Two-Kidney, Two Clip Renovascular Hypertensive Rats Can Be Used as Stroke-prone Rats

Jinsheng Zeng, MD, PhD; Yiqin Zhang, MD, MSc; Jianwei Mo, MD, MSc; Zhenpei Su, MD, MSc; Ruxun Huang, MD

Background and Purpose—The cerebrovascular lesions in stroke-prone spontaneously hypertensive rats are not only dependent on high blood pressure but partly related to pressure-independent genetic factors. The aim of the present study was to observe whether spontaneous stroke occurred in renovascular hypertensive rats without a genetic deficiency.

Methods—The 1-kidney, 1 clip (1k1c); 2-kidney, 1 clip (2k1c); and 2-kidney, 2 clip (2k2c) methods were used to induce hypertension in male Sprague-Dawley rats with a ring-shaped silver clip. Sham-operated rats were used as controls. Blood pressure and neurological symptoms were observed in the rats without any artificial inducement. Brain sections stained with hematoxylin-eosin and phosphotungstic acid–hematoxylin were examined under a microscope to determine stroke foci.

Results—The attack rate of stable hypertension was 100% (55/55) in the 2k2c group, which was significantly higher than that in the 1k1c (23/30, 76.7%) and 2k1c (21/30, 70%) groups (P<0.01). None of the rats in the 2k2c group died of acute renal failure or suffered from diffuse cerebral lesions postoperatively. Forty weeks after renal artery constriction, the incidence of spontaneous stroke in the 2k2c group was 61.8% (34/55), which was significant higher than that in the 1k1c (7/30, 23.3%) and 2k1c (5/30, 16.7%) groups (P<0.01). Stroke foci were not observed in normotensive controls.

Conclusions—We conclude that 2k2c renovascular hypertensive rats with proper renal artery constriction can be used as stroke-prone renovascular hypertensive rats independent of a genetic deficiency. (Stroke. 1998;29:1708-1714.)

Key Words: cerebrovascular disorders ■ hypertension ■ rats

Most of the animal models mimicking stroke are established on normotensive animals with occlusion or rupture of cerebral artery to artificially induce infarction or hemorrhage in brain.1,2 It is well accepted that hypertension is one of the most important risk factors causing cerebrovascular disorders. The majority of patients with stroke clinically have extensive cerebral arteriosclerosis induced by hypertension.3,4 There are great differences in structure of the cerebral artery, autoregulation of cerebral blood flow, extent of lesion in brain tissue, and reaction to medication after ischemia between subjects with extensive cerebral arteriosclerosis and subjects with normal cerebral blood vessels.5-7 The relevance of animal models with normal cerebrovascular structure to human conditions remains dubious.8

The stroke-prone spontaneously hypertensive rats (SHRSP) bred from spontaneously hypertensive rats (SHR) are the most utilized animal model of spontaneous stroke and are regarded as a unique animal model in which prevention of stroke can be studied experimentally because the incidence of spontaneous occurrence of stroke lesions reached ≈80% in males and 60% in females with extensive cerebral arteriosclerosis.9,10 However, the structural abnormalities of the cerebral artery in SHR and SHRSP are not only dependent on high blood pressure (BP) but at least to some extent are related to BP-independent genetic factors.10,11 There are great genetic differences of the cerebral structure between normotensive control strains (Wistar-Kyoto rats) and SHR.12-14 Obviously, it is possible that the genetic deficiency may obscure some study results on cerebrovascular disorders in SHR and SHRSP.

