

end-stage renal disease per million population, the highest prevalent rates in the world, and about 5% of them receive CAPD [1].

Stroke is the second or third leading cause of death in patients on CAPD next to cardiac diseases, accounting for about 10% of deaths during CAPD [3–5]. CAPD has been considered to be superior to hemodialysis (HD) for cardiovascular and cerebrovascular protection, because CAPD was reported to keep better control of hypertension [6, 7]. Recently, long duration of CAPD, however, has been reported to be disadvantageous to cardiac function compared with HD, possibly because of the development of hypervolemia [8]. Thus, it is in dispute which dialysis has an advantage for the prevention of stroke. To resolve this question, detailed analysis for patients on CAPD with stroke, such as of the type of stroke and alteration of blood pressure, is necessary.

Our clinic has monitored all patients who had started CAPD in the Kidney Center since 1980. The first goal of this study was to clarify clinical features of stroke in CAPD patients, and to determine factors to predict the onset of stroke during chronic CAPD. The second goal was to determine whether CAPD had an advantage over HD for the prevention of stroke.

## Patients and Methods

From May 1980 to October 2001, 188 patients started CAPD and 1,681 started HD in our Kidney Center. The choice between CAPD and HD depended on each patient's desire in principle, except for some contraindications to CAPD, including documented loss of peritoneal function or extensive abdominal adhesions, patients being physically or mentally incapable of performing CAPD in the absence of a suitable assistant, and uncorrectable mechanical defects that prevent effective CAPD like surgically irreparable hernia [9]. In addition, we often recommended HD, not CAPD, to patients over 70 years of age because they might have troubles to perform the exchange of dialysate by themselves, and to patients with diabetes because glucose loading via dialysate might worsen glucose tolerance. Because residual kidney function contributes significantly to total solute and water removal during the first few years of CAPD therapy, we often introduced CAPD while patients still kept somewhat good kidney function.

Among 188 patients on CAPD, we identified patients who developed stroke while they received CAPD. To assess indicators for the occurrence of stroke, clinical characteristics at initiation of CAPD and during chronic CAPD listed in table 1 were analyzed for all the patients with or without stroke. Characteristics during chronic CAPD were evaluated after 4 years of CAPD, or at the last day in the outpatient clinic if duration of CAPD (before the onset of stroke) was less than 4 years. Blood urea nitrogen, serum creatinine, and mean arterial pressure (MAP) were measured by averaging 3 consecutive data at the clinic every 2–4 weeks. Residual renal urea clearance normalized to total body water,  $K_t/V_{\text{urea}}$ , was measured to evaluate

the quality of dialysis. The maximal cardiac diameter divided by the maximal internal thoracic diameter, i.e. cardiothoracic ratio, was measured on the chest roentgenogram at the standing position. For patients with stroke, the type and etiology of their strokes were assessed according to the classification of cerebrovascular diseases III by NINDS [10].

The advantage of CAPD over HD for the prevention of stroke was tested as follows. First, to determine selection bias between patients with CAPD and HD, we examined baseline characteristics of the patients at initiation of HD; age, sex, and original kidney diseases for all 1,681 patients who started HD in our Kidney Center, and duration of renal insufficiency, blood urea nitrogen, serum creatinine, and MAP for 200 among the 1,681 patients selected at random. Second, we examined 137 consecutive HD patients who developed acute stroke and were hospitalized in our institute among the 1,681 patients and compared their clinical characteristics at initiation of dialysis and during chronic dialysis with those of CAPD patients with stroke. Third, to assess the prevalence of young-onset stroke during dialysis, we analyzed the incidence of stroke within 10 years after initiation of the dialysis therapy in patients who had started CAPD or HD under 45 years of age. Finally, we determined clinical severity of stroke for patients on CAPD or HD. Neurological deficits on admission were evaluated using the National Institute of Health Stroke Scale (NIHSS). Functional outcome 8 weeks after stroke onset was evaluated using the Barthel Index. Patients with a Barthel Index score <50 were regarded as dependent. For 3 patients on CAPD and 34 patients on HD who developed stroke before 1993, NIHSS and the Barthel Index were assessed afterwards based on their medical records. NIHSS seems to be abstracted from medical records with a high degree of reliability and validity for the purposes of retrospective studies of acute stroke outcome [11].

Values are expressed as mean  $\pm$  SD. Student's *t* test and  $\chi^2$  test were used as univariate analysis. Multivariate analysis was performed by logistic regression using the significant factors.  $p < 0.05$  was accepted as statistically significant.

## Results

### *Features of Stroke for Patients on CAPD*

We enrolled 188 patients on CAPD, 126 men and 62 women, aged  $46 \pm 14$  years. The duration of observation was 892.9 person-years. Twelve patients developed stroke in total 14 times; 6 brain infarction, 7 brain hemorrhage, and 1 subarachnoid hemorrhage (table 2). One patient (patient 6) developed brain hemorrhage 13 months after brain infarction. Another patient (patient 11) suffered brain hemorrhage twice at an interval of 9 days. The incidences of total stroke, brain infarction, brain hemorrhage, and subarachnoid hemorrhage were 15.7, 6.7, 7.8, and 1.1 per 1,000 person-years, respectively.

Brain infarction consisted of 2 atherothrombotic, 3 lacunar, and 1 cardioembolic infarction. Infarcts were located in the frontal cortex, parietal subcortex, basal ganglia, putamen, and pons (2 patients). All patients with

**Table 1.** Characteristics of patients

Variable	CAPD		p value CAPD-S (-) vs. CAPD-S (+)	HD stroke (+) (n = 137)	p value CAPD-S (+) vs. HD-S (+)
	stroke (-) (n = 176)	stroke (+) (n = 12)			
<i>Baseline characteristics at initiation of dialysis</i>					
Age, years	46 ± 14	48 ± 12	NS	55 ± 13	NS (0.06)
Duration of renal disease, years	13.2 ± 8.5	11.5 ± 8.3	NS	-	-
Male sex, %	66	75	NS	65	NS
Blood urea nitrogen, mg/dl	85.8 ± 24.2	73.3 ± 22.5	NS	-	-
Serum creatinine, mg/dl	9.9 ± 2.9	9.9 ± 2.8	NS	-	-
MAP, mm Hg	107 ± 15	103 ± 8	NS	-	-
Original kidney disease, %			NS		0.003
Chronic glomerulonephritis	70	75		25	
Diabetic nephropathy	11	17		42	
Hypertensive nephrosclerosis	5	0		17	
Lupus nephritis	2	8		1	
Polycystic kidney disease	1	0		2	
Others	11	0		13	
<i>Characteristics during chronic dialysis (before onset of stroke)</i>					
Duration of dialysis, years	4.7 ± 3.5	4.6 ± 3.5 <sup>a</sup>	NS	6.7 ± 5.8 <sup>a</sup>	NS
Hypertension, %	81	92	NS	85	NS
Diabetes, %	21	33	NS	48	NS
Dyslipidemia, %	25	33	NS	13	NS
Atrial fibrillation, %	5	17	NS	14	NS
Ischemic heart disease, %	12	25	NS	13	NS
K <sub>t</sub> /V <sub>urea</sub>	2.19 ± 0.16	2.17 ± 0.32	NS	-	-
MAP, mm Hg	107 ± 14	115 ± 16	0.05	-	-
ΔMAP <sup>b</sup> , mm Hg	0 ± 13	13 ± 15	0.002	-	-
Cardiothoracic ratio, %	46.9 ± 4.9	50.0 ± 3.8	0.04	-	-
<i>Medication</i>					
Antihypertensives, %	76	83	NS	-	-
Antihypertensives, n of agents	1.3 ± 0.9	1.4 ± 1.2	NS	-	-
Aspirin, %	9	0	NS	-	-
Warfarin, %	2	0	NS	-	-
<i>Characteristics on stroke</i>					
Age at stroke onset, years	-	52 ± 12	-	62 ± 11	0.008
Type of stroke, %			-		NS
Brain infarction	-	43		58	
Brain hemorrhage	-	50		38	
Subarachnoid hemorrhage	-	7		4	
Subtype of brain infarction, %			-		NS
Atherothrombotic	-	33		25	
Lacunar	-	50		48	
Cardioembolic	-	17		27	
NIH-SS at admission, %			-		NS
0-6	-	42		49	
7-15	-	17		24	
16-42	-	42		27	
Dead or dependent (Barthel Index ≤ 50) at discharge, %	-	67		43	NS (0.10)

<sup>a</sup> Until stroke onset.<sup>b</sup> Increase in MAP during chronic CAPD compared with initiation of CAPD (mean ± SD).

**Table 2.** Characteristics of stroke patients on CAPD

Patient No.	Age <sup>1</sup>	Sex	Original kidney disease	Risk factors	Antihypertensives	Profiles of stroke				
						type	etiology	location	NIHSS <sup>1</sup>	prognosis <sup>2</sup>
1	59	m	CGN	HT, DL, IHD	Ca (2 agents)	BI	atherothrombotic	subcortex	12	ID
2	61	f	CGN	HT, DL	Ca	BI	atherothrombotic	cortex	2	100
3	70	m	CGN	HT, DM, af, IHD	Ca	BI	cardioembolic	basal ganglia	10	ID
4	34	f	CGN	HT	Ca, $\beta$	BI	lacunar	pons	2	100
5	65	m	CGN	HT, IHD	none	BI	lacunar	pons	5	ID
6-i	39	m	CGN	HT, DL	Ca, ACEI	BI	lacunar	putamen	1	100
6-ii	40	m				BH	HT	putamen	>16	DD
7	54	m	diabetic	HT, DM, DL, af	Ca, ACEI	BH	HT	thalamus	4	90
8	49	f	lupus	HT	$\beta$	BH	HT, (vasculitis)	putamen	>16	35
9	44	m	diabetic	HT, DM	Ca	BH	HT	pons	>16	DD
10	48	m	CGN	HT	Ca, ACEI, $\beta$	BH	HT	putamen	>16	DD
11-i	40	m	CGN	HT	Ca, $\alpha$	BH	HT	thalamus	1	100
11-ii	40	m				BH	HT	putamen	4	100
12	68	m	CGN	DM, LD	none	SAH	unknown	-	>16	ID

Patient 6 developed brain hemorrhage 13 months after brain infarction. Patient 11 suffered brain hemorrhage twice at an interval of 9 days. CGN = Chronic glomerulonephritis; HT = hypertension; DM = diabetes mellitus; DL = dyslipidemia; IHD = ischemic heart disease; af = atrial fibrillation; Ca = calcium channel blocker;  $\alpha$  =  $\alpha$  blocker;  $\beta$  =  $\beta$  blocker; ACEI = angiotensin-converting enzyme inhibitor; BI = brain infarction; BH = brain hemorrhage; SAH = subarachnoid hemorrhage; ID = indirect death; DD = direct death.

