

## Hypercholesterolemia Accelerates Vascular Calcification Induced by Excessive Vitamin D via Oxidative Stress

F. T. Tang, S. R. Chen, X. Q. Wu, T. Q. Wang, J. W. Chen, J. Li, L. P. Bao, H. Q. Huang, P. Q. Liu

Laboratory of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510080, People's Republic of China

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**Abstract.** Hypercholesterolemia plays an important role in the initiation and progression of atherosclerosis and has a positive correlation with cardiovascular disease. Calcification is a common feature of atherosclerotic lesions and contributes to cardiovascular dysfunctions. The present study investigated the role of hypercholesterolemia in vascular calcification and its potential mechanism. Models of vascular calcification were established by administering vitamin D<sub>2</sub> (VD) to rats alone or combined with a high-cholesterol diet (HCD) and by treating rat aorta smooth muscle cells (RASMCs) with  $\beta$ -glycerophosphate (GP) alone or combined with oxidized low-density lipoprotein (oxLDL) *in vitro*. In rats, the combination of VD with HCD significantly enhanced vessel calcium deposition and the activity and mRNA expression of vessel alkaline phosphatase (ALP) compared to treatment with VD alone. This combination also enhanced serum levels of total cholesterol, oxLDL, and malondialdehyde as well as vascular production of superoxide anion, while it reduced the vascular activity of superoxide dismutase. Both simvastatin, a cholesterol-lowering agent, and antioxidant vitamin E antagonized the effects of the above combination. In RASMCs, oxLDL accumulation dependently accelerated calcium deposition in cell layers initiated by GP alone. Also, oxLDL stimulated ALP activity and mRNA expression in RASMCs in a concentration-dependent manner. Taken together, these results suggest that acceleration of vascular calcification by hypercholesterolemia might be attributed to oxidative stress and such calcification may be another target of statin or antioxidant action in antiatherosclerosis.

**Key words:** Hypercholesterolemia — Vascular calcification model — Oxidized low-density lipoprotein — Simvastatin — Vitamin E

Vascular calcification is a common and clinically significant component of several human diseases, including atherosclerosis, aortic stenosis, and diabetes [1] and is known to be present in 80% of vascular lesions and

>90% of patients with coronary artery disease [2]. Vascular calcification is an important risk factor for cardiovascular events because it causes decreased aortic compliance and elastic recoil, which results in cardiac ischemia in severe cases due to impaired reverse aortic flow and coronary perfusion [3]. Vascular calcification has been considered an end-stage process of “passive” mineral precipitation. However, there is a growing awareness that vascular calcification is a biological phenomenon similar to bone formation and osteoporosis. Those similarities are supported by accumulating evidence, such as increased activity of alkaline phosphatase (ALP), a marker of osteogenic differentiation of osteoblastic cells, in the calcified vessels [4]. Hypercholesterolemia plays an important role in the initiation and progression of atherosclerosis and has a positive correlation with cardiovascular disease, largely depending on the oxidation of low-density lipoprotein (LDL), the main cholesterol carrier in plasma [5]. Atherosclerotic calcification is seen as early as the second decade of life, just after the fatty streak stage; and many studies have demonstrated a clear association between lipid accumulation and atherosclerotic calcification [6]. Calcification colocalizes with cholesterol crystals in human atherosclerosis [7] and is present in the intima of cholesterol-fed rabbits [8] and apolipoprotein E (ApoE) knockout mice [9]. Microscopic calcification in aortic valves seems to form in the area in which lipoproteins have been retained [10]. Early deposits of calcification occur within and around isolated vascular smooth muscle cells (VSMCs) from the lipid core. Despite these close links, little is known about the role that hypercholesterolemia plays in regulating vascular calcification. Studies *in vitro* have indicated that oxidized LDL (oxLDL) is involved in the calcification of VSMCs by stimulating the osteoblastic differentiation of vascular cells [11, 12]. Whether oxLDL bridges hypercholesterolemia and vascular calcification *in vivo* is still unknown. In addition, it remains unclear whether the beneficial effects of cholesterol-lowering agents such as simvastatin (Sim) and antioxidants like vitamin E (VE)

on patients with cardiovascular diseases could be attributed to their action of anticalcification in vessels.

The present study was designed to explore the effects of hypercholesterolemia on vascular calcification and the involved mechanisms in rats. In the study *in vivo*, a calcified vessel model of the rat was established by administering to rats high doses of vitamin D<sub>2</sub> alone or combined with a high-cholesterol diet (HCD). In the study *in vitro*, cultured rat aorta smooth muscle cells (RASMCs) was treated with  $\beta$ -glycerophosphate (GP) alone or combined oxLDL to duplicate the calcified cell model. The results showed that hypercholesterolemia accelerates vascular calcification by means of oxLDL.

## Materials and Methods

### Chemicals and Reagents

Sim was from Hangzhou Merk Company (Hangzhou, PR China). Vitamin E (VE) was produced by Guangzhou Baiyunshan Pharmaceutical Company (Guangzhou, PR China). Kits for the determination of total cholesterol (TC), free cholesterol (FC), LDL cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and calcium and phosphorus ions were from Beijing Zhongsheng Bioengineering Company (Beijing, PR China). Kits for the detection of superoxide dismutase (SOD), malondialdehyde (MDA), alkaline phosphatase (ALP), and protein were produced by Nanjing Ji-ancheng Bioengineering Company (Nanjing, PR China). Detecting kits for serum oxLDL and 25-hydroxyvitamin D (25-OHD) were the products of BPB Biomedicals (Bionewtrans Pharmaceutical Biotechnology Co., Ltd., Franklin, USA) and IDS (Immunodiagnostic Systems Ltd., Tyne & Wear, UK), respectively. Dihydroethidium bromide (DHE), vitamin D<sub>2</sub> (VD), and GP tetraethoxypropane were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) and Trizol were from GIBCO (Gaithersburg, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Deoxynucleotide triphosphate (dNTP), Moloney murine leukemia virus transcriptase (MMLV), *Taq* DNA polymerase, RNAsin, oligo(dT)<sub>15</sub> primer, and oligonucleotides for ALP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Sangong Biotechnology (Shanghai, PR China). The sequences of oligonucleotide primers were as follows: ALP-S, 5'-GCC CTC TCC AAG ACA TAT A-3'; ALP-A, 5'-CCA TGA TCA CGT CGA TAT CC-3'; GAPDH-S, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; GAPDH-A, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. Other chemicals and reagents were of analytical grade.

### Animals and Vascular Calcification Procedures

Male Sprague-Dawley rats (220  $\pm$  20 g) aged 6 weeks were purchased from the Experimental Animal Center, Sun Yat-sen University. The rats were housed under standard conditions (room temperature 20  $\pm$  1°C, humidity 60  $\pm$  10%, lights from 6 a.m. to 6 p.m.) and given water freely. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China (1998). Rats were randomly divided into a control (Con) group, a VD group, a VD+HCD group, a VD+HCD+Sim group, and a VD+HCD+VE group, containing eight animals in each group. The HCD was composed of standard chow (94.3%), cholesterol (2%), lard (3%), cholic acid (0.5%), and propylthiouracil (0.2%). The preparation for vascular calcification was as described by Kitagawa et al. [13] with modification. In

short, except for rats in the Con group, all rats were orally administered with 300,000 IU/kg VD daily for 4 consecutive days, followed by *ad libitum* standard chow in the VD group, HCD in the VD+HCD group, and HCD combined with oral administration of Sim (5 mg/kg) in the VD+HCD+Sim group or VE (100 mg/kg) in the VD+HCD+VE group for 12 weeks. Rats in the Con group consumed standard chow. All rats were weighed every 2 weeks. At the end of VD oral administration for 4 days, blood was collected from eyes of fasting rats (12 hours) under light anesthesia by ether, from which serum was separated for determination of 25-OHD. After fasting rats (12 hours) were anesthetized with 30 mg/kg pentobarbital sodium at the end of the twelfth week, blood was collected from the abdominal aorta, from which serum was separated by centrifugation at 3,000 rpm for 30 minutes and stored at -70°C until use for biochemical analysis. Thoraco-abdominal aorta was isolated and put into ice-cold phosphate-buffered saline (PBS). After removing the connected tissue carefully, it was stored at -70°C. The aortic arch was removed and fixed in 10% formalin.