Renovascular hypertensive rats (RHR) are commonly used as experimental models for the study of hypertension. There are many different methods to induce hypertension with renal arterial constriction, eg, one-kidney one clip (1k1c); two-kidney one clip (2k1c); and two-kidney two clip (2k2c) methods.6,12-18 Until now, however, little attention has been given to the incidence of spontaneous stroke in RHR, especially in RHR induced with the 2k2c method. To find a type of RHR with a high incidence of spontaneous stroke independent of a genetic deficiency, the present study was designed to observe the incidence of spontaneous stroke and the relationship between stroke onset and level and duration of high BP in different RHR.
Materials and Methods

Surgery

The experimental protocol was approved by the local ethical committee for animal research. A total of 115 male Sprague-Dawley rats weighing 80 to 100 g underwent an operation of renal artery constriction with 3 different methods: 30 rats each with the 1k1c and 2k1c methods and 55 rats with the 2k2c method. Under anesthesia with 3% sodium pentobarbital (36 mg/kg body wt IP), a median longitudinal incision on abdominal skin was performed, then a ring-shaped silver clip with an inner diameter of 0.30 mm was placed around the root of right renal artery, followed by a left nephrectomy in rats in the 1k1c group. In the 2k2c group, rats underwent the same surgical procedure in the right renal artery as the 1k1c group, but the left contralateral kidney remained untouched. The roots of both right and left renal arteries were constricted by placing ring-shaped silver clips with an inner diameter of 0.30 mm to induce hypertension in the 2k2c group. The ring part of the clip was placed around the root of each artery, and then the outer gap of the clip was shut. During the operation, the remaining kidneys, liver, chyloycst, and renal veins were undamaged. Thirty-five sham-operated rats underwent the same experimental procedures as the test rats except for placement of renal artery clip and nephrectomy; they served as normotensive controls. All rats were allowed an ordinary rat chow diet (plant protein 15.9%, nonfish animal protein 5.4%, fish protein 1.7%; carbohydrate 52.5%; unsaturated fat 3.4%, saturated fat 1.3%; Na+ 0.24%, K+ 1.0%) and tap water as desired and kept on a 12-hour light/dark cycle.

Blood Pressure Measurement and Stroke Symptom Observation

Systolic BP was measured by an indirect tail-cuff sphygmomanometer (MRB-III, Shanghai Institute of Hypertension) in preheated (37°C, 15 minutes) conscious rats before and at weekly intervals after renal artery constriction for 40 weeks. For 40 weeks after renal arterial constriction, movement of limbs, respiration, diet, fur, and consciousness of the rats were examined twice daily (at 8 AM and 6 PM).

Histology

On the third day after neurological symptoms occurred, the rats were terminally anesthetized with sodium pentobarbital and perfused through the ascending aorta with 0.9% saline for 1 minute, followed by 4% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 5 minutes at room temperature. At 40 weeks after renal artery constriction, all surviving rats were perfused with the aforementioned methods. The brain was removed, then photographed and immersed in the same fixative overnight at 4°C. In the rats that died abruptly, the brain was removed without perfusion and then immersed in 4% formaldehyde in 0.1 mol/L phosphate buffer over 2 nights at 4°C. Then the brains were sectioned into 1.0-mm-thick coronal sections in a brain cutter and were dehydrated and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin-eosin; some sections were stained for periodic acid–Schiff (PAS) detection and some for phosphotungstic acid–hematoxylin (PTAH) for routine light microscopic observation.

Statistical Analysis

Values are expressed as mean±SD. ANOVA followed by Student’s t test was performed for the comparison of BP level and days in which peak BP occurred. The χ² test was used for comparison of incidence of hypertension and stroke between two groups. P<0.05 was considered significant.

Results

Blood Pressure

The mean systolic BP in male Sprague-Dawley rats weighing 80 to 100 g was 110 mm Hg before renal artery constriction, rose to 150±8, 122±7, and 125±7 mm Hg at the end of first week, then exceeded 170, 150, and 150 mm Hg at 3 weeks in the 1k1c, 2k1c, and 2k2c groups, respectively, after renal artery constriction. Afterward, it rose progressively and reached a peak mean value of 196±18, 172±25, 215±23, and 143±9 mm Hg at 47±23, 47±12, 172±48, and 273±25 days postoperatively in 1k1c, 2k1c, 2k2c, and control groups, respectively. The peak BP in the 2k2c group was significantly higher than that in the 1k1c and 2k1c groups (P<0.01), and the time to reach peak BP in the 2k2c group was longer than in the other test groups within 40 weeks after operation (P<0.01).

