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Effects of Benidipine Hydrochloride on Cerebrovascular Lesions in Salt-Loaded Stroke-Prone Spontaneously Hypertensive Rats: Evaluation by Magnetic Resonance Imaging

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ABSTRACT—We determined possible protective effects of benidipine hydrochloride (benidipine), a dihydropyridine calcium antagonist, on cerebrovascular lesions in salt-loaded stroke-prone spontaneously hypertensive rats (SHRSP). The animals were orally treated with benidipine at 1, 3 and 10 mg/kg daily for 7 weeks, and their neurological symptoms, body weight changes, systolic blood pressure and cerebrovascular lesions on magnetic resonance imaging (MRI) were determined at various time points of treatment. Moreover, the brains of the rats that showed cerebrovascular lesions on MRI in the course of treatment or completed 7-week treatment were examined histopathologically. Control rats presented such symptoms as sedation, ataxia and aggressiveness, while their MRI analysis revealed high signals over wide areas from the occipital to frontal cortex and from the corpus callosum to external capsule. These high signal areas corresponded in location to edematous or softening lesions revealed by the histopathological observation. Treatment with benidipine at 3 and 10 mg/kg ameliorated neurological symptoms, significantly suppressing cerebrovascular damages on MRI. Benidipine at 3 mg/kg significantly decreased blood pressure for the first four weeks but it did not thereafter. These findings demonstrate that benidipine can protect salt-loaded SHRSP from cerebrovascular injury as assessed by MRI.

Keywords: Benidipine, Calcium channel blocker, Magnetic resonance imaging, Stroke-prone spontaneously hypertensive rat, Cerebrovascular lesion

Hypertension is one of the risk factors for cerebrovascular and cardiovascular diseases, and its severity is highly correlated with the incidence of stroke, ischemic heart diseases and renal insufficiency (1, 2). The stroke-prone spontaneously hypertensive rats (SHRSP), established by Okamoto et al., develop serious hypertension, frequently exhibiting strokes (3). Similarity of cerebrovascular lesions between SHRSP and humans (4) makes this animal a useful experimental model for investigating the pathogenesis of stroke and its treatment measures in humans (3, 5). Calcium channel blockers have been reported to suppress development of stroke and to prolong survival time in SHRSP (6 – 10). These observations suggest a possibility that the antihypertensive treatment improves the morbidity and mortality of the patients with stroke. Assessment of calcium channel blocker against cerebrovascular injuries in SHRSP has been made mainly by using histopathological techniques, and none of the previous studies have employed magnetic resonance imaging (MRI) to examine the time-related effect of calcium channel blockers on cerebral damages.

Benidipine hydrochloride (benidipine) is a long-acting calcium channel blocker, which is widely used in clinical practice for the treatment of essential hypertension, renoparenchymal hypertension and angina pectoris (11, 12). This compound has been shown to protect against cerebral damages in a rat model of cerebral ischemia (13, 14). In this study, we determined the time-related effects of benidipine on cerebrovascular lesions in salt-loaded SHRSP, by using the noninvasive diagnostic means MRI (15), focusing on the development of cerebral injuries and the site, nature and importance of intracerebral lesions. Moreover, we examined the cerebral damages histopathologically and compared those with the MRI data.

MATERIALS AND METHODS

Animals used

Six-week-old male SHRSP were obtained from a commercial source (Hoshino Experimental Animal Farms Co.,

Ltd., Saitama), and those that were considered healthy according to their weight changes and general condition during four-week adaptation were used for the experiments. They had free access to the pellets (FR-2; Funabashi Farms Co., Ltd., Chiba). For drinking water, they were given, ad libitum, tap water before the start of treatment (up to 9 weeks of age) and 1% saline throughout the treatment period (starting from 10 weeks of age through 17 weeks of age). Since the previous studies indicated no difference in overall or final incidence of stroke between SHRSP on salt-loaded diet and those on ordinary diet (3, 16), we used 1% saline to accelerate hypertension, ensuring homogeneous occurrence of stroke in SHRSP. Animals were divided into four groups so that the SHRSP in each group showed almost the same mean values of systolic blood pressure before the treatment period.

