Establishment and evaluation of a reversible two-kidney, one-clip renovascular hypertensive rat model

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Abstract. The aim of the present study was to establish and evaluate a novel and reversible two-kidney, one-clip renovascular hypertensive rat model with a titanium vascular clip. A total of 40 male Sprague-Dawley rats were evenly and randomly divided into a sham-operated group, and 3, 7, 12 and 28D groups (namely removing the vascular clip in the renovascular hypertensive model after 3, 7, 12 and 28 days, respectively). The systolic blood pressure (SBP) and plasma renin activity (PRA) were measured, and color duplex imaging was conducted before placing the clips, as well as before and after removing them. After placing the vascular clips, SBP and PRA in the 3, 7, 12 and 28D groups were significantly increased (SBP: Sham-operated vs. 3D groups, P=0.020; 3 vs. 7D groups, P=0.008; 7 vs. 28D groups, P=0.019; 12 vs. 28D groups, P=0.039, and between other groups P<0.001. PRA: 3 vs. 7D groups, P=0.001; 7 vs. 12D groups, P=0.004; 12 vs. 28D groups, P=0.040, and between other groups, P<0.001). After removing the clips, SBP were significantly reduced in the 3 and 7D groups (P=0.023, 0.040, 0.066 and 0.314 in the 3, 7, 12 and 28D groups, respectively), but were still significantly higher than that before placing clips in the 7, 12 and 28D groups (P=0.067, P=0.005, P<0.001 and P<0.001 in the 3, 7, 12 and 28D groups, respectively). After removing the clips, PRA was significantly reduced in each group (P<0.001, P<0.001, P=0.012 and P=0.049 in 3, 7, 12 and 28D groups, respectively), but still higher than that before placing the clips (P<0.001, P=0.001, P=0.001 and P=0.003 in 3, 7, 12 and 28D groups, respectively). Vascular imaging also indicates this model has a reversible property. In conclusion, a reversible renovascular hypertension rat model is feasible, and provides a basis for research on clinical ischemic nephropathy and renal artery revascularization.

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Introduction

Renovascular hypertension is clinically important. Previous high-quality studies suggest that interventions, including stenting for renovascular hypertension, are not as effective long term compared to medication (1,2). In addition, the vast majority of cases of renovascular hypertension are atherosclerotic, and the disease is systemic and complex. However, these studies have been criticized for several reasons, including the inclusion of a large number of cases with minor vascular occlusive disease, numerous complications, poor technique and inexperienced operators (3). Therefore, the usefulness of endovascular therapy for reducing blood pressure and preventing renal and cardiovascular events remains uncertain. Furthermore, the development of precise methods to identify critically severe renovascular disease, such as plasma biomarkers, will be essential to bring the advantages of revascularization to those most likely to benefit. Experimental models of hypertension, including the two-kidney, one-clip Goldblatt model have been clearly described in basic research on hypertension (4), cardiovascular (5) and kidney disease (6). In the Goldblatt model of hypertension, the kidney is considered to be a vital organ in the pathogenesis of renovascular hypertension, as it is the origin of the pressor rennin-angiotensin system and controls water and sodium balance (7). Blood flow is decreased in the clipped kidney contributing to ischemia, and the non-clipped kidney is characterized by increased shear stress (8). The present study hypothesized that a novel model with a reversible property could simulate renal artery revascularization, which means that the blood flow of the renal artery could return to normal following removal of renal artery stenosis. Although a local clip in this renovascular hypertensive model is unlikely to represent the same conditions as in a clinical situation, we hope that this model provides a basis for further research on changes in biomarkers of renal fibrosis, including neutrophil gelatinase-associated lipocalin, serum creatinine and cystatin C. In addition, the aim of the present study is to identify a plasma biomarker with satisfactory specificity and sensibility in order to instruct the prognosis of endovascular therapy, indicating whether patients' hypertension can be relieved following renal artery revascularization.

