

## Original Article

# Comparison study on antihypertensive drugs for treatment of pre-hypertension in rats

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**Abstract:** Background: Pharmacological treatment from pre-hypertension may benefit to long-term blood pressure and target organ. Purpose: To compare the effects of losartan and amlodipine on systolic blood pressure (SBP) and brain for treatment of pre-hypertension in stroke-prone spontaneously hypertensive rats (SHRSP). Methods: SHRSP were administered with losartan (6 or 16 weeks, respectively), amlodipine (6 or 16 weeks, respectively) or saline from pre-hypertension. Wistar-Kyoto rats were used as a control. Rats were followed up until 40-weeks-old. SBP was determined by tail-cuff method. Stroke was determined with the clinical score of stroke and optical microscope. Apoptosis was analyzed by electron microscopy and TdT-mediated dUTP-biotin nick end labeling. Protein expressions of gp91<sup>phox</sup>, superoxide dismutase (SOD), and angiotensin II type 1 (AT1R) and type 2 (AT2R) receptor in the cerebral cortex were detected by western blot. Results: The progressions of BP were more slowly in losartan treated SHRSP. Compare with amlodipine, losartan further improved structure and morphology of brain, decreased the clinical score of stroke, and reduced stroke. However, amlodipine showed superior in delaying the peak of apoptosis. Losartan further decreased the protein expression of AT1R and increased the protein expression of AT2R but amlodipine further decreased the protein expression of gp91<sup>phox</sup> and increased the protein expression of SOD. Longer administration, much effects. Conclusions: Treatment with losartan from pre-hypertension is more effective than amlodipine on delaying long-term BP increase, ameliorating cerebral structure, and reducing stroke due to its stronger inhibition of AT1R and activation of AT2R. However, amlodipine shows superior in delaying the peak of apoptosis because of its stronger anti-oxidative stress.

**Keywords:** Pre-hypertension, amlodipine, losartan, stroke

## Introduction

Pre-hypertension, a transitional phase from normal blood pressure (BP) to hypertension, which is defined by a systolic BP (SBP) of 120 to 139 mmHg or a diastolic BP of 80 to 89 mmHg [1]. Evidence showed that individuals with pre-hypertension were at increased risk of developing hypertension and suffering cardiovascular events [2]. Lifestyle modification is recommended as basis for all subjects belonging to this blood pressure category by current guidelines of hypertension, and drug therapy is considered for individuals concomitant with diabetes or kidney disease if a trial of lifestyle modification fails to reduce their BP to 130/80 mmHg or less [1]. But in real life, individual habit, work and living environment, and other

factors are usually difficult to change. So it is hard to implement lifestyle modification, especially in young and middle-aged patients with pre-hypertension [3]. Pre-hypertension could also be an early marker of adverse cardiometabolic risk in disease-free adults [4], and pre-hypertensive preconditioning improves adult antihypertensive and cardio-protective treatment [5]. Recently published TROPHY, DHyPP and PHARAO trials demonstrated that pre-hypertensive treatment with renin-angiotensin system (RAS) inhibitor reduced the risk of incident hypertension and was well tolerated [6-8]. Thus, some researchers have suggested that anti-hypertensive therapy may be recommended for everyone with pre-hypertension and have addressed the resulting cost-benefit issues [9].

## Comparison of antihypertensive drugs for pre-hypertension

Losartan, an angiotensin receptor blocker, and amlodipine, a calcium channel blocker, are widely used clinically as antihypertensive drugs to lower BP [10]. However, which drug is better in delaying the progress of BP and protecting the brain remains unclear. To date, few studies have addressed the treatment of losartan and amlodipine from the onset of pre-hypertension to prevent the progress of BP and protect brain. Furthermore, it is unknown whether these benefits show a 'persistent effect' [11, 12]. Stroke-prone spontaneously hypertensive rats (SHRSP), which are cultivated from Wistar-Kyoto rats (WKY), tend to develop severe hypertension from the age of 4 weeks and display a pre-hypertensive state from the age of 4 to 10 weeks, and more than 95% of them will suffer stroke at 40 weeks of age, making them widely used as models to study cerebral lesion [13].

The aims of this study were: 1) to assess the effects of losartan and amlodipine administered from pre-hypertension on long-term BP and cerebral protection to SHRSP, and 2) to elucidate whether the effects of losartan and amlodipine show a 'persistent effect'. To address these aims, we studied the effects of losartan and amlodipine (different intervention time: 6 or 16 weeks, respectively.) on SHRSP (as a model of hypertension and pre-hypertension) and WKY rats (as control group) from the onset of pre-hypertension. We examined the Systolic BP (SBP), the clinical score of stroke, stroke, cell apoptosis in the cerebral cortex, and protein expressions of gp91<sup>phox</sup>, superoxide dismutase (SOD), angiotensin II type 1 (AT1R) and type 2 (AT2R) receptor in untreated WKY rats and SHRSP treated with losartan, amlodipine, or saline.

### Materials and methods

#### *Animals and grouping*

A total of 48 male, 4-week-old SHRSP and WKY rats (Shanghai Slac Laboratory Animal Co. Ltd, Shanghai, China) were used in this study. Rats were randomly divided into the following 6 groups (n = 8 rats in each group): i) WKY, untreated WKY as a control group; ii) SHRSP-vehicle (Veh) group, SHRSP treated with saline; iii) SHRSP-Los6 group, SHRSP treated with 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> losartan from 4-weeks-old to 10-weeks-old; iv) SHRSP-Los16 group, SHRSP treated

with 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> losartan from 4-weeks-old to 20-weeks-old (Merck, Sharp & Dohme, New Jersey, USA); v) SHRSP-Aml6 group, SHRSP treated with 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> amlodipine from 4-week-old to 10-week-old; vi) SHRSP-Aml16 group, SHRSP treated with 10 mg·kg<sup>-1</sup>·d<sup>-1</sup> amlodipine from 4-week-old to 20-week-old (Pfizer, New York, USA) [14].