Drugs used

Benidipine (Lot No. P-009; Kyowa Hakko Kogyo Co., Ltd., Tokyo) was prepared before each use with 0.3% carboxymethyl cellulose-Na in distilled water (0.3% CMC-Na), and 0.5 ml per 100 g body weight was given to each animal. In the MRI study, animals were anesthetized with pentobarbital-Na (Tokyo Kasei Co., Ltd., Tokyo) and halothane (Fluothane®; Takeda Chemical Industries Co., Ltd., Osaka).

The doses of benidipine examined were 1, 3 and 10 mg/kg, which showed antihypertensive effects in SHR and SHRSP (11, 17, 18) and suppressed renal lesions in SHRSP (17, 18). Control animals were given 0.3% CMC-Na solution. Drug or vehicle was administered once a day for 7 weeks starting from 10 weeks of age. The control and 3 mg/kg benidipine groups consisted of 7 animals each, and the 1 and 10 mg/kg benidipine groups consisted of 8 animals each.

Neurological observation

Neurological observation was carried out every day before the administration of drug or vehicle according to the description by Nagaoka et al. (19). Incidence of neurological symptoms in SHRSP was estimated at each week of age by the method of Ogiku et al. (20).

Body weight measurement

Body weight was measured once a week before the administration of drug or vehicle.

Systolic blood pressure

Systolic blood pressure was determined once a week by the tail-cuff technique with a noninvasive manometer (TK-350; Unicom, Chiba) 3 to 5 h after the administration of drug or vehicle.

MRI

MRI was performed once every two weeks under anesthesia with pentobarbital Na (40 mg/kg, i.p.) and halothane (0.5 – 1.0%) with room air. For the animals that presented neurological symptoms in the course of treatment, MRI was performed when such symptoms appeared.

An MRI equipment of 2.0-tesla superconductive magnet type (SIS 85/310; Varian, Palo Alto, CA, USA) was used for imaging. The area from the tip of the frontal lobe (#8) to the end of the cerebellum (#1) was sliced into 8 portions upon T_1-weighted images (TR/TE = 520/20 ms; TR: time of repetition; TE: time of echo) and T_2-weighted images (TR/TE = 1600/80 ms) obtained with a spin-echo pulse sequence, and frontal sections were visualized by sequential multi-slice imaging. The other imaging parameters were determined according to the following conditions: thickness of slice: 2.5 mm, field of view (FOV): 50 × 50 mm, number of matrices: 256 × 128 and number of integrations: 8 for T_1-weighted imaging and 6 for T_2-weighted imaging.

A high signal on T_2-weighted images was considered to indicate the presence of cerebrovascular lesions such as edema or softening lesions (15, 21).

Histopathology

The SHRSP that exhibited MRI-confirmed cerebrovascular lesions or that completed treatment were bled to death under ether anesthesia and immediately subjected to craniotomy to excise the brain (cerebrum, cerebellum and medulla oblongata). The organs thus obtained were then fixed in a 10% neutral buffered formalin solution, and the area from the tip of the frontal region to the end of the cerebellum was divided into 8 portions at frontal section in correspondence with the MR images prior to the usual paraffin embedding. Histopathological examination was carried out by light microscopy on preparations double-stained with hematoxylin-eosin (HE) and Klüver-Barrera (KB).

Statistical analyses

Results of the experiments were expressed as mean values ± standard errors of the mean or as incidences. Difference in mean values was evaluated first by the Bartlett's test for variance and then by the one-way analysis of variance (ANOVA) if the variance was found to be uniform ($P>0.05$) or by the Kruskal-Wallis's rank sum test if it was not uniform ($P\leqq 0.05$). When ANOVA or the Kruskal-Wallis's test revealed a significant difference ($P\leqq 0.05$), then a comparison was made between the control and experimental groups by Dunnett's test (either parametric or non-parametric analysis). As for the incidence of cerebrovascular lesions, the χ^2 test was applied to compare control and treated animals.

