

*Full Length Research Paper*

# Interference of amlodipine combined with ALT-711 on arteriosclerosis in spontaneously hypertensive rats

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The objective of this study was to investigate the effect of amlodipine combined with ALT-711 on hypertension and the prevention of the complication of hypertension such as arteriosclerosis. Ten-week-old male spontaneously hypertensive rats (SHR) were randomly divided into four groups with six in each group: the blank control group (Group A, physiological saline 1 ml/kg/d), the amlodipine group (Group B, amlodipine 1 mg/kg/d), the ALT-711 group (Group C, ALT-711 10 mg/kg/d), and the ALT-711 + amlodipine group (Group D amlodipine 1 mg/kg/d and ALT-711 10 mg/kg/d). Eight week later, the aorta was harvested and sliced with stain of Masson solution, Collagen volume fraction was measured by image analysis. The expressions of advanced glycation end-products (AGEs), integrin  $\beta$ 1, fibronectin (FN) on the slice in each group were measured by immunohistochemistry and western blot were assessed. The artery mechanical strength in all groups was compared and the carotid artery rupture pressure was measured. Compared to Group A, the blood pressure of Group B, C and D significantly decreased. The positive expressions of AGEs, intergrin  $\beta$ 1 and FN in Group D significantly decreased compared to Group A, B or C ( $P < 0.0134$ ), and the pressure rupture of carotid artery, the blank control group (group A) were the lowest, and amlodipine combined with ALT-711 group (D group) is the highest among 4 groups and higher than amlodipine and ALT-711 alone ( $p < 0.0012$ ). However, ALT-711 combined with amlodipine can improve artery mechanical strength and prevent the aortic wall from extracellular matrix remodel in SHRs than amlodipine alone.

**Key words:** ALT-711, amlodipine, arteriosclerosis, age.

## INTRODUCTION

Hypertension is a major risk factor for cardiovascular disorders which is a leading cause of morbidity and mortality worldwide. Many of the complications of hypertension, such as stroke, coronary heart disease and aneurysm formation are themselves a direct result of the vascular damage induced by prolonged blood pressure elevation. Hypertensive end-organ damage in the myocardium, the renal glomeruli and vasculature remodeling is also characterized by the appearance of fibrosis. Fibrosis is caused by the accumulation of extracellular matrix (ECM) proteins, such as collagens,

advanced glycation end-products (AGEs) and fibronectin (FN). If the accumulation of vascular collagen leads to excessive stiffness, this would be maladaptive, since increased stiffness of conduit arteries, as evidenced by increases in pulse pressure and pulse-wave velocity, is in itself an independent risk factor for cardiovascular disease, especially for stroke (Van Bortel et al., 2001). Therefore, when considering new strategies for the prevention and treatment of hypertension and its complications, just only lowering blood pressure to target is not enough, preventing ECM from production and cross-linking in hypertension may therefore, be important. Study has shown that hypertensive arteriosclerosis is closely correlated with the deposition of AGEs and the cross-links among them (Bakker et al., 2004).

ALT-711 is a type of protein inhibitor which can interrupt

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the cross-links among AGEs and inhibit cross-link formation and break the cross-links caused by AGEs, thereby, maybe reversing one of the main mechanisms of aging and stiffness (Guo et al., 2009). Thus, in this study, we hypothesize that combining amlodipine with ALT-711 had better effects in lowering blood pressure, the mechanical strength of the intact CA and intra-aortic collagen volume and ECM than amlodipine and ALT-711 alone, in order to explore an optimum scheme for the preventing and treating of hypertensive arteriosclerosis.

## MATERIALS AND METHODS

### Grouping

Twenty-four 10-week-old spontaneously hypertensive rats (SHRs) were randomly divided into the control group (n = 6), the amlodipine group (n = 6), the ALT-711 group (n = 6) and the ALT-711 + amlodipine group (n = 6). Drugs were dissolved in distilled water. Experimental groups were intragastrically administered with drug solution for 8 weeks while the control group was administered with the same volume of saline, Group A were gavaged with physiological saline at 1 ml/kg/d, Group B were gavaged with amlodipine at 1 mg/kg/d, Group C with ALT-711 at 10 mg/kg/d and Group D with amlodipine at 1 mg/kg/d and ALT-711 at 10 mg/kg/d. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Zhongnan Hospital, Wuhan University.