Rats (4 animals per cage) were housed in a room with controlled temperature (23 ± 2°C) under a 12-hour light/12-hour dark cycle. Animals were allowed access to standard food and tap water ad libitum. All procedures were approved by the Animal Ethics Committee of Fujian Medical University and performed in accordance with institutional guidelines.

#### *Experimental protocol*

Rats were administered from the 4<sup>th</sup> to the 10<sup>th</sup> or 20<sup>th</sup> week after birth and followed up until 40 weeks of age. Losartan and amlodipine were dissolved in drinking water and immediately offered to the experimental SHRSP by gavage once daily. The amount of losartan and amlodipine were adjusted to maintain a constant dosing based on the body weight, which was measured weekly.

#### *Measurement of systolic blood pressure*

SBP was measured noninvasively in unanesthetized rats using the tail-cuff method with a specialized pressure transducer (PowerLab ML125/R NIBP System, AD Instruments, Sydney, Australia) from 4 weeks of age until 40 weeks of age, as described previously [15]. Briefly, SBP was measured when the cuff pressure corresponded to the restoration of the first caudal artery pulse, and three consecutive readings were averaged for each determination.

#### *Determination of clinical stroke score*

The investigators that scored the rats for stroke were blinded to the experimental groups. The clinical score of stroke was determined as described previously [16]. Briefly, 0 point: Normal activities; 1 point: Less active--Slightly reduced exercise or mild excitement; 2 points: Very few activities--Significantly reduced exercise or irritation; 3 points: Prone fixed--Unable walk or depressive symptoms; 4 points: paralysis--Unable stand or limb paralysis.

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### *Examination with naked-eye and optical microscope*

The brain of rats was removed quickly and photographed after rats were anesthetized with 10% chloral hydrate. After fixed in 10% formalin for 24 h, the right cerebral cortex of each rat was cut into 6  $\mu\text{m}$  thick coronal slices and stained with hematoxylin and eosin. Finally, cerebral cortex slices were observed and photographed under an optical microscope (OLYMPUS CX41-32RFL, Tokyo, Japan;  $\times$  100 magnification).

### *Detection of apoptosis in cerebral cortex cells with electron microscopy*

The left cerebral cortex of each rat was removed quickly after the rats were anesthetized with 10% chloral hydrate. The specimen was first fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0) for more than 4 h, washed three times in the phosphate buffer, and then post-fixed with 1%  $\text{OsO}_4$  in phosphate buffer (pH 7.0) for 1 h and washed three times in the phosphate buffer. The specimen was dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%) for about 15 to 20 min at each step and transferred to absolute acetone for 20 min; then placed in 1:1 mixture of absolute acetone and the final Spurr's resin mixture for 1 h at room temperature, and then transferred to 1:3 mixture of absolute acetone and the final resin mixture for 3 h and to final Spurr's resin mixture for overnight. Specimen was placed in capsules contained embedding medium and heated at 70°C for about 9 h. Finally, the specimen sections were stained by uranyl acetate and alkaline lead citrate for 15 min respectively and observed in TEM (Philips 208, Amsterdam, Holland;  $\times$  5000 magnification).

### *Detection of apoptosis in cerebral cortex cells with TdT-mediated dUTP-biotin nick end labeling*

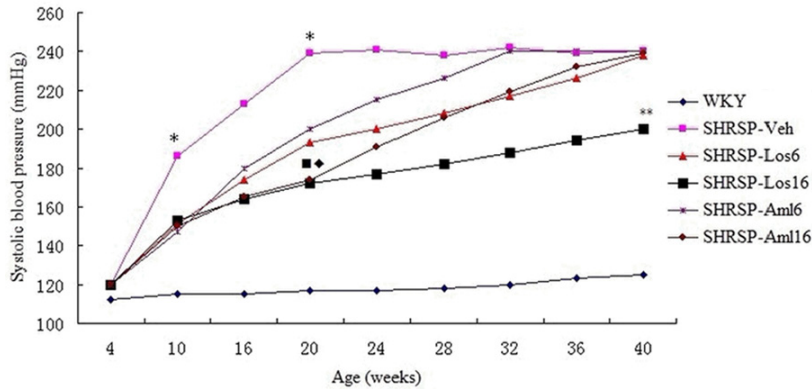
The right cerebral cortex of each rat was removed quickly after the rats were anesthetized with 10% chloral hydrate. After fixing in 10% formalin for 24 hours, the cerebral cortices were cut into coronal slices with a thickness of 6  $\mu\text{m}$ . Apoptosis in the cerebral cortex was analyzed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) via fluorescence micros-

copy (In Situ Cell Death Detection Kit POD. Cat No. 11684817910, Roche, Basel, Switzerland). Experimental procedures were strictly according to kit instructions. Briefly, paraffin-embedded cortical sections were de-waxed, rehydrated, protease digested, and then incubated in the TUNEL reaction mixture. Slides were viewed and photographed using a fluorescent microscope (OLYMPUS CX41-32RFL, Tokyo, Japan) with a standard fluorescein filter of 500 nm. Apoptosis was analyzed by light microscopy (OLYMPUS CX41-32RFL, Tokyo, Japan). Five sections per animal were for analysis. The quantities of apoptosis in four randomly selected fields of vision were averaged for each determination.

### *Western blot analysis*

Western blot analysis for protein expressions of gp91<sup>phox</sup>, SOD, AT1R and AT2R in the cerebral cortex was performed at the age of 40 weeks. Briefly, 50 mg of the cerebral cortex was homogenized in lysis buffer. An equal amount of protein was applied to SDS-polyacrylamide gels and electrophoresed under 10% reducing gel conditions. Proteins were transferred to a nitrocellulose membrane, blocked with 5% non-fat milk in TBS containing 0.05% Tween-20 (TBST), and then incubated with a rabbit polyclonal anti-NOX<sub>2</sub>/gp91<sup>phox</sup> antibody (1:500, Abcam, Cambridge, Britain), a rabbit polyclonal anti-SOD 1 antibody (1:500, Abcam, Cambridge, Britain), a rabbit polyclonal anti-AT1R antibody, a rabbit polyclonal anti-AT2R antibody (1:500, Abcam, Cambridge, Britain), or a rabbit polyclonal anti- $\beta$ -actin antibody (1:1000, sc-1616, Santa Cruz Biotechnology, California, the United States) overnight at 4°C. After washing for 3 times with Tris-Buffered Saline and Tween 20, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (Goat anti-rabbit IgG-CFL 488, Santa Cruz Biotechnology, California, the United States). Next, the samples were washed 3 times with TBST, and then the protein bands were visualized with electrochemiluminescence (ECL; sc-2048, Santa Cruz Biotechnology, California, the United States) on high-performance chemiluminescence film. Band intensity was quantified by densitometry with image analysis software. The results were expressed as a ratio of gp91<sup>phox</sup>, SOD, AT1R, and AT2R over  $\beta$ -actin.