RESULTS

Effects on neurological symptoms

Control animals showed sedation, ataxia or aggressiveness after 12 weeks of age, and at 17 weeks of age, neurological symptoms were observed in 6 of 7 rats. One of 8 animals at 1 mg/kg was dead, showing neurological symptoms at 13 weeks of age. In animals treated with benidipine at 1 mg/kg, symptoms including aggressiveness and convulsion appeared after 11 weeks of age, and 6 of 7 animals had these symptoms at 17 weeks of age, showing similar incidence to that in control animals. Treatment with benidipine at 3 mg/kg caused sedation in one of 7 animals at 11 weeks of age, and neurological symptoms were seen in only 2 animals at 17 weeks of age. In the 10 mg/kg benidipine group, no neurological symptoms appeared up to 16 weeks of age except convulsion in one 11-week-old animal. At 17 weeks of age, 2 of 8 rats were neurologically affected (Table 1).

Effects on body weight changes

In control animals and those treated with benidipine at 1 mg/kg, individual body weights were decreased in the animals showing neurological symptoms. One of 7 animals treated with benidipine at 3 mg/kg lost weight as neurological symptoms got worse, while all 8 animals in the 10 mg/kg benidipine group gained weight.

Effects on systolic blood pressure

In animals treated with benidipine at 1 mg/kg, blood pressure was slightly lowered by 18 mmHg as compared with controls on the day of the first dose (at 10 weeks of age), but was not changed after 11 weeks of age. Treatment with benidipine at 3 mg/kg significantly lowered blood pressure by 43 mmHg on the first dosing day and by 61 mmHg one week later (at 11 weeks of age), compared with the control; although the blood pressure increased with aging, such a significant antihypertensive effect lasted until 13 weeks of age; thereafter, blood pressure continued

Table 1. Effects of repeated administration of benidipine hydrochloride on symptoms and MRI of brain in SHRSP

Drugs	Dose (mg/kg per day)	Animal No. (age weeks)	Main symptom	Hyperintensity of each slice in brain by multislice MR images (T_2-WI)							
				#1	#2	#3	#4	#5	#6	#7	#8
Control		1 (16W)	aggression	ND	ND	ND	CC	CC	CC, FC	FPC	ND
		2 (13W)	ataxia	ND	ND	ND	OC	FC	FC	ND	ND
		3 (14W)	aggression	ND	ND	ND	OC	CC, CAE	CC, CAE, CP	FPC	FPC
		4 (12W)	sedation	ND	ND	ND	OC	CC, CAE	CC, CAE	FPC	FPC
		6 (15W)	aggression	ND	ND	ND	OC	ND	CP	FPC	ND
		7 (17W)	normal	ND	ND	ND	OC	CC	CC, CP	FPC	ND
		8 (13W)	aggression	ND	ND	ND	OC	CC, CAE	CC, CAE	FPC	ND
Benidipine	1	9 (14W)	aggression	ND	ND	ND	ND	CC	CP	FPC	ND
		10 (13W)	convulsion	ND	ND	ND	OC	CC, CAE	CC, CAE	FPC	ND
		11 (16W)	aggression	ND	ND	ND	OC	FC	CC, CAE	FPC	ND
		12 (12W)	aggression	ND	ND	OC	OC	CC, CAE	CC	FPC	FPC
		13 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		14 (13W)	convulsion	ND	ND	ND	OC	CC	ND	FPC	ND
		15 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		16 (13W)	death								
Benidipine	3	18 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		19 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		20 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		21 (17W)	convulsion	ND	ND	ND	ND	ND	ND	ND	ND
		22 (15W)	convulsion	ND	ND	ND	ND	ND	FC	FPC	ND
		23 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		24 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
Benidipine	10	25 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		26 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		27 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		28 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		29 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		30 (11W)	convulsion	ND	ND	ND	ND	CP	ND	ND	ND
		31 (17W)	normal	ND	ND	ND	ND	ND	ND	ND	ND
		32 (17W)	convulsion	ND	ND	ND	ND	ND	ND	ND	ND

SHRSP were loaded with 1% salt in drinking water for 8 weeks from 10 weeks of age. Vehicle or benidipine hydrochloride (1, 3 or 10 mg/kg per day) was orally administered for the same period. #1 – #8: slice No. ND: not detected, CC: corpus callosum, CAE: external capsule, FC: frontal cortex, FPC: frontoparietal cortex, OC: occipital cortex, CP: caudate putamen.

