Resveratrol has effects to improve atherosclerosis in vivo study

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Abstract

Aim: The aim of this study is to explain the effects and mechanism of resveratrol to improve atherosclerosis in vivo study.

Methods: SD rats were divided randomly into control group, vascular calcification model group and treatment group. Vascular calcification models were made by subcutaneous injection of warfarin plus vitamin K1 for two weeks. Rats in the treatment group were subcutaneously injected with resveratrol (10 mg/kg) at the end of the first week and consecutively for two weeks. We observed the morphological changes by HE staining and the calcium deposition by Alizarin red staining in the artery vascular wall. The AT1R, RUNX2 and BMP2 proteins expressions were evaluated by WB assay. The apoptosis of smooth muscle cells (SMCs) were detected by TUNEL.

Results: The aortic vascular calcification was induced by warfarin and vitamin K1. Compared with the vascular calcification model group，the protein expressions of AT1R, BMP2 and RUNX2 were significantly downregulated in the aorta in the resveratrol treatment group. Furthermore, the apoptosis of SMCs was obviously decreased in resveratrol treatment group.

Conclusion: Resveratrol inhibited apoptosis of SMCs via regulation AT1R; It downregulates the BMP2 and RUNX2 expressions in the vascular calcification process.

Key words: vascular calcification; angiotensin II receptor 1 (AT1R); Resveratrol; BMP2; RUNX2

Introduction

Vascular calcification is a common pathological change in atherosclerosis, diabetes mellitus and chronic renal failure. It is closely related to cardiovascular disease, high morbidity and mortality (1, 2). In the past, vascular calcification is a passive process of calcium and phosphorus deposition in the vascular wall. In recent years, studies have shown that vascular calcification is an active, controllable biological process similar to bone development (3). Vascular calcification occurs between the elastic membranes of the large and medium-sized arteries, such as the aorta, the carotid artery, and the coronary artery. Various components of the vascular wall, such as vascular smooth muscle cells (SMC), pericytes, and macrophages, may participate in the calcification process. Bone morphogenetic protein 2 (BMP2) is a member of the transforming growth factor beta superfamily, which regulates osteoblast differentiation and bone formation (4). High expression of BMP2, bone matrix proteins and other related proteins were found on the calcified vessel wall, indicating that BMP2 plays an important role in the process of vascular calcification (5). Runt related transcription factor 2 (RUNX2) is an osteoblast specific transcription factor, which plays an important role in the differentiation of osteoblasts and the expression of bone matrix proteins (6). Blockade of the renin angiotensin system can reduce morbidity and mortality in atherosclerosis, diabetes, and chronic kidney disease (7-9).

Resveratrol (Res) is a polyphenolic compound widely found in plants. It has an obvious protective effect on cardiovascular system, such as ischemia-reperfusion injury, myocardial injury, atherosclerosis, and coronary heart disease (10). Res has antioxidant, scavenging free radicals, inhibiting platelet aggregation, regulating blood lipids, preventing atherosclerosis, anti-inflammatory and anti-tumor effects (11, 12). In our present study, we evaluated the effects and mechanism of Res in vascular calcification.

Materials and methods

Materials

Resveratrol, warfanin and alizann were purchased from Sigma (USA), mice anti-rat RUNX2 and rabbit anti-rat ATLR and BMP2 were purchased from Abcam (USA), Rabbit anti-rat GAPDH was purchased from Cell Signaling Technology (USA). HRP labeled Goat anti mouse IgG and HRP labeled Goat anti rabbit IgG were purchased from Biosharp company (USA). The rats were purchased from Nanjing Medical University.

Establishment of rat model of vascular calcification

A rat model of vascular calcification was established by reference to the literature (13). 27 SD rats were randomly divided into 3 groups: normal control group (NC group), vascular calcification group (MC group) and resveratrol treated vascular calcification group (Treatment group). MC group: warfarin (150 mg/kg) combined with vitamin K1 (15 mg/kg) subcutaneous injection at 8 a.m.; warfarin was re injected once again at 8 p.m., continued for 2 weeks (13, 14). Treatment group: Rats were given subcutaneous injections of warfarin for first weekends and treated with resveratrol (100 mg/kg) for 1 week (15, 16). After being treated, rats were sacrificed to take the aorta, and specimens were collected by liquid nitrogen cryopreservation or 40 g/L paraformaldehyde fixation.