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**Figure 1.** Systolic blood pressure in untreated Wistar Kyoto rats (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP) treated with losartan, amlodipine, or vehicle. Values are expressed as means  $\pm$  SD ( $n = 8$  animals per group). \* $P < 0.05$  vs. drug-treated SHRSP group;  $\blacklozenge P < 0.05$  vs. SHRSP-Los6 group;  $\blacklozenge P < 0.05$  vs. SHRSP-Aml6 group; \*\* $P < 0.05$  vs. SHRSP-Veh.

the progression of SBP in the SHRSP-Los16 group and the SHRSP-Aml16 group were slower ( $P < 0.05$ ). When compared with the SHRSP-Los16 group, the progression of SBP in the SHRSP-Aml16 group was accelerated after 20-week-old. At 40-week-old, the SBP was still lower in the SHRSP-Los16 group, not other drug-treated SHRSP groups, when compared with the SHRSP-Veh group ( $P < 0.05$ ) (Figure 1).

### Effects of losartan and amlodipine on clinical

### Statistical analysis

Data are reported as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed with the SPSS 13.0 software program. Differences between groups were compared by one-way analysis of variance (ANOVA), followed by the LSD-t test for multiple comparisons. The clinical score of stroke was analysis by the Kruskal-Wallis H test, followed by the Mann-Whitney U test. A value of  $P < 0.05$  was considered to indicate statistical significance.

## Results

### Effects of losartan and amlodipine on SBP

In all groups, the values of SBP were similar at 4-weeks-old. The SBP in the SHRSP-Veh group was increased when compared with the pre-treatment value, and stabilized at around 240 mmHg at about 20-week-old, whereas the SBP in WKY rats stayed at a nearly constant level, indicating hypertension was well established in the SHRSP-Veh group. Losartan and amlodipine treatments were equivalent in delaying the increase of SBP in the intervening period. When compared with the SHRSP-Los16 group and the SHRSP-Aml16 group, the progression of SBP in the SHRSP-Los6 group and the SHRSP-Aml6 group, especially for the SHRSP-Aml6 group, was accelerated after 10-weeks-old. At 20-week-old, the SBP in all drug-treated SHRSP groups were still lower when compared with the SHRSP-Veh group. When compared with the SHRSP-Los6 group and the SHRSP-Aml6 group,