### Body Weight and Systolic Blood Pressure

Body weight was monitored every 2 weeks, and systolic blood pressure was measured at the end of the experiment by placing a cuff which was connected to a sphygmomanometer (Chendu Technology & Market Corp., Chendu, PR China) on the base of the tail.

### Serum Levels of Lipid Files and Calcium

Serum was diluted with 150 mmol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA, pH 7.4) so that the optical density (OD) measurement and lipid concentrations were brought into the normal range. Serum concentrations of TC and triacylglycerol (TG) were assayed enzymatically using commercial kits, and those of HDL-C and LDL-C were determined by precipitation with phosphotungstic acid/magnesium chloride and heparin/sodium citrate, respectively. Serum calcium content was determined colorimetrically by the *o*-cresolphthalein complexone method.

### Serum Levels of oxLDL and 25-OH VD

Serum levels of oxLDL and 25-OHD were assayed by competitive enzyme-linked immunosorbent assay method following the manufacturer's instruction. Briefly, 50  $\mu$ L of standards, control, and samples containing oxLDL were added to wells of a microplate, which were coated with high-affinity anti-rat oxLDL antibodies, and then 50  $\mu$ L of biotin conjugate reagent were added in the case of oxLDL or 200  $\mu$ L of calibrator, control, or samples diluted 40 times with 25-OH biotin solution were added to the appropriate wells of the plate coated with 25-OHD sheep polyclonal antibody in the case of 25-OHD. The microplate was incubated at 37°C for 60 minutes in the case of oxLDL and at 20°C for 2 hours in the case of 25-OHD. After washing the wells using washing solution 5 times or 3 times, respectively, in automatic washer to remove the unbound components from the microplate, 50 or 200  $\mu$ L of enzyme conjugate reagent were added to the wells and the microplate was incubated at 37°C for 30 minutes in the case of oxLDL or at 20°C for 30 minutes in the case of 25-OHD. After another washing for 5 or 3 times, 50  $\mu$ L of color A and color B reagents or 200  $\mu$ L of tetramethyl benzidine (TMB) substrate were added, respectively, followed by incubation at 37°C for 15 minutes in the case of oxLDL or at 20°C for 30 minutes in the case of 25-OHD. Then, 50 or 100  $\mu$ L of an acidic stop solution were added, respectively, and the absorbance (OD) of yellow solution was read at 450 nm within 20 minutes using a microplate reader. A dose-reverse response curve of OD vs. concentrations of oxLDL or 25-OHD was generated

using the values obtained from standard or calibrate. Serum levels of oxLDL or 25-OHD were determined directly from this curve.

#### *Serum MDA Content*

Serum MDA content was measured as thiobarbituric acid-reactive substances (TBARS) according to Yagi [14]. In short, serum was added 10% (w/v) trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA), followed by incubation at 95°C for 1 hour. After centrifugation, TBARS in the supernatant were determined at 532 nm. Concentrations of TBARS were calculated using tetraethoxypropane as a reference standard.

#### *Vessel Levels of Lipids and Calcium*

Accumulations of vascular lipids and calcium were measured using a kit and atomic absorption spectrometry, respectively. Briefly, thoracic aorta was freeze-dried to a constant weight using a low-temperature freeze drier (Heto fd2.5; Heto Lab Equipment, Tokyo, Japan). After weighted, the lipids were extracted at 50°C for 20 minutes with chloroform-methanol (2:1) and the extracts used for the determination of TC and FC by kits. Cholesterol ester (CE) was the subtraction between TC and FC. The precipitation was dissolved in HNO<sub>3</sub> and then dried in an oven and redissolved with blank solution (27 nmol/L KCl, 27 μmol/L LaCl<sub>3</sub> in deionized water). Calcium content was measured using an atomic absorption spectrophotometer at 422.7 nm (AA-670; Shimadzu, Kyoto, Japan). Lipid and calcium contents were expressed as milligrams per gram dry tissue.

#### *Vessel Levels of Superoxide Anion and Autofluorescence of Elastic Fiber in Media*

Frozen, enzymatically intact, 30-μm-thick sections of abdominal aorta were incubated with DHE (10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide anion to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. For ethidium bromide detection, a 543 nm He-Ne laser (FV500 Laser Scanning Confocal Microscope; Olympus, Tokyo, Japan) combined with a 560 nm long-pass filter was used. Image Pro Plus software (Media Cybernetics, Inc., Silver Spring, MD, USA) was employed to analyze the images and to transform them into fluorescence intensity. For detecting the autofluorescence of the elastic fiber in media, a 488 nm argon laser combined with a 500- to 550-nm bandpass filter was simultaneously used. Elastic fiber fluoresced green [15].

#### *Vessel Activities of SOD and ALP*

Homogenates, 10% (w/v), of about 30 mg abdominal aortas (homogenized buffer: 20 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid [HEPES] containing 0.2% NP-40 and 20 mmol/L MgCl<sub>2</sub>) were prepared using a Polytron (Tsao Hsin Enterprise Co. Ltd., Shanghai, PR China) followed by centrifugation at 8,000 ×g for 10 minutes. The supernatant was used for determination of SOD and ALP activities. SOD activity was determined using the hydroxylamine reduction assay of Oyanatui [16]. In this method, the reduction of hydroxylamine by superoxide anion was monitored at 550 nm utilizing the hypoxanthine/xanthine oxidase system as the source for superoxide anion. One unit of SOD activity was defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. For ALP activity determination [17], the sample was mixed with reaction mixture, followed by incubation for 30 minutes at 37°C. The yellow color was determined at 405 nm, and ALP activity was calculated with U-

nitrophenol as the standard according to the manufacturer's instructions. One unit was defined as the activity producing 1 nmol/L of U-nitrophenol for 30 minutes. Protein concentrations were determined using a kit. SOD and ALP activities were normalized to the protein content.

#### *Vessel mRNA Expression of ALP*

Expression of ALP mRNA was assessed by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA of about 30 mg abdominal aorta was extracted following standard techniques. Isolated total tissue RNA was then quantified using an ultraviolet spectrophotometer (DU-640; Beckman, Fullerton, CA). Reverse transcription to cDNA was accomplished by priming 2 μg of total RNA samples with MMLV and oligo(dT)15 primer. The products were then used for the following PCR amplification: the PCR mixture was in a 25 μL volume containing 2.5 mM dNTP 1 μL, 10-PCR buffer (20 mM MgCl<sub>2</sub>, 500 mM KCl, 1.5 M Tris-HCl, pH 8.7), 2.5 μL cDNA, 200 nM of the appropriate rat ALP-paired primers, and 1.25 units of *Taq* DNA polymerase. Amplification was performed in the Perkin-Elmer (Foster City, CA) Gene Amp PCR System 9600 and consisted of 30 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds (terminal extension at 72°C for 7 minutes) after an initial denaturation step at 95°C for 10 minutes. The amplified products were visualized in a 1.5% agarose gel by staining with ethidium bromide under ultraviolet transillumination. OD was analyzed using the Molecular Analyst computer program (Bio-Rad, Vilbert Lourmat, France). The final results are expressed as the ratio of ALP PCR product to GAPDH PCR product for each sample.

#### *Vessel Morphological Observation*

The aortic arch was fixed in 10% formalin, followed by dehydration and embedding in paraffin. Sections, 6 μm thick, were cut and some of the slides were stained with hematoxylin and eosin (H&E). Other slides were deparaffinized and dehydrated before being immersed in light-protected 5% AgNO<sub>3</sub> for 30 minutes and then immersed in a solution of 5% sodium thiosulfate for 2 minutes, followed by counterstaining with eosin (von Kossa staining).

#### *Preparation of oxLDL*

Whole blood was obtained by venipuncture from healthy volunteers after 12 hours of fasting and processed for LDL separation within 1 day by sequential flotation in NaBr solution containing 1 mg/mL EDTA. Cu<sup>2+</sup>-modified LDL (1.0 mg of protein/mL) was prepared by exposure of LDL to 5 mM CuSO<sub>4</sub> for 18 hours at 37°C. The extent of LDL oxidation was determined by TBARS, and the TBARS content of ox-LDL used in our experiment was 8.6 vs. 0.56 nmoles/100 mg protein in the native LDL preparation. Protein was measured by the Comas protein assay reagent.