In the 1k1c group, 7 rats (23.3%) died of acute renal failure 1 week postoperatively. In the 2k1c group 6 rats had not developed hypertension, and the high BP of 3 rats returned to normal level after 6 postoperative weeks. Chronic hypertension (BP ≥150 mm Hg after 3 postoperative weeks) was induced in 23 and 21 of 30 rats in the 1k1c (76.7%) and 2k1c (70%) groups, respectively, which was significantly lower than that in the 2k2c group (55/55, 100%; P<0.01). The peak BP of rats that displayed symptoms or died after 2 postoperative weeks was significantly higher than that of rats that survived without symptoms in each test group (Table 1).

Table 1. Peak BP in RHR That Displayed Symptoms or Died and in Rats That Survived Without Symptoms in Different Groups

<table>
<thead>
<tr>
<th>Test group</th>
<th>Peak BP</th>
<th>n</th>
<th>Days postoperative (PW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1k1c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS or died 2–5 PW</td>
<td>8</td>
<td></td>
<td>210±7*</td>
</tr>
<tr>
<td>DS or died ≥6 PW</td>
<td>6</td>
<td></td>
<td>212±7*</td>
</tr>
<tr>
<td>NS survivors</td>
<td>9</td>
<td></td>
<td>178±12</td>
</tr>
<tr>
<td>2k1c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS or died 2–5 PW</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS or died ≥6 PW</td>
<td>3</td>
<td></td>
<td>212±10*</td>
</tr>
<tr>
<td>NS survivors</td>
<td>27</td>
<td></td>
<td>167±15</td>
</tr>
<tr>
<td>2k2c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS or died 2–5 PW</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DS or died ≥6 PW</td>
<td>35</td>
<td></td>
<td>225±13*</td>
</tr>
<tr>
<td>NS survivors</td>
<td>20</td>
<td></td>
<td>192±11</td>
</tr>
</tbody>
</table>

n indicates number of rats; DS, displayed symptoms; NS, nonsymptomatic; and PW, postoperative week. Values are mean±SD.

*P<0.01 compared with NS survivors in each group.

Neurological Symptoms

From postoperative weeks 2 to 5, 8 rats in the 1k1c group displayed symptoms, including seizures, bleeding from nose and mouth, changes of respiratory rhythm, and coma, or died without preceding overt symptoms. No rat displayed symptoms or died in the 2k1c, 2k2c, or control groups within the first 5 postoperative weeks.

From weeks 6 to 34, 6, 3, and 35 rats in the 1k1c, 2k1c, and 2k2c groups, respectively, displayed neurological symptoms, including right or left hemiplegia, quadriplegia, bleeding from nose and mouth, and seizure, or died without preceding overt symptoms. Several hours after the occurrence of the symptoms, the signs of brain stem or whole brain damage (eg,
changes of respiratory rhythm and coma) were observed in 4 rats in the 2k2c group. Swelling of head skin was noted in 9 of the rats after the symptoms occurred.

Cerebral Lesions

No gross brain lesions were observed in the rats that died during week 1 after renal artery constriction. The rats that displayed symptoms or died during postoperative weeks 2 to 5 showed diffuse cerebral swelling and disseminated petechial hemorrhages on the surface of the brains (Figure 1A). In the 1k1c, 2k1c, and 2k2c groups, the gross foci of focal brain damage were noted in 4, 2, and 18 rats, respectively, that displayed symptoms or died abruptly after postoperative week 6, including cerebral infarction (Figure 1B), cerebral hemorrhage (Figure 1C), and subarachnoid hemorrhage. On coronal section, the lateral ventricle and midline structures were pressed toward the contralateral side by a large hematoma in 2 rats with cerebral hemorrhage.

