemia, and rabbits for total ischemia. Owing to the difficulty in operative manipulation to produce ischemic lesions, the precise temporal profile of different grades of pure ischemic insult was not investigated until the appearance of the ischemic model using rodents. In 1972, Kahn [7] reported the selection of stroke-positive gerbils by using the stroke index score, which measures the various behaviors shown by animals after unilateral carotid occlusion. Therefore, we switched from using rhesus monkeys to using Mongolian gerbils at the NIH to investigate the precise histo-pathological temporal profile of various parts of the cerebral hemisphere after various durations of temporary ischemia. After investigating thousands of gerbils, we discovered that the rapid death of neurons did not occur soon

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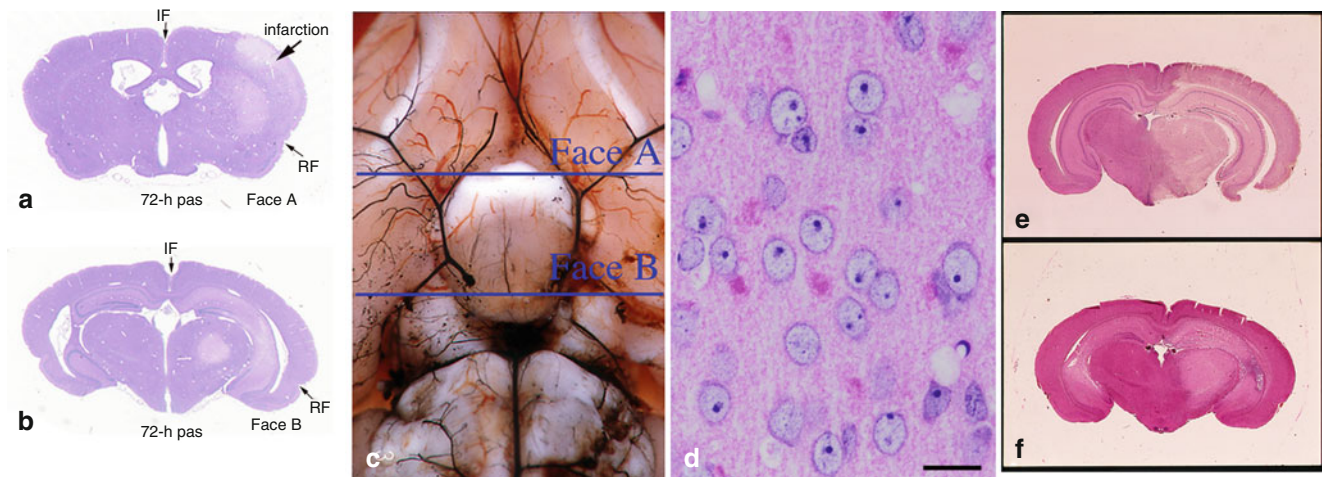e-mail: yamguchi@toshima-hp.metro.tokyo.jp; ohno.nsrg@tmd.ac.jp

Fig. 1 Coronal sections at (**a**) the chiasmatic (*Face A*) and (**b**) the infundibular (*Face B*) levels, 72 h post-ischemia; PAS staining. (**c**) Carbon-black perfused Willis's circle of a stroke-positive, unilaterally carotid-occluded gerbil, and the cutting line of *Face A* and *B*. Right anterior and bilateral posterior communications are lacking. (**d**) Light microscopy of post-ischemic cerebral cortex (*Face B*), 8 weeks; PAS staining: Bar=12.5 μm. The PAS-positive dead neurons (ghost cells) are seen in disseminated fashion among the surviving, normal-appearing neurons. (**e**) Homogeneous infarction in the left cerebral hemisphere. Unilateral left carotid occlusion for 5 h. H&E staining. (**f**) Heterogeneous focal infarctions in the bilateral cerebral hemisphere. Five hours after temporary bilateral carotid occlusion for 20 min. HE staining

after the ischemic insult. Rather, the animals suffered various post-ischemic neuronal changes ranging from degeneration and recovery to death, and further to focal infarction beyond the threshold of the ischemic insult. Also, the intensities of ischemic injuries and the speed of their appearance were directly related to the duration of ischemia. We referred to this phenomenon as the "maturation phenomenon of ischemic injuries" [3]. Later, using a bilateral carotid occlusion model of gerbils, Kirino reported the same kinds of changes confined to CA1 pyramidal cells of the hippocampus and described this process as "delayed neuronal death" [8]. This phenomenon provided a therapeutic window and boosted research in this field to rescue dying neurons and to reduce the size of the infarction. In this present study, we compared gerbils in which the unilateral carotid artery was permanently occluded and stroke-positive animals were selected by calculating the stroke index score [12], and gerbils in which bilateral carotid arteries were permanently occluded but the selection was not made.