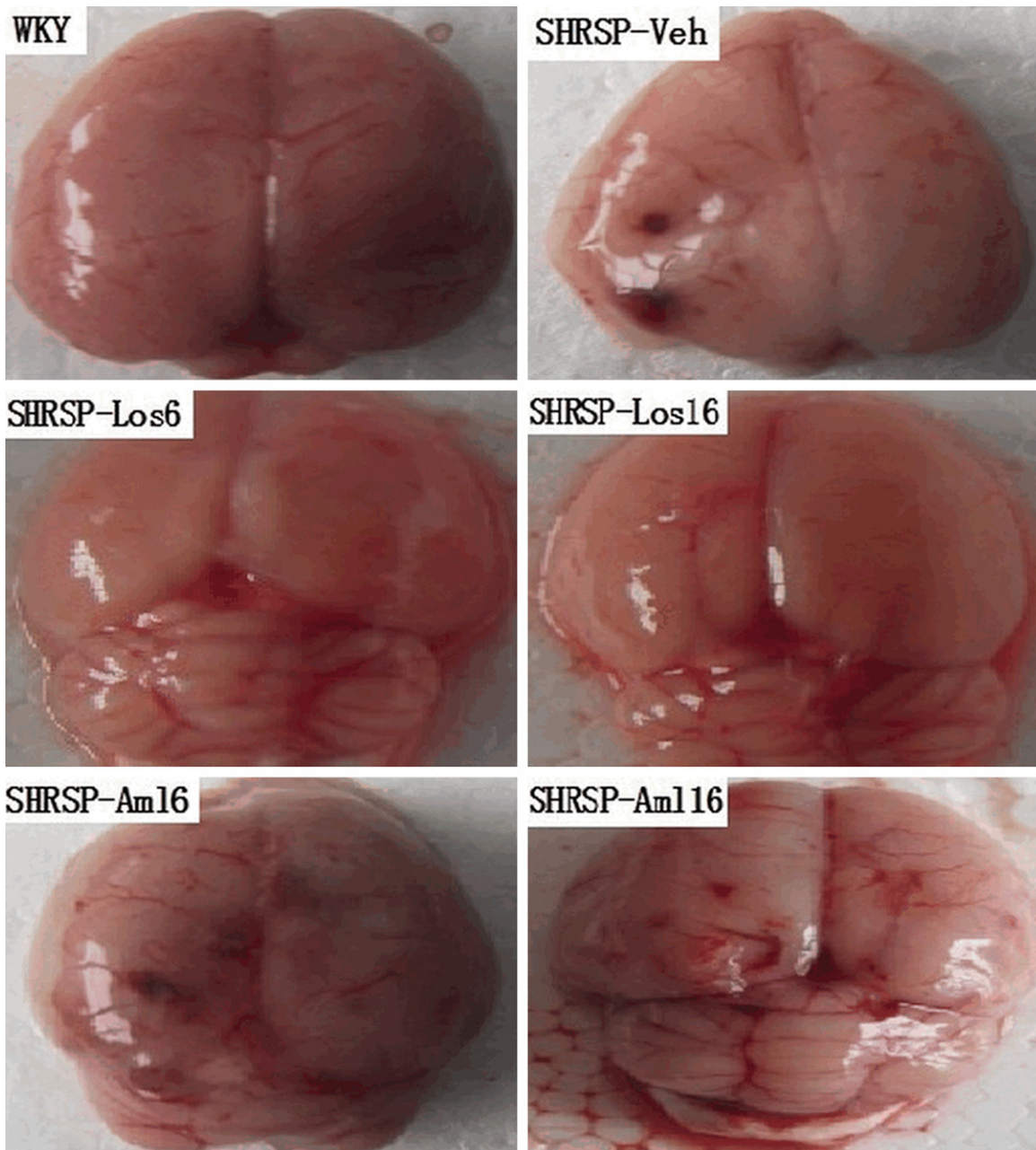
### score of stroke

At 40-weeks-old, the clinical score of stroke was 0 in every rat of the WKY rats and 4 in every rat of the SHRSP-Veh group. In the SHRSP-Los6 group, it was 1 in three rats, 2 in four rats and 3 in one rat. In the SHRSP-Los16 group, it was 0 in every rat. In the SHRSP-Aml6 group, it was 3 in two rats and 4 in six rats. In the SHRSP-Aml16 group, it was 2 in two rats, 3 in four rats and 4 in two rats. The clinical score of stroke in the SHRSP-Veh group was increased when compared with the WKY rats ( $P < 0.05$ ). When compared with the SHRSP-Veh group, the clinical score of stroke in the SHRSP-Los6 group and SHRSP-Los16 group, especially in SHRSP-Los16 group, was significantly decreased ( $P < 0.05$ ). However, no significant difference was observed between the SHRSP-Veh group and the SHRSP-Aml6 group or the SHRSP-Aml16 group ( $P > 0.05$ ).

### Examination results with naked-eye and optical microscope

Results of naked-eye examination showed that, in the brains of WKY rats, the morphology of gyri and sulci were normal, and there was no edema, hemorrhage and offset midline. However, in the brains of SHRSP-Veh group, the gyri were flattening and the sulci were shallow, and there were local tissue edema, hemorrhage and offset midline. In the brains of SHRSP-Los6 group, the morphology of gyri and sulci were nearly normal, and there were mild local tissue edema and occasionally hemorrhage, but no

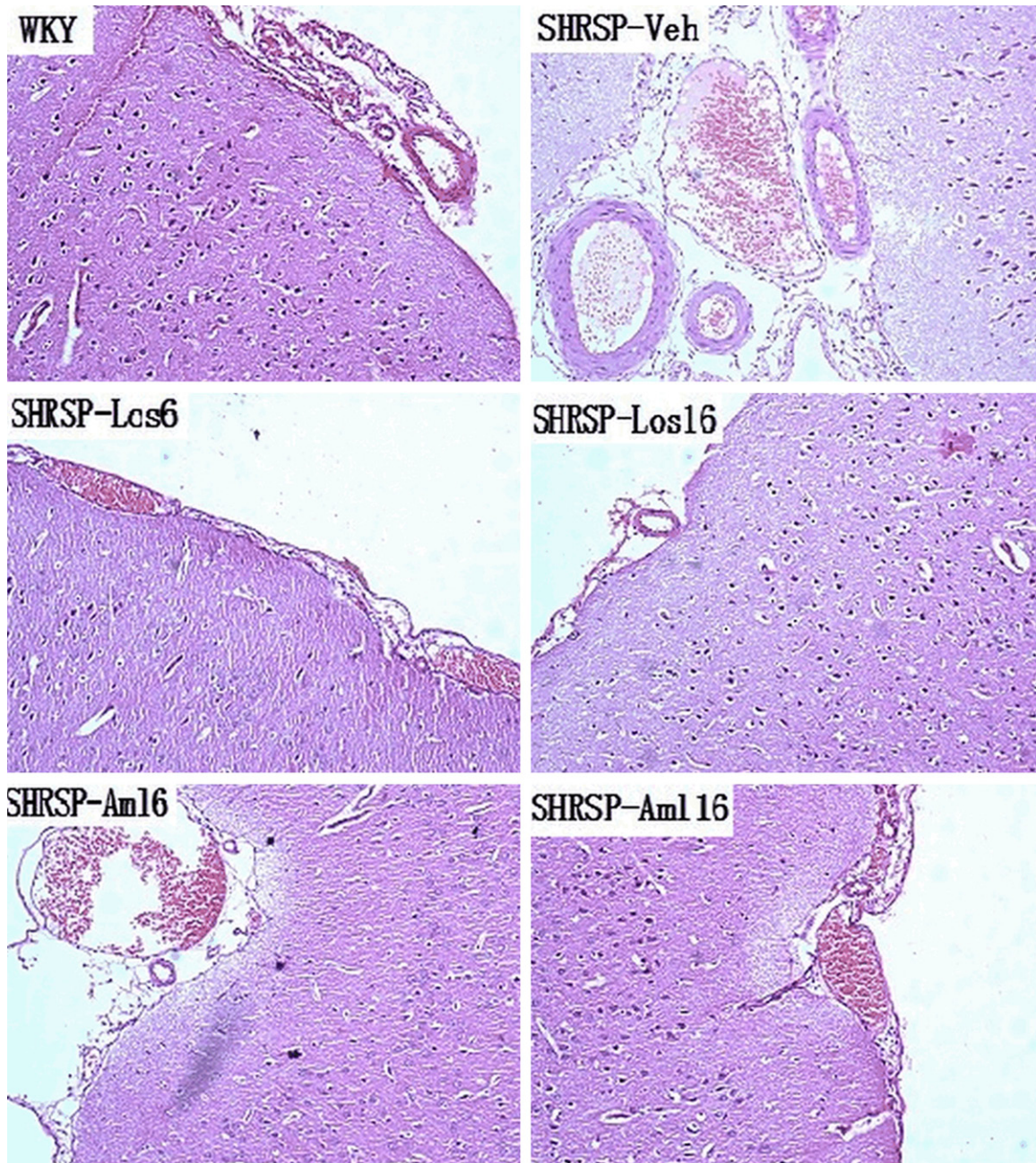
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**Figure 2.** Changes in the brain structure of Wistar Kyoto rats (WKY), stroke-prone spontaneously hypertensive rats (SHRSP)-Veh and the four drug treatment SHRSP groups at the age of 40 weeks. A global view of brains dissected from rats of each group investigated. The brains of WKY rats were normal and no obvious bleeding spots were observed in the brains from the SHRSP-Los6 and -Los16 groups.

offset midline. In the brains of SHRSP-Los16, the morphology of gyri and sulci were nearly normal, and there was no edema, hemorrhage and offset midline. However, in the brains of SHRSP-Am16 group and SHRSP-Am116 group, the gyri were flattening and the sulci were shallow, and there were significantly local tissue edema, many hemorrhages and some offset midline (**Figure 2**).