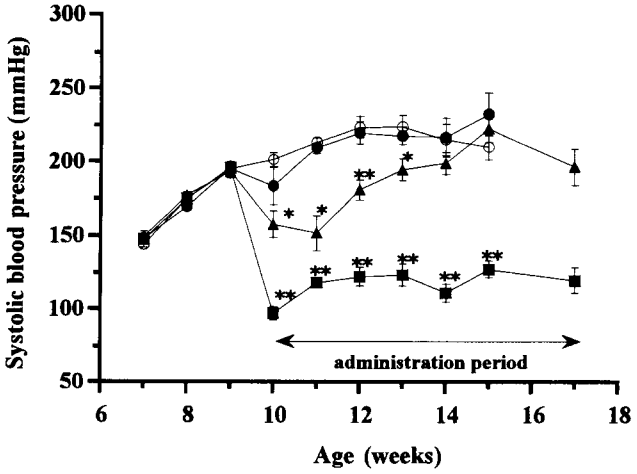

Fig. 1. Effects of repeated administration of benidipine hydrochloride on systolic blood pressure in SHRSP. SHRSP were loaded with 1% NaCl drinking water for 8 weeks from 10 weeks of age. Benidipine hydrochloride or vehicle was administered for the same period. ○: vehicle, ●: 1 mg/kg, ▲: 3 mg/kg, ■: 10 mg/kg. Each point represents the mean ± S.E.M. of 3 to 8 animals. * and **: Significant difference from the control at $P<0.05$ and $P<0.01$, respectively.

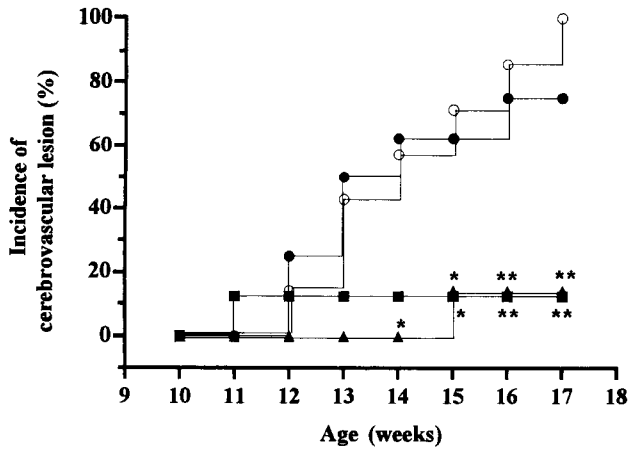

Fig. 2. Effects of repeated administration of benidipine hydrochloride on incidence of cerebrovascular lesion in SHRSP. SHRSP were loaded with 1% NaCl drinking water for 8 weeks from 10 weeks of age. Benidipine hydrochloride or vehicle was administered for the same period. The cerebrovascular lesion was determined by T_2-weighted MR images. ○: vehicle, ●: 1 mg/kg, ▲: 3 mg/kg, ■: 10 mg/kg. * and **: Significant difference from the control at $P<0.05$ and $P<0.01$, respectively.

increasing and remained as high as 198 to 223 mmHg through the end of treatment (at 17 weeks of age). A significant antihypertensive effect was observed in the animals treated with benidipine at 10 mg/kg throughout the treatment period, the pressure being lowered by 80 to 100 mmHg as compared with control animals (Fig. 1).

Brain MRI findings

Figure 2 shows the incidence of cerebrovascular lesions and Table 1 summarizes the affected regions in brain slices.

In control animals, cerebral T_2-weighted images taken 1 to 2 days after onset of neurological symptoms showed high signal departments (abnormal signal regions) suggestive of cerebrovascular lesions in areas from the occipital to frontoparietal cortex (slices #4 to #8) and from the corpus callosum to external capsule (slices #5 and #6) after 12 weeks of age. Cerebrovascular lesions continued to increase with age to the extent that all 17-week-old control animals had cerebrovascular damages. Figure 3 represents a typical control MRI recording. In this T_2-weighted image of animal No. 8 at 13 weeks of age, high signal departments were detected in the frontal cortex and the area involving the corpus callosum and external capsule (slice #5).