<sup>1</sup> At the onset of stroke.

<sup>2</sup> Number indicates Barthel Index scores 8 weeks after stroke onset.

brain hemorrhage were hypertensive, with possible lupus vasculitis in 1 patient with systemic lupus erythematosus. Hematomas were distributed in the putamen in 4 patients, in the thalamus in 2, and in the pons in 1 patient. The cause of subarachnoid hemorrhage was unknown.

#### Comparison between CAPD Patients with and without Stroke

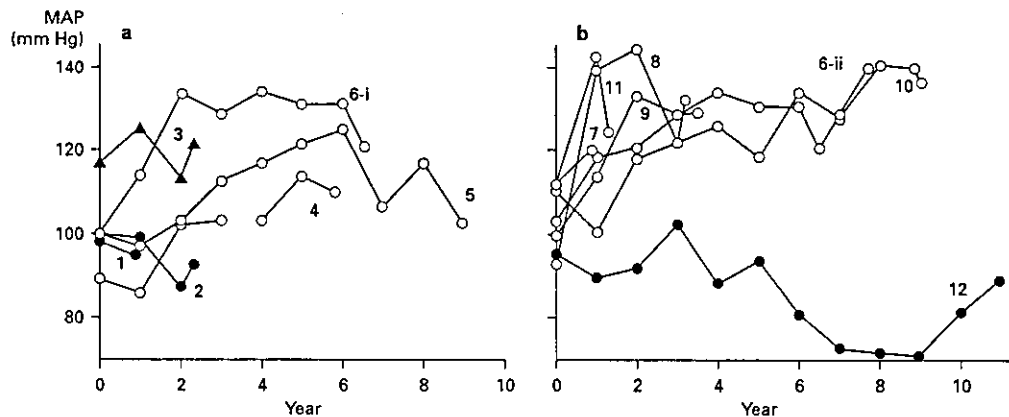
Clinical characteristics at initiation of CAPD were similar between CAPD patients with and without stroke (table 1). Among the characteristics during chronic CAPD, the cardiothoracic ratio in patients with stroke was greater than that without stroke ( $p = 0.04$ ). MAP of chronic CAPD patients with stroke increased from  $103 \pm 8$  mm Hg at the initiation of CAPD to  $115 \pm 16$  mm Hg in the chronic stage ( $p = 0.01$ ), whereas that of CAPD patients without stroke did not change ( $107 \pm 15$  to  $107 \pm 14$  mm Hg; fig. 1). Increase in MAP in patients with stroke during chronic CAPD was greater than that in patients without stroke ( $p = 0.002$ ). There were no differences, however, in the use of antihypertensives between patients with and without stroke. As antithrombotic agents, aspirin or warfarin were used for a few patients without stroke and none for patients with stroke. For patients with high blood pressure, we often used 2.5% glucose dialysate as hypertonic CAPD solution. For 10 among 12 patients with stroke (except for patients 4

and 7), we used the dialysate 1–3 times out of 4 times exchange per day according to the severity of hypertension.

After the multivariate analysis using the cardiothoracic ratio and increase in MAP as independent variables among all characteristics at initiation of CAPD and during chronic CAPD, increase in MAP alone was an independent indicator for the occurrence of stroke (odds ratio for an increase in 10 mm Hg = 2.04, 95% CI 1.21–3.43). In CAPD patients with brain hemorrhage, MAP increased from  $105 \pm 8$  mm Hg at the initiation of CAPD to  $128 \pm 5$  mm Hg during chronic CAPD ( $p = 0.003$ ), and was an independent indicator for the occurrence of brain hemorrhage (odds ratio for an increase in 10 mm Hg = 5.05, 95% CI 1.94–13.12). In CAPD patients with brain infarction, MAP changed from  $101 \pm 9$  to  $111 \pm 16$  mm Hg ( $p = 0.18$ ). Among them, MAP for 3 patients with lacunar infarction changed from  $96 \pm 6$  to  $118 \pm 15$  mm Hg ( $p = 0.07$ ).

#### Comparison between Patients on CAPD and HD

In our institute, patients initiated HD ( $52 \pm 18$  years) at an older age than CAPD ( $46 \pm 14$  years,  $p < 0.0001$ ). Male patients tended to be less frequent for HD (60%) than CAPD (67%,  $p < 0.08$ ). The prevalence of major original kidney diseases, i.e. chronic glomerulonephritis (39%), diabetes (31%), and hypertensive nephrosclerosis



**Fig. 1.** Changes in MAP of 12 patients with stroke during chronic CAPD until just before the onset of stroke. **a** Ischemic stroke. ○ = Lacunar infarction; ● = atherothrombotic infarction; ▲ = cardioembolic infarction. **b** Hemorrhagic stroke. ○ = Brain hemorrhage; ● = subarachnoid hemorrhage. Numbers in panels (1–12) correspond to patient numbers in table 2.

(9%), was significantly different from that of CAPD ( $p < 0.0001$ ). Based on data at initiation of HD for 200 patients selected at random, the duration of renal insufficiency was shorter ( $8.7 \pm 8.6$  vs.  $13.0 \pm 8.5$  years,  $p = 0.0003$ ), levels of blood urea nitrogen ( $96.4 \pm 18.0$  vs.  $84.4 \pm 24.3$  mg/dl,  $p < 0.0001$ ) and serum creatinine ( $10.5 \pm 2.4$  vs.  $9.9 \pm 2.8$  mg/dl,  $p = 0.04$ ) were higher, and MAP was higher ( $115 \pm 13$  vs.  $106 \pm 14$  mm Hg,  $p < 0.0001$ ) in patients on HD compared with CAPD.

The duration of observation for all 1,681 patients who started HD in our Kidney Center was approximately 11,500 person-years. Among the 1,681 patients, 79 developed brain infarction, 52 brain hemorrhage, and 6 subarachnoid hemorrhage. The incidences of total stroke, brain infarction, brain hemorrhage, and subarachnoid hemorrhage for HD patients were approximately 12, 7, 5, and 0.5 per 1,000 person-years, respectively. Patients on HD who developed stroke tended to be older at the initiation of dialysis than those on CAPD ( $p = 0.06$ , table 1). Patients on HD were older than those on CAPD at the onset of stroke ( $62 \pm 11$  vs.  $52 \pm 12$  years for all stroke,  $p = 0.008$ ;  $65 \pm 10$  vs.  $54 \pm 14$  years for brain infarction,  $p = 0.01$ ;  $57 \pm 12$  vs.  $46 \pm 6$  years for brain hemorrhage,  $p = 0.03$ ). For patients who started dialysis under 45 years of age, 6 (8 strokes) among 97 CAPD patients (6.2%) and 10 among 625 HD patients (1.6%) developed stroke within 10 years after the initiation of dialysis; the incidences

being 12.2 and about 2.2 per 1,000 person-years, respectively. As an original kidney disease for stroke patients, chronic glomerulonephritis was less common and diabetes was more common in patients on HD compared with CAPD ( $p = 0.008$ ).

NIHSS at stroke onset for CAPD patients was similar to that for HD patients. Eight weeks after stroke onset, 8 among 12 (67%) patients on CAPD and 43% of those patients on HD ( $p = 0.10$ ) were dead or dependent. Seven patients on CAPD died after stroke onset; 3 died directly from brain hemorrhage, and 4 indirectly from peritonitis, pneumonia, pancreatitis, and myocardial infarction. These patients accounted for 27% of 26 patients who died during chronic CAPD.

## Discussion

This is the first study that clarified incidence, etiology, and outcome of stroke in CAPD patients. We stress the following 4 major findings. First, annual incidence of stroke in CAPD patients was high, exceeding 15/1,000. Second, CAPD patients developed stroke while they were relatively young, on average about 52 years of age. Third, blood pressure of patients with stroke significantly increased during chronic CAPD. Increase in MAP was related independently to the occurrence of total stroke or

brain hemorrhage in CAPD patients. Fourth, the majority of patients were dead or dependent in the chronic stage of stroke.

#### *Stroke Incidence*

According to the Framingham Study [12], stroke incidence for the general population was 6.03 per 1,000 person-years for men and 4.53 for women. Other community-based studies reported similar or smaller values [13]. In the Hisayama Study, a community-based study in our suburban town, the incidence of brain infarction was 6.4 per 1,000 person-years for men and 3.4 for women [14], that of brain and subarachnoid hemorrhages being 1.2 and 0.96, respectively [15, 16]. The age structure of these community-based studies was generally higher than that of the present study. Thus, it may not be ideal to directly compare values of stroke incidence in the present and previous studies. It is highly probable, however, that CAPD patients have much more risk of developing any type of stroke compared with the general population. In CAPD patients, the proportion of brain hemorrhage to all strokes was high, and the ratio of subtypes of brain infarction was similar compared with the general population in the Hisayama Study [14, 15].