H＆E staining

The paraffin sections of rat aorta after routine H＆E staining under light microscope to observe the histological characteristics.

Alizarin red staining

The aortic tissue was fixed by 10 mL/L formalin and embedded in paraffin; Section as 4 μm, the slices were stained with alizarin red for 30 min to detect calcium deposition, and the morphological characteristics were observed by microscope.

TUNEL assay

Paraffin embedded sections of aorta as 4 μm, Following the instructions of the apoptosis detection kit (Roche) (17), Finally, color with DAB. Under the microscope,

the cells with brown granules in the nucleus of the vascular smooth muscle cells were positive cells, namely apoptotic cells. Each slice was randomly selected at 10 high-power visual fields (×200) to count apoptotic cells, and the percentage of apoptotic cells per view was calculated.

WB assay

The aorta was removed from liquid nitrogen, suspended with protein extract, placed in boiling water for 10 min, and centrifuged at a low speed. The sample is separated by 80 g /LSDS-PAGE and transferred to the PVDF film; After 50 g /L skimmed milk powder, room temperature closed for 30 min, adding rabbit anti-mice BMP2 antibody (1:800), ATLR antibody (1:800), RUNX2 antibody (1:500) and GAPDH antibody (1:1000), cultured overnight at 4℃; Washing clearly, Goat labeled anti goat IgG and HRP labeled anti mountain rabbit IgG (1: 5000) were incubated with HRP markers, incubated at room temperature for 1 h, and were detected by ECL kit. Image J software was used for image analysis.

Results

Res has effects to warfarin combined with vitamin K1 induced vascular calcification

Warfarin combined with vitamin K1 could induce vascular calcification in vivo, By the H&E staining, the pathology of MC group was shown that the wall of the vessel is loose, the thickness of the vessel wall increases and the brittleness decreases. Some areas of the vessel wall are stratified. These are typical lesions of vascular calcification (Figure 1). After alizarin red staining, some blood vessels in the model group showed deep red stripe like calcium deposits, which were caused by calcified plaques (Figure 2). With Res treatment, the vascular wall structure and calcium deposition pattern all decrease (Figure 1&Figure 2).