Microscopic examination of hematoxylin-eosin–stained brain sections in the rats that died during week 1 after renal artery constriction showed no obvious infarcted or hemorrhagic lesions. Diffuse small foci of necrosis and hematoma in cortex and rarefaction of white matter but no local foci of stroke were observed in the rats that died or showed symptoms during postoperative weeks 2 to 5. These lesions in the brain are similar to those of hypertensive encephalopathy in humans.19,20 Stroke foci were observed in all rats in the 1k1c and 2k1c groups and in 31 of 35 rats in the 2k2c group that died suddenly or with symptoms after postoperative week 6. Stroke foci were also observed in 1 of 8, 2 of 27, and 3 of 20 surviving RHR without symptoms in the 1k1c, 2k1c, and 2k2c groups, respectively. The attack rate of spontaneous stroke in the 2k2c group was higher than that in other groups (Table 2). The subtypes of stroke included cerebral infarction, cerebral hemorrhage, both cerebral infarction and hemorrhage in the same brain, and subarachnoid hemorrhage (Table 2). No obviously different brain lesions were observed between rats that displayed symptoms and rats that died without symptoms. Three rats in the 2k2c group had hemorrhagic infarcts together with ischemic infarcts in the same brain. No stroke focus was found in any control rat, and 4 RHR died abruptly in the 2k2c group. There were no significant differences (P>0.05) among the onset time of cerebral infarction (108±55 days), hemorrhage (98±31 days), and mixed stroke (106±57 days) in the 2k2c group.

The stroke foci were often observed in the cerebral cortex and white substance of the parietal and occipital areas of telencephalon, less often in cerebellum and basal ganglia. On the coronal sections, the largest diameter of hematomas was 50 μm to 3.5 mm, and the largest diameter of infarcts was 100 μm to 4.0 mm. The hemorrhagic lesions were caused by bleeding from the arteriolar wall of fibrinoid necrosis (Figure 2A) or ruptured microaneurysms. In the infarct area, arterioles or small arteries occluded by thrombi were observed (Figure 2B). In the small infarct in which the largest diameter was <1 mm on the coronary section, 1 or 2 occluded arteries were observed. However, in the infarct in which the largest diameter was >1 mm, several lesioned small arteries were occluded. Some large infarcts were found in parietal or/and
occipital areas with a majority of occluded arterioles or small arteries. Swollen cells were observed around the foci of hematoma or infarct, but the obvious disseminated edema signs in whole brain were only found in the brain with large stroke foci. Some small cysts with cells in the wall, which originated from old hemorrhagic or infarcted lesions, were observed in cortex. Rarefaction of the white matter in these areas was also observed. In some instance these lesions contained lakes of PAS-positive material within the tissue, and multilocular cysts developed. In sham-operated rats, no infarcted or hemorrhagic foci were found in the brain.

The cerebrovascular lesions in RHR with stroke in different groups were similar, mainly showing fibrinoid necrosis, hyaline degeneration, and hyperplasia of the wall of arterioles or small arteries with or without enhanced mural thickness, cell proliferation in the external layer, and stenotic lumen (Figure 2A, 2B, and 2D). Fibrinoid necrosis of the wall of cerebral arteries showed as granular and eosinophilic with hematoxylin-eosin staining and blue with PTAH staining. Hyaline degeneration appeared homogeneous or granular, light eosinophilic with hematoxylin-eosin staining and tan with PTAH staining. Sometimes the formation of microaneurysms and thrombotic vascular occlusions was observed (Figure 2C). The predilection sites of the segmental vascular lesions were the same as stroke foci. These lesioned arteries were distributed mainly in the superficial or deep layers of cerebrum, especially in parietal or occipital areas, not only in stroke foci but in normal cortex or subarachnoid space. Sometimes different kinds of arterial lesions were observed in the brain (Figure 2D). The hypertensive arteriolosclerosis in RHR that experienced stroke within 10 weeks after renal artery constriction was mainly shown as fibrinoid necrosis of intracerebral arterioles or small arteries without distinct mural thickening and luminal narrowing. In other rats that had stroke 12 weeks after renal artery constriction, the hyperplasia of cerebral arterioles or small arteries was very obvious, with mural thickening and luminal narrowing. The lesions of large cerebral arteries showed only enhanced medial thickness of the wall without atherosclerosis. The cerebral arterioles and small arteries in RHR without brain lesions showed only medial thickening without fibrinoid necrosis or hyalinosis. Examination of hematoxylin-eosin–and PTAH-stained brain sections in the RHR with diffuse brain lesions showed obvious fibrinoid necrosis of the wall of cerebral vessels.
without cell proliferation in the external layer and occlusion of the lumen by thrombi. No abnormal histological findings of cerebrovascular structure were observed in sham-operated animals.