Materials and Methods, Results, and Discussion

Unilaterally Carotid-Occluded, Stroke-Positive Model

As the cerebral arterial system lacks various anterior and posterior communicating arteries at the Willis circle (Fig. 1c),

Table 1 Modified stroke index

Signs	Stroke index
Hair roughed up or tremor	1
Obtundent or paucity of movement	1
Hypesthesia of ear	1
Head cocked	3
Eye fixed open	3
Ptosis	1
A splayed out hind limb	3
Circulation movement	3
Seizures or abrupt explosive movement	3
Extreme weakness	6
Total	25

After C.P. McGraw

permanent occlusion of the unilateral occlusion of the carotid artery induced infarction uniformly involving the caudal two thirds of the cerebral hemisphere in only about 40 % of animals (Fig. 1e).

During various surgical procedures, light anesthesia with ether and air or 2 % halothane or 3 % isoflurane in a mixed gas of 70 % nitrous oxide and 30 % oxygen was applied. The unilateral cerebral ischemia induced various abnormal behaviors of the conscious animals. Each behavior could be scored as points, and the total score of each animal was calculated (maximal total score: 25 points; Table 1). We classified the gerbils according to the total score: 0 as stroke-negative, 1–9 as pseudo-positive, and more than 10 as stroke-positive animals. Of the 282 animals evaluated, 42–43 % were positive for stroke, 47–53 % were negative, and the

remainder were indefinite. We permanently occluded the left common carotid arteries of male Mongolian gerbils of 60–80 g and various ages. After recovery of the animals from anesthesia and determination of McGraw's modified stroke index score [12], we compared the percentage of stroke-positive and -negative animals between the 6-week age group and the 25- to 32-week age group. There was no statistical difference in the occurrence of stroke-positive and -negative animals between these different age groups. At 1 h after permanent unilateral cerebral occlusion of five stroke-positive animals, 1.0 ml of carbon-black suspension (Pelican, carbon-black ink) was perfused from a femoral vein for 30 s prior to decapitation, and then their brains were immediately immersion-fixed in 10 % phosphate-buffered formaldehyde. All five animals showed a non-perfused area involving the caudal two thirds of their left cerebral hemisphere. Animals that survived more than 5 h after left carotid occlusion were investigated. We examined 109 animals microscopically by observing HE-stained coronal sections of brains from animals perfusion-fixed via the heart with 10 % phosphate-buffered formaldehyde. All 35 stroke-positive animals showed a homogeneous cerebral infarction involving the caudal two thirds of the left cerebral hemisphere (Fig. 1e). In the 50 gerbils in the stroke-negative group, 48 of them showed no infarction, with the other 2 animals having small foci of infarcts. Of the 24 animals in the indefinite group, 2 of them showed large infarctions, 7 showed small infarctions in their left cerebral hemisphere, and 15 had no infarction.

1. We measured the rCBF of the cerebral cortex, hippocampus, and diencephalon of conscious animals by using the ³H-nicotine scintillation method [12] at 1 h after the start of permanent ischemia due to left carotid occlusion. In the control group (six animals), the rCBF was 1.099±0.189 ml/g/min in the cerebral cortex, 0.575±0.056 ml/g/min in the hippocampus, and 0.686±0.035 ml/g/min in the diencephalon. In the stroke-positive group (five animals), the corresponding values were 0.091±0.016, 0.035±0.22, and 0.149±0.014 ml/g/min, respectively. All values of the stroke-positive group were less than 10 % of those of the control group, and they showed minimal variation. In the stroke-negative group (five animals), all values of these three portions of the cerebral hemisphere showed only a slight decrease. In the indefinite group (three animals), there was a large variety among animals. In the right non-ischemic hemisphere of the stroke-positive animals, there was a slight decrease in rCBF in all portions. All data were presented as the average±SEM.

2. We analyzed the EEG using the computerized wave-form recognition method [4, 11] in five stroke-positive animals in the awake state at 1 h after the start of permanent ischemia induced by the left carotid occlusion. After the injection of a muscle relaxant and with controlled respiration, mono polar electrodes were placed on the bilateral parietal dura mater; and the EEG was recorded bilaterally for 1 min. We analyzed by computer the average number of waves multiplied by the average amplitude in the lower frequency (0.5–8.5 Hz) and higher frequency (9.0–30Hz) groups. For the left hemisphere of the control group, the above value for the entire frequency range (0.5–30 Hz) was 1,029±180, whereas in the stroke-positive group, it had decreased to 370±124 with minimal variation. The stroke-negative group showed a very slight decrease, whereas the indefinite group showed marked variation. In the right non-ischemic hemisphere of the stroke-positive animals, the above value for the control group was 1,052±152, whereas in the stroke-negative group, there was a moderate decrease to 739.4±153.