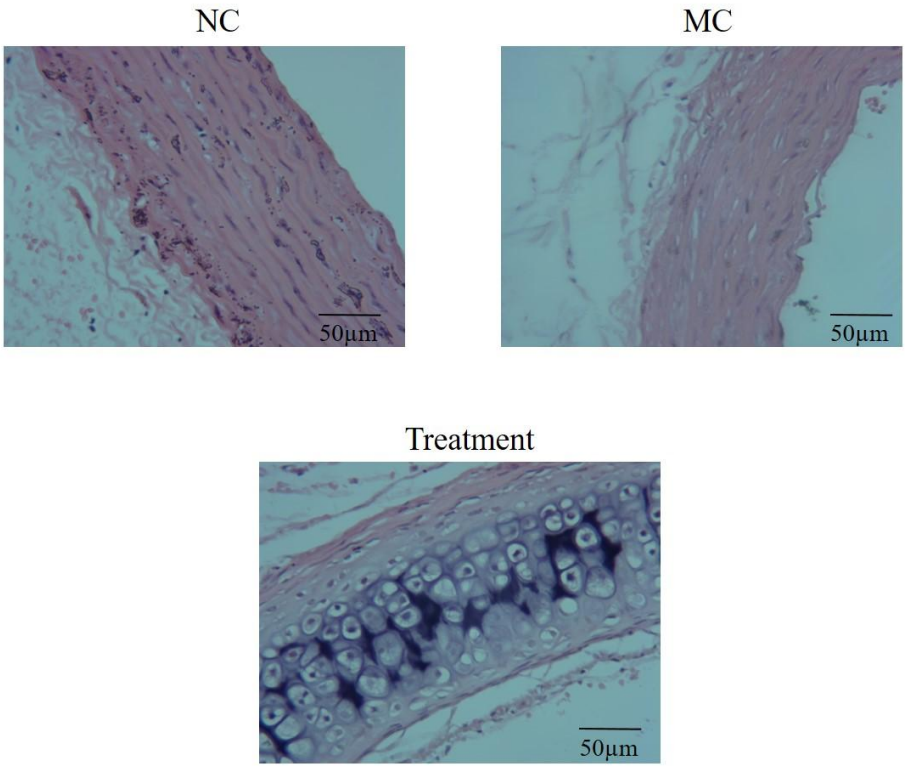

Figure 1. The pathology of difference groups by H&E staining (×200)

NC: normal control group; MC: model control group; Treatment: the Res treated group

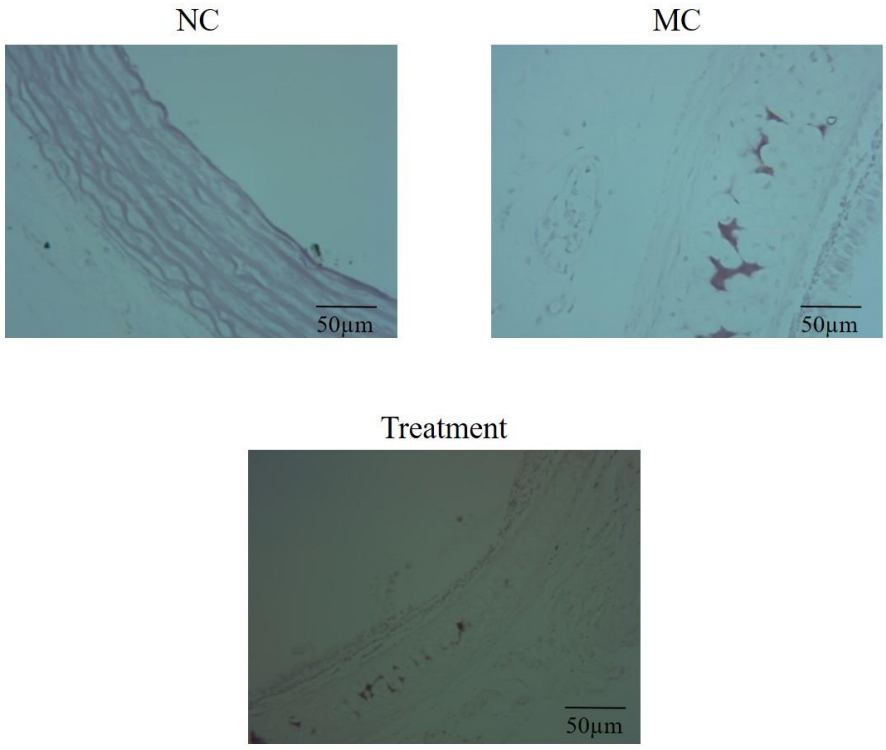

Figure 2. The alizarin red staining of difference groups (×200)

NC: normal control group; MC: model control group; Treatment: the Res treated group

Res suppressed vessel wall SMC apoptosis

We detected the apoptosis of SMC in the vessel wall by TUNEL assay. Compared with NC group, the cell apoptosis rate of MC group was significantly increased ($P < 0.05$); however, the cell apoptosis rate of treatment group was significantly improved compared with MC group ($P < 0.05$). The relative data were shown in Figure 3.