Hypertension is the most powerful risk factor for stroke in most of community-based studies [17, 18]. Thus, higher incidence of stroke in the present CAPD patients than in the general population may partly result from higher frequency of hypertension in CAPD patients. In the Hisayama Study, stroke incidence for residents with systolic blood pressure >140, 160, and 180 mm Hg was 6.0, 14.5 and 17.0 per 1,000 person-years, respectively [Tanizaki et al., pers. commun.]. These values were somewhat close to stroke incidence of the present CAPD patients, whose systolic blood pressure averaged 149 mm Hg.

In the Framingham Study [12], the mean age of stroke patients was between 65 and 74 years. The present CAPD patients who had stroke were more than 10 years younger than them, although it appears at least partly because of the population of CAPD patients being younger than the general residents. Again in the Framingham Study [12], stroke incidence for residents aged 45–54 years, similar distribution of age to the present patients on CAPD, was 1.79 per 1,000 person-years for men and 0.99 for women. Thus, patients on CAPD seem to develop stroke much more frequently than the general population while they are relatively young.

The incidence of brain infarction was similar between patients on CAPD and HD in our series, and that of hem-

orrhagic stroke was higher in CAPD patients than HD patients. Incidence of total stroke for HD patients was previously reported to be 13.2 [19] and 17.6 per 1,000 person-years [20], similar to that for the present CAPD patients. In the previous and present studies on HD patients [19, 20], age at onset of brain infarction was 64–66 years and that of brain hemorrhage was 55–57 years. Judging simply from these data, CAPD patients who developed brain infarction were about 10 years younger, and those with brain hemorrhage were about 5 years younger than HD patients. Analysis using patients under 45 years of age, to avoid effects of different distribution of age between patient population on CAPD and HD, showed much more prevalent young-onset stroke in CAPD patients compared with HD patients.

#### *Etiological Mechanism*

Patients with kidney diseases have dual risk factors for cardiovascular diseases; 'traditional' factors including hypertension and diabetes, and 'uremia-related' ones including anemia, hyperparathyroidism and decline in kidney function [21]. Even mild renal insufficiency with serum creatinine concentration between 1.4 and 2.3 mg/dl increases the risk of cardiovascular events including stroke [22]. Among the factors, hypertension was reported as an independent predictor of stroke for HD patients [20].

In this study, hypertension itself was not a predictor of stroke. The majority of patients with end-stage renal disease have hypertension by several mechanisms including stimulation of the renin-angiotensin aldosterone system [23]. Such patients may already have had arteriosclerotic change in cerebral circulation at the initiation of CAPD. MAP increased during CAPD for few patients in this study, but the increase seemed to frequently cause stroke, especially brain hemorrhage. As indicated in table 2, some patients with stroke did not take multiple antihypertensives for accelerated high blood pressure, and seemed to be undertreated for their hypertension. CAPD patients with stroke had a greater cardiothoracic ratio than patients without stroke. Because increase in this ratio indicates cardiomegaly essentially due to overhydration, poor control of hypertension in the present patients with stroke seems to result, at least in part, from overhydration during chronic CAPD. Takeda et al. [8] reported that patients on long-term CAPD of >60 months' duration had a greater cardiothoracic ratio, greater left ventricular mass index on echocardiogram, and higher arterial pressure than those on HD or short-term CAPD. They discussed that peritoneal dysfunction after long-term CAPD might cause

overhydration and consequent hypertension. Thus, poor control of hypertension seems to be a good indication for changing CAPD to HD or renal transplantation.

#### Outcome

Although stroke outcome for 12 patients on CAPD varied widely as shown in table 2, dead or dependent patients on CAPD in the chronic stage of stroke tended to be more frequent than among HD patients.

#### Advantage for Stroke Protection

Patients who selected CAPD in our institute were younger and less diabetic, and kept better residual kidney function and lower MAP at the initiation of the dialysis therapy than those who selected HD, essentially because of our criteria for choice between CAPD and HD stated above. The longer duration of renal insufficiency for patients on CAPD compared with HD may be due to different frequency of original kidney diseases between patients on CAPD and HD, because kidney function of diabetic patients often worsens for a shorter period than that of patients with chronic glomerulonephritis. Our patients on CAPD accordingly seemed to have less risk factors for arteriosclerosis at the initiation of dialysis compared with those on HD. Stroke incidence for patients on CAPD, however, was not smaller than that for HD patients, and was much higher using young patients who started dialysis therapy before 45 years of age. Thus, we could not prove the advantage of CAPD for cerebrovascular protection over HD from the present results.

The present study was retrospective, and was based on a small number of patients from one kidney center. Accordingly, there are some limitations in the study. The most essential limitation is the selection bias for CAPD.

Because indication for CAPD is widely different among countries or among institutes, influenced by several medical and nonmedical factors [2], features of stroke in CAPD patients may also be different among countries to some extent. For example, in the institutes where aged patients and diabetic patients can choose CAPD without any limitation, stroke incidence for CAPD patients might be higher than the present result. In the United States, stroke caused approximately 20 deaths per 1,000 patient-years in diabetic and about 10 deaths in nondiabetic patients on CAPD [5]. Different frequency of diabetic and aged patients in other institutes may also influence results of stroke subtype, including the frequency of large artery disease, and stroke outcome. In addition, because this study was based on patients from one kidney center, there seemed to be some biases of management of patients, including equipments and techniques of dialysis, and choice of antihypertensives and other drugs. At least, our kidney center maintained good quality of chronic CAPD, because  $K_t/V_{urea}$  for the patients averaged 2.2.

In conclusion, CAPD patients seem to have a greater risk of stroke than the general population primarily because of poor control of hypertension presumably, in part, due to volume overload. Increasing pressure during CAPD is associated with an increased risk of stroke. Although there is a lack of controlled research studies, it seems reasonable to be particularly aggressive in the anti-hypertensive treatment of such patients.

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## Free radical scavenger, edaravone, in stroke with internal carotid artery occlusion

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### Abstract

**Background:** Edaravone has potent free radical quenching and antioxidant actions. The agent has been recently in commercial use for acute ischemic stroke patients. In this study, we investigated the therapeutic effect of edaravone on severe carotid-territorial stroke. **Methods:** Stroke patients with internal carotid artery occlusion and baseline NIH Stroke Scale Score  $\geq 15$  were treated for 14 days with drip intravenous infusion of edaravone ( $n=30$ ) and were compared with a historical control cohort of similar patients ( $n=31$ ). Glycerol was also administered to all patients in both groups. **Results:** Infarct volume ( $P<0.02$ ) and midline shift ( $P<0.02$ ) on CT performed on day 2 of the patients treated with edaravone were smaller than those without edaravone. For patients with edaravone, infarct volume ( $P<0.0001$ ) and midline shift ( $P<0.0001$ ) on days 5–7 were greater than those on day 2. Hemorrhagic transformation of infarcts on day 2 was less severe in patients with than without edaravone ( $P<0.03$ ). Within 14 days after the onset of stroke, 6 patients with edaravone (20%) and 14 without edaravone (45%) died directly of stroke ( $P<0.03$ ). Among all patients, only two treated with edaravone were independent without any assistance 8 weeks after the onset. **Conclusions:** Edaravone was associated with delayed evolution of infarcts and edema in patients with severe carotid-territorial stroke and decreased mortality during the acute stage. The agent, however, failed to prevent evolution of infarcts and edema on later days, and did not significantly improve functional outcome among the surviving patients.

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**Keywords:** Brain infarction; Stroke management; Stroke outcome; Brain edema; Hemorrhagic transformation; Neuroprotective agent

### 1. Introduction

Free radical generation after cerebral ischemia seems to contribute to the neuronal injury mainly by activating the lipoxygenase pathway in the arachidonate cascade. 3-Methyl-1-phenyl-2-pyrazolin-5-one (edaravone, formerly MCI-186) inhibits both nonenzymatic lipid peroxidation and lipoxygenase pathway of the arachidonate cascade, and has potent antioxidant action [1]. Thus, the agent has several protective effects against ischemia/reperfusion-induced dam-

ages, including vascular endothelial cell injury [2], delayed neuronal death [3], brain edema [4–6], infarct [7,8], and concomitant neurological deficits [8].

After a phase III, multicenter, placebo-controlled, double-blind trial [9], edaravone was accepted in clinical use for management of acute ischemic stroke in Japan in June 2001. In the trial, patients with semicomatose or comatose were excluded from the trial, and 64% of patients were alert at the onset of stroke. At the 3-month assessment, >95% of patients survived and  $\approx 70\%$  of patients were independent without any assistance in both edaravone- or placebo-treated groups. Thus, most of the patients in the trial had mild or moderate stroke. Therapeutic effect of edaravone for severe stroke has not yet been clearly identified.

Abrupt occlusion of the carotid axis often ends in fatal outcome. Thrombolytic therapy often fails to recanalize

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acute internal carotid artery (ICA) occlusion, and more than half of the patients died even after this therapy [10]. Thus, we have not yet obtained available therapeutic strategy for severe stroke due to ICA occlusion. Extensive infarction in the unilateral middle cerebral artery (MCA) territory occasionally accompanied by the ipsilateral anterior or posterior cerebral artery infarction, so-called malignant MCA infarction, is almost always caused by occlusion of the distal ICA or the proximal MCA trunk, and has the mortality rate as high as 80% [11]. Trans-tentorial herniation with concomitant brain death was the cause of fatal outcome. The maximal midline shift documented on CT occurred between days 2 and 4 in all patients who died, and between days 3 and 7 in the surviving patients with malignant MCA territory infarction. These findings suggest that delay in evolution of space-occupying infarct and edema is an essential factor for prevention of fatal prognosis of the acute patients. Post-ischemic treatment with edaravone significantly decreased infarct size in rats with MCA occlusion [8]. In addition, edaravone markedly diminished cortical water content during and after massive carotid-territorial ischemia in rats, even more effectively than glycerol [4]. We hence hypothesized that edaravone reduced space-occupying mass effect of patients with severe carotid-territorial infarction and improved their outcome.