Discussion

Since the pioneering working of Goldblatt and colleagues, 1k1c and 2k1c methods have been used to induce hypertension in rats for different purposes. However, it has been found that 1k1c RHR often died of acute renal failure and mainly suffered from diffuse lesions in the brain but not stroke because of malignant hypertension. In 2k1c RHR it has been observed that BP is relatively low, and even at 16 weeks it is lower than BP at 4 weeks, which means that at a late stage there is a tendency to return to normal BP levels in 2k1c RHR. In the present study, which included a large number of animals, 23.3% (7/30) of rats died of renal failure during postoperative week 1 in the 1k1c group, and 30% (9/30) of rats did not develop stable hypertension in the 2k1c group. Because there is only one silver clip in each of these RHR and the outer diameters of renal arteries in different rats are not the same, the constrictions with clips of renal arteries in different rats to the same internal diameter might be relatively too tight or too loose, and therefore renal failure, lack of hypertension, or return of normotension will occur in 1k1c and 2k1c rats. However, in 2k2c RHR there are two silver clips and 2 remaining kidneys in each rat, so the deficiency inherent in the one clip method can be avoided. If the proper internal diameter of silver clip is used, the expected high BP is easy to achieve in a group of 2k2c RHR with very few or no deaths as a result of acute renal failure. The ring-shaped clips with 0.30-mm internal diameter should be suitable to induce stable and reliable hypertension in Sprague-Dawley rats weighing 100 g because after constriction of renal arteries with the clips, none of the operated rats died of renal failure in the early stage. In addition, all of the rats became hypertensive, and the incidence of spontaneous stroke was 61.8%, which was much higher than the incidence in other RHR, based on hypertension without any other artificial inducement such as salt loading or low-protein or hypercholesterolemic diets. All of the stroke foci were easily determined under microscopic examination. Disseminated edema and petechial hemorrhages in the brain, which usually occur in hypertensive encephalopathy, were not found in the RHR with stroke. Therefore, the 2k2c RHR with a high incidence of spontaneous stroke can be used as stroke-prone renovascular hypertensive rats (RHRSP) independent of a genetic deficiency.

It has been well accepted in experimental and epidemiological studies that hypertension is one of the most important risk factors for stroke. In SHRSP, stroke often occurs spontaneously in rats with higher BP, although some pressure-independent effects in the lesions of blood vessels have been identified. In 1k1c RHR, it has been found that BP is higher in rats with cerebral lesions than in those without brain lesions. In the present study the peak BP of RHR with diffuse brain lesions or spontaneous stroke was higher than that in RHR without brain lesions in a different group. BP was relatively lower in the 2k1c group than in other test groups, and therefore fewer rats showed brain lesions in the group. In 1k1c RHR, however, BP reached a peak at ≈6 weeks postoperatively, and diffuse brain lesions similar to hypertensive encephalopathy occurred in this period because BP was elevated to a level close to that of malignant hypertension. BP reached a peak in 2k2c RHR at ≈25 weeks postoperatively, which was significantly longer than in 1k1c RHR. Although the peak BP was higher in 2k2c RHR than in 1k1c RHR, a lower incidence of diffuse brain lesions and a higher incidence of spontaneous stroke were observed in the former than in the latter. Because genetic deficiency did not play a role in the onset of stroke, these results suggest that the level and duration of high BP are the main factors that affect stroke onset in RHR.