### Non-invasive blood pressure measurement

Rats were warmed up at 40°C for 15 min, and then the systolic blood pressure (SBP) of the caudal artery in a conscious and resting state was measured by PBP-1B blood pressure measuring apparatus. Blood pressure was, respectively measured before drug administration and 1 week later after administration. Then, blood pressure was measured at an interval of 2 weeks.

### Mechanical strength

The mechanical strength of the intact CA was characterized by determining the *in vitro* intraluminal pressure leading to vascular wall rupture as previously described (Cohuet et al., 2001). The pressure rupture was measured in each group rats. The reproducibility was 5 ± 2% (intra-observer coefficient of variation of rupture pressure: left versus right CA. Parietal ruptures were confirmed on "en face" preparations.

### Intravascular collagen detection

Tissues were stained by Masson solution and fixed with neutral formalin. Paraffin slices were made. Slices were deparaffinized and rehydrated routinely, and then stained by Masson compound solution. Collagen fibers appeared blue, muscle fibers appeared red and the nucleuses were in blue brown. Slices were routinely mounted and observed under Olympus BH2 microscope. Pictures were taken. Collagen volume fraction (CVF) was calculated based on the quantitative analyses of the results conducted by HPIAS-2000 MED image analysis system (Kappert et al., 2000).

### Detection of the expressions of AGEs, integrin $\beta$ 1 and FN

The expressions of AGEs, integrin  $\beta$ 1 and FN were detected by immunohistochemistry (SP method) and image analyses. Paraffin-embedded slices were taken, and then detection was conducted according to the instructions of the immunohistochemical kit (Kits of AGEs and FN were bought from Beijing Biosynthesis Biotechnology Co. LTD. The kit of intergrin $\beta$ 1 was bought from WuHan Boster Bio-engineering Limited Company). Three visual fields were randomly chosen for each slice under Olympus BH2 microscope, and analyses were carried out by high-definition picture and text analysis system. The expressions of AGEs, integrin  $\beta$ 1 and FN were obtained from the average of three optical density (OD) values in each group.

### Western blot and immunoprecipitation

Rats aortae were ground in ice-cold lysis buffer containing 20 mM Tris-HCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, and 0.1% Tween 20 and protease inhibitors. Detergent-soluble fractions were retained, and protein concentrations in samples were equalized using a Bradford protein in assay. For western blot analysis, lysates containing 25  $\mu$ g of protein were electrophoresed on polyacrylamide gels and transferred to nitrocellulose membranes (Amersham ECL). Membranes were incubated with AGEs antibody or FN antibody or integrin  $\beta$ 1 antibody. For immunoprecipitation experiments, 100  $\mu$ g of protein were first pre-adsorbed with protein A/G-Sepharose for 2 h at 4°C, incubated with 5  $\mu$ g second- antibody (Santa Cruz) and, then with protein A/G-Sepharose (Santa Cruz) for 2 h. The immunocomplexes were subjected to Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes. An enhanced chemiluminescence system was used as the detection method (ECL+, Amersham).

### Statistical analysis

All values were averaged and expressed as mean  $\pm$  SEM. Data were analyzed by SPSS13.0 software. Analysis of variance (ANOVA) was carried out for the comparison between groups. The significance of differences among groups was determined by t-test.  $P < 0.05$  was considered significant.

## RESULTS

### Comparisons of SBP before and after treatment among groups

There were no significant differences in SBP among groups before treatment ( $P > 0.1436$ ). There were significant differences before and after treatment in both the amlodipine group and the amlodipine + ALT-711 group. But no significant difference was found between the amlodipine + ALT-711 group and the amlodipine group after treatment ( $P > 0.1105$ ) (Table 1). There was light decrease for SBP in ALT-711 group comparing with control group, but SBP was significantly higher than the amlodipine group and the amlodipine + ALT-711 group.