3. We measured various parameters of energy metabolism by using the Lowry method at 1 h after permanent ischemia had been established in conscious animals. Each animal brain was fixed instantaneously by using microwave irradiation, and the posterior two thirds of the left ischemic and right non-ischemic hemisphere were homogenized. We measured ATP, PCr (phosphocreatine), lactate, and glucose content in the cerebral cortex, hippocampus and diencephalon of each cerebral hemisphere [10]. We measured these values in five animals in each group. In the control group, the ATP content was 9.25±0.14 μmol/g protein; PCr, 17.76±0.56 μmol/g protein; lactate, 6.04±0.57 μmol/g protein; and glucose, 6.26±0.76 μmol/g protein. In the stroke-positive group, the ATP value decreased to 1.72±0.20 μmol/g protein; PCr, to 3.08±0.48 μmol/g protein; and glucose, to 2.23±0.10 μmol/g protein. In contrast, the lactate increased to 25.22±3.58 μmol/g protein. Thus, we found a marked decrease in the values of ATP and PCr and a marked increase in lactate. In the right non-ischemic hemisphere, a slight decrease in ATP to 6.74±0.61 μmol/g protein and a slight decrease in PCr to 12.17±1.57 μmol/g protein occurred. In the stroke-negative group, all values for the ischemic hemisphere were almost the same as those of the control group.

Bilaterally Carotid-Occluded Model (Without Selection of Stroke-Positive Gerbils)

Under light anesthesia (the same as for unilateral occlusion), bilateral carotid arteries were permanently occluded. Immediately after occlusion, all 66 ischemic animals showed abnormal respiration and cyanosis owing to paralysis of their respiratory muscles, which are innervated bilaterally from the cerebral cortex, and 32 % of them died within 20 min. The surviving animals showed abnormal hyper-respiration.

At 15 min after bilateral carotid occlusion of 10 animals, we perfused each of them with 1.0 ml of carbon-black

Table 2 rCBF measurement with ³H-nicotine at 1 h of ischemia (bilateral carotid occlusion)

Side of cerebral hemisphere	Gerbil number	Part of cerebral hemisphere		
		Cortex	Hippocampus	Diencephalon
	Control	1.099 ± 0.189	0.575 ± 0.056	0.686 ± 0.035
Left	Cbb 1	0.21	0.18	0.28
	Cbb 2	0.01	0.02	0.28
	Cbb 3	0.06	0.02	0.14
	Cbb 4	0.56	0.34	0.50
Right	Cbb 1	0.41	0.32	0.44
	Cbb 2	0.01	0.01	0.40
	Cbb 3	0.50	0.32	0.44
	Cbb 4	0.15	0.12	0.12

suspension (Pelican, carbon-black ink) via a femoral vein for 30 s prior to decapitation, and then immediately immersion-fixed the brains in 10 % phosphate-buffered formaldehyde. Carbon-black was perfused in various parts of the cerebral cortex and the diencephalon irregularly, depending on the animal and the hemisphere, right or left.

As the percentage of animal deaths was very high in the bilateral occlusion group, we temporarily occluded the bilateral carotid arteries for 20 min and then selected 43 animals that survived for more than 5 h. We investigated all 43 brains by microscopically observing HE-stained sections cut coronally at three different levels, i.e., pre-chiasma, infundibulum, and caudal to the mammillary body (Fig. 1c). We measured the size of the infarct foci in each cerebral hemisphere (Fig. 1f), and classified them into one of two categories: (1) large infarction involving the cortex, hippocampus, diencephalon, and basal ganglia; and (2) small infarction covering less than two of the above three cerebral portions. A large infarction evolved in the bilateral hemispheres of 7 animals; and a small infarction, in those of 25 animals. A small infarction also evolved in the right or left hemisphere of 4 animals. In the remaining 7 animals, a small infarction evolved in either the right or left hemisphere, and a large infarction in the opposite hemisphere. Thus, we were unable to obtain a homogeneous distribution of infarctions in the bilateral carotid occlusion model.