In animals treated with benidipine at 1 mg/kg, cerebrovascular lesions were observed after 12 weeks of age, as in control animals, and continued to increase with age up to 17 weeks of age, when 6 of 7 animals showed lesions. The areas affected were as wide as those in control animals, involving the occipital to the frontoparietal cortex (slices #4 to #8) and the corpus callosum to the external capsule (slices #5 and #6). Benidipine at 3 mg/kg significantly suppressed development of cerebrovascular lesions at and after 14 weeks of age, when only one 15-week-old animal had damaged vessels. A typical MR image of benidipine-treated animals is given in Fig. 4. This T_2-weighted image from animal No. 20 at 17 weeks of age failed to reveal any high signal departments (slice #5). One of 8 animals treated with benidipine at 10 mg/kg developed cerebrovascular lesions at 11 weeks of age, but at and after 15 weeks, this dosage level protected all the remaining animals from brain injury.

Effects on histopathological changes

Five of 7 control animals developed edema in the cerebral cortex, particularly in the frontal lobe and parietal lobe; it was mild in 3 animals while, in 2 animals, it formed extensive softening lesions accompanied by degeneration, necrosis and loss of neurons. Spongiosis observed in the corpus callosum of 7 animals was located mainly in the circumference of edema. Hemorrhage ranging in severity from mild (2 animals) to moderate (3 animals) occurred mostly in the cerebral cortex and rarely in the corpus callosum and white matter. It was fresh bleeding in the majority of cases (Fig. 5).

Animals treated with benidipine at 1 mg/kg developed minimal edema (1 of 7 animals) and softening lesions (4 animals), which were located mainly in the cerebral cortex, particularly in the frontal and parietal lobe. Only one animal had edema in the corpus callosum. As for hemorrhage, fresh bleeding was seen, mild in two animals and moderate in another two. Four rats showed spongiosis

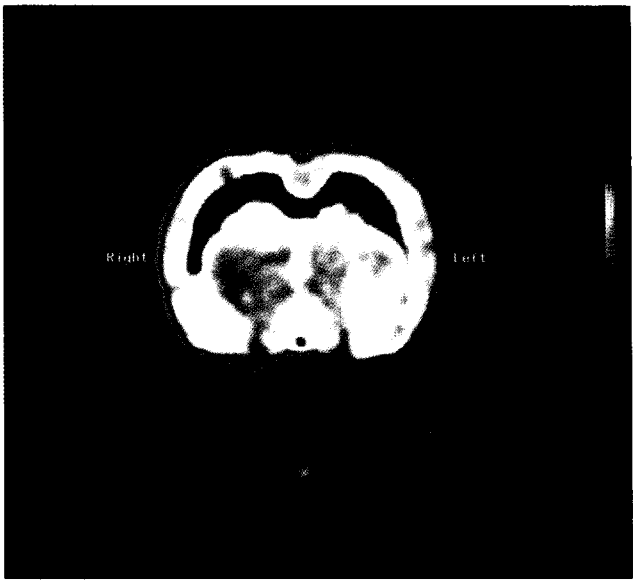

Fig. 3. Coronal T_2-weighted image of brain (slice #5) of the untreated SHRSP (Animal No. 8, 13 weeks of age). High signal (red) department rate was detected in the frontal cortex, corpus callosum and the external capsule.