Materials and methods

Animals. A total of 40 specific pathogen-free male Sprague-Dawley rats (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were housed individually with constant humidity and temperature (25˚C). Rats were acclimatized for at least one week and had unrestricted water intake under such conditions before the start of the experiments. Furthermore, the experiments were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. In addition, all efforts were made to relieve rats' suffering and to reduce the number of animals used. The animal use protocol has been approved by the Institutional Animal Care and Use Committee of Capital Medical University (Beijing, China).

Surgical procedure. Rats (weight, 350-400 g; age, 8-10 weeks) were evenly and randomly divided into sham-operated, 3, 7, 12 and 28D groups. All rats were anesthetized by a peritoneal injection of 0.3 ml/100 g 10% chloral hydrate (Jiang Lai Biotech., Beijing, China). After being shaved, the abdominal wall was coated with povidone-iodine (Yuande Biomeidical Engineering Co., Beijing China) and 70% alcohol (Jiang Lai Biotech.). The left renal hilum was revealed through a flank abdominal incision (Fig. 1A), and the renal artery and vein were carefully isolated by blunt dissection. In each of the rats in the 3, 7, 12 and 28D groups an insulin needle tip whose outer diameter was 0.23 mm was used in order to prevent vessel compression during clip placement. In addition, a titanium vascular clip was gently placed around the left renal artery (Fig. 1B and C). The needle tip was then carefully removed (Fig. 1D). Rats in sham-operated group underwent the same surgical procedure, except for the application of the vascular clip. Abdominal contents were carefully returned to their original location, and the abdominal wall and skin was sutured. In addition, rats in the 3, 7, 12 and 28D groups received another operation after 3, 7, 12 and 28 days, respectively. The left renal artery was exposed via back incision, carefully isolated and the vascular clip was removed (unclipping, UC). After each operation, intramuscular injection of 50,000 units/100 g penicillin (North China Pharmaceutical Co., Ltd., Shijiazhuang, China) was used for three days. All rats were then observed for 1 h following surgery, then individually housed and allowed free access to food and water. The sham-operated group also underwent these procedures but without a clip compared to the operation group.

Systolic blood pressure (SBP). SBP of every unanesthetized rat was measured before placing clips and before UC by means of the tail-cuff method. SBP was measured three times, and the average value was then obtained. In addition, SBP of rats in 3, 7, 12 and 28D groups were measured after UC for 3, 7, 12 and 28 days, respectively. Prior to the blood pressure measurement, the rats were warmed to 35˚C in a cupboard for 10 min.

Color duplex imaging. Immediately posterior to the SBP measurement of all the timepoints, the rats were anesthetized by 0.3 ml/100 g 10% chloral hydrate (Jiang Lai Biotech.) and sonography was performed with a Vevo 2100 high-frequency, high-resolution ultrasound system (Fujifilm VisualSonics,

Inc., Toronto, ON, Canada) with a transducer frequency of 12.5-15 MHz. Color Doppler Mode was selected for detection of blood vessels including flow directional information and peak systolic velocity (PSV) at the place of the vascular clip and aorta at the origin of the renal artery.

Plasma renin activity (PRA). Immediately following undergoing color duplex examination of each time, the rat tail was disinfected, and was then cut with operation scissors. Blood flowed out the tail end and 1 ml was collected in K3 EDTA tubes (North Institute of Biological Technology, Beijing, China). After centrifugation and storage at 4˚C, the EDTA-plasma samples were analyzed using an iodine (^{125}I) angiotensin I radioimmunoassay kit (North Institute of Biological Technology). PRA was then determined by comparing the samples to a standard curve. At the end of the study period, the rats were sacrificed by carbon dioxide asphyxiation.

Statistical analysis. Values are expressed as the mean ± standard error of the mean. All data were analyzed using SPSS 22.0 software (IBM SPSS, Armonk, NY, USA). Differences in placing and removing clips in every group were assessed by analysis of variance in a randomized block design. Where a significant difference was identified, the groups were compared by Student's t-test. P<0.05 was used to indicate a statistically significant difference.