The purpose of this study was to determine the therapeutic effect of edaravone on clinical and radiological findings of patients with ICA occlusion and severe neurological deficits.

## 2. Subjects and methods

Four stroke units (see Appendix A) joined this study. Between October 1999 and July 2002, 1564 patients with brain infarction admitted to the units within 7 days after the onset (845 before June 2001 and 719 after July 2001). Among them, we examined 61 consecutive patients who developed ischemic stroke caused by ICA occlusion and underwent an acute cranial CT scan within 6 h after onset of the stroke. Occlusion of ICA was diagnosed on the day of stroke onset using digital subtraction angiography, MR angiography, or duplex carotid ultrasonography. On ultrasonogram, ICA occlusion was diagnosed according to Yasaka et al.'s criteria [12]. Patients with a fresh infarct on the initial CT or baseline NIH Stroke Scale (NIHSS) score <15 were excluded from the study. As a major early ischemic sign on CT, we recorded existence of parenchymal hypoattenuation in >33% of the MCA territory [13], but did not exclude patients with this sign from analysis.

As therapeutic agents, 10% glycerol ( $\approx 10$  ml/kg body wt./day) was administered intravenously for initial  $\approx 14$  days for all patients. Thirty consecutive patients, who admitted to our hospitals after July 2001, were treated with

drip intravenous infusion of 30 mg of edaravone diluted with 100 ml of saline twice a day for initial 14 days. The initial use of edaravone was within 6 h after the stroke onset, and generally right after the initial CT. For 31 patients admitted before June 2001, edaravone was not used. Thrombolytic therapy immediately after the initial CT, anticoagulant therapy using unfractionated or low-molecular-weight heparin for prophylaxis of intracardiac or deep vein thrombi after the 24-h follow-up CT, and decompressive craniectomy were at each investigator's discretion. If reopening of the occluded ICA was radiologically ascertained within an hour after the thrombolysis, the patient was excluded from the study. Patients who underwent decompressive craniectomy were excluded from analysis of CT study on days 5–7.

Each patient had 24-h and 5- to 7-day follow-up CT scan. Hypodense area indicating infarct or edema was calculated for 12 axial 5- or 10-mm-thick slices. Infarct volume (accurately speaking "infarct plus edema volume") was calculated by multiplying the hypodense area by the slice thickness for 12 slices and by summing them. Horizontal displacement of the pineal body and the septum pellucidum was measured as midline shift in the slice of maximum displacement. For some patients who could not take CT on days 5–7 because of acute death or severe physical condition or who received decompressive craniectomy before day 5, we prepared censored data by imputing the worst observed value of infarct volume and midline shift on days 5–7 to the above patients and then deriving a multivariate model that predicted the CT parameters on days 5–7 from those on 24-h follow-up CT and clinical factors listed in Table 1. Using this model, we calculated the missing data for the above patients.

Table 1  
Characteristics of patients

Variable	Edaravone (+) (n=30)	Edaravone (-) (n=31)	P value
Male gender	15 (50%)	11 (35%)	NS
Age, years	77 $\pm$ 10	77 $\pm$ 10	NS
Onset to CT, h	2.0 $\pm$ 1.3	2.0 $\pm$ 1.2	NS
Left side infarct	17 (57%)	17 (55%)	NS
Baseline NIHSS	21 $\pm$ 4	21 $\pm$ 3	NS
<i>Potent cardiac embolic source</i>			NS
Atrial fibrillation	21 (70%)	22 (71%)	
Others	3 (10%)	4 (13%)	
None	6 (20%)	5 (16%)	
<i>Risk factors for arteriosclerosis</i>			
Hypertension	17 (57%)	13 (42%)	NS
Diabetes	10 (33%)	5 (16%)	NS
Dyslipidemia	4 (13%)	4 (13%)	NS
<i>Treatment</i>			
Tissue plasminogen activator	2 (7%)	0	NS
Urokinase	6 (30%)	3 (10%)	NS
Heparin*	16 (53%)	11 (35%)	NS
Decompressive surgery	0	3 (10%)	NS

\*: Including low molecular weight heparin.

According to definitions in the ECASS study [14], hemorrhagic transformation of the infarct on CT was classified as hemorrhagic infarction (HI) types I (small petechiae along the margins of the infarct) and II (more confluent petechiae within the infarcted area) and parenchymal hemorrhage (PH) types I (blood clot not exceeding 30% of the infarcted area with some mild space-occupying effect) and II (dense blood clot exceeding 30% of the infarct volume with significant space-occupying effect).

Clinical outcome was evaluated by mortality within 14 days and functional prognosis quantified using a modified Rankin scale (mRS) at 8 weeks after the onset. Dead patients at 8 weeks were scored as grade 6 of mRS.

### 2.1. Statistics

Values are expressed as mean  $\pm$  S.D. Comparison of variables between patients with and without edaravone use was performed using chi-square test, Mann–Whitney's *U*-test, and Wilcoxon rank sum test. Survival curves for the two groups were estimated by Kaplan–Meier method and compared using the log-rank test.  $P < 0.05$  was accepted as statistically significant, and  $0.05 \leq P < 0.1$  as marginally significant.

## 3. Results

### 3.1. Baseline characteristics (Table 1)

Between 30 patients who were treated with edaravone and 31 without edaravone, there were no differences in gender, age, onset-to-CT interval, laterality of the infarct, baseline NIHSS on admission, and frequency of atrial fibrillation, hypertension, diabetes, and dyslipidemia. Two patients were treated with intraarterial tissue plasminogen activator (tPA) and nine with intravenous urokinase, none of whom showed reopening of the occluded ICA an hour after thrombolysis. Two patients received decompressive craniectomy on day 2, and one did on day 3; one of them died 12 weeks after stroke and two were dependent in the chronic stage. All of these three patients were not treated by edaravone.

### 3.2. Infarct and edema

On the baseline CT, the major early ischemic sign was present in seven patient with edaravone (23%) and 6 without edaravone (19%,  $P > 0.1$ ). Two patients missed the second CT on day 2. Both of them were not treated by edaravone, had too severe condition to perform CT, and died on day 3. Infarct volume of the patients with edaravone on day 2 ( $n = 30$ , 21.4  $\pm$  6.8 h after stroke onset) was smaller than that without edaravone ( $n = 29$ , 21.7  $\pm$  6.4 h) ( $156 \pm 126$  vs.  $241 \pm 140$  cm<sup>3</sup>,  $P < 0.02$ ). Midline shift of the patients with edaravone on day 2 was also smaller than that without

edaravone ( $3.5 \pm 3.3$  vs.  $7.7 \pm 7.3$  mm,  $P < 0.02$ ). The third CT on days 5–7 was performed for 24 patients treated with edaravone ( $115.3 \pm 23.0$  h after stroke onset) and 18 without edaravone ( $108.7 \pm 16.3$  h). The remaining patients died within 4 days after stroke onset ( $n = 13$ ), did not undergo CT because of severe condition ( $n = 3$ ), or were excluded from analysis because they received decompressive craniectomy ( $n = 3$ ). After calculating data for these missing patients using the multivariate models, infarct volume of the patients with edaravone on days 5–7 was smaller than that without edaravone ( $241 \pm 149$  vs.  $321 \pm 151$  cm<sup>3</sup>,  $P < 0.05$ ), and midline shift of the patients with edaravone on days 5–7 tended to be smaller than that without edaravone ( $8.2 \pm 6.4$  vs.  $11.5 \pm 7.0$  mm,  $P < 0.07$ ). In patients with edaravone, infarct volume ( $P < 0.0001$ ) and midline shift ( $P < 0.0001$ ) on days 5–7 became greater than those on day 2, and so did the parameters in patients without edaravone ( $P < 0.0001$  and  $P < 0.0005$ , respectively) (Fig. 1).

### 3.3. Hemorrhagic transformation

Incidence of hemorrhagic transformation was smaller in patients treated with edaravone than without on day 2 (10% vs. 34%,  $P < 0.03$ ), and was not significantly different on days 5–7 (42% vs. 67%). After grading the severity of hemorrhage in the order of none, HI type I, HI type II, PH type I, and PH type II, patients with edaravone had less severe hemorrhage than those without edaravone on day 2 ( $P < 0.03$ ) (Fig. 2).

### 3.4. Survival outcome

Within 14 days after the onset of stroke, 6 patients treated with edaravone (20%) and 14 patients without edaravone (45%) died directly of stroke ( $P < 0.03$ ). Survival on day 14 had no correlation with gender, age, onset-to-CT interval,

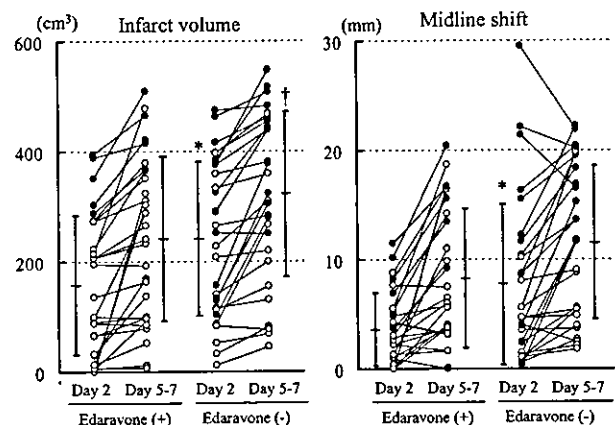


Fig. 1. Infarct volume and midline shift on cranial CT. Closed circle shows a patient who missed 5- to 7-day follow-up CT. For the patient, censored data was calculated using the multivariate model which was introduced in Subjects and Methods. Bar shows mean  $\pm$  S.D. \* $P < 0.02$ , † $P < 0.05$ , vs. "edaravone (+), Day 2".