#### *RASMC Culture*

RASMCs were acquired following the method originally described by Ross [18] with modification. Briefly, the tunica media was isolated from the rat aorta. The tissue was fragmented into small pieces (1–2 mm<sup>3</sup>) and placed in a 10 cm culture dish containing DMEM with 4.5 g/L of glucose supplemented with 20% FBS and 10 mM sodium pyruvate to grow for several weeks in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. Cells that had migrated from explants were collected and maintained in growing medium. Immunocytochemical examination showed positive staining in all cells for α-smooth muscle actin.

### RASMC Calcification and Treatments

RASMC calcification was induced by the method of Wada et al. [19]. In short, after RASMCs grew confluent, they were seeded in DMEM containing 20% FBS and 10 mM sodium pyruvate in the presence of 5 mM GP (calcifying medium) alone or combined with different doses of oxLDL. The calcifying medium was refreshed every 2 days. Cells were grown in medium as follows: (1) cells of the Con group grew in normal medium; (2) cells of the calcifying group grew in calcifying medium; (3) cells of the oxLDL groups grew in calcifying medium supplemented with oxLDL (25, 50, 100 mg/L). After 10 days of incubation, the following measurements were performed.

**Quantitation of calcium deposition in RASMCs.** Calcification was detected following the method described by Jono et al. [20] with modification. Briefly, cultures were decalcified with 0.6 N HCl for 24 hours. The calcium content of the HCl supernatant was determined colorimetrically by the *o*-cresolphthalein complexone method. After decalcification, cultures were washed with PBS and solubilized in 0.1 N NaOH/0.1% sodium dodecyl sulfate. Total protein content was measured with a Comas protein assay. The calcium content of the cell layer was normalized to the protein content.

**Phosphorus and calcium ions in culture medium of RASMCs.** Concentrations of phosphorus and calcium in the culture medium were measured by the phosphomolybdate complex and the *o*-cresolphthalein complexone methods, respectively, following manufacturer's instruction.

**von Kossa staining for calcification.** von Kossa calcium staining of cultured RASMCs was performed using the conventional method. Briefly, cell monolayers were fixed in 0.1% glutaraldehyde for 15 minutes at room temperature. Cells were then washed twice with ddH<sub>2</sub>O and incubated with 5% silver nitrate for 30 minutes at room temperature in the dark. Silver nitrate was removed, and the cells were rinsed twice with ddH<sub>2</sub>O. After being dried by air, cultures were exposed to sunlight until color development was complete.

**ALP activity in RASMCs.** RASMCs were rinsed three times with cold PBS and scraped into 200  $\mu$ L of lysis buffer (0.2% NP-40 in 1 mM MgCl<sub>2</sub>). The cells were sonicated for 10 seconds and then centrifuged. Supernatants of the cells were collected for determination of ALP activity and protein concentration using a kit as above. The data were normalized to the protein content.

**Expression of ALP mRNA in RASMCs.** Isolated cellular RNA was used for RT-PCR as mentioned above. The final results are expressed as the ratio of ALP PCR product to GAPDH PCR product for each sample.

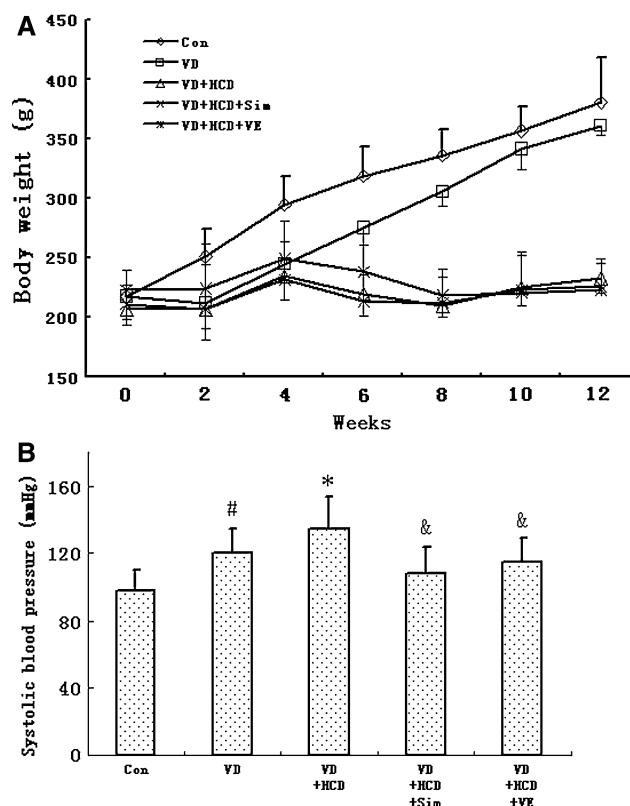
### Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation (SD) and were compared by Student's *t*-test. A significant difference was considered as  $P < 0.05$ .

## Results

### Body Weight and Systolic Blood Pressure

The body weights of rats aged 6 weeks showed no differences among the five groups at the beginning of the

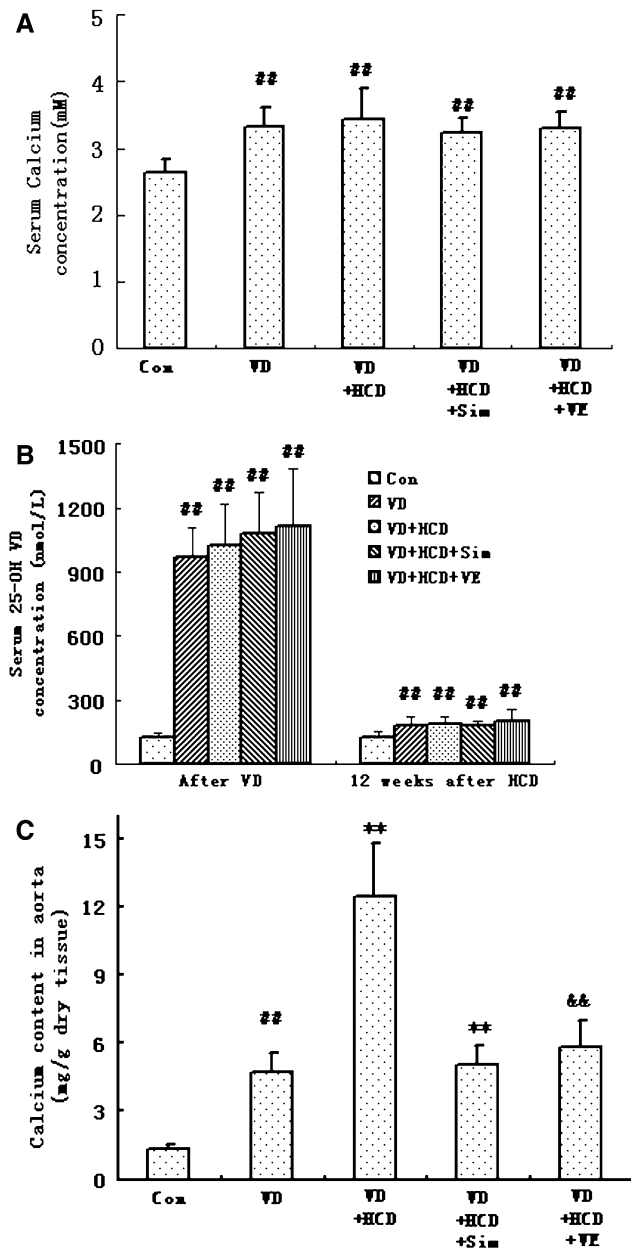


**Fig. 1.** Body weight (A) and blood pressure (B) of rats. Rats were treated and body weight and blood pressure were monitored as described in Materials and Methods. Mean  $\pm$  SD,  $n = 8$ . <sup>#</sup> $P < 0.05$  compared with Con group, <sup>\*</sup> $P < 0.05$  compared with VD group, <sup>&</sup> $P < 0.05$  compared with VD+HCD group.

experiment. Rats in the Con group grew gradually, with body weight of about 380 g at the end of 12 weeks. VD alone-treated rats experienced a slight reduction in body weight in the first 2 weeks due to VD toxicity-induced reduction of food intake, followed by fast growing in the rest of the experimental period. There was no difference between VD-treated rats and rats in the Con group at the end of the experiment. The body weight of rats in the other three groups was similar to that of VD-treated rats in the first 4 weeks, and then it remained stable because of poor appetite caused by HCD (Fig. 1A). Rats in all five groups had similar levels of blood glucose (data not shown). Systolic blood pressures in VD-treated rats was higher than that in the Con group. Rats cotreated by VD and HCD exhibited a much higher blood pressure than those in the VD group, implying a dysfunction of vessel contraction and relaxation. Sim and VE significantly reduced systolic blood pressure, suggesting an improvement of vascular motor activity (Fig. 1B).