Results

SBP. As summarized in Fig. 2, after placing the vascular clips, SBP in the 3, 7, 12 and 28D groups significantly increased to 125±13, 143±13, 174±14 and 160±11 mmHg, respectively (sham-operated vs. 3D group, P=0.020; 3 vs. 7D group, P=0.008; 7 vs. 28D group, P=0.019; 12 vs. 28D group, P=0.039 and between other groups P<0.001). Following UC, SBP in the 3 and 7D groups were significantly reduced to 109±13 and 129±11 mmHg (in 3, 7, 12 and 28D groups, P=0.023, 0.040, 0.066 and 0.314, respectively). SBP after UC were significantly higher than that before placing the clips in the 7, 12 and 28D groups (in 3, 7, 12 and 28D groups, P=0.067, P=0.005, P<0.001 and P<0.001, respectively).

PRA. As summarized in Fig. 3, after placing the vascular clips, the PRA in 3, 7, 12 and 28D groups increased significantly to 18.74±2.68, 14.43±2.56, 10.96±2.49 and 8.53±1.62 ng/ml, respectively (3 vs. 7D group, P=0.001; 7 vs. 12D group, P=0.004; 12 vs. 28D group, P=0.040, and between other groups P<0.001). After UC, the PRA in each group was significantly reduced to 10.46±2.49, 8.47±2.60, 7.89±2.13 and 6.77±1.48 ng/ml, respectively (in the 3, 7, 12 and 28D group, P<0.001, P<0.001, P=0.012 and P=0.049, respectively), but remained higher than that before placing the clips (in the 3, 7, 12 and 28D groups, P<0.001, P=0.001, P=0.001 and P=0.003, respectively).

Color duplex imaging. PSV at the location of the vascular clip and the aorta at the origin of the renal artery were obtained, and the resulting renal-aortic ratio (RAR) >3.5 indicated a hemodynamically significant renal artery stenosis (60-99%) (9,10). Before placing clips, the PSV values at
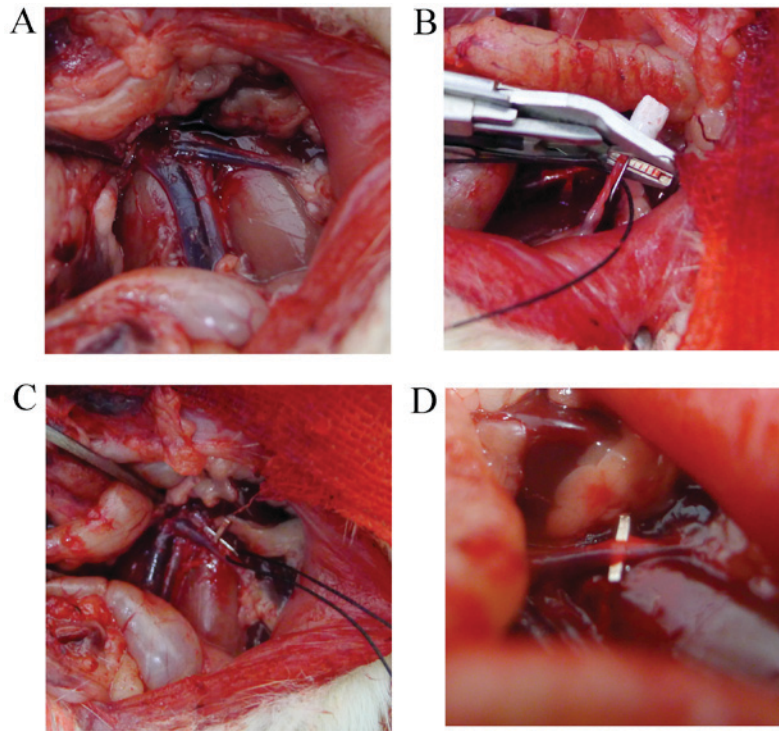
Figure 1. A renovascular hypertensive model was established using the 2K1C method. (A) View through a flank abdominal incision where the left renal hilum was revealed. (B) View of the renal artery and vein that were carefully isolated by blunt dissection. (C) A titanium vascular clip is shown that was successfully placed on the left renal artery, with the aid of an insulin needle tip whose outer diameter was 0.23 mm. (D) Image showing that the insulin needle tip was carefully removed.
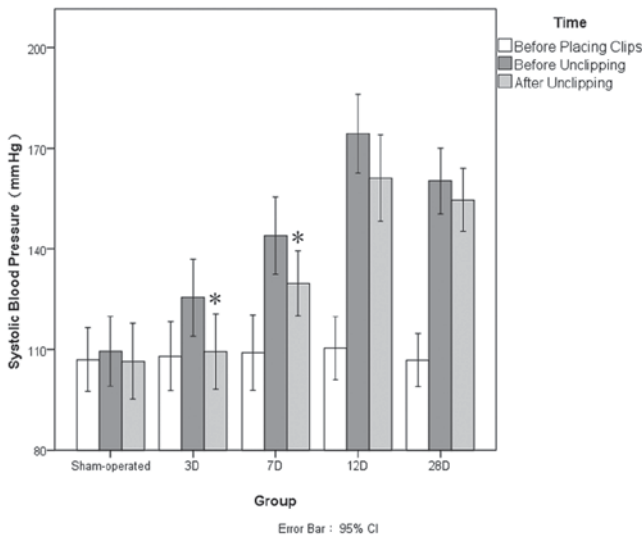