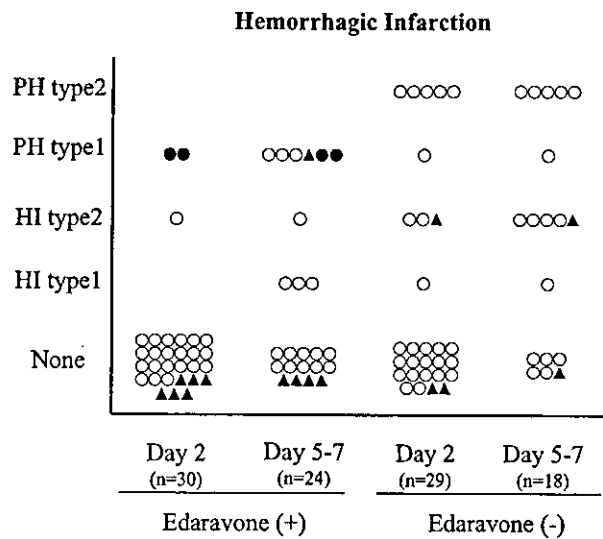


Fig. 2. Hemorrhagic transformation of infarct on cranial CT. Open circle shows a patient without thrombolysis. Closed circle shows a patient with intraarterial tissue plasminogen activator use. Closed triangle shows a patient with intravenous urokinase use.  $P < 0.03$  between “edaravone (+), Day 2” and “edaravone (-), Day 2”.  $P > 0.1$  between “edaravone (+), Days 5–7” and “edaravone (-), Days 5–7”.

laterality of the infarct, baseline NIHSS, frequency of atrial fibrillation, or other risk factors. The major early ischemic sign on the initial CT and thrombolysis on the day of stroke onset may be decisive factors for acute survival. Although all 6 patients with the early ischemic sign who were not treated with edaravone died or received decompressive craniectomy within 14 days, 4 among 7 patients with the early sign and treatment by edaravone survived the acute stroke. Regarding 11 patients undergoing thrombolysis, 1 among 8 patients with edaravone and 1 among 3 patients without edaravone died within 14 days without hemorrhagic transformation on CT by days 5–7. Survival curve estimated by Kaplan–Meyer method still tended to be different between patients with and without edaravone after exclusion from the analysis of patients with the early sign (survival rate on day 14: 87% vs. 64%,  $P < 0.06$ ) or with thrombolysis (77% vs. 54%,  $P < 0.08$ ) (Fig. 3).

3.5. Functional outcome

In the chronic stage within 8 weeks after the stroke onset, two patients with edaravone died of congestive heart failure and one patient without edaravone died of disseminated intravascular coagulation. Among all the patients, only two who were treated with edaravone were independent without any assistance 8 weeks after the onset, corresponding to mRS score of 2 or less. One of them who received intravenous urokinase use right after the initial CT repeated ultrasonographic examination. His ICA was still occluded on day 2, and was recanalized on day 5. For another one without thrombolysis, occlusion site shifted to the insular segment of MCA on MRA taken

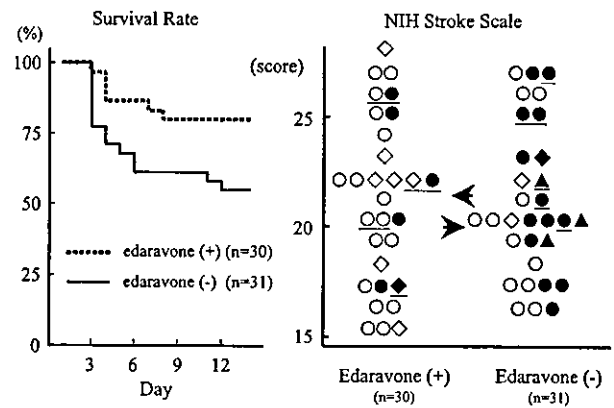


Fig. 3. Survival outcome in the acute stage. Left panel: Survival rate within 14 days after the stroke onset.  $P < 0.03$  between edaravone (+) and edaravone (-). Right panel: Contribution of baseline NIH Stroke Scale score, the major early ischemic sign on the initial CT, and hyperacute thrombolytic therapy to survival outcome. Arrow shows the median score of NIH Stroke Scale. Open circle/diamond shows a surviving patient and closed circle/diamond shows a dead patient on day 14. Closed triangle shows a patient who underwent decompressive craniectomy. Diamond shows a patient with hyperacute thrombolysis. Underline shows a patient with the early ischemic sign.

on day 20. Frequency of independent patients differ marginally significantly between patients with and without edaravone use (7% vs. 0%,  $P < 0.1$ ). Functional outcome evaluated by mRS was better in patients with edaravone than without ( $P < 0.03$ ). If the same analysis was done using only surviving patients at 8 weeks after stroke onset, mRS was not significantly different between the two patient groups (Fig. 4).

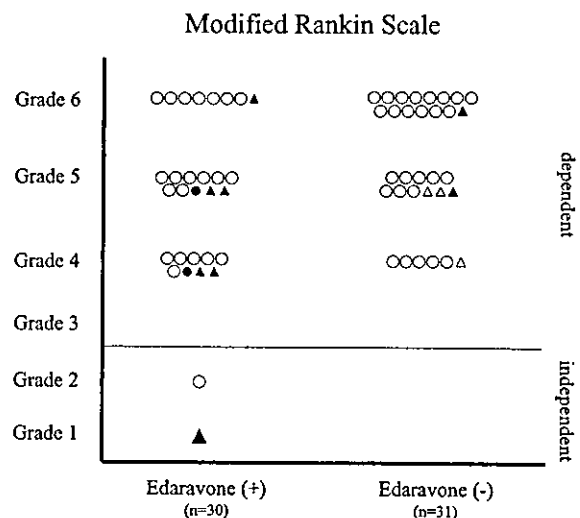


Fig. 4. Modified Rankin Scale (mRS) score 8 weeks after the stroke onset. Closed circle shows a patient with intraarterial tissue plasminogen activator use. Closed triangle shows a patient with intravenous urokinase use. Open triangle shows a patient who underwent decompressive craniectomy.  $P < 0.03$  between edaravone (+) and edaravone (-) in severity of mRS.  $P < 0.1$  between edaravone (+) and edaravone (-) in frequency of independent patients.

## 4. Discussion

This is the first clinical study to examine the therapeutic effect of edaravone, the free radical scavenger and antioxidant, for fatal ischemic stroke. Because this is not a double-blind trial, there are some limitations stated later for interpretation of the results. This study, however, has several important suggestions on edaravone use for management of acute stroke as follows. First, edaravone, together with glycerol use, was associated with delayed evolution of infarct and edema after ICA occlusion in stroke patients on day 2. Second, edaravone decreased severe hemorrhagic transformation on day 2. Third, edaravone was associated with decreased mortality during the acute stage of stroke. However, edaravone did not seem to contribute much to improvement of the functional outcome among the surviving patients.

### 4.1. Therapeutic effect on edema

A 56% reduction in acute mortality rate of the present patients after edaravone use, from 45% to 20%, presumably resulted from potent antiedematous activity of edaravone as shown in Fig. 1. The effect against space-occupying brain edema was already proven using animal models for which edema was induced by pancerebral or regional ischemia [4,6], intracarotid injection of polyvinyl acetate [5], or intraparenchymal injection of arachidonate [6], and here ascertained using patients. Subsequent to energy failure during brain ischemia, several polyunsaturated fatty acids, including arachidonate, were released from membrane phospholipids, followed by activation of arachidonate cascade including lipoxygenase pathway and consequent production of oxygen radicals and lipid peroxidation [15]. Arachidonate itself and lipoxygenase products from arachidonate cascade induce brain edema [16–18], partly due to disruption of microvascular integrity. Because edaravone has radical scavenging and antioxidant activity [3,8], prevents peroxidative cell damage due to hydroperoxyeicosatetraenoic acids from arachidonate cascade [2], and attenuates arachidonate-induced edema in a rat model [6], an essential antiedematous mechanism for this agent seems to result from inhibition of ischemic neuronal and cerebrovascular cell injury by products of arachidonate cascade.

Early decompressive craniectomy and induced hypothermia were reported to be the only available strategies for space-occupying supratentorial ischemic stroke [19,20], although hypothermia might not be as effective as craniectomy. Use of edaravone may be another available treatment for such type of stroke, at least regarding survival outcome.

### 4.2. Therapeutic effect on ischemia

Besides the antiedematous effect, edaravone protects brain against ischemia/reperfusion-induced injury by free radical quenching and antioxidant actions. The protective

effect was proven in the transient hippocampal ischemia [3] and MCA branch occlusion model [7,8]. Thus, decrease in the infarct volume in this study may partly result from the activities of edaravone other than antiedematous effect.

### 4.3. Therapeutic effect on hemorrhagic transformation

Frequency of hemorrhagic transformation in the present patients with edaravone (42% on days 5–7) was almost identical but that in the patients without edaravone (67%) was relatively high compared with previous CT studies (41–43%) [21–23]. Because a large infarct with mass effect augments hemorrhagic transformation [21–24], difference in infarct volume and midline shift between patients with and without edaravone seems to be a leading cause of difference in incidence and severity of hemorrhagic transformation on day 2 between them.

Cerebrovascular integrity is provided by the microvascular intima, which contains two barriers to solute transport and transmigration of circulating blood cells, i.e. the blood–brain barrier and basal lamina [25]. For the initiation of ischemic brain injury, ischemic insults to these barriers are as necessary as those to neuronal cells [26]. This cascade of microvascular events includes fibrin accumulation, endothelium expression of leukocyte adhesion receptors, breakdown of the basal laminae with loss of astrocyte and endothelial cell contacts leading to blood–brain barrier disruption, and consequently edema formation and hemorrhagic transformation [27–29]. Because edaravone prevented hydroperoxide-induced injury of vascular endothelial cell cultures [2], the agent might have protected microvascular barriers against ischemic injury to some extent and prevented severe hemorrhagic transformation as well as edema formation in this study. Thrombolysis using tPA treatment has been recently reported to rapidly aggravate ischemia-induced damage to microvascular barriers, thereby enhancing hemorrhagic transformation [30]. Thus, edaravone may be an indispensable combined agent for hyperacute thrombolytic therapy.