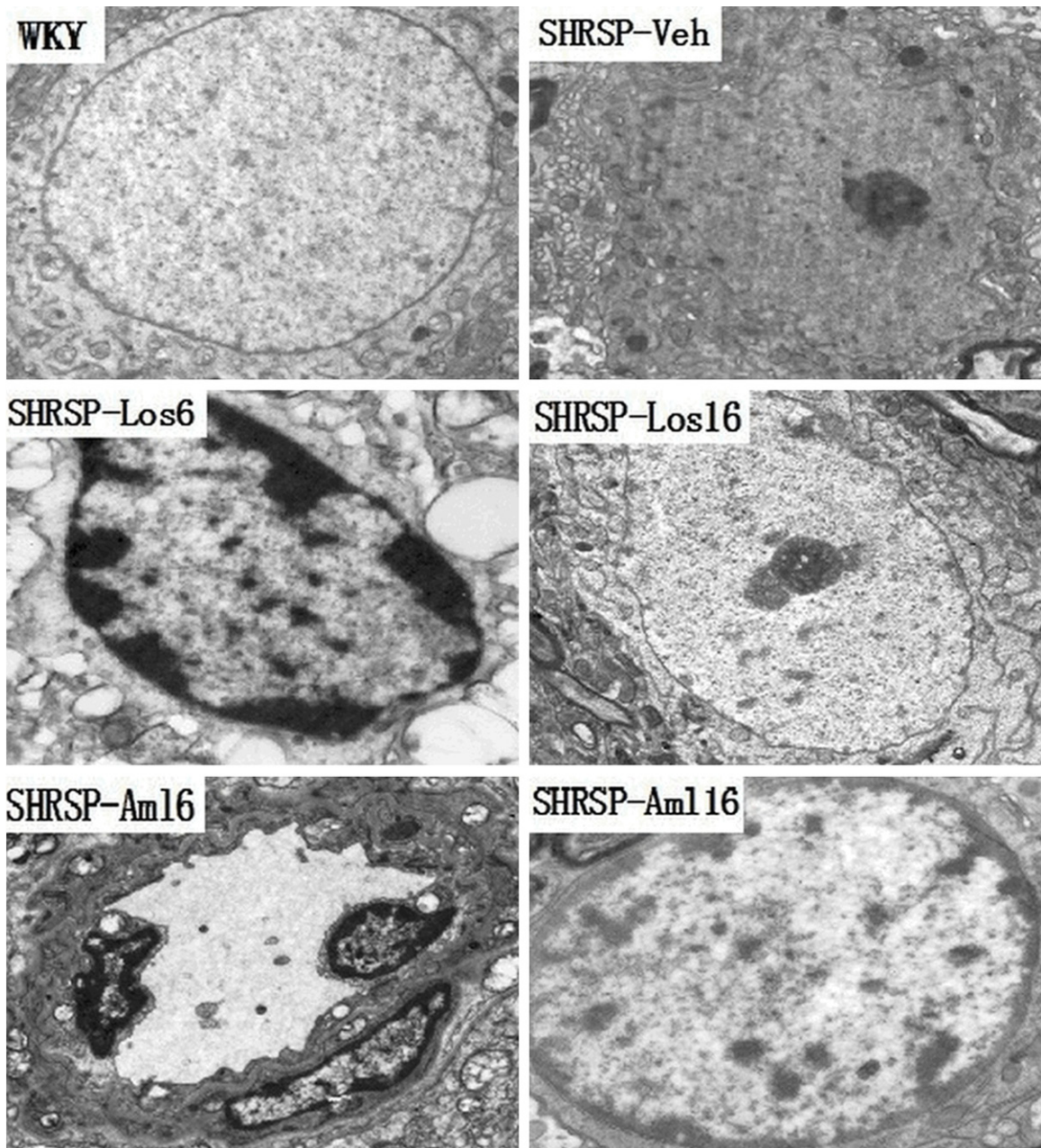
Results of optical microscope examination showed that, in the cerebral cortex of WKY rats, the tissue structure was slightly loose, but cell outline was clear and morphology of neurons was normal, and no bleeding and edema was observed. In the cerebral cortex of SHRSP-Veh group, the tissue structure was loose, and cell outline was fuzzy and morphology of neurons was disappeared. The old and fresh hemor-



**Figure 3.** Changes in the brain structure of Wistar Kyoto rats (WKY), stroke-prone spontaneously hypertensive rats (SHRSP)-Veh and the four drug treatment SHRSP groups at the age of 40 weeks. Examination results of brain with optical microscope. Hematoxylin and eosin (H&E) staining shows bleeding in the SHRSP-Veh, SHRSP-Los6, and two amlodipine treatment groups, but not the SHRSP-Los16 group.

rhages and particulate matters like hemosiderin were observed. In the cerebral cortex of SHRSP-Los6 group, the tissue structure was slightly loose, but cell outline was clear and morphology of neurons was normal, and occasionally bleedings and edema were observed. In the cerebral cortex of SHRSP-Los16 group,

the tissue structure was slightly loose, but cell outline was clear and morphology of neurons was normal, and no bleeding and edema was observed. In the cerebral cortex of SHRSP-Am16 group, the tissue structure was loose and vacuolar changes, and cell outline was fuzzy and morphology of neurons was disappeared.