### Mechanical strength

The pressure rupture of carotid artery, the blank control

**Table 1.** Comparisons of SBP before and after treatment among groups ( $\bar{x} \pm s$ ) (mmHg).

Group (n = 6)	Pre-treatment (mmHg)	Post-8 weeks (mmHg)
A	163.00 ± 17.47	194.72 ± 15.14
B	165.00 ± 25.88	108.00 ± 10.19*
C	168.00 ± 18.11	170.22 ± 12.31 <sup>▲</sup> <sup>△</sup>
D	166.22 ± 24.74	99.83 ± 12.27*

\*, indicated a significant difference compared to the blank control after treatment (\*P < 0.05); <sup>▲</sup>, indicates a statistically significant difference compared to the amlodipine group (<sup>▲</sup> P < 0.05); <sup>△</sup>, indicates a statistically significant difference compared to the amlodipine combined with ALT-711 group (<sup>△</sup> P < 0.05).

**Table 2.** Rupture blood of carotid artery in SHRs.

Group (n = 6)	BP (mmHg)
A	1408 ± 61
B	1780 ± 59*
C	1608 ± 62* <sup>▲</sup>
D	1980 ± 48* <sup>▲</sup>

\*, Indicates a statistically significant difference compared to the control group (\*P < 0.05); <sup>▲</sup>, indicates a statistically significant difference compared to the amlodipine group (<sup>▲</sup> P < 0.05).

group (Group A) is the lowest, and amlodipine combined with ALT-711 group (Group D) is the highest among four groups and higher than amlodipine and ALT-711 alone ( $p < 0.0012$ ) (Table 2).

### Collagen detection

The collagen fibers, muscle fibers and nucleuses exhibited distinct colors after Masson staining (blue, red and blue brown, respectively) (Figure 1). The distribution area of collagen fibers in the blank control group was the largest while that in the amlodipine + ALT-711 group was the smallest. Semiquantitative results by image analyses in the control, amlodipine and amlodipine + ALT-711 groups were  $6.25 \pm 0.19\%$ ,  $3.56 \pm 0.035\%$  and  $1.32 \pm 0.025\%$ , showing significant differences between the experimental groups and the control group ( $P < 0.0134$ ) (Table 3).

### The expressions of AGEs, integrin $\beta_1$ and FN

After immunohistochemical staining, AGEs were presented as buffy or brown granules between cells, and integrin  $\beta_1$  and FN were presented as buffy granules between the cellular membrane and cytoplasm under light microscope. The expressions of all the mentioned indexes in Group A were obviously higher than those in

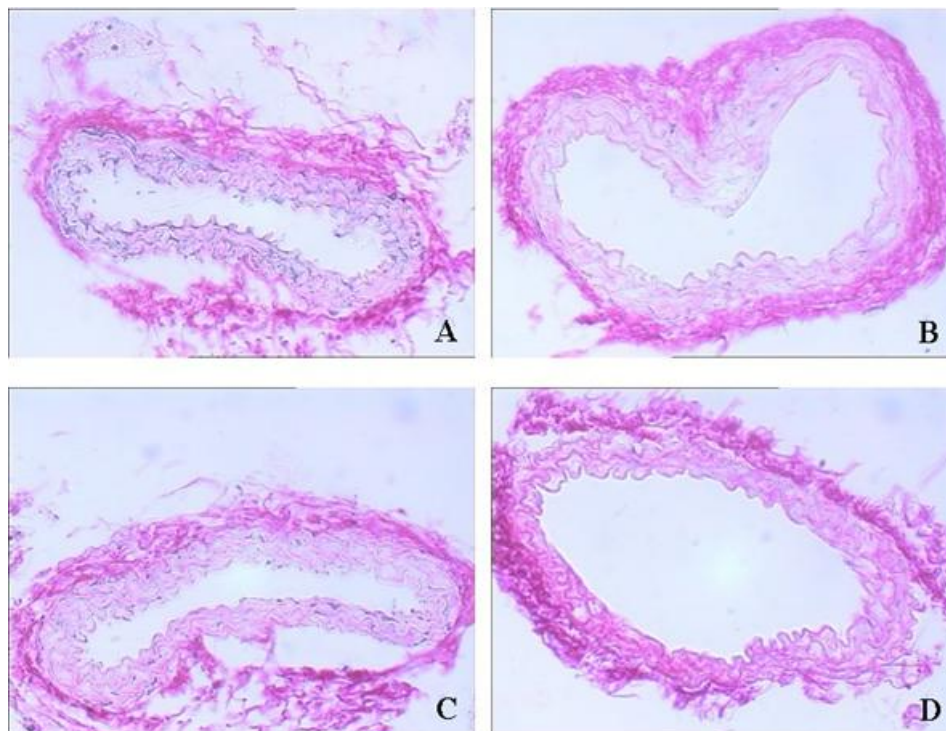
any other experimental group and those in Group C were notably lower than those in any other group (Figures 2 and 3). Expression results were analyzed by image analyzing system (HPIAS2000 image analysis system), and then, the positive expression rates were, respectively obtained (the positive expression rate = the number of positive cells in the visual field / the number of all cells in the same field  $\times 100\%$ ) (Table 3).