Figure 2. Vascular clipping and unclipping resulted in systolic blood pressure changes. After placing clips, systolic blood pressure in the 3, 7, 12 and 28D group significantly increased. In addition, after removing the clips, SBP in the 3 and 7D group were significantly reduced. Systolic blood pressure after unclipping were significantly higher than that before placing clips in the 7, 12 and 28D groups. *P<0.05 after unclipping vs. befor unclipping.
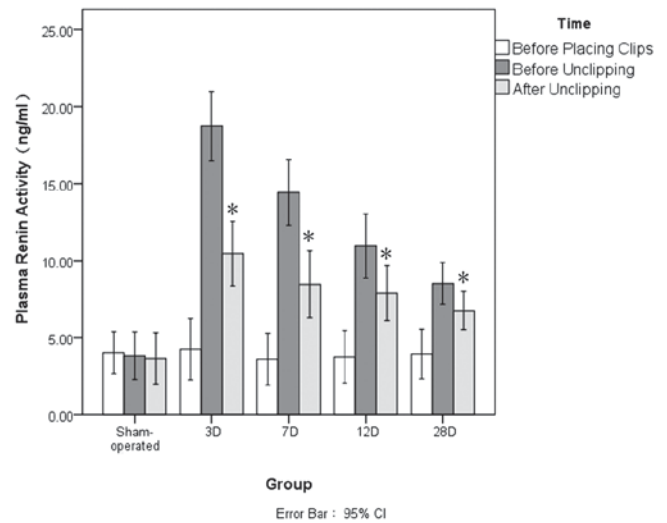

Figure 3. Vascular clipping and unclipping resulted in plasma renin activity changes. After placing the clips, plasma renin activity in 3, 7, 12 and 28D group significantly increased. After unclipping, plasma renin activity in each group were significantly reduced but was still higher than that before placing the clips. *P<0.05 after unclipping vs. before unclipping.