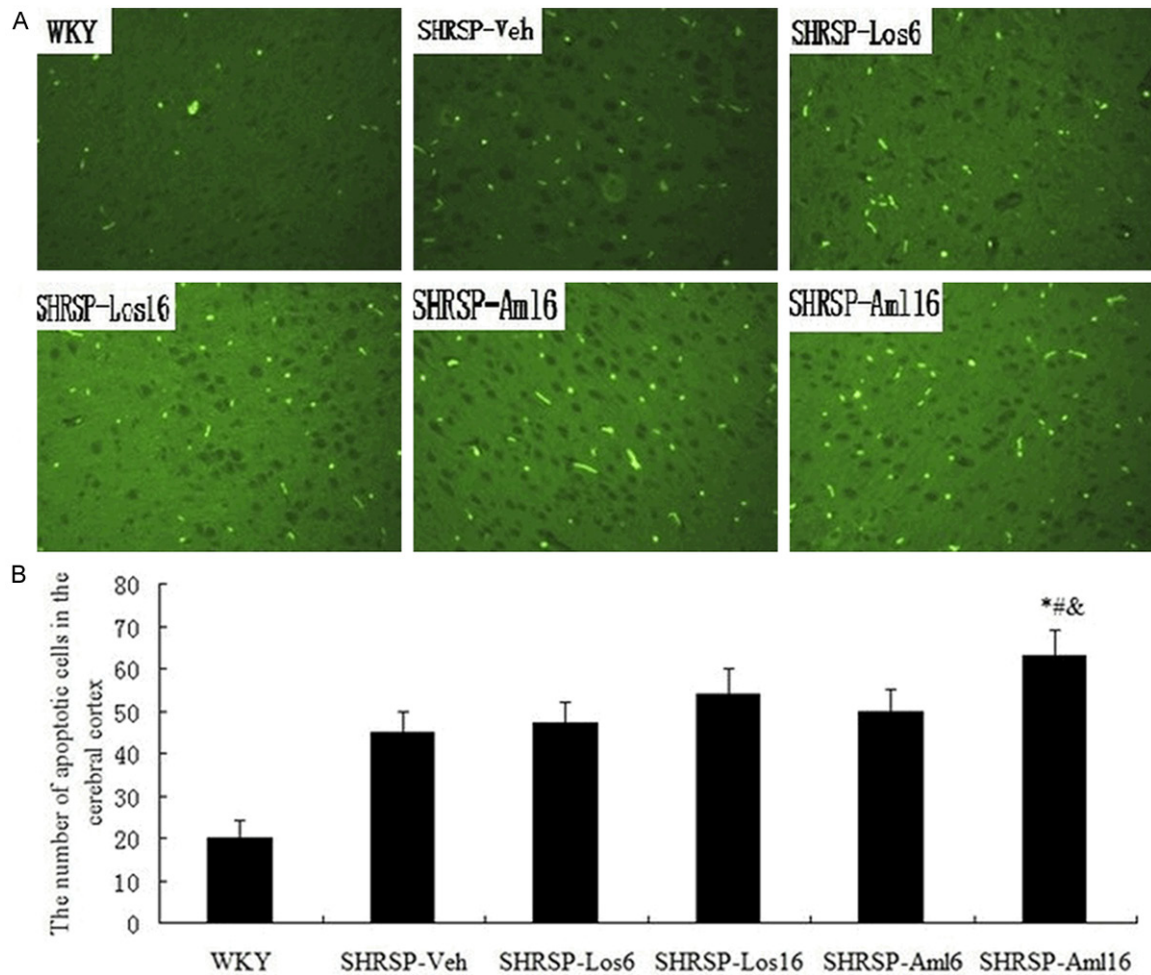
**Figure 4.** Changes in the brain structure of Wistar Kyoto rats (WKY), stroke-prone spontaneously hypertensive rats (SHRSP)-Veh and the four drug treatment SHRSP groups at the age of 40 weeks. Examination results of cell apoptosis with transmission electron microscopy (TEM). Ultrastructural analysis of brain tissues from the six groups by TEM shows swollen mitochondria and disrupted cristae in SHRSP-Veh. Similar pathological changes are present in the SHRSP-Aml6, SHRSP-Aml16 and SHRSP-Los6 groups, but not in the SHRSP-Los16 and WKY groups.

The old and fresh hemorrhages and particulate matters like hemosiderin were observed. In the cerebral cortex of SHRSP-Aml16 group, the tissue structure was slightly loose, but cell outline was clear and morphology of neurons was normal. The old and fresh hemorrhages and particulate matters like hemosiderin were observed (**Figure 3**).

*Effects of losartan and amlodipine on cell apoptosis of cerebral cortex*

The electron microscopy showed that, in the cerebral cortex of WKY rats, the chromatin was still evenly distributed and nuclear membrane was integrity in most neurons. But in some neurons, nuclear membrane was denser, cytoplas-

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**Figure 5.** Comparison of apoptosis in the brains of Wistar Kyoto rats (WKY), stroke-prone spontaneously hypertensive rats (SHRSP)-Veh and the four drug treatment groups at different ages (weeks). TUNEL staining was performed on 6  $\mu$ m sections of brains prepared from these experimental groups as indicated. TUNEL-positive cells were scored from three independent fields per sample. Values are expressed as means  $\pm$  SD ( $n = 8$  animals per group). \* $P < 0.05$  vs. SHRSP-Veh group; # $P < 0.05$  vs. SHRSP-Los16 group; & $P < 0.05$  vs. SHRSP-Aml6 group.