### Calcium Contents in Serum and Thoracic Aorta and Serum 25-OHD Level

Compared with the Con group, serum calcium contents in the other four groups were significantly higher,



**Fig. 2.** Serum levels of calcium (A) and 25-OHD (B) and calcium content in thoracic aorta (C). Rats were treated and calcium levels in serum and thoracic aorta were measured as described in Materials and Methods. Mean  $\pm$  SD,  $n = 8$ . <sup>##</sup> $P < 0.01$  compared with Con group, <sup>\*\*</sup> $P < 0.01$  compared with VD group, <sup>&&</sup> $P < 0.01$  compared with VD+HCD group.

suggesting that VD-treated rats had hypercalcemia. However, no differences in calcium content were found among the four groups (Fig. 2A). 25-OHD, the active form of VD after being given orally, is responsible for calcium absorption from the intestinal track. At the end of VD oral administration, serum levels of 25-OHD in VD-treated rats were about eight to nine times those in control rats and no significance was found among the four VD-treated groups. At the end of the experiment,

serum levels of 25-OHD in VD alone-treated rats were about 1.5 times those in control rats. VD+HCD, HCD+Sim, and HCD+VE had no effects on serum 25-OHD production compared with VD alone treatment (Fig. 2B). Vessel calcium content represents the degree of calcification. The calcium content of thoracic aorta in the VD and HCD+VD groups increased to about 3.6- and 9.6-fold that of the Con group, respectively, suggesting excessive VD initiated vascular calcification and HCD strongly accelerated VD-induced calcification. Sim and VE significantly reduced calcium deposition in the aorta by 59% and 53%, respectively, without affecting serum levels of calcium and 25-OHD (Fig. 2C).

#### *Histopathological Observation by H&E and von Kossa Staining*

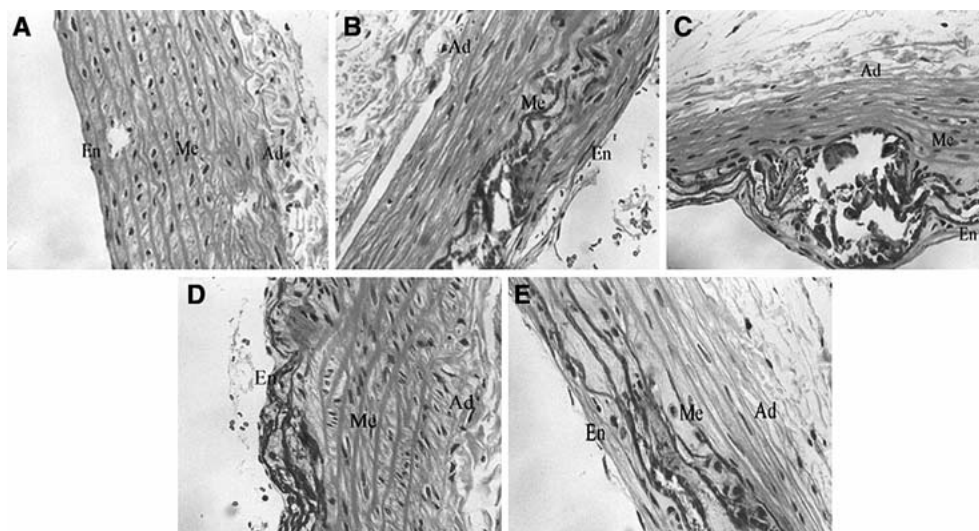
H&E staining of aortic arch calcification represented by blue color showed that no pathological changes were found in rats of the Con group (Fig. 3A). While rats treated with VD alone developed mild or moderate aortic calcification which was characterized by light blue color mainly in media, disorders or disruption of the elastic fiber was found (Fig. 3B). This calcification was further enhanced by combination of VD with HCD, and severe atherosclerotic calcification occurred predominantly in both the intimal area adjacent to the media and in the media, represented by dark blue color. Much more severe disorders or disruptions of elastic fiber and lipid deposition were seen under the intimal layer (Fig. 3C). Sim and VE could significantly decrease the aortic atherosclerotic calcification (Fig. 3D and E), protect elastic fiber from fragmentation, and reduce lipid deposition. von Kossa staining, represented by yellow color, exhibited similar results to H&E staining (Fig. 4).

#### *Autofluorescence of the Elastic Fiber*

The autofluorescence of elastic fiber by laser confocal assay clearly demonstrated that the elastic fiber in normal vessel lined up in order (Fig. 5A). VD-treated rats showed moderate disorders or fragmentations of elastic fiber (Fig. 5B), while these changes were aggravated by cotreatment with VD and HCD (Fig. 5C). Sim and VE treatment significantly improved these disorders and protected elastic fiber from disruptions (Fig. 5D and E). The results matched those observed by H&E staining.

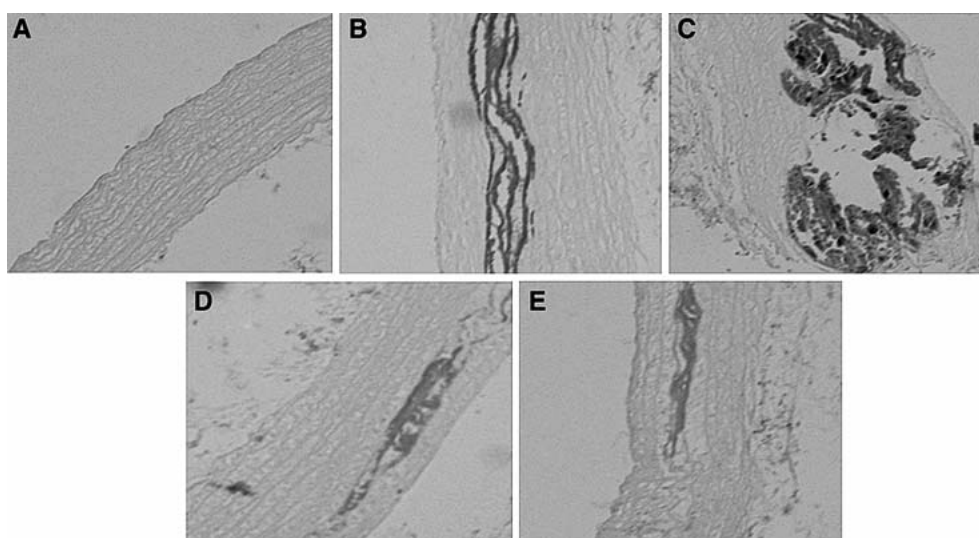
#### *Lipids in Serum and Thoracic Aorta*

No significant differences in the serum concentrations of TC, LDL-C, and HDL-C were detected between the VD and Con groups, suggesting that VD alone treatment did not affect serum lipid. However, TC and LDL-C in the VD+HCD group increased by 5.5- and 28-fold, respectively, while HDL-C decreased by 46% compared



**Fig. 3.** H&E staining for calcium deposition in aortic arch. Rats were treated and calcium deposition was assessed by H&E staining as described in Materials and Methods. Blue color indicates calcium deposition. (A) Con group, no calcium deposition was present and elastic fiber lined up in order. (B) VD group, mild to moderate calcium deposition represented by light blue color, and disorders or disruptions of elastic fiber

are seen. (C) VD+HCD group, severe calcium deposition represented by dark blue color, much more severe disorders or disruptions of elastic fiber and lipid deposition under intimal layer. (D, E) VD+HCD+Sim and VD+HCD+VE groups, calcium deposition was reduced and elastic fiber was improved. En, endothelia; Me, media; Ad, adventitia. Original magnification  $\times 200$ .