the middle section of the renal artery and at the aorta were 390.4±96.9 and 413.7±77.1 mm/sec ($PSV_{renal\ artery}$ vs. PSV_{aorta}, P=0.947) with a RAR of every subject <1.5:1. After placing the clips, the PSV near the location of vascular clip and aorta confirmed renal artery stenosis directly on the basis of an increase in $PSV_{renal\ artery}$ of 1,576.4±120.5 mm/sec and PSV_{aorta} of 375.2±86.3 mm/sec ($PSV_{renal\ artery}$ vs. PSV_{aorta}, P<0.001), and with RAR of every subject >3.5 (Fig. 4). Following UC, Doppler waveforms and PSV had a normal recovery with $PSV_{renal\ artery}$ of 426.0±79.8 mm/sec and PSV_{aorta} of 408.2±76.3 mm/sec ($PSV_{renal\ artery}$ vs. PSV_{aorta}, P=0.892), which was the same as that before placing vascular clips with RAR of every subject <1.5:1.
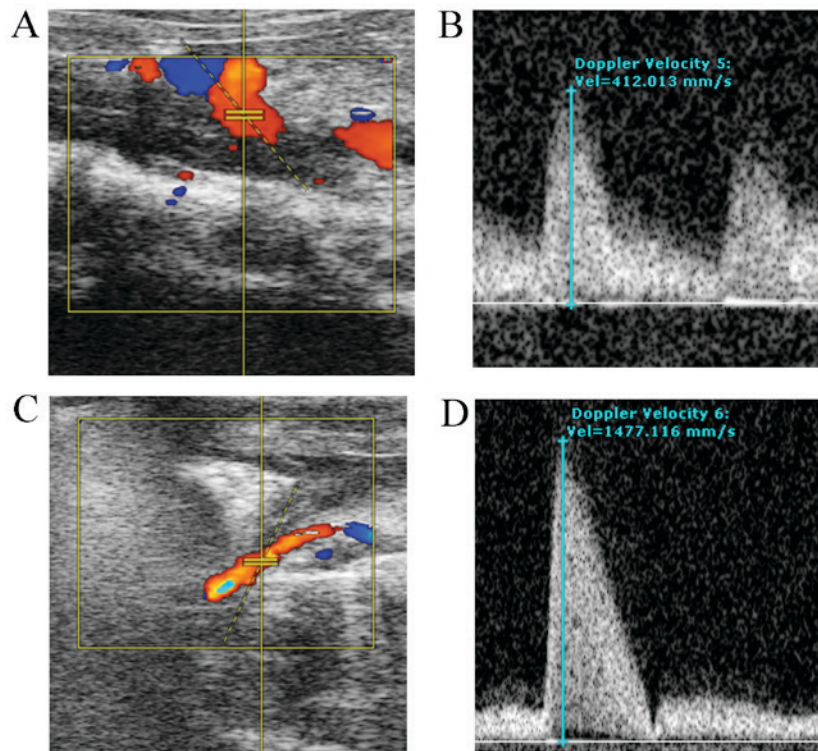
Figure 4. Waveforms and peak systolic velocity changes in the 2K1C model observed by color duplex imaging. (A) Image from color duplex imaging. Color duplex imaging was performed at the aorta near the site of the left renal artery. (B) Waveforms and peak systolic velocity at the aorta are shown that were obtained near the site of the left renal artery. (C) Image from color duplex imaging. Color duplex imaging was performed at the vascular clip site of the left renal artery. (D) Waveforms and peak systolic velocity are shown that were obtained at the vascular clip site of the left renal artery.

Discussion

Renal artery stenosis (RAS), which causes narrowing of the renal artery or its main branches, is produced by a heterogeneous group of conditions, including atherosclerosis, fibromuscular dysplasia, congenital bands, extrinsic compression, vasculitis, neurofibromatosis and radiation (11). Renovascular hypertension refers to the rise in arterial pressure led by reduced renal artery perfusion, as the most common secondary forms of hypertension. In addition, reduced kidney perfusion can also lead to ischemic nephropathy, manifesting to loss of kidney size and function, when RAS is severe and prolonged (12). Due to these conditions the benefit from vascular intervention remains a controversial topic (13), and thus establishing an animal model that simulates renal artery revascularization is required.