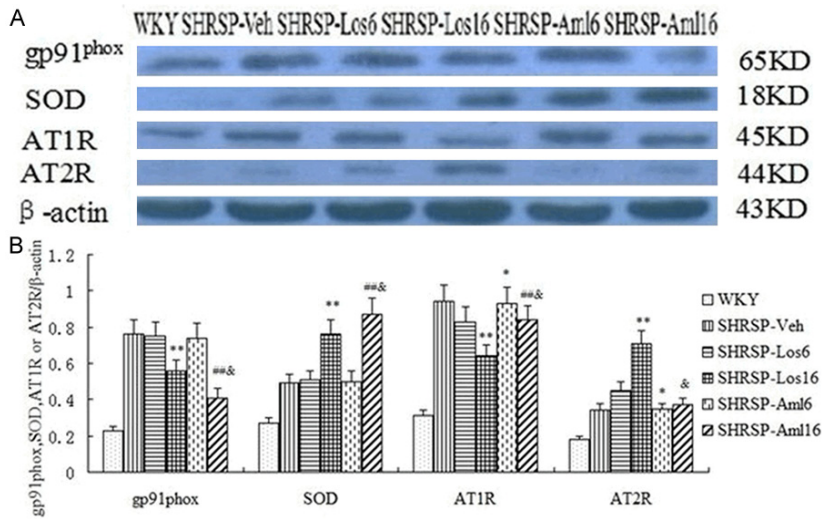
mic endoplasmic reticulum was mild expansion, mitochondrial was mild swelling, and part cellular ridge was fracture or reduced. Cell apoptosis was observed. In the neurons of cerebral cortex of SHRSP-Veh group, electron density was increased, nucleuses were pyknotic, and nuclear heterochromatin was increased. Chromatin was loose in the nuclear center but thick in the nuclear periphery, which presented 'chromatin margination'. Some nuclear membrane lost a two-tier structure, and endoplasmic reticulum was expanded and mitochondrion was swelling. Cellular ridge was fracture and reduced, or even disappear and vacuolar changes. The structure of myelin was distorted and some neurons were degeneration and necrosis. The treat-

ments of losartan and amlodipine could improve the structure of neurons, and longer administer time, more protective effects (**Figure 4**).

The treatments of losartan and amlodipine could delay the peak of cell apoptosis of cerebral cortex. At 40 weeks of age, no significant difference in the level of cell apoptosis of cerebral cortex was found between the SHRSP-Veh group and the SHRSP-Los6 group, the SHRSP-Los16 group or the SHRSP-Aml6 group. However, when compared with the SHRSP-Los16 group or the SHRSP-Aml6 group, the level of cell apoptosis of cerebral cortex was still higher in the SHRSP-Aml16 group ( $P < 0.05$ ) (**Figure 5**).



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**Figure 6.** Changes in the protein expression levels of gp91<sup>phox</sup>, SOD, AT1R and AT2R in cerebral cortex in untreated Wistar Kyoto (WKY) rats and stroke-prone spontaneously hypertensive rats (SHRSP) treated with losartan, amlodipine, or vehicle. Western blot was performed on protein lysates extracted from brains of rats from each group.  $\beta$ -actin served as the control. Values are expressed as means  $\pm$  SD ( $n = 8$  animals per group). \*\* $P < 0.05$  vs. SHRSP-Los6 group; ## $P < 0.05$  vs. SHRSP-Aml6 group; \* $P < 0.05$  vs. SHRSP-Los16 group; & $P < 0.05$  vs. SHRSP-Los6 group.

### Effects of losartan and amlodipine on protein expressions of gp91<sup>phox</sup>, SOD, AT1R and AT2R in cerebral cortex

At the age of 40 weeks, protein expressions of gp91<sup>phox</sup>, AT1R and AT2R in the SHRSP-Veh group significantly increased when compared with those in the WKY group ( $P < 0.05$ ). The protein expression of SOD in the SHRSP-Veh group was decreased when compared with that in the WKY group ( $P < 0.05$ ). The protein expressions of AT1R was not different between the SHRSP-Veh group and the SHRSP-Aml6 group, while it is different between the SHRSP-Los6 group and the SHRSP-Aml6 group, and between the SHRSP-Los16 group and the SHRSP-Aml16 group ( $P < 0.05$ ). When compared with the SHRSP-Los6 group and SHRSP-Aml6 group, the protein expression of AT1R was significantly decreased in the SHRSP-Los16 group and the SHRSP-Aml16 group, respectively ( $P < 0.05$ ). When compared with the SHRSP-Veh group, the protein expression of AT2R was higher in the losartan-treated SHRSP groups ( $P < 0.05$ ) while not different in the amlodipine-treated SHRSP groups. The protein expression of AT2R was different between the SHRSP-Los6 group and the SHRSP-Los16 group ( $P < 0.05$ ), while not different between the SHRSP-Aml6 group

and the SHRSP-Aml16 group. When compared with the SHRSP-Aml6 group and SHRSP-Aml16 group, the protein expression of AT2R was significantly increased in the SHRSP-Los6 group and the SHRSP-Los16 group, respectively ( $P < 0.05$ ).

The protein expressions of gp91<sup>phox</sup> and SOD were not different between the SHRSP-Veh group and the SHRSP-Los6 group or the SHRSP-Aml6 group, while those were different between the SHRSP-Los16 group and the SHRSP-Aml16 group ( $P < 0.05$ ). When compared with the SHRSP-Los6 group and SHRSP-Aml6 group, the pro-

tein expression of gp91<sup>phox</sup> was significantly decreased while the protein expression of SOD was significantly increased in the SHRSP-Los16 group and the SHRSP-Aml16 group, respectively ( $P < 0.05$ ) (Figure 6).

### Discussion

Early pharmacological intervention may delay BP progression and protect target organs in spontaneous hypertension. Different antihypertensive drugs may protect divergent target organs through the same BP reduction process [17]. Better strategies for cerebral protection from pre-hypertension have not been systematically studied. In the present study, we investigated the therapeutic aspects of two of the most commonly used anti-hypertensive drugs, losartan and amlodipine, from pre-hypertension in SHRSP.

This study is a follow-up study from our previous study [18]. In this study, we found that the SBP in the SHRSP-Veh group was increased when compared with the pre-treatment value, whereas the SBP in WKY rats stayed at a nearly constant level. With the given dosage, both losartan and amlodipine effectively delayed the progression of SBP in SHRSP in the intervening

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period. Longer administration time, slower the progression of BP, and the progression of SBP in the losartan-treated SHRSP groups were significantly slower after the drug intervention was stopped when compared to the amlodipine-treated SHRSP groups. At 40-weeks-old, the SBP was still lower in the SHRSP-Los16 group, not other drug-treated SHRSP groups when compared with the SHRSP-Veh group. This indicates that the application of either drug delayed the progression of BP in SHRSP and both losartan and amlodipine, especially losartan, demonstrated a 'persistent effect' in delaying the progression of BP within a certain time period.