### Western blot and immunoprecipitation

Figure 4 showed the difference in the levels of AGEs, FN and integrin  $\beta_1$  in four groups. AGEs, FN and integrin  $\beta_1$  were increased by 2- to 3-folds, respectively in blank control group compared with amlodipine combined with ALT-711 group. There were differences between amlodipine with ALT-711 group and amlodipine alone (Figure 4 and Table 4).

### DISCUSSION

Target organ remodeling caused by hypertensive such as arteriosclerosis includes structure and function remodel. The vascular remodeling associated with hypertension may contribute both to the pathogenesis of its hypertension and to its pathophysiological consequences. ECM responses to the elevated wall stress in hypertension are



**Figure 1.** Comparisons of intravascular collagen volume among different groups under OLYMPUS BX50 microscope ( $\times 200$ ). A, The control group; B, the amlodipine group; C, the amlodipine + ALT-711 group.

**Table 3.** Comparing of the results of AGEs, integrin  $\beta 1$  and FN among different groups by immunohistochemistry ( $n = 6$ ) ( $\bar{X} \pm s$ ) %.

Group	AGEs (%)	Integrin $\beta 1$ (%)	FN (%)
A	$31.48 \pm 7.30$	$29.24 \pm 5.37$	$28.18 \pm 5.66$
B	$20.55 \pm 5.91^*$	$19.57 \pm 6.20^*$	$17.26 \pm 5.83^*$
C	$25.88 \pm 2.64^{*\Delta}$	$23.97 \pm 0.34^{*\Delta}$	$22.24 \pm 1.04^{*\Delta}$
D	$7.48 \pm 0.98^{*\Delta}$	$7.39 \pm 0.15^{*\Delta}$	$18.20 \pm 1.44^{*\Delta}$

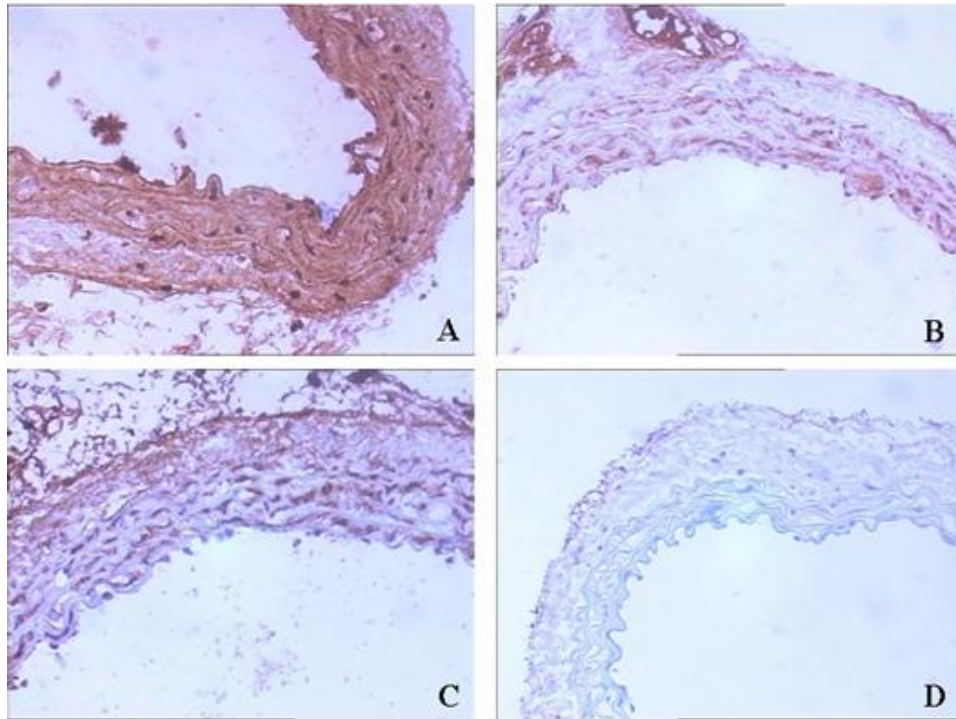
\*, Indicates a statistically significant difference compared to the control group (\*  $P < 0.05$ );  $\Delta$ , indicates a statistically significant difference compared to the amlodipine group ( $\Delta P < 0.05$ ).