**Fig. 4.** von Kossa staining for calcium deposition in aortic arch. Rats were treated and calcium deposition was assessed by von Kossa staining as described in Materials and Methods. Yellow color indicates calcium deposition. (A) Con group, no calcium deposition was present. (B) VD group, mild to mod-

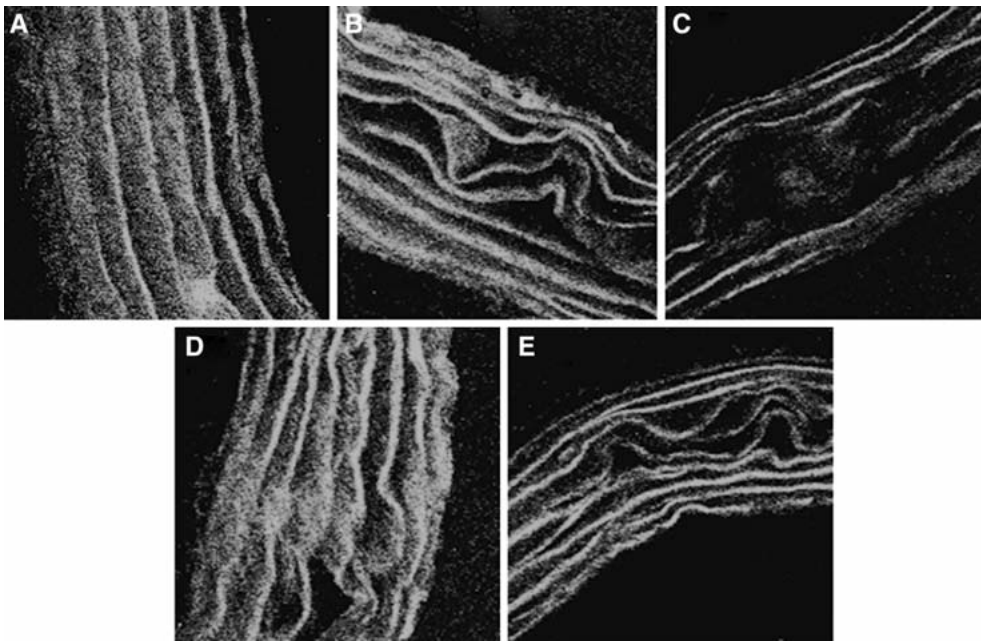
erate calcium deposition represented by light yellow color. (C) VD+HCD group, severe calcium deposition represented by dark yellow color. (D, E) VD+HCD+Sim and VD+HCD+VE groups, reduced calcium deposition. Original magnification  $\times 100$ .

with the Con group, implying that HCD induced hypercholesterolemia in rats. TC and LDL-C decreased while HDL-C increased in the VD+HCD+Sim group compared with the VD+HCD group. VE had no effect on serum lipid (Fig. 6A). Vascular lipid, especially CE content, represented the formation of foam cells and degree of atherosclerosis. TC and CE levels of thoracic aorta in the VD+HCD group were significantly higher than those in the Con or VD group. Both Sim and VE

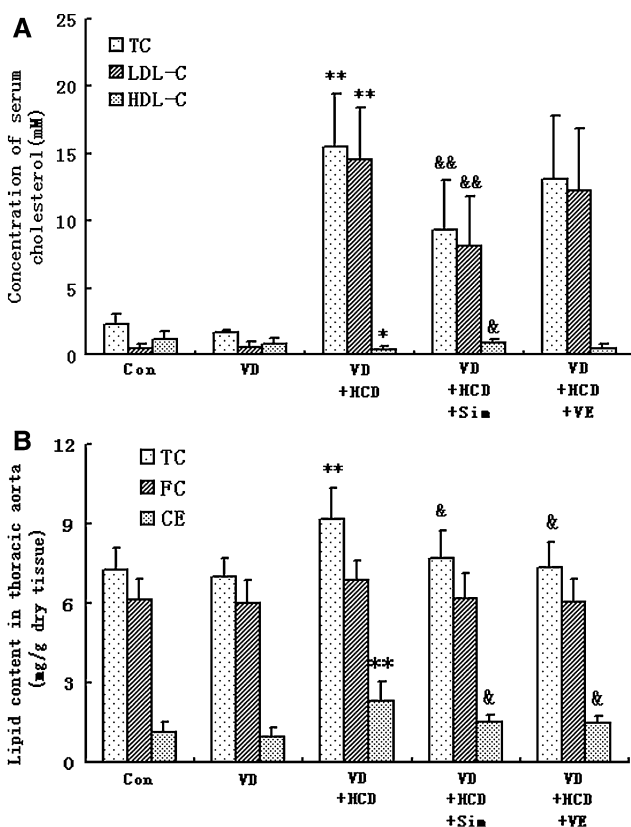
treatments significantly reduced TC and CE content compared with VD+HCD treatment. FC content was similar in the five groups (Fig. 6B).

#### *Serum Contents of oxLDL and MDA*

Because oxLDL is mainly formed by oxidized modification of LDL under the vessel endothelium and releases to blood, the serum oxLDL concentration indirectly



**Fig. 5.** Autofluorescence of elastic fibers determined using laser confocal assay. Rats were treated and autofluorescence of elastic fiber was detected as described in Materials and Methods. Green color represents the elastic fiber. (A) Con group, elastic fiber lined up in normal order. (B) VD group, mild to moderate disorders or disruptions of elastic fiber. (C) VD+HCD group, severe disorders or disruptions of elastic fiber. (D, E) VD+HCD+Sim and VD+HCD+VE groups, improved disorders of elastic fiber. Original magnification  $\times 200$ .



**Fig. 6.** Lipid levels in serum (A) and thoracic aorta (B). Rats were treated and lipid levels in serum and thoracic aorta measured as described in Materials and Methods. Mean  $\pm$  SD,  $n = 8$ . \*\* $P < 0.01$  compared with VD group, & $P < 0.05$  compared with VD+HCD group.

represents its production in vessels. Serum MDA content partly reflects the amount of serum oxLDL. The results showed that levels of oxLDL and MDA in the

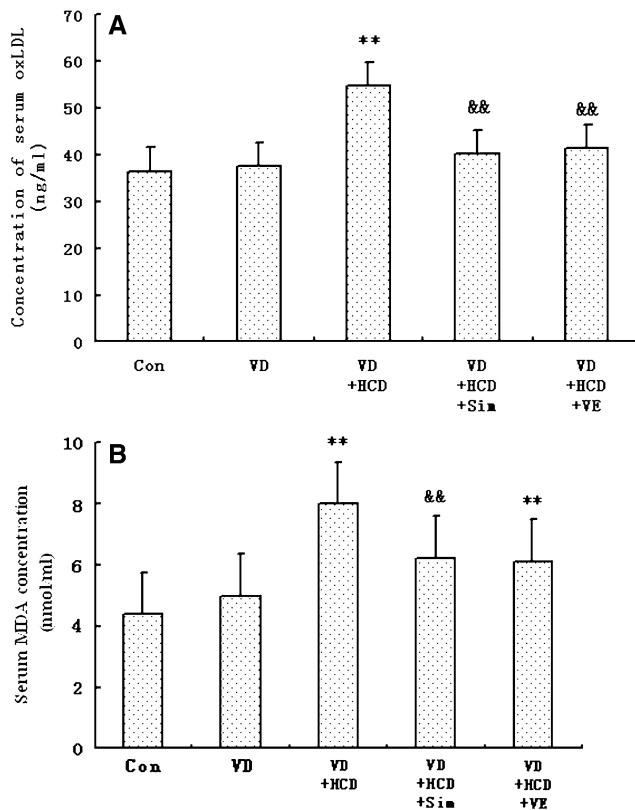
VD group were comparable with those in the Con group. However, oxLDL and MDA increased by 46% and 48%, respectively, in the VD+HCD group compared with the VD group, suggesting increased production of oxLDL under vessel endothelia. Sim and VE treatments decreased oxLDL content, leading to a decreased MDA content compared with VD+HCD treatment (Fig. 7).

#### Superoxide Anion Production and SOD Activity in Abdominal Aorta

Superoxide anion derived from various vessel cells is the main source for the oxidation of LDL into oxLDL, and SOD is the specific scavenger of superoxide anion. The results indicated that the production of superoxide anion, represented by lightness of red color (Fig. 8A–E) and expressed as fluorescence intensity (Fig. 8F), as well as SOD activity (Fig. 8G) in abdominal aorta was comparable between the Con and VD groups. However, the production of superoxide anion in aorta significantly increased while SOD activity decreased in the VD+HCD group compared with the VD group, suggesting an imbalance between vessel oxidation and antioxidation states, which was prone to LDL oxidation. Both Sim and VE treatments remarkably increased SOD activity, leading to a decreased superoxide anion production.