1. In the same way as in the left unilateral carotid occlusion model, we measured the rCBF of the cerebral cortex, hippocampus, and diencephalon after 10 min of permanent ischemia of the bilateral carotid occlusion in four gerbils. In one of them, the rCBF of the left cerebral cortex was 0.56 ml/g/min, whereas that of the right cerebral cortex was 0.15 ml/g/min. In another gerbil, the rCBF of the left cerebral cortex was 0.01, while that of the right cerebral cortex was 0.01 ml/g/min. The decrease in the rCBF of the cerebral cortex thus showed quite a difference with respect to the animal and to the right or left side (Table 2).

2. In the same way as in the left unilateral carotid occlusion model, we obtained the EEG of five conscious, bilaterally occluded animals at 30 min after permanent ischemia. In one gerbil, the number of waves over the entire frequency (0.5–30 Hz) was 446 in the left hemisphere, but 965 in the right. In another gerbil, these numbers were 148 and 44 respectively. The decrease in the number of waves of the entire frequency from the control value were quite different according to the animal and to the right or left side. The values of the number of waves multiplied by the amplitudes showed the same tendencies as the number of waves, but were exaggerated.

3. In the same way as in the left unilateral carotid occlusion model, after 20 min of permanent ischemia due to bilateral carotid occlusion, we measured various parameters of energy metabolism of six stroke-positive conscious animals by using the Lowry method. In one animal, the ATP content was 7.18 µmol/g protein in the left hemisphere, and 2.37 µmol/g protein in the right. The respective PCr contents were 12.52 and 2.38 µmol/g protein. In another animal, ATP was 0.79 µmol/g protein in the left hemisphere and 1.93 µmol/g protein in the right; and PCr was 0.81 in the left, and 3.92 in the right. These values were quite different according to the animal as well as to the hemisphere involved (Table 3).

Conclusion

1. Infarction size, decrease in rCBF, in the number of waves in the EEG, and in the various parameters of the energy state showed great variation in each animal and in each portion and side of the cerebral hemisphere in the bilateral carotid occlusion model. However, all parameters were uniform among the gerbils of the unilateral carotid occlusion model using stroke-positive gerbils, and the operative manipulation of this model does not injure the cerebral cortex.

2. The death rate of animals due to impaired respiration was very high in the bilateral carotid occlusion model. For these reasons, we have discontinued the use of the bilateral model.

Table 3 Energy metabolism 20 min after ischemia (bilateral carotid occlusion)

Side of cerebral hemisphere	Gerbil number	ATP	PCr	Lactate	Glucose
	Control	9.25 ± 0.14	17.76 ± 0.56	6.04 ± 0.57	6.76 ± 0.76
Left	246	1.08	0.82	57.39	2.33
	248	2.53	5.55	67.62	2.73
	251	0.79	0.81	50.34	6.13
	255	4.78	5.62	46.98	2.90
	257	7.18	12.52	6.89	17.3
	259	1.19	1.92	24.91	2.25
Right	246	1.26	2.07	50.7	2.62
	248	1.64	3.43	72.37	4.02
	251	1.93	3.92	48.15	5.59
	255	3.80	3.24	31.95	1.75
	257	2.37	2.38	50.7	2.50
	259	1.08	2.26	23.24	1.82

Modified Unilateral Carotid-Occlusion Model

We have had to answer a question regarding the maturation phenomenon of ischemic injury after temporary ischemia, i.e., whether the transition from maturing disseminated selective neuronal necrosis (DSNN) (Fig. 1d) to the abrupt onset of focal infarction (Fig. 1a) is continuous or not after temporary ischemia [1]. As the mechanism of the transition is an enigma, we devised a gerbil model suitable for studying the transition after the threshold level of ischemic injury in inducing focal infarction had been reached. By changing the duration of the unilateral carotid occlusions and intervals, we found that two 10-min unilateral carotid occlusions with a 5-h interval between them achieved a threshold ischemic insult for producing uniform cortical focal infarctions at the coronal surface sectioned at the chiasmatic level (Fig. 1a) that evolved in the maturing DSNN (Fig. 1d) [2]. This model showed a marked reduction in the occurrence of ischemic epilepsy and death, which had long been considered as a problem with the use of the unilateral carotid occlusion model of gerbils. With this model we found that a focal cerebral–cortical infarction developed in the maturing DSNN after temporary ischemia. This infarction was induced by the delayed occurrence of the temporary microvascular obstruction due to compression of micro-vessels by swollen astrocytic end-feet at 3 ~ to 8 h after the restoration of blood flow, and it later resulted in tissue pan-necrosis at 12 ~ to 24 h post-ischemia [5, 6]. By changing time length of each of the two occlusions and the interval between them, we could obtain a different intensity of maturing ischemic injuries [1, 2].

Conflict of Interest   We declare that we have no conflict of interest.

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