### 4.4. Limitations

An important limitation for this study is that this is not a double-blind trial. Because all the patients who developed stroke after July 2001 used edaravone and any patients before June 2001 did not, we cannot deny contribution of recent improvement of medical instruments to the present results to some extent. Regarding stroke with ICA occlusion, we did not obtain efficient therapeutic strategy in a recent couple of years, and thus the lag of hospitalization year between the two patient groups does not seem to influence the present results decisively.

Another limitation is that some choices of stroke therapy including hyperacute thrombolysis, anticoagulation after day 2, and decompressive surgery were at each investigator's discretion. This limitation may make interpretation of

the results on hemorrhagic transformation and clinical outcomes difficult to some extent.

#### 4.5. Implications

Some clinical features in the present study, including abrupt onset of stroke, high frequency of atrial fibrillation, and high incidence of hemorrhagic transformation, suggest that cardiogenic embolism is the most prominent etiology in our patients. Because reperfusion injury highly connects with embolic stroke, free radical quenching and antioxidant actions of edaravone seem to work efficiently for patients with this type of stroke.

Although edaravone markedly improved the acute survival rate for stroke patients with ICA occlusion, it did not significantly improve chronic functional outcome among the survivors. This limitation seems to have causal relation with failure of edaravone in prevention of evolution of infarcts and edema on days 5–7. Thus, edaravone alone is not sufficient for acute management of severe stroke. Combination of edaravone with additional stroke therapy including antithrombosis or thrombolysis may be needed.

In conclusion, early treatment with edaravone was associated with delayed evolution of infarcts and edema in patients with severe carotid-territorial infarction and decreased mortality of the acute patients. Edaravone may be a useful antiedematous and anti-ischemic agent for the treatment of acute ischemic stroke with large infarct size and mass effect.

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#### Appendix A

This study was performed with the cooperation of stroke units in the following four hospitals in Japan: National Kyushu Medical Center, Fukuoka Red Cross Hospital, National Ureshino Hospital, and Hakujyuji Hospital.

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Protocols

# Krypton laser-induced photothrombotic distal middle cerebral artery occlusion without craniectomy in mice

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## Abstract

Recent advances in genetical engineering of the mouse have highlighted the importance of reproducible and less invasive models of cerebral ischemia in mice. In this paper, we developed minimally invasive and reproducible model of distal middle cerebral artery (MCA) occlusion in mice using krypton (Kr) laser-induced photothrombosis.

C57BL/6 or BALB mice ( $n=8$  each) were anesthetized with halothane. The skin was cut, the temporal muscle was retracted, and the right distal MCA was observed through the skull. A Kr laser beam of wavelength 568 nm was focused onto the MCA over the intact skull. Upon laser irradiation, intravenous administration of a rose bengal solution was begun. After 4 min of irradiation, the laser beam was refocused on the MCA just proximal to the first spot, and another 4-min irradiation was performed. Then, the right common carotid artery (CCA) was ligated. Three days later, the brain was removed, and infarct volume was determined.

Infarction confined almost solely to the cortical area was produced in each mouse. Mean infarct volume in C57BL/6 mice was  $25.2 \pm 13.7 \text{ mm}^3$ . The BALB mice group showed significantly larger and more reproducible infarction ( $44.1 \pm 5.2 \text{ mm}^3$ ; the coefficient of variation was 12%) than did C57BL/6 mice ( $P<0.005$ ).

Our photothrombosis model of stroke in mice can be performed without craniectomy, and its reproducibility is satisfactory when using BALB mice.

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*Theme:* Disorders of the nervous system

*Topic:* Ischemia

*Keywords:* Mouse; Cerebral ischemia; Focal; Photochemistry

## 1. Type of research

Focal ischemia model in mice [8,11].

Thrombotic occlusion induced by krypton (Kr) laser and rose bengal solution [4,23,26].

## 2. Time required

The time required for the experiment greatly depends on the skills and practice of the researcher:

Stroke surgery: 45 min

Harvesting and sectioning the brain: 15 min

Staining slices of the brain: 30 min

Postfixing the slices: 3 days

Infarct volume measurement: 15 min including image capturing

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### 3. Materials

All procedures were done in accordance with the Animal Care Guidelines at Kyushu University and the law (No.105) and notification (No.6) of the Japanese government.

Adult male BALB/cA Jcl (BALB) mice or C57BL/6 Jcl (C57BL/6; 8–9 weeks old, body weight 25–30 g) purchased from Nippon CLEA (Shizuoka, Japan) were used in this study.

#### 3.1. Special equipment

##### 3.1.1. Stroke surgery and harvesting the brain

Micro dissection forceps [Fine Science Tools (FST), Heidelberg, Germany]

Micro dissecting scissors (FST)

Micro spring scissors (FST)

6-0 silk suture (Ethicon, Somerville, NJ)

6-0 monofilament nylon suture (Ethicon)

Stereoscopic microscope for surgery (S21, Zeiss, Germany), magnification  $\times 10$  through  $\times 25$

Heparinized saline (2 U/ml)

Rose bengal dye (Wako Pure Chemical Industries, Osaka, Japan)

Krypton laser (643-Y-A01; Melles Griot, Tokyo, Japan)

Mirror

Convex lens (KPX082AR.14, Newport, Irvine, CA)

Digital thermometer (PTM 100A, Unique Medical, Tokyo, Japan)

Heating lamp

##### 3.1.2. Sectioning and staining the brain

Blades for slicing the brain

Mouse brain matrix (RBM-2000C, ASI Instruments, Warren, MI)

2,3,5-triphenyltetrazolium chloride (TTC) solution (Wako Pure Chemical Industries)

10% buffered formalin (Wako Pure Chemical Industries)

##### 3.1.3. Supplementary experiments

3.1.3.1. *Precise temperature measurement.* Infrared thermography system (TVS-8500, Nippon Avionics, Tokyo, Japan).

3.1.3.2. *Transmission of the laser beam study.* Power indicator (Lasermate 3, Coherent, Tokyo, Japan).

3.1.3.3. *Physiological study.* Transducer for blood pressure monitoring (CitiFlo TA1017, Nippon Becton Dickinson, Tokyo, Japan)

Bridge amp (WT645G) and recorder (AP601-G, Both Nihon Kohden, Tokyo, Japan)

Arterial blood gases (i-STAT 300F, Fuso Pharmaceutical Industries, Tokyo, Japan)

### 4. Detailed procedure

#### 4.1. Stroke surgery

Focal cerebral infarction was produced by permanent occlusion of the right middle cerebral artery (MCA) by a laser-induced photochemical reaction (see review by Watson [22]) modified from the rat model developed in our laboratory [4,26]. This method enables us to make a pinpoint occlusion of a vessel leaving the dura and the skull intact and without producing massive heat. The mice were anesthetized by inhalation of 2% halothane in 70% N<sub>2</sub>O and 30% O<sub>2</sub>, and anesthesia was then maintained by 0.8–1% halothane. With a midline neck incision, the right common carotid artery (CCA) was exposed, and a 6-0 silk suture was loosely put around it. The thyroid gland was pulled aside, and the right jugular vein was exposed. The right jugular vein was carefully dissected from the surrounding tissue, and two pieces of sutures were put around the vein. The distal suture was fastened and a small hole on the vein was made between the sutures. A catheter was inserted into the superior vena cava through the hole of the right jugular vein for intravenous administration of a solution, and the proximal suture was tied tightly. Then, the skin was cut between the right eye and ear, and the skull and right temporal muscle were exposed. The temporal muscle was retracted until the distal part of the right MCA was observed through the skull moistened with normal saline (Fig. 1A). Temporal muscle temperature was monitored continuously and maintained at  $36.0 \pm 0.3$  °C during MCA occlusion (MCAO) by a heating lamp.

A krypton laser (643-Y-A01, Melles Griot), operating at 568 nm wavelength and 6 mW of emitted power, was used to irradiate the MCA. The laser beam was positioned with mirror and focused with a convex lens (Newport KPX082AR.14) onto the MCA just distal to the cerebral vein (Fig. 1B and C). Upon laser irradiation, intravenous administration of a photosensitizing rose bengal dye solution (20 mg/kg) over 90 s was begun. After 4 min of irradiation, the laser beam was refocused on the MCA just proximal to the first spot, and another 4 min of irradiation was performed. After the second irradiation, the temporal muscle was put in its normal position, and the skin was sutured. Then, the suture around the right CCA was tied and thereby ligated. The jugular vein catheter was removed, the jugular vein was ligated, and the wound was closed with sutures.