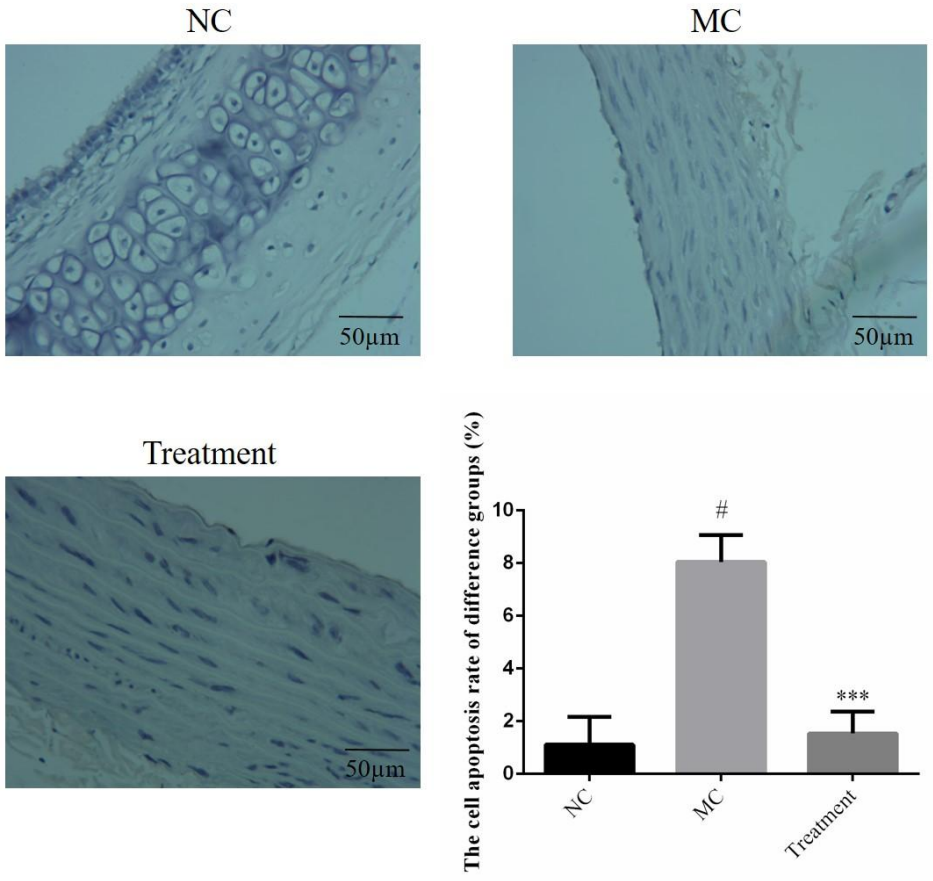

Figure 3. The cell apoptosis of difference groups by TUNEL assay (×200)

NC: normal control group; MC: model control group; Treatment: the Res treated group.#: $P < 0.05$, compared with NC group; ***: $P < 0.05$, compared with MC group.

Res suppressed the AT1R, RUNX2 and BMP2 proteins expression

By the WB assay, compared with NC group, the AT1R, RUNX2 and BMP2 proteins expression of MC group were significantly up-regulation ($P < 0.05$, Figure 4);

Compared with MC group, after Res treatment, the AT1R, RUNX2 and BMP2 proteins expression of Treatment group were significantly down-regulation (P＜0.05, Figure 4). The relative data were shown in Figure 4.