#### ALP Activity and mRNA Expression in Abdominal Aorta

ALP, a marker of osteogenic differentiation of osteoblastic cells, is able to reflect the degree of vessel calcification. ALP activity and its mRNA expression in



**Fig. 7.** Serum levels of oxLDL (A) and MDA (B). Rats were treated and serum levels of oxLDL and MDA measured as described in Materials and Methods. Mean  $\pm$  SD,  $n = 8$ . \*\* $P < 0.01$  compared with VD group, && $P < 0.01$  compared with VD +HCD group.

abdominal aorta in the VD group were upregulated by 103% and 57%, respectively, compared with the Con group. This upregulation was strongly enhanced by cotreatment with HCD by 85% and 109%, respectively, compared with VD alone treatment. Sim and VE treatments downregulated significantly both the activity and mRNA expression of ALP (Fig. 9).

#### RASMC Calcification Assayed by von Kossa Staining

Calcium mineral deposits formed in cultured RASMCs were assessed by the silver nitrate method (von Kossa staining). The results showed that RASMCs grown in growth medium showed normal morphology typical for these cells without any calcified area (Fig. 10A). Incubation of RASMCs in calcifying medium alone for 10 days induced calcium deposition (Fig. 10B), which was further enhanced by addition of oxLDL (100 mg/L) (Fig. 10C).

#### Calcium Content in RASMC Cultures and Concentrations of Calcium and Phosphate in Culture Medium

Calcium content in RASMC cultures increased significantly after treatment with calcifying medium

containing GP alone. Correspondingly, calcium concentration in culture medium significantly decreased, while phosphate concentration increased, suggesting that calcium deposition occurred in RASMCs. This calcium deposition was concentration-dependent, which was enhanced by addition of oxLDL. oxLDL further decreased calcium concentration in culture medium and increased phosphate concentration in a concentration-dependent manner (Fig. 11).

#### ALP Activity and mRNA Expression in RASMCs

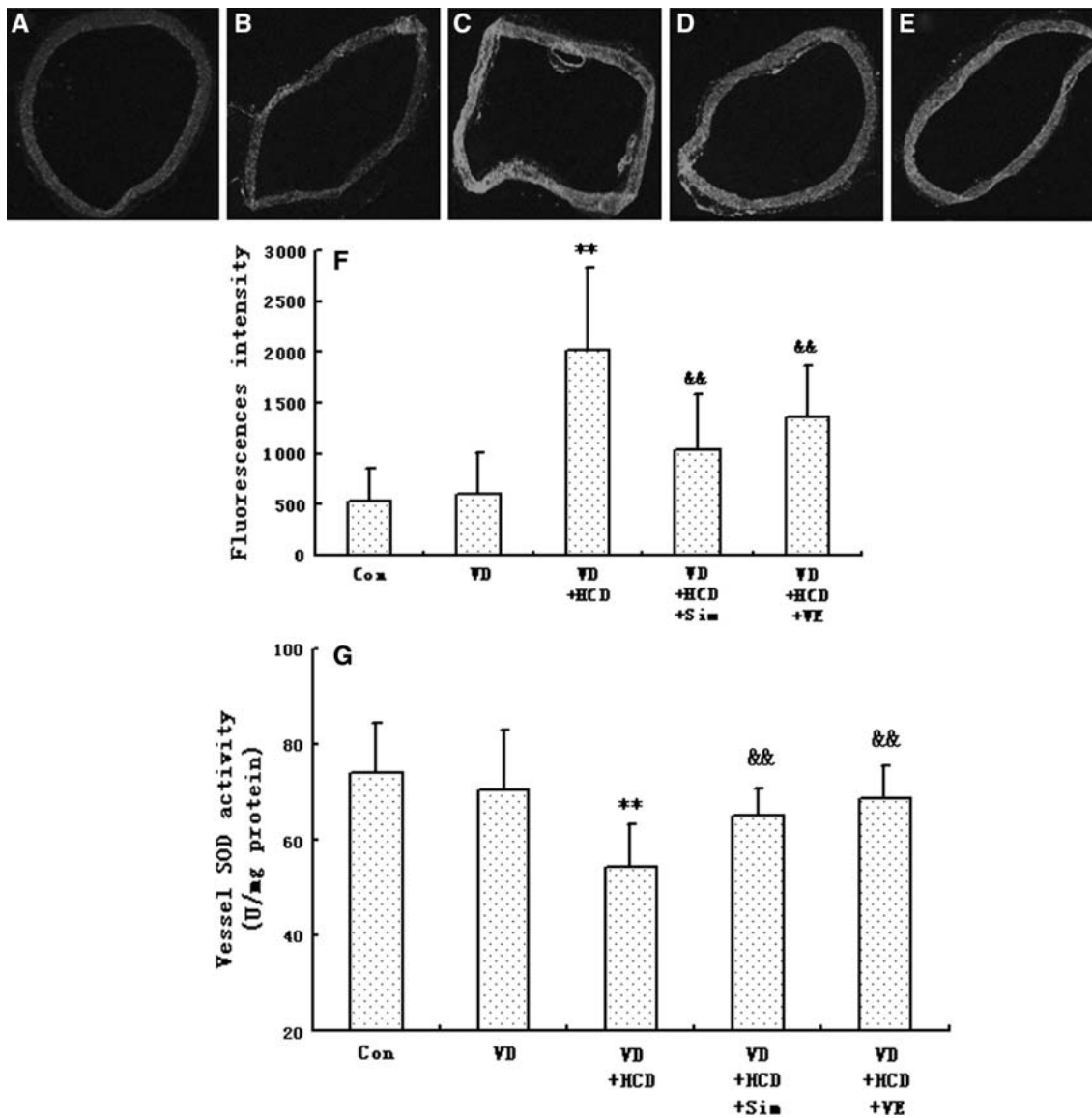
ALP activity and mRNA expression in RASMCs in the GP group were significantly upregulated compared with the Con group. This upregulation was concentration-dependent and enhanced by cotreatment with oxLDL compared to GP alone (Fig. 12).

#### Discussion

Vascular calcification is a common feature of atherosclerotic lesions. In the past, it was considered a passive, degenerative process of aging and presumed to be unregulated, inevitable, and untreatable. In recent years, vascular calcification has been regarded as an actively regulated, cell-mediated process that shares several features with skeletal bone formation at the cellular and molecular levels. Evidence for this hypothesis includes the presence of osteoblast-like calcifying vascular cells in the artery wall that undergo osteoblastic differentiation and calcification *in vitro* [21]. Pathological examinations reveal the colocalization of vascular calcification with atherosclerotic lesions in general, which is termed *atherosclerotic calcification*. The close link between atherosclerotic lesion and vascular calcification suggests that atherogenic lipids may regulate vascular cell differentiation and mineralization [22]. Nevertheless, the underlying mechanism remains unclear. Hypercholesterolemia is a major risk factor for atherosclerosis, which largely depends on the production of oxLDL derived from oxidized modification of LDL, the main carrier of cholesterol. A large body of evidence shows that oxLDL is involved in the very early yet critical steps of atherogenesis, such as endothelial injury, expression of adhesion molecules, and leukocyte recruitment and retention, as well as foam cell and thrombus formation [23–25]. Studies *in vitro* demonstrated that oxLDL could promote vascular calcification [11]. Whether oxLDL is a bridge *in vivo* to link hypercholesterolemia and vascular calcification and the involved mechanism needs to be elucidated.