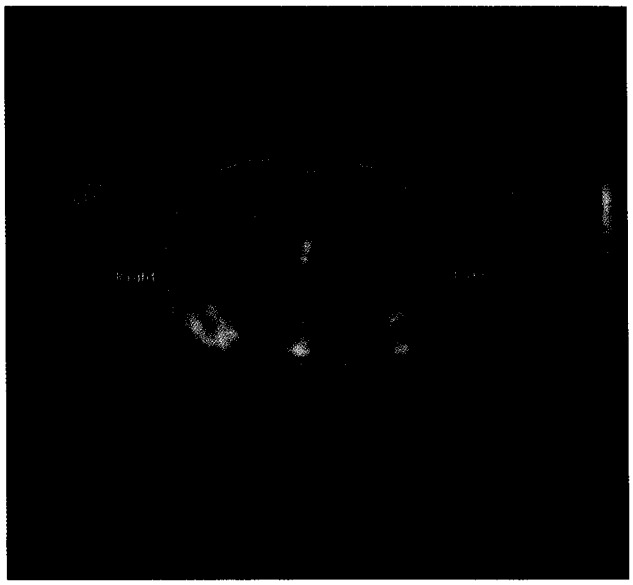

Fig. 4. Coronal T_2-weighted image of brain (slice #5) of the benidipine-treated SHRSP (Animal No. 20, 17 weeks of age). High signal (red) department rate was not detected.

mainly in the cerebral cortex, mild in 3 and moderate in one. Slight and moderate cellular reactions involving glia cells occurred in two animals each. The remaining two animals had no histopathological changes. In the 3 mg/kg benidipine group, only one animal developed histopathological changes such as mild edema, spongiosis in the frontal lobe of the cerebral cortex and moderate localized fresh hemorrhage. No abnormal findings were obtained in the remaining 6 animals (Fig. 6). All the animals treated with benidipine at 10 mg/kg were normal.

Fig. 5. The brain of the untreated SHRSP. Vehicle control: Animal No. 8 (13 weeks of age). Softening (arrow heads) was observed in the cortex and edema, hemorrhage (arrow) observed in the corpus callosum. HE-KB staining, ×17.

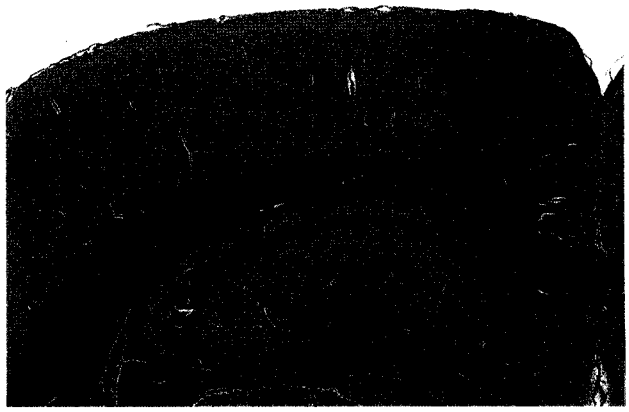

Fig. 6. The brain of the benidipine-treated SHRSP. Benidipine hydrochloride, 3 mg/kg: Animal No. 20 (17 weeks of age). No abnormal finding was observed in the cortex and the hippocampus. HE-KB staining, ×21.

Comparison of histopathology and MRI analysis

Locations of edema or softening lesions in the cerebral cortex as detected by histopathology (Fig. 5) accorded in many cases with high signal departments on MR T_2-weighted images (Fig. 3). T_2-weighted imaging clearly visualized edema in the corpus callosum that was hardly detectable by light microscopy following HE-KB double staining. However, the hemorrhage, cellular reactions involving glia cells and vascular lesions revealed by histopathology were

scarcely detected as abnormal signals on T_1- or T_2-weighted images. On the other hand, the abnormal signals in the cerebral basal nuclei (nucleus caudatus, putamen, pallidum, thalamus, etc.) on T_2-weighted images were not accompanied by abnormal histopathological findings.

DISCUSSION

This study assessed the influence of benidipine on salt-loaded SHRSP through general behavioral observation, histopathology and MRI, and demonstrated beneficial effects of the prophylactic treatment with this compound at 3 and 10 mg/kg against development of cerebrovascular lesions. Benidipine at minimal hypotensive doses also proved to be very effective in protecting animals from cerebral injury. This is the first study that evaluated the time-related effects of a calcuim channel blocker in SHRSP by means of MRI.