rapid and sensitive (Keeley and Bartoszewicz, 1995). The synthesis of ECM is governed by both mechanical stimuli and biochemical stimuli. Hypertension can lead to arteriosclerosis, and the change of ECM is also closely correlated with arteriosclerosis (Intendant and Schifrin, 2000). The loss of aortic elasticity is an independent risk factor which can induce death due to cardiovascular diseases (McNulty et al., 2007). Thus, to reverse arteriosclerosis and improve vascular remodeling at the same time has become a new orientation in exploration into the effective method for hypertension treatment.

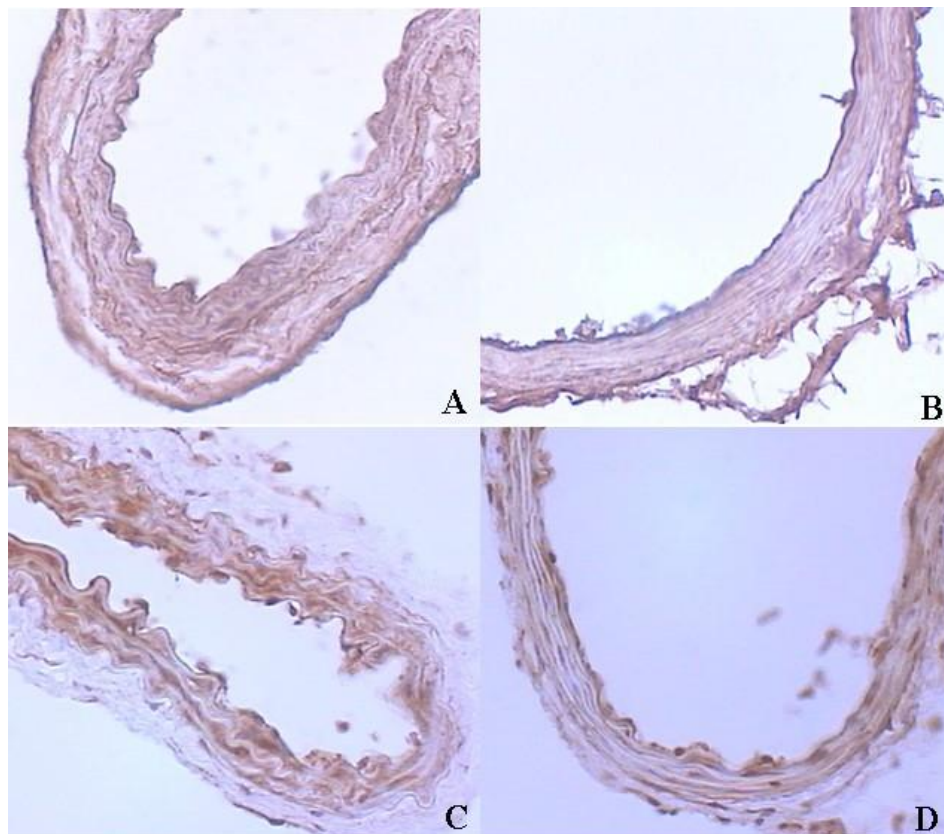
In this study, SHR were used as animal models. The history of SHR used for medical study can be traced back to 1963. As SHR have high incidence rates of

hypertension and cardiovascular diseases but without obvious primary renal or adrenal injury, they are quite suitable for study on human hypertension. Even more, SHR can show reactions to antihypertensive drugs, which enable them to become one of the most ideal animal models for antihypertensive drug screening.