In 1934, Goldblatt *et al* (14) established the association between renal artery and sustained hypertension in dogs by placing arterial occlusive clamps. The physiopathological mechanism of the sustained hypertension is complex and multifactorial, and its basis is the activation of the renin angiotensin aldosterone system (RAAS). Reduced perfusion to the kidney contributes to the release of renin from juxtaglomerular cells to the afferent arteriole (15,16). A cascade reaction amplified the signal to produce both local and systematic angiotensin II, whose biochemical reactions are complex. Angiotensin II raises arterial pressure by direct vascular effects and by changing renal sodium homeostasis, in addition to having wide effects on other pressure systems, fibrosis and remodeling (17,18). The one-kidney, one-clip

(1K1C), two-kidney, one-clip (2K1C) and two-kidney, two-clip (2K2C) are methods used to induce renovascular hypertension (19). In rabbits (20) and rats (21), 2K1C induces a chronic and gradual rise in the arterial pressure, which peaks after two weeks and decreases subsequently in 10-20% of the subjects. In general, rats develop hypertension more easily than rabbits, and become the primary choice to establishing a 2K1C renovascular hypertensive model.

However, perhaps due to the design flaws in traditionally used silver clips, early studies on establishing a 2K1C renovascular hypertensive model often concluded in low success rates of 40-60% (22-24). The gap widths of traditionally designed silver clips cannot be well maintained during handling or these clips can also be dislodged from the renal artery following placement. Furthermore, silver is malleable and easily reshaped. However, this ductile nature may lead to the unreliability of the U-shape clip, thereby resulting in different changes in the renal artery lumen diameters. In addition, if there are silver dioxides on the surface of the clip during sterilization and handling, the clip could cause perivascular inflammation, intimal proliferation and tissue granulation, which may further alter the renal artery diameter, particularly in chronic phases (25). Furthermore, Kraft *et al* (26) illustrated that silver or stainless steel implants may elicit in leukocyte extravasation and venular dilation to some degree. In an attempt to solve the problems mentioned above, a titanium vascular clip was used in the 2K1C model. Chelko *et al* (27) reported that the application of clips with a gap of width of 0.23 mm could induce reliable and consistent hypertension in rats. Therefore, the needle tip of insulin with a 0.23 mm outer

diameter was used to measure the residual diameter of the left renal artery in the 2K1C model.

Following placement of the clip, the factor maintaining the subsequent rise in arterial pressure is characterized by an increased activity of the RAAS (28). However, controversy surrounds the role of the sympathetic nervous system (SNS) in the maintenance of 2K1C hypertension. In a six-week observation, the arterial pressure of 2K1C was almost reduced to normal levels by administration of the angiotensin receptor blocker (ARB). Furthermore, arterial pressure was not significantly affected by sympathetic blockade, and the combination of sympathetic blockade and ARB were not more effective than ARB alone (29). Therefore, the influence of SNS on 2K1C hypertension in this phase is negligible. UC in the 2K1C model returns arterial pressure to almost normotensive levels within a few hours. It has been suggested that the reduction in arterial pressure after UC is caused by the release of a putative renal medullary depressor hormone (30). Furthermore, UC or removal of the ischemic kidney resulted in similar reductions in arterial pressure, indicating that the reduction in arterial pressure observed after UC is mainly attributed to elimination of angiotensin II secretion from the ischemic kidney and reduced plasma concentration of renin following the normalization of renal perfusion pressure. No clear link between UC and the decreased activity of SNS observed in the 2K1C model was identified. In summary, both maintenance and reversal of hypertension in a 2K1C hypertensive model are controlled by the RAAS (29).