In SHRSP, hypertension exceeding 200 mmHg accompanies cerebrovascular lesions mainly involving perforating arterioles (22, 23), and further rise in blood pressure damages the blood-brain barrier, leading to extravasation of plasma components. Damaged endothelial cells and smooth muscle cells in damaged cerebral vessels are replaced by fibrinoids, basal membrane and collagenous fiber (24), resulting in dysfunction of endothelial cells and necrosis of arterioles (25, 26). Such necrosis is a starting point toward rupture of arteriolar microtubule aneurysm and thrombotic occlusion of aneurysmal arterioles, which lead to cerebral hemorrhage and infarct, respectively (22, 23). These previous observations were confirmed by the present study: control animals had a systolic blood pressure of over 200 mmHg in and after the 2nd week of treatment with the vehicle (at 12 weeks of age) and developed neurological symptoms such as aggressiveness and convulsion in the following week (at 13 weeks of age), all of them being more or less affected neurologically at the end of treatment (at 17 weeks of age). The histopathological examination of these animals revealed edema and degeneration and loss of neurons as described in previous studies.

MRI is a noninvasive diagnostic technique. It has been used to study development, location, nature and importance of cerebrovascular lesions in SHRSP (15), and the correlation between MRI and histopathology data has been investigated (with regard to edema, softening and hemorrhage) (27). The MRI T_2-weighted images of the control SHRSP displayed abnormal signals in the occipital cortex, frontal cortex and the frontoparietal cortex and over a wide area from the corpus callosum to the external capsule. The histopathological analysis identified these signals as edema and softening lesions with degeneration, necrosis and loss of neurons. This observation supports the notion that high signals on T_2-weighted images originate from edematization that increases water content and consequently prolongs T_2 relaxation time (28).

Although histopathological examination on the cerebral cortex from control animals showed mild to moderate fresh hemorrhage, MRI T_1- and T_2-weighted images failed to give any corresponding abnormal signals. It is known that MRI is so sensitive to cerebral hemorrhage as to emit different signals according to the phase of hematoma (29, 30). Intracerebral hematoma proceeds from the superacute phase to acute, subacute and chronic phases. Hemoglobin is oxidized into oxyhemoglobin, deoxyhemoglobin, methemoglobin and finally into hemosiderin; and it is the oxidated hemoglobin in its advanced level of oxidation that visualizes T_2-weighted imaging clearly. Absence of clear T_2-weighted images corresponding to the hemorrhage, which was confirmed to be fresh bleeding by histopathology, suggests the possibility that the MRI signals reflected the superacute phase of hematoma. In the basal nuclei, however, T_2-weighted images gave abnormal signals, while histopathology was unable to detect any corresponding abnormality. Thus, it is suggested that MRI is useful for detecting slight or localized lesions that are beyond the limit of histopathology. Moreover, MRI seems to be an excellent method that ensures not only time-related but highly sensitive and precise observation of cerebrovascular lesions.

Treatment with benidipine at 3 and 10 mg/kg prevented stroke symptoms and suppressed development of edema and other degeneration, as was observed in the behavioral and histopathological examinations. Moreover, the T_2-weighted image analysis showed that development of cerebrovascular lesions was significantly suppressed in rats that were 14-week-old and over. In this study, blood pressure was maintained at a lower level in the SHRSP treated with benidipine at 3 and 10 mg/kg than that in control animals, thus suggesting involvement of the hypotensive effect in suppressing strokes and cerebral injury. In the latter half of the treatment period, however, benidipine at 3 mg/kg suppressed development of cerebrovascular lesions despite a rise in blood pressure to 200 mmHg or higher. In fact, hydralazine, a vasodilator agent, which prevents the progression of hypertension, showed no beneficial effects on survival of animals (31). Suppression of cerebrovascular lesions by benidipine at minimal hypotensive doses may indicate involvement of some other mechanisms, responsible for the beneficial effect of this drug. Whether these direct effects of benidipine on cerebrovascular injury are involved in prevention of cerebrovascular lesions remains to be clarified.

In summary, the present observation of general behavioral conditions, histopathology and MRI in salt-loaded SHRSP showed that benidipine could prevent development of cerebral injury.

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