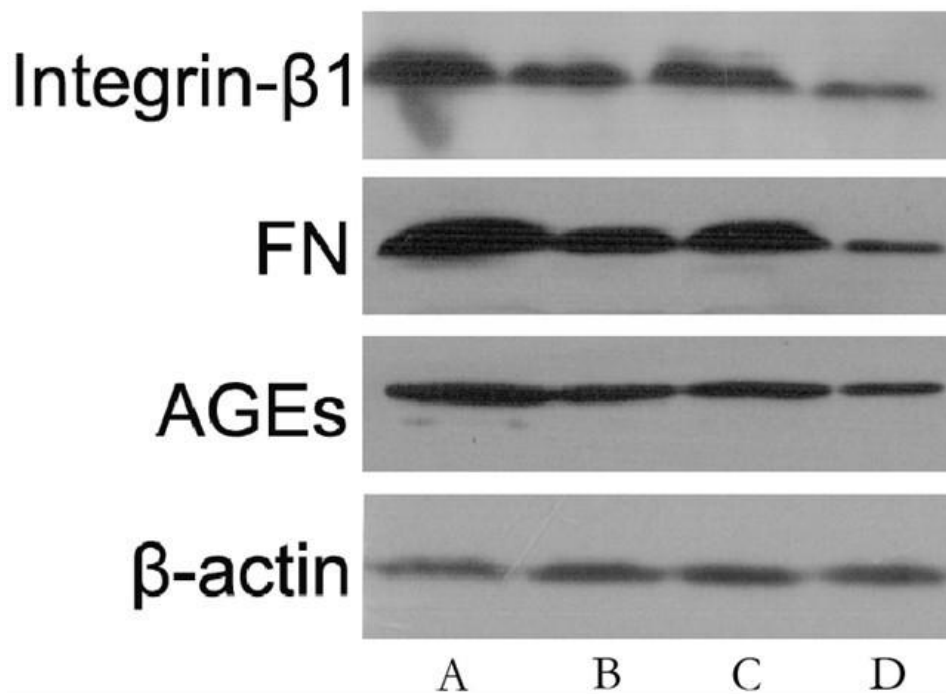
In this study, the manifestation of hypertensive arteriosclerosis is obvious increased interstitial collagen fibers around artery, AGEs, FN, Integrin  $\beta 1$ . These appeared prominently in blank control group (Group A), and amlodipine alone or combined with ALT-711 relieved this remodel, and the effects of amlodipine combined with ALT-711 were better than amlodipine alone, however, the effects of ALT-711 alone was not significantly different



**Figure 2.** Photos of AGEs in different groups under OLYMPUS BX50 microscope ( $\times 200$ ). A, The control group; B, the amlodipine group; C, the amolodipine + ALT-711 group.



**Figure 3.** Photos of integrin  $\beta 1$  in different groups under OLYMPUS BX50 microscope ( $\times 200$ ). A, The control group; B, the amlodipine group, C, the amolodipine + ALT-711 group.



**Figure 4.** Photos of FN in different groups under OLYMPUS BX50 microscope ( $\times 200$ ). A, The control group; B, the amlodipine group; C, the amlodipine + ALT-711 group.

**Table 4.** Comparisons of AGEs, integrin  $\beta 1$ , FN by western blot among groups ( $\bar{x} \pm s$ )%.

Group	N	AGEs (%)	Integrin $\beta 1$ (%)	FN (%)
A	6	$0.85 \pm 0.09$	$1.15 \pm 0.15$	$1.08 \pm 0.54$
B	6	$0.69 \pm 0.67^*$	$0.57 \pm 0.10^*$	$0.62 \pm 0.15$
C	6	$0.61 \pm 0.08^{*\Delta}$	$0.84 \pm 0.10^{*\Delta\Delta}$	$0.82 \pm 0.08^{*\Delta\Delta}$
D	6	$0.44 \pm 0.02^{*\Delta}$	$0.24 \pm 0.25^{*\Delta}$	$0.39 \pm 0.22^{*\Delta}$