Hypertension is an independent risk factor for stroke, and cerebral protection of antihypertensive treatment is well established. Previous studies have found that treatment with ARB just in pre-hypertension can reduce long-term brain damage in SHRSP, and it is may be related with ARB's sustained inhibition of renin-angiotensin-aldosterone system (RAAS) [19, 20]. In the present study, in WKY rats, the clinical score of stroke is low, the morphology and structure of the brain tissue and cerebral cortical cells were normal. While in the SHRSP-Veh group, the clinical score of stroke is high, the morphology and structure of the brain tissue changed obviously, the morphology and structure of the cerebral cortical cells was disappeared, and stroke was present. Losartan and amlodipine could effectively delay the progression of SBP, improve structure and morphology of brain and cerebral cortical cells and inhibit brain edema. The results showed that delaying the progression of BP is the key of cerebral protection. Furthermore, this study found that after drug intervention was stopped, the SBP showed a slower rise in the losartan-treated SHRSP groups when compare with amlodipine-treated SHRSP groups. Meanwhile, losartan treatment could significantly reduced stroke regardless of the administer time, while the effect of amlodipine is not obvious. These results showed that the progression of BP is an important factor for stroke, and the advantage of losartan in delaying the progression of BP is one of the important reasons of its advantage in reducing stroke. Cerebral protection of antihypertensive drugs beyond lowering BP is also one of focus. Circulation and local brain tissue are present RAAS [21]. In SHRSP, the increase of activity of RAAS in local brain tissue is one of factors of cerebral damage and stroke. The

effects of losartan and amlodipine to RAAS in cerebral cortex are different.

In this study, administration with losartan and amlodipine (except for SHRSP-Aml6) decreased the protein expression of AT1R in the cerebral cortex, which indicated that the protein expression of AT1R in the cerebral cortex was main related with BP, and lower BP, less the protein expression of AT1R. Losartan showed superior in inhibiting AT1R regardless of administration time, which may be related with its direct inhibition of AT1R. This is another important factor that losartan is superior to amlodipine in cerebral protection. Furthermore, losartan significantly up-regulated the protein expression of AT2R in cerebral cortex, but the effect of amlodipine is weak. This result showed that the protein expression of AT2R in cerebral cortex was main related with direct activation of losartan. This is also one of important factors that losartan is superior to amlodipine in cerebral protection [22, 23]. These results indicate that 'persistent effect' of cerebral protection of antihypertensive drugs treatment in inhibiting RAAS is within a certain time period.

The main target organs of hypertension are present apoptosis. Cardiomyocyte apoptosis is related with cardiac remodeling and heart function [24], and the acceleration of neuronal apoptosis is associated with senile dementia [25]. Therefore, although apoptotic is a physiological death, pathological factors can induce or cause increased apoptosis. It has been shown that an increase in BP causes apoptosis in the heart and blood vessels [26]. In the present study, the number of apoptotic cells was significantly greater in the cerebral cortex of the SHRSP-Veh group when compared with that in the WKY group, which indicated that the progression of BP led to an increase of apoptosis in the cerebral cortex. Apoptosis in cerebral cortex is active in early stage and diminished in the later stage in SHRSP. In the present study, at the age of 40 weeks (later stage), the structure of neurons were significantly improved and cell apoptosis of cerebral cortex was still active in losartan and amlodipine, especially amlodipine, treated SHRSP. These results indicate that the treatment with losartan and amlodipine, especially amlodipine, can delay the peak of cell apoptosis in cerebral cortex. Longer administration, more obvious those affects, especially amlodipine. These results indicate that 'persistent effect' of anti-hypertensive drugs

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treatment in delaying the peak of cell apoptosis is within a certain time period.

Cell apoptosis is related to BP, RAAS and oxidative stress [27, 28]. In this study, the advantages of losartan in delaying the progression of BP and inhibiting RAAS did not translate into the benefit of anti-apoptosis, which indicated that brain cell apoptosis and stroke not share the same pathological basis, and apoptosis may mainly associated with oxidative stress. Oxidative stress can lead to apoptosis [28], and the gp91<sup>phox</sup> induced oxidative stress and SOD can inhibit oxidative stress [29]. In this study, the protein expression of gp91<sup>phox</sup> was high while SOD was low in the SHRSP-Veh group, which indicated that high oxidative stress is present in the cerebral cortex of SHRSP. This is consistent with previous findings [30]. Losartan and amlodipine, especially amlodipine, can down-regulated the protein expression of gp91<sup>phox</sup> and up-regulated the protein expression of SOD in cerebral cortex. This may be related to the pharmacological features of anti-oxidative stress of amlodipine beyond lowering BP. Previous studies have shown that spontaneous neuronal apoptosis is associated with excessive Ca<sup>2+</sup> influx, leading to a further increase in intracellular Ca<sup>2+</sup> and the generation of reactive oxygen species [31]. Thus, agents such as amlodipine that block voltage-sensitive Ca<sup>2+</sup> channels and inhibit cellular oxidative stress may be effective in inhibiting apoptosis [32]. Therefore, amlodipine with its superior anti-oxidative stress properties may be the main mechanism of delaying the peak of cell apoptosis in the cerebral cortex. In addition, this study found that longer administration, more obvious of anti-oxidative stress, especially amlodipine. These results indicate that 'persistent effect' of anti-hypertensive drugs treatment in anti-oxidative stress is within a certain time period.

In conclusion, our present study indicates that treatment with losartan from pre-hypertension was more effective than amlodipine on delaying long-term BP increase. Losartan shows superior in improving structure and morphology of brain and cerebral cortical cells, inhibiting brain edema, and reducing stroke within a certain time. This may be related with its stronger inhibition of RAAS. However, amlodipine shows superior in delaying the peak of cell apoptosis in cerebral cortex. This may be related with its stronger anti-oxidative stress properties.

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### Disclosure of conflict of interest

None.

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