The present study showed that VD alone-treated rats developed moderate and localized vascular calcium deposition, while rats treated with VD +HCD



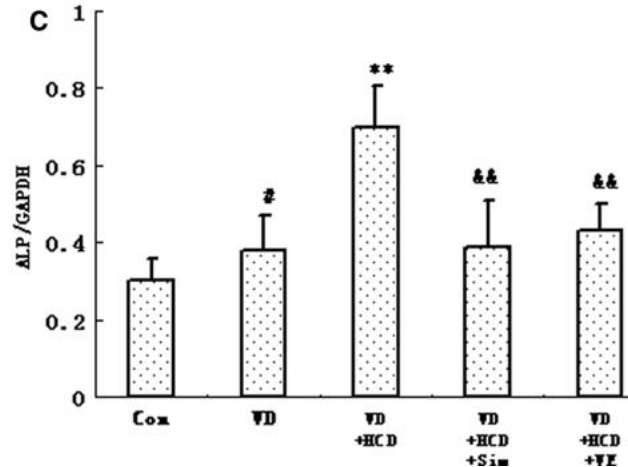
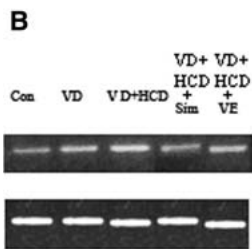
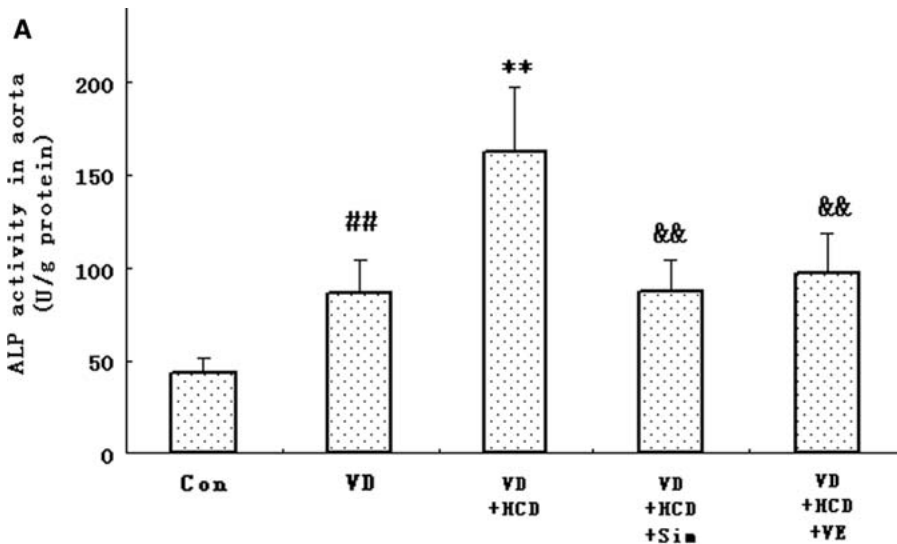


**Fig. 8.** Superoxide anion production represented by laser confocal microscope photos (A–E). Fluorescence intensity was calculated by Image Pro Plus software (F) and SOD activity (G) in abdominal aorta. Rats were treated and superoxide anion production and SOD activity in abdominal aorta detected as described in Materials and Methods. (A) Con

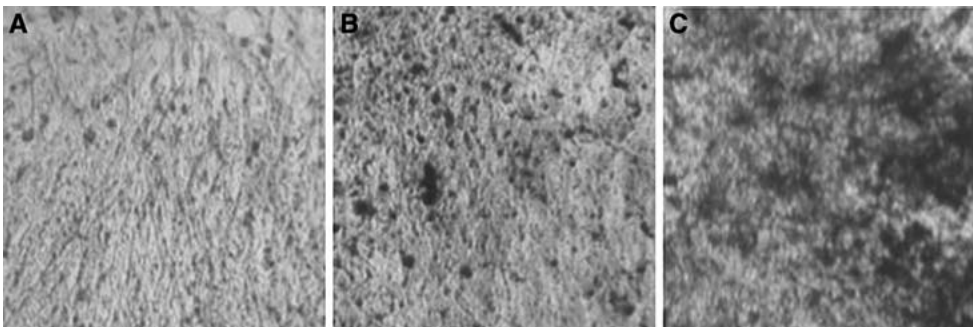
group. (B) VD group. (C) VD+HCD group. (D) VD + HCD + Sim group. (E) VD + HCD + VE group. Original magnification  $\times 50$ . Mean  $\pm$  SD,  $n = 8$ . \*\* $P < 0.01$  compared with VD group, && $P < 0.01$  compared with VD+HCD group.

developed both hypercholesterolemia and extended severe calcium deposition in vessels. Sim, a cholesterol-lowering agent, significantly reduced calcium deposition in vessels. The results implied that hypercholesterolemia might accelerate vascular calcification initiated by VD. The results of both H&E and von Kossa staining indicated that calcium deposition was mild to moderate and localized only in the media of the vessel in VD-treated rats. However, the calcium deposition was severe and extended to the intimal area adjacent to the media of the vessel in rats cotreated with HCD and VD. In addition, lipid deposition represented by foam cells under

endothelium coexisted with calcium deposition in cotreated rats, suggesting a close relationship between lipid deposition and vascular calcification. Autofluorescence of elastic fiber assayed by laser confocal microscopy showed a mild to moderate disorder or disruption in VD-treated rats and greater severity in cotreated rats, indicating a dysfunction of vascular tone due to the calcification. Cotreatment with VD and HCD increased vessel calcium content, vascular activity, and mRNA expression of ALP, two markers of calcification, compared with VD treatment. Meanwhile, vessel calcium content increased in parallel with vessel CE in cotreated



**Fig. 9.** ALP activity (A) and mRNA expression (B, C). Rats were treated and activity and mRNA expression of ALP measured as described in Materials and Methods. (A) ALP activity. (B) Representative electrophoresis of ALP mRNA. (C) Analyzed ALP mRNA expression. Mean  $\pm$  SD,  $n = 8$ . # $P < 0.05$  compared with Con group, \*\* $P < 0.01$  compared with VD group, && $P < 0.01$  compared with VD+HCD group.

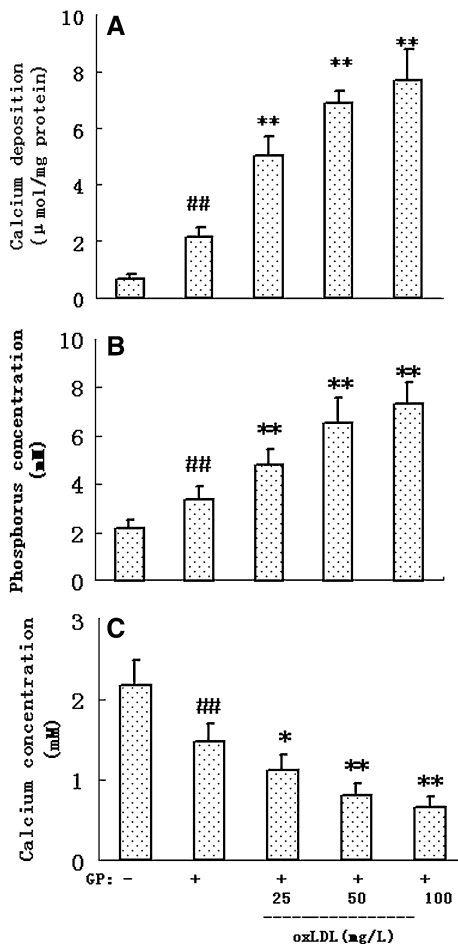


**Fig. 10.** von Kossa staining for RASM cell calcification. Cells were treated and von Kossa staining was performed as described in Materials and Methods. Black represents staining of calcified areas. (A) Cells cultured in growth medium showed no

calcified areas. (B) Cells cultured in calcification medium showed mild to moderate calcified areas. (C) Cells cultured in calcification medium combined with oxLDL (100 mg/L) showed severe calcified areas. Original magnification  $\times 100$ .

rats, further supporting the above possibilities. Sim inverted the changes of the above parameters, resulting in alleviation of calcium deposition, simultaneous reduction of both calcium and CE content in vessel, and downregulation of the activity and mRNA expression of ALP. The beneficial effect of Sim on patients with