\*, Indicated a significant difference compared to the blank control after treatment (\* $P < 0.05$ );  $\Delta$ , indicates a statistically significant difference compared to the amlodipine group ( $\Delta P < 0.05$ );  $\Delta\Delta$ , Indicates a statistically significant difference compared to the amlodipine combined with ALT-711 group ( $\Delta P < 0.05$ ).

with the blank control group, which means arteriosclerosis in Group A SHR in our study. This proves again that effective antihypertensive treatment is the key to prevention of vascular remodel, but results show that such effects were even better when amlodipine combined with ALT-711 compared with amlodipine alone and ALT-711 alone. On the other hand, for artery mechanical strength, the pressure rupture of carotid artery, the blank control group (Group A) is the lowest, and amlodipine combined with ALT-711 group (Group D) is the highest among four groups and higher than amlodipine and ALT-711 alone, which means this is a better way to prevent and treat hypertension and complication by combining lowering blood pressure with inhibiting ECM cross-linking than amlodipine alone to

antihypertension. The stiffening of the aortic wall is closely correlated with AGEs formed by non-enzymatic glycosylation between collagen fibers of reducing sugar and ECM as well as the formation and accumulation of cross-links among these produced AGEs. And meanwhile, it is also correlated with the interaction between FN and integrin  $\beta 1$ , which can prime the intra- and extra-cellular signal transduction to result in matrix deposition (Bezie et al., 1998). The extracellular collagen matrix plays an essential role in the structural integrity and the normal functions of the cardiovascular system. Physiologically, collagen can generate matrix for collagen cross-links and fibrous cross-links via enzymatic reactions to maintain the necessary intension and dilatancy of the aorta. Collagen cross-links can cause the



formation of AGEs via non-enzymatic reaction (Alberts et al., 2002; Lodish et al., 2000; Furber, 2006). The specific binding between AGEs and receptors for advanced glycation end products (RAGE) in endothelial cells and smooth muscle cells can lead to a series of vascular injuries. Immunohistochemical results in our study showed that there were statistically significant differences in the positive expression of AGEs between the control group and the experimental groups ( $P > 0.05$ ).

ALT-711 is a kind of compound developed by Vasani (Adams and Blizard, 1991). It has a stable structure and a high activity (5, 8-Dimethyl-3-(2-oxo-2-phenylethyl)thiazolium chloride). ALT-711 combines AGEs which have formed and is prone to build a kind of spontaneous broken structure, which can cut off the bridges formed by collagen and other biological macromolecules cross-linking. When bridges connecting protein molecules do not exist, AGEs cross-linked is broken. In our study, amlodipine or ALT-711 alone relieved AGEs expression in aortic tissues, which means they are all effective ways to prevent artery from remodel by decreasing mechanical stimuli or biochemical stimuli, but the Group D (amlodipine combined with ALT-711) had the best effect among four groups, understanding results indicates amlodipine adding up ALT-711 had superior effect to relieve excessive AGEs composition by high blood pressure.

Integrin family members (Wolfenbuttel et al., 1998) are cell adhesion receptors mediating the interaction between fibroblasts and ECM. FN is a kind of integrin ligand existing in the ECM of most of tissues. Its interaction with integrin  $\beta 1$  can influence multiple cellular functions such as adherency, growth, migration, tissue repair, etc (Kawano et al., 2000). When there is hypertension, the deposition of FN will exert impacts on the deposition of collagens as well as the structure of the ECM. Hypertension can stimulate fibroblasts to generate more integrins, ECM (such as collagens), FN, etc, and the interaction between the proliferated FN and integrin  $\beta 1$  can mediate the intra- and extra-cellular signal transduction, which ultimately aggravate arteriosclerosis (Caiado et al., 2011). In our study, results by immunohistochemistry and western blot showed that the expressions of AGEs, integrin  $\beta 1$  and FN in the ALT-711 + amlodipine group significantly decreased compared to any one of four groups, and had the highest rupture blood pressure among four groups, which indicate that the combination of amlodipine and ALT-711 can effectively improve arteriosclerosis caused by hypertension. Therefore, we believe the combination of amlodipine and ALT-711 may be optimal way to prevent and treat hypertension and its complications.

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