#### 4.2. Harvesting and staining the brain

Three days after MCAO, the mice were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with heparinized saline. Then, the brains were quickly removed. Eight fresh coronal sections (each 1 mm thick) at bregma levels +2.8 through –4.2 mm were obtained using a mouse brain matrix (ASI Instru-



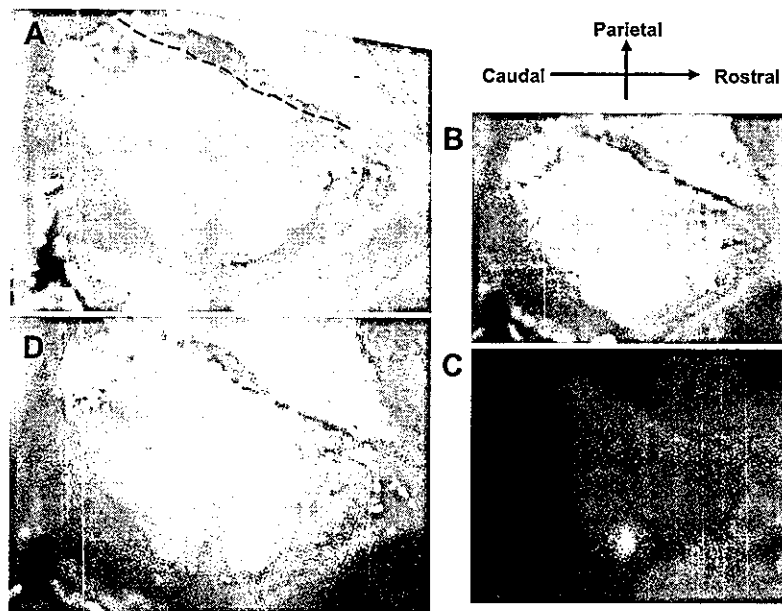


Fig. 1. Laser photothrombotic occlusion of the right MCA in mouse. (A) The right MCA was observed through the skull. A broken line denotes the linea temporalis. (B) Laser beam (greenish yellow light) was focused onto the MCA. (C) A picture taken through a 568 nm laser line rejection filter. A rose bengal solution administered intravenously was activated by the laser beam, emitted fluorescent light, and formed a platelet-rich thrombus. (D) After photothrombosis, no arteries distal to the irradiated spot were observed, and blood flow cessation was confirmed.

ments). Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) and postfixed with 10% buffered formalin for 3 days. Images of the sections were captured with a digital camera, and infarcted areas were measured with NIH image software (Ver. 1.63).

#### 4.3. Infarct volume measurement

Infarct volume was determined by the “indirect method”, as described elsewhere [13,21]. Specifically, the infarct area in each slice was determined by subtracting the area of intact tissue in the ischemic hemisphere from the total area of the intact contralateral hemisphere to correct for brain edema. Infarct area was summed among slices and multiplied by slice thickness (1 mm) to provide infarct volume ( $\text{mm}^3$ ). To validate the infarct area determined by TTC staining at 3 days after ischemia, three BALB mice were selected. Then, six sections at bregma levels +1.8 through –3.2 mm of each mouse were stained by both TTC and H&E staining and were compared.

#### 4.4. Supplemental experiment and statistical analysis

##### 4.4.1. Precise temperature measurement and transmission of the laser beam study

In one C57BL/6 mouse, the alteration of the brain temperature during laser irradiation was determined by an infrared thermography system (TVS-8500, Avio System Technology), with temperature sensitivity of 0.025 °C and spatial resolution of 0.2 mm. In addition, the temporal part of the skull was dissected on sacrifice

and was measured with a power indicator (Lasermate 3; Coherent).

##### 4.4.2. Physiological parameter measurement

Since arterial sample drawing alone causes substantial hypotension in mice [2], physiological parameters were determined in a separate group. In the separate group of C57BL/6 or BALB mice, a polyethylene catheter was inserted into the right femoral artery in addition to the procedure inducing focal ischemia. Mean arterial blood pressure (MABP) and arterial blood gases (i-STAT 300F; Fuso Pharmaceutical Industries) were measured before and after cerebral ischemia.

##### 4.4.3. Statistical analysis

All data were expressed as mean  $\pm$  standard deviation and were analyzed by the unpaired *t*-test or analysis of variance (ANOVA), followed by Fisher’s PLSD test. A *P*-value under 0.05 was considered statistically significant.

## 5. Results

In the present study, we applied Kr laser-induced photothrombosis to mice and developed a model of distal MCA occlusion while leaving the skull and dura intact. To validate this method, we produced focal ischemia in the widely used C57BL/6 Jcl and BALB/cA Jcl mouse strains with laser photothrombosis; and then, we determined whether the differences of infarct size were compatible with the results in other reports using direct cauterization of MCA.

By Kr laser-induced photothrombosis, the distal part of the MCA was effectively occluded, and the cessation of blood flow in the MCA was confirmed by microscopy (Fig. 1). The total time duration of each surgery did not exceed 45 min, and no mortality was observed during this experiment.

Within the range from 4 through 10 mW of emitted power of laser beam (568 nm of wavelength), the transmission of the laser beam through the temporal part of the skull was estimated to be 50%. The diameter of the focused laser beam was about 0.2 mm on the surface of the brain. Therefore, the corresponding average intensity at the focal plane (brain surface) for an incident power of 6 mW was approximately 10 W/cm<sup>2</sup> in this experiment. As shown in Fig. 2, a rise in the skull temperature was transient and less than 1 °C, even at the focal point of irradiation. In a separate group of C57BL/6 or BALB mice ( $n=4$  to 5), physiological variables were monitored, and no differences were found in the MABP and arterial blood gases between both strains of mice, before or after cerebral ischemia (Table 1).

Infarction was confined to the cortical area of the cerebrum (Fig. 3A–F) in both strains of mice. For comparison of infarct area determined by TTC and H&E staining, a percent ratio of infarct area to the area of intact contralateral hemisphere was calculated in each slice by both methods. Linear regression analysis revealed a good correlation between the ratios determined by the two staining methods ( $N=18$ ,  $y=0.9+93.1x$ ,  $R=0.85$ ,  $P<0.01$ ; see Fig. 3G).

Since infarct volume in BALB mice is larger and more reproducible than that of C57BL/6 mice after direct

Table 1

Physiological variables

	Before ischemia	After 1st irradiation	After ischemia
<i>Mean arterial blood pressure, mm Hg</i>			
C57BL/6	66.6 ± 7.9	73.6 ± 8.2	75.4 ± 6.8
BALB	73.0 ± 4.8	77.4 ± 7.0	75.4 ± 5.0
<i>Temporalis muscle temperature, °C</i>			
C57BL/6	36.2 ± 0.05	36.0 ± 0.17	36.2 ± 0.20
BALB	36.1 ± 0.21	36.2 ± 0.17	36.0 ± 0.09
<i>Arterial blood gases</i>			
pH			
C57BL/6	7.29 ± 0.06		7.28 ± 0.04
BALB	7.30 ± 0.06		7.28 ± 0.05
pCO <sub>2</sub> mm Hg			
C57BL/6	43.9 ± 3.8		50.9 ± 4.6
BALB	46.1 ± 4.5		52.0 ± 2.00
pO <sub>2</sub> mm Hg			
C57BL/6	205.6 ± 23.6		146.8 ± 22.8
BALB	215.3 ± 34.5		159.2 ± 19.4

$n=4$  to 5 in each mouse strain. Values are mean ± S.D.

No statistical difference in each variable was found between the both groups of mice.

MCAO [15], we compare the infarct volumes of the both strains following laser-induced photothrombosis. In C57BL/6 mice, the mean infarct volume was 25.2 ± 13.7 mm<sup>3</sup>. BALB mice showed significantly larger infarction (44.1 ± 5.2 mm<sup>3</sup>, with a coefficient of variation of 12%) than did the C57BL/6 mice ( $P<0.005$  by unpaired Student's  $t$ -test). The infarct area on every slice of BALB mice was larger than that of C57BL/6, and these differences reached statistical significance, except for Slices 4

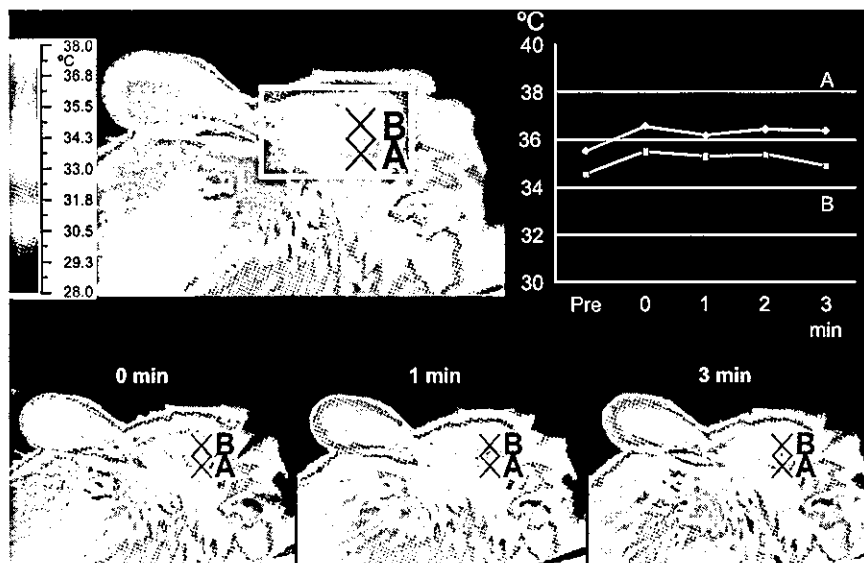


Fig. 2. Alteration of brain temperature during laser photothrombosis (emitted power = 8 mW). Left upper panel shows initial state of brain temperature. Point A is the focus of laser irradiation and point B is 2 mm dorsal to point A. A white frame almost corresponds to Fig. 1A–D. Right upper graph shows temporal changes in the brain temperature. Lower panels depict the brain temperature at 0, 1, and 3 min after starting laser irradiation.