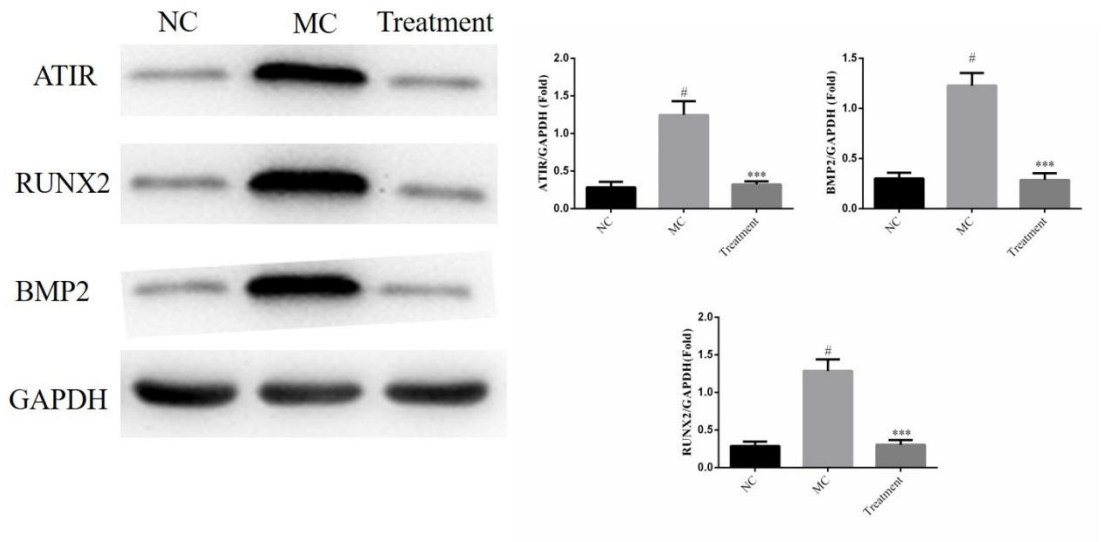

Figure 4. The relative proteins expression of difference groups (×200)

NC: normal control group; MC: model control group; Treatment: the Res treated group. #: P＜0.05, compared with NC group; ***: P＜0.05, compared with MC group.

Discussion

Vascular calcification is characterized by thickening of the elastic membrane of the arteries, calcium phosphate deposits and calcification of the intermediate elastic fibers, causing the tube wall to become less resilient and harder. We used a combination of warfarin and vitamin K1 induced by HE staining, alizarin red staining showed that the rat aorta appeared internal elastic membrane thickening, calcification and mineral deposition of calcium salt and other changes, establish the model of vascular calcification. Warfarin as vitamin K inhibitors, by regulating vascular calcification inhibitor of matrix Gla protein (MGP) activation play its induced arterial calcification, which is consistent with clinical reports.

Vascular calcification is an active regulation process in which the vascular smooth muscle cells (VSMC) are transformed into osteoblast like cells and accompanied by hydroxyapatite deposition (17), BMP2 expression can promote the

differentiation of VSMC into osteoblast like cells (18, 19), and is one of the markers of osteogenic transformation of VSMC (20, 21). Our experiments showed that the expression of BMP2 protein was up-regulated after model vessel calcification in rats, and confirmed that BMP2 participates in the regulation of vascular calcification, which is consistent with the previous studies. RUNX2 is a member of the runt domain family and is one of the major osteogenic transcription factors. Knockout of RUNX2 gene in rats completely blocked bone formation, indicating that RUNX2 is an important regulator of bone calcification (22) and one of the target genes of BMP2 signaling pathway (23). We found that vascular calcification after upregulation of RUNX2 and BMP2 expression had the same trend with the reported BMP2 activation of Drosophila mothers against decapentaplegic protein (Smads) l /5 /8, start RUNX2 expression consistent. Our results support reports that BMP2 / Smad L /RUNX2 pathway activation is involved in vitamin D3 and nicotine induced renal artery calcification in rats (23).

Some previous studies found that Res had effects to suppress renin angiotensin system (24, 25). In our present study, we found that vascular calcification after resveratrol treatment, calcium deposition in the vessel wall was reduced, AT1R, ossification marker BMP2 and transcription factor RUNX2 expression, suggesting that resveratrol inhibits vascular calcification or anti arterial calcification (26), and correlated with down-regulation of AT1R, BMP and RUNX2, to suppress VSMC apoptosis (27).

Apoptosis occurs during calcification (28, 29), and we found that the rate of apoptosis increases after vascular calcification. The up regulation of BMP2 may be related to the process of calcification induced by BMP2. At the same time, we also found that the expression of AT1R increased after vascular calcification, and AT1R and SMA were co expressed and increased. It showed that SMC proliferated and activated AT1R expression during the process of vascular calcification. Our findings are consistent with reports of angiotensin II activation, nuclear factor κ B receptor activation, ligand modulation, vascular calcification (30, 31), and AT1R involvement

in vascular calcification regulation (32, 33).

The present study showed that the aortic vascular calcification caused upregulation of BMP2 and RUNX2, apoptosis of vascular SMC and upregulation of AT1R expression; however, Res can reduce the expression of BMP2, RUNX2 and other vascular calcification markers, and inhibit the expression of AT1R in the calcified region and SMC apoptosis to alleviate the development of vascular calcification, and provide a new approach for the treatment of vascular calcification.

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