In RHR with stroke, cerebrovascular lesions included hyalinosis, fibrinoid necrosis, and hyperplasia of internal or external (or both) layers of cerebral arterioles or small arteries with enhanced mural thickness and stenotic lumen. Sometimes the formation of microaneurysms and thrombotic vascular occlusions was observed. These kinds of vascular lesions are similar to those in SHRSP and in hypertensive patients. The predilection sites of the vascular lesions were the same as stroke foci in our results. Although some lesioned or occluded vessels were found in normal cortex or subarachnoid space, and it has been reported that not all artery occlusions will produce cerebral infarction because of the collateral circulation supply in rats, we consider that cerebrovascular lesions induced by hypertension are the pathological basis of stroke onset in RHR. The lesioned small artery or arteriole with thrombotic occlusion is the main cause of cerebral infarction in 2k2c RHR that may be similar to lacunar infarction in human brain, although platelet aggregation on the damaged endothelial surfaces of the artery has not been identified in RHR. However, in RHRSP we observed several occluded small arteries in a large infarcted area. The greater the number of occluded arterioles or small arteries, the more extensive was the size of ischemic lesions of cerebral tissue around the blood vessels. Since no distinct stenosis or occlusion was found in large cerebral arteries, it is obvious that a large number of occluded small arteries in an area in the brain will induce a large infarct focus. In addition, some PAS-positive materials within multilocular cysts in white matter were observed in the RHR brain in the present study. It has also been found that chronic cystic lesions were associated with occluded arterioles and edema fluid around blood-brain barrier leakage sites in SHRSP. Taken together, these findings indicate that similar mechanisms exist in the lesions of cerebral tissue in 2k2c RHR and SHRSP.

In SHRSP, the main cause of cerebral hemorrhage is rupture of fibrinoid necrotic arteries or cerebral microaneurysms. In 2k2c RHR, we found that cerebral hemorrhage had the same cause as in SHRSP, and one rat suffered from subarachnoid hemorrhage without hematomas in the brain. Bleeding in the subarachnoid space was perhaps the result of a ruptured vessel on the brain surface. Although the vascular lesions with proliferated cells of arterial wall occurred later than fibrinoid necrosis alone, there was no
significant difference between the onset of cerebral infarction and hemorrhage. A possible explanation for the results is that the microaneurysms and fibrinoid necrotic arteries were not only occluded but also ruptured in the present study, and different kinds of cerebral lesions were encountered in the rat brain. Furthermore, the rats were killed 72 hours after neurological signs occurred; both infarction and hemorrhage occurred in the brain of 11 RHR in the 2k2c group. Although not all of the stroke foci in the rats were fresh, it appears possible that cerebral hemorrhage and infarction, as a result of rupture and occlusion of lesioned small arteries or arterioles in the RHR brain, occurred simultaneously or at almost the same time in different areas of the brain. This kind of mixed stroke may have some features that distinguish it from cerebral infarction or hemorrhage. Detailed mechanisms of mixed stroke remain to be explored.

RHRSP have a high incidence of spontaneous stroke based on cerebrovascular lesions induced by hypertension without any other artificial inducement or genetic deficiency. This animal model is easy to establish at a low cost, and normotensive rats may conveniently be used as controls. These factors highlight some of the advantages of studying the association between arterial lesions and BP and stroke onset in RHRSP. We also found that stroke prevention can be studied experimentally in RHRSP (J. Zeng, MD, PhD, et al, unpublished data, 1997). For these reasons, we recommend that RHRSP can be used as a stroke-prone rat model independent of a genetic deficiency.

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model of stroke in humans. It is well recognized that the structural abnormalities in the cerebral vessels in these animals are also influenced by genetic factors that are blood pressure independent. In the accompanying article, Zeng and colleagues report a high incidence of spontaneous stroke in rats rendered hypertensive by applying a clip to each renal artery. Accordingly, this preparation provides another animal model useful for the study of stroke that is independent of the genetic factors prevalent in the SHRSP.

It must be remembered, however, that the 2k2c hypertensive rat model may present other complications of a different nature. Obviously, hypertension in this model is dependent on increased generation of angiotensin. It is well known that angiotensin has vascular effects independent of the associated increase in BP. Therefore, this new model for stroke, although a clearly useful addition, does not obviate the need for the development of yet additional models based on different mechanisms.

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