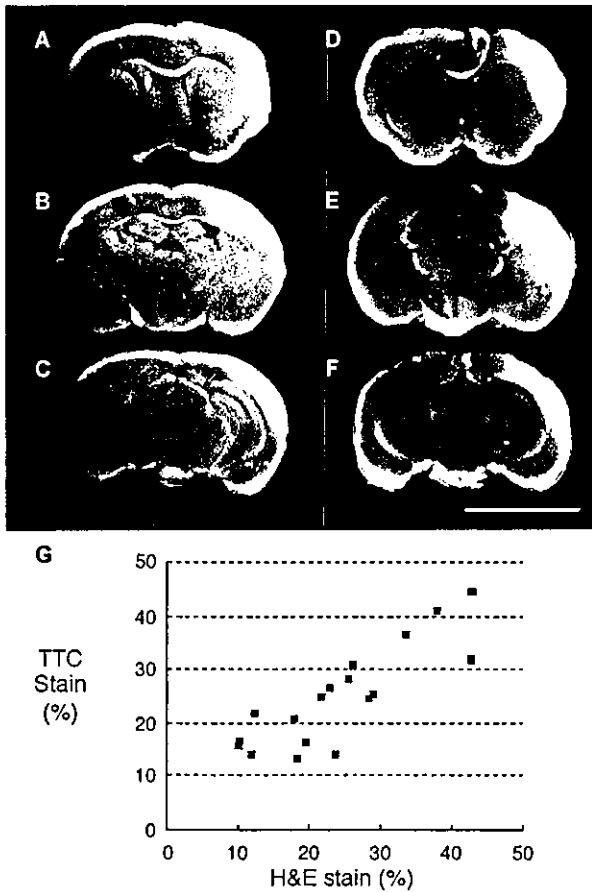


Fig. 3. (A)–(F): Representative infarct in a C57BL/6 or BALB mouse determined by TTC staining. Left (A–C) and right panels (D–F) show infarction of a C57BL/6 mouse and a BALB mouse, respectively. Scale bar=5 mm. (G) Infarct areas determined by H&E and TTC staining in selected animals. Values are expressed as percent ratios of infarct area to the area of intact contralateral hemisphere (see text for detail). Infarct area measured by TTC staining is linearly correlated with the areas determined by H&E.  $N=18$ ,  $y=0.9+93.1x$ ,  $R=0.85$ ,  $P<0.01$ .

and 5. The mean values of infarct area in each slice and total infarct volumes are depicted in Fig. 4.

6. Discussion

Recent advances in genetical engineering of the mouse and availability of a draft sequence of the mouse genome [17], as well as the availability of “knockout” or “transgenic” mice, have enabled investigators to examine effects of a given gene and its translated product in vivo. In the field of cerebral ischemia, Kinouchi et al. [11] employed the *SOD1* transgenic mouse and induced focal cerebral ischemia by electric cauterization of the distal middle cerebral artery (MCA). Huang et al. [8] utilized knockout mice deficient in neuronal nitric oxide synthase in a focal ischemia study using suture occlusion of the MCA. Both the direct cauterization and the suture occlusion methods,

however, are surgically demanding and require a skilled and well-experienced animal surgeon. Other models, including bilateral carotid occlusion, have been also introduced into mouse studies; yet, these methods have disadvantages of their own, mostly due to technical difficulties, short survival times, or variance in the extent and severity of ischemia. Thus, the importance of a consistent ischemia model that allows longer survival times in mice has been highlighted recently.

Photothrombotic occlusion of small cerebral vessels induced by the interaction of a photosensitizing dye rose bengal and a filtered xenon arc lamp was first introduced by Watson et al. [23]. This technique then evolved to occlude arteries by a more sophisticated technique employing an argon or argon-dye laser beam as the light source [16,24]. In terms of focal ischemia, we have established a reproducible model of distal MCA occlusion without injury to the dura in spontaneously hypertensive rats using the simple krypton (Kr) laser system and rose bengal [4,26].

A few researchers have occasionally used the photothrombotic technique in mouse studies [10,18,20]. However, some caution is necessary in applying photochemistry to focal cerebral ischemia. In an advanced study by Boquillon et al. [3], they described a shift of the absorption spectrum peak of a rose bengal solution, i.e., 548 nm, when dissolved in normal saline, to 562 nm, when dissolved in mouse plasma (due to protein binding). Thus a Kr laser of 568-nm wavelength is quite efficient for activating rose bengal and forming a platelet-rich (almost entirely platelet) thrombus, resulting in the occlusion of an artery in vivo. In addition, heat production is almost negligible, as shown in Fig. 3. In the case of an incoherent light source of 540-nm wavelength, the efficiency of light absorption reduces to approximately 30%, as compared with a 568-nm beam, so that thrombus formation likely takes a much longer time and may produce excess heat.

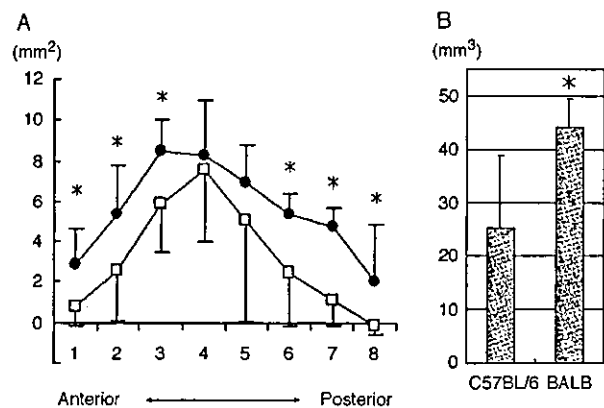


Fig. 4. Infarct volume in C57BL/6 and BALB mice ( $n=8$  vs. 8). (A) Infarct area by slice. Open squares ( $\square$ ) and closed circles ( $\bullet$ ) represent C57BL/6 and BALB mice, respectively.  $F=16.65$ ,  $P<0.001$  by ANOVA. (B) Total infarct volume.  $*P<0.005$  by unpaired Student's *t*-test.

The lack of need to open the skull and leaving the dura intact are other advantages of the laser method. Because the mouse skull is very thin, drilling out the skull without traumatic or heat injury to the brain parenchyma is truly challenging. More importantly, Glazier et al. [7] unexpectedly found that the cortical expression of heat shock protein 72 is induced, and thereby, ischemic tolerance is observed following sham operation of direct (mechanical) distal MCAO. Their finding suggests that craniotomy and opening the dura alone may cause a substantial amount of stress to the brain cortex in rats. In addition, we found that microclip occlusion of distal MCA induces a small amount of subarachnoid hemorrhage, whereas laser-induced photothrombosis and reperfusion does not make any injury to the distal MCA [27]. In laser photothrombosis in mice, the thinness and translucency of the skull are not at all problematic but are quite helpful in locating and irradiating the MCA beneath the skull. With the suture occlusion technique, craniotomy is unnecessary. Instead, careful measurement of cerebral blood flow during ischemia [8,9] and an evaluation of posterior communicating artery patency [12] may be required to obtain less variable infarct size.

Theoretically, laser light illumination alone could induce tissue damage. Our laser method has several advantages regarding this issue. First of all, laser method enables a pinpoint irradiation (diameter of irradiated area is as small as 0.2 mm) so that illuminated area is practically confined to the distal segment of the MCA. Therefore, possible damage on the brain tissue can be minimized. Second, several researchers have histologically shown that direct laser irradiation (through the dura matter) does not cause any damage on the irradiated vessel [24,27]. Third, spectrin breakdown, which is fairly sensitive to tissue damage as compared with histological evaluation, was the same level both in the illuminated and unilluminated areas when not administering rose bengal solution [25]. We employed smaller power, and irradiation was performed through the skull in this mouse model. Taken altogether, we could at least say that laser illumination alone causes negligible, if any, damage on the brain tissue.

C57BL/6 is one of the most frequently used strains as a background strain in transgenic or knockout mouse studies. Although several researchers investigated the degree of cerebral ischemia in different strains of mice, including C57BL/6, the results are somewhat controversial. Fujii et al. [6] showed that C57BL/6 mice are more susceptible to global ischemia by bilateral carotid artery occlusion than are SV129 mice. In addition, infarct volume after permanent or transient focal ischemia by the suture occlusion technique is larger in C57BL/6 than in SV129 [5], and Maeda et al. [14] demonstrated that the area perfused by the MCA (MCA territory) is bigger in C57BL/6 than in SV129. Prass et al. [19], however, reported that C57BL/6 mice show similar size of infarction as SV129 mice do when ischemia is induced by suture occlusion.

Recently, BALB mice were proven to produce a much larger infarct than CFV or BDW following direct MCAO [1]. Majid et al. [15] demonstrated that among BALB, C57BL/6, and SV129 mice, BALB shows much bigger infarction than do the other two strains after direct distal MCAO. Our results in the current study corresponded very well with these results using direct MCAO models. Both Barone et al. [1] and Majid et al. [15] proposed that the differences may be attributed to the patency of the posterior communicating artery, which is relatively undeveloped in BALB mice. In addition to efficacy of collateral circulation, difference in induction of the tumor necrosis factor is suggested to be important in the strain-related difference of infarct size after focal ischemia. In the present study, we performed “tandem occlusion” of the right MCA and the ipsilateral CCA and obtained similar results, i.e., bigger infarction in BALB mice than in C57BL/6. As Barone et al. [1] have already described, the addition of CCA occlusion facilitated a fairly constant and larger size of infarction as compared with MCAO only in this study (unpublished observation).

In conclusion, Kr laser-induced photothrombosis could efficiently occlude the distal segment of the MCA, even when irradiating through the intact skull, and the experimental variance in infarct volume is satisfactorily small when using BALB mice. This model may be useful when applied to a long-term experiment, owing to its reproducibility and minimal invasiveness.

### 6.1. Troubleshooting

Due to its tiny size, the cannulation of vessels in a mouse may be a difficult part of the experiment. However, practice and use of good microscope of large magnification solve this problem. A small amount of hemorrhage substantially weakens a mouse, and particular attention should be paid to avoid hemorrhage from vessels or muscles. Air bubble, injected along with a rose bengal solution, may cause air embolism and lead to the loss of an animal. Awareness of the trouble will efficiently prevent air embolism. Lastly, the distal part of the MCA may hardly be identifiable because of variation in some mouse. If the MCA cannot be found, even with moistening the skull, exclusion of the mouse is recommended.

### 6.2. Alternative and support protocols

For focal ischemia in mice, there are currently two major methods: the direct cauterization and the suture occlusion. Both methods, however, have disadvantages of their own, mostly due to technical difficulties, short survival times, or variance in the extent and severity of ischemia. The embolic stroke model in mice described by Zhang et al. [28] can be a good alternative. They made stroke by putting a clot of blood at the origin of MCA through a catheter. Compared with our model, it requires a