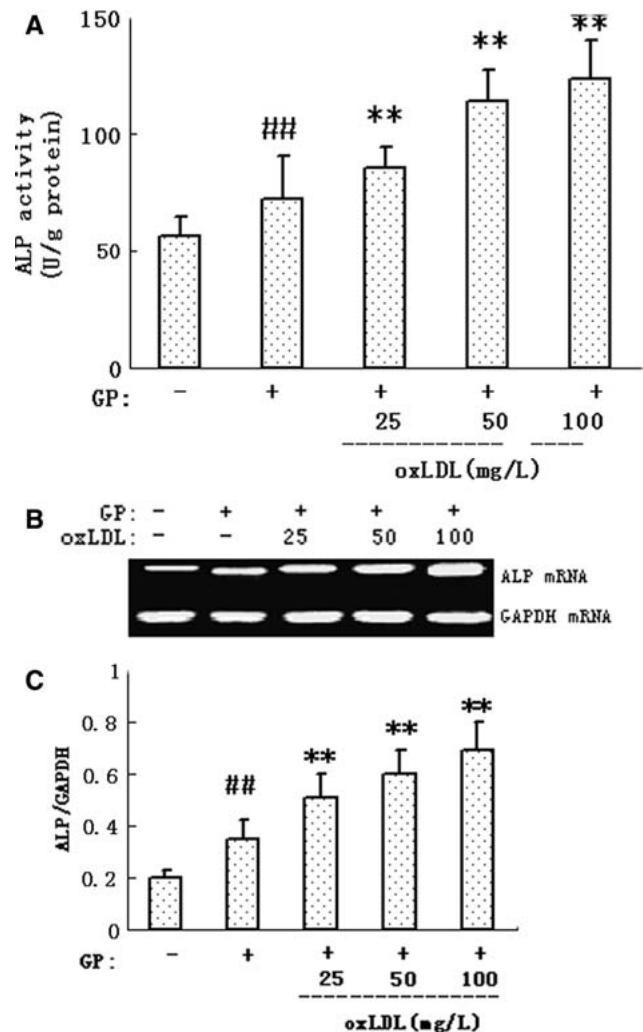
calcified cardiovascular diseases could be at least partly explained by its reduction of vascular calcification. The present results are consistent with those of Kitagawa et al. [13], in which VD alone induced vascular calcification in rats and VD combined with HCD aggravated this calcification. Taken together, the present results suggest



**Fig. 11.** Calcium deposition in the cell layer (A) and medium phosphorus (B) and calcium (C) concentrations in cultured RASMCs. Cells were treated and calcium and phosphorus measured as described in Materials and Methods. Mean  $\pm$  SD,  $n = 6$ . <sup>##</sup> $P < 0.01$  compared with growth medium group, <sup>\*\*</sup> $P < 0.05$  compared with GP group.

that both VD and HCD are involved in vascular calcification and HCD accelerates the calcification.

Administration of excessive VD to rats could enhance intestinal calcium absorption and bone resorption, leading to hypercalcemia [26, 27]. The present study showed that the calcium deposit in the aorta was accompanied by a rise of serum calcium level, suggesting a causal relationship between hypercalcemia and aortic calcification. However, serum calcium levels in all VD-treated rats were identical, although HCD significantly increased aortic calcium content compared with VD alone. Sim and VE treatment decreased the levels of serum calcium compared with the combination of VD and HCD, suggesting that serum calcium level could not explain these effects and that other factors might be responsible. This was consistent with the report by Hass et al. [28], which showed that there was no persistent rise in serum calcium level in VD-treated rats, although the rats developed more and more severe arterial calcification. Porta et al. [29] found in the rat model treated with



**Fig. 12.** ALP activity (A) and mRNA expression (B, C) in RASMCs. Cells were treated and ALP activity and mRNA expression assayed as described in Materials and Methods. Mean  $\pm$  SD,  $n = 6$ . <sup>##</sup> $P < 0.01$  compared with growth medium group, <sup>\*\*</sup> $P < 0.01$  compared with GP group.

VD that the calcium antagonist nifedipine, rather than defibrotide, could reduce serum calcium, although both remarkably prevented aortic calcinosis. In addition, age-dependent aortic calcification in humans is not related to changes in blood calcium levels. The present study and other reports suggest a poor correlation between serum and tissue calcium concentrations and that other factors should be responsible for vessel calcification. Serum 25-OHD, the active form of VD when orally given, is a major regulator of intestinal calcium absorption. The present results showed that VD alone-treated rats demonstrated significantly higher serum 25-OHD levels than control rats both at the end of VD administration and at the end of the experiment. However, combination of VD+HCD, HCD+Sim, or HCD+VE did not influence serum levels of 25-OHD compared with VD alone treatment, suggesting that the accelerating action

of HCD on vascular calcification and the beneficial effects on vascular calcification of both Sim and VE might be independent of circulating levels of 25-OHD.

To further investigate the mechanism by which hypercholesterolemia accelerated vascular calcification, serum levels of oxLDL, MDA, and lipids and vessel levels of SOD and superoxide anion were evaluated. Because oxLDL is mainly formed by oxidized modification of LDL, the main carrier of cholesterol, under the vessel intima and releases to blood, serum oxLDL concentration indirectly represents its production in vessels. Meanwhile, serum MDA content partly reflects the amount of serum oxLDL. The present study showed that cotreatment of VD and HCD rather than VD alone increased serum oxLDL concentration, suggesting that oxLDL was induced by hypercholesterolemia instead of VD. However, VD might play an important role in oxLDL formation because calcium overload induced by excessive VD increased permeability of vessel intima, leading to accumulation of LDL with high blood concentration underneath the intima, where LDL is oxidized to oxLDL. Sim was shown to lower serum oxLDL levels, partly depending on its LDL-lowering action. VE, an antioxidant, was also shown to reduce serum oxLDL level without affecting serum lipid files, suggesting that VE inhibited oxLDL formation by a different mechanism from Sim. Changes of serum MDA content were consistent with changes of oxLDL level under different treatments, giving further evidence for the formation and change of oxLDL. More and more reports support the hypothesis that the modified forms of LDL are involved in atherosclerotic calcification. Proudfoot et al. [1] found that *in situ* hybridization of human atherosclerotic plaques demonstrated that subsets of lipid-filled VSMCs adjacent to sites of calcification expressed ALP, bone Gla protein, and bone sialoprotein, suggesting an osteogenic phenotype. They also found that treatment of cultured VSMCs with acetylated LDL increased nodule calcification by threefold, whereas lipoprotein-deficient serum significantly inhibited nodule calcification. These findings are further supported by other studies in which minimally oxLDL and isoprostanes stimulated ALP activity, which is known to be a key regulator/initiator of matrix vesicle calcification via generation of local phosphate ions, in calcifying vascular cells [11–12, 22]. There are several potential mechanisms by which oxLDL could enhance calcification. It has been postulated that oxLDL might mimic the effect of VD in the vessel wall or that oxLDL might act as a transport vehicle to deliver VD to the vessel wall [30]. Another possibility is that oxLDL could produce a functional vitamin K deficiency in the arterial wall, leading to inactivation of matrix Gla protein, an inhibitor of bone morphology protein that could enhance vessel calcification [31, 32].

As far as this model was concerned, vessel oxidative stress might be responsible for the increase of oxLDL. LDL is oxidized underneath the vessel intima mainly by superoxide anion produced in various cells of the vessel. SOD is the specific scavenger of superoxide anion. The present results showed that cotreatment of VD and HCD rather than VD alone decreased vessel activity of SOD and increased the vessel production of superoxide anion, which favors LDL oxidation in the vessel. Both Sim and VE increased SOD activity and reduced superoxide anion production, leading to less oxLDL formation. Studies have shown that the degree of LDL oxidation is directly proportional to the rate of superoxide production [33]. Several studies have shown that an increase in SOD activity is capable of preventing LDL oxidation in cultured cells. Heinecke et al. [34] reported that addition of SOD to the culture medium inhibits the LDL oxidation induced by human and monkey smooth muscle cells (SMCs). Steinbrecher [35] reported that SOD attenuates LDL oxidation in rabbit endothelial cells and cultured SMCs. Guo et al. [36] indicated that overexpression of Cu/Zn SOD reduced cell-mediated LDL oxidation.

To verify the role of oxLDL in vascular calcification, calcified RASMCs induced by GP were incubated with different concentrations of oxLDL. von Kossa staining showed that oxLDL significantly aggravated RASMC calcification. Treatment of RASMCs with oxLDL enhanced calcium deposition initiated by GP and phosphorus concentration in a concentration-dependent manner and decreased calcium concentration in culture medium. This is most likely due to ALP, the activity and mRNA expression of which were also upregulated by oxLDL, by means of liberating inorganic phosphate from its substrate, GP, to raise the local concentration of phosphorus. The combination of phosphorus and calcium stimulated the formation of calcified nodules in cells, leading to decreased calcium level in culture medium [19]. These results are consistent with the explanation of the bridge role of oxLDL in linking hypercholesterolemia and atherosclerotic calcification.

In summary, the present study has demonstrated that hypercholesterolemia enhances the vascular calcification induced by excessive VD in rats and oxLDL might play a pivotal role in linking hypercholesterolemia and atherosclerotic calcification. This hypothesis is further supported by *in vitro* results showing that oxLDL enhances RASMC calcification initiated by GP. These results indicate a potential therapeutic role for agents such as statins, which reduce LDL, and antioxidants, which inhibit the oxidation of LDL.

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