In a previous study and as mentioned above, the blood pressure after UC declined in a few hours (30). However, none of the data of long time observation has been previously reported. In the present study, SBP increased and peaked in the 12D group after placing titanium vascular clips. Following UC, SBP in the 3 and 7D groups were significantly reduced whereas in the 12 and 28D groups SBP did not decline. Moreover, SBP in 7, 12 and 28D groups after removing clips were significantly higher than normal. This result indicates that renal ischemia injury was irreversible in the 12 and 28D groups, and it is likely that this irreversible change happened in 7-12D group. The present study indicates that the placement of a titanium vascular clip could establish renal artery stenosis and induce renovascular hypertension. The removal of the clip could eliminate renal artery stenosis and lower the blood pressure in the early phase. However, despite the patency of renal artery, the SBP cannot return to normal in chronic phases. It was hypothesized that the reason that renal hypertension was dominant instead of renovascular hypertension at some time point was due to the renal fibrosis as the prolonged renal ischemia time (31).

However, arterial pressure may be influenced by numerous factors, including animal age, clip type, postoperative stress, measuring time, environmental changes and diet (32). In order to supply more evidence to verify the fact that stenosis exists after the placement of a clip and disappears after the removal of the clip, PRA was measured soon after the measurement of SBP each time.

The PRA change in each group of the rat models studied is shown in the graph in Fig. 3. Although the degree of change varied, it was significant between each group after placing clips for 3, 7, 12 and 28 days, with the highest value observed in the 12D group. In each group, PRA declined significantly after UC, but was still significantly higher than that prior to the surgery. Hypertension in the 2K1C model may be influenced by renin released from the stenotic kidney. Kidney-borne renin, as the key regulator of RAAS, plays an essential part in extracellular fluid volume, sodium and blood pressure homeostasis (16). Renin release is suppressed and sodium excretion is increased from the contralateral kidney, which tends to reduce blood pressure (33-35). As follows, stenotic renal perfusion pressure is decreased and renin is again released, which leads to elevated blood pressure (36-38). Several reasons may be responsible for the subsequent decline of systemic PRA (39,40). If the increase in systemic pressure restores the perfusion pressure of the stenotic kidney, as in slight or moderate renal artery stenosis, PRA and angiotensin II can be sustained with relatively low levels or even return to normal levels (41,42). However, in severe renal artery stenosis, these series of the reaction cannot be cycled, which may result in volume depletion and renal failure. Due to a wide range of severity of stenosis, coexistent morbidities, age, posture and sodium intake, PRA and other hormones have a considerable variability in PRA. This partly explains the unpredictability of the blood pressure response to vascular intervention by measuring a single pathway in humans (43).

Therefore, prior to placing a vascular clip as well as before and after UC, every rat received color duplex imaging examination. To date, there is no criterion to diagnose renal artery stenosis in rats. In addition, rats have a different circulation feature, which is that the normal PSV at each site of renal vascular is approximately 400 mm/sec. Thus, it has been proposed that the PSV in the aorta may be associated to that at the origin of the renal artery. Therefore, the resulting renal-aortic ratio (RAR) >3.5 indicated significant hemodynamic renal artery stenosis (60-99%). The RAR was found to have a surprisingly high sensitivity of 83-100% and a specificity of 73-97% (9,10). Furthermore, following removal of the clips, PSV in the sonography at each site returned normal. In conclusion, to evaluate the recovery of blood flow in the renal artery after removing the clips, SBP in early phase, PRA measurements and image indications of renal artery stenosis indicate reversible characteristics were exhibited and levels were returned to normal. The results proved that after removal of the clip, the blood pressure and PRA significantly decreased in the 2K1C rat model. In addition, a reversible renovascular hypertensive rat model is feasible and plausible. However, SBP in a later phase has no such reversible properties. We hypothesized that the main cause is that renovascular hypertension was replaced by renal hypertension as the process of renal fibrosis was induced by renal ischemia. Establishment of this model provides the basis for further research on changes in biomarkers of renal fibrosis, including neutrophil gelatinase-associated lipocalin, serum creatinine, and cystatin C when the vascular clip is placed and removed. Finally, we hope that a plasma biomarker with satisfactory specificity and sensibility can be found to instruct the prognosis of renovascular hypertension and ischemic nephropathy. In other words, a specific and sensitive biomarker is required in order to assess whether hypertension could be relieved following renal artery revascularization.

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