

Anti-aging Activity of *Aralia Cordata Thunb.* by Inhibiting Oxidized Low-density Lipoprotein Production in Rats

Min Kyung Hyun, Ji Cheon Jeong*

Department of Internal Medicine, College of Oriental Medicine, Dongguk University

Aralia cordata Thunb. (Araliaceae, ACT) is an remarkable herbal plant that has been widely used in traditional oriental medicine for the treatment of inflammatory diseases and cardiovascular disorders. In this study, we have established a vascular aging model in rats by orally administrating excessive vitamin D₂ (500,000 IU/kg/day) for 4 days followed by feeding high cholesterol diet for 16 weeks and then rats were randomly divided into control group, high cholesterol diet (HCD) group, HCD+ACT (30 mg/kg) and HCD+ACT (60 mg/kg) group. ACT (30, 60) significantly reduced total cholesterol (TC) content compared with HCD, but no significant differences in the serum lipids. Secondly, we measured the serum levels of Oxidized Low-density Lipoprotein (OxLDL) and malondialdehyde (MDA) in order to further investigate the anti-vascular aging mechanism of ACT. The results, ACT (30, 60) treatments decreased OxLDL, MDA content and increased Cu/Zn superoxide dismutase activity compared with HCD treatments. The results suggested that ACT inhibited OxLDL production rather than serum lipids lowering and that ACT could be used as potential anti-atherosclerotic agent in aged cells.

Key words : *Aralia cordata Thunb.*, Oxidized Low-density Lipoprotein, malondialdehyde, Cu/Zn superoxide dismutase, Cytotoxicity

Introduction

Aralia cordata Thunb. (Araliaceae, ACT) is an remarkable herbal plant that has been widely used in traditional oriental medicine for the treatment of inflammatory diseases and cardiovascular disorders in China, Japan, Taiwan and Korea¹. It is known that its nonpolar extracts inhibit platelet aggregation and protect myocardium against ischemia-induced derangement.

In recent study, ACT has also been confirmed as an anti-inflammatory agent². Since then, several diterpenes, polyacetylenes, lipid glycerol, and sterols have been isolated from the methyl chloride fraction of the root of ACT. These compounds selectively inhibited cyclooxygenase (COX)-1 and COX-2 activities. In addition, the compounds inhibited COX-1 dependent conversion of the exogenous arachidonic acid to Prostaglandin E₂ (PGE₂) and weakly inhibited COX-2 dependent PGE₂ generation. These compounds were significantly effective regarding analgesics, hypothermia³.

Vascular aging is the major source of morbidity and

mortality and claims more lives than all types of cancer combined, plus the economic costs are considerable. vascular aging is characterized by the accumulation of cholesterol deposits within macrophages in large- and medium-sized arteries, as well as the vascular calcification and proliferation of vascular smooth muscle cells⁴. Oxidized Low-density Lipoprotein (OxLDL), the oxidized form of low-density lipoprotein (LDL), which is the main carriers of cholesterol, is presumed to play a pivotal role in initiation and progression of vascular aging⁵. It was indicated that enhanced serum levels of OxLDL are strong prediction of coronary heart disease closely linked to atherosclerosis^{6,7}.

In this study, we investigated the in vivo effect of ACT on vascular aging in rat model induced by excessive vitamin D₂ and high cholesterol diet.

Materials and Methods

1. Reagents

Kits for the determination of total cholesterol (TC), low-density lipoprotein cholesterol(LDL-C), high-density lipoprotein cholesterol (HDL-C) and calcium ion were purchased from YD Diagnostics. Kits for the detection of Cu/Zn superoxide dismutase (Cu/Zn SOD), malondialdehyde

* To whom correspondence should be addressed at : Ji Cheon Jeong,
Department of Internal Medicine, Gyeongju Korean Hospital of Dongguk
University, 1090-1, Seokjang-dong, Gyeongju-si, Gyeongbuk, Korea

· E-mail : jjcjh@paran.com, · Tel : 054-770-1265

· Received : 2007/08/23 · Accepted : 2007/09/19

(MDA) and protein were produced by Sigma Co. (USA). An Elisa kit for detection of OxLDL was the products of BPB Biomedicals, Inc. (USA). Dihydro-ethidium bromide (DHE), vitamin D₂, tetraethoxypropane (TEP) were purchased from Sigma. Other chemicals and reagents were of analytical grade.

2. Process for preparation of ACT extracts

The plant was collected in Kyungju city, Korea, and sample and voucher are kept in the herbarium of the College of Oorean Medicine, Dongguk University. The water extracts of ACT were prepared immediately before use by boiling 10 g of dried leaves in 50 ml ultrapure water for 15 min. The boiled materials were filtered and boiled until the final volume was equal to 1 ml. The plant samples were extracted 3 times with methanol at 70°C for 5 h. The extracts were filtered through a 0.45 µm filter and lyophilized. The w/w yield of extracts was about 5.5%. The methanol extract (50 g) was suspended in water (500 ml) and successively reextracted by 500 ml each of ethanol (yield: 3.5%; 1.75 g), methyl chloride (yield: 30.8%, 15.4 g) and ethyl acetate (yield: 23.2%, 11.6 g) and butanol (yield: 7.5%, 3.8 g) each 3 times. All fractions including the final remaining water fraction (yield: 9.0 g) were concentrated under reduced pressure using a rotary evaporator and then freeze dried. For the bioassay test, samples were dissolved in dimethylsulfoxide (DMSO) and further diluted in culture media.

3. Animal groups and rat model of vascular aging

Male Sprague - Dawley rats (220±20 g) aged 3 weeks were purchased from the Korea Experimental Animal Co. (Seoul, Korea). The rats were housed under standard conditions (room temperature 22±1°C, humidity 60±10%, lights from 6 am to 6 pm) and given water freely. All experimental procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, Korea NIH (1999). Rats were randomly divided into control (Con) group, high cholesterol diet(HCD) group, HCD+ACT (30 mg/kg) and HCD+ACT (60 mg/kg) group, each containing 10 animals. HCD is composed of standard chow (92.2%), cholesterol (3%), lard (3%), cholic acid (0.5%), propylthiouracil (0.3%). The preparation for rat model of vascular aging was as described by Park et al⁸⁾. with some modifications. In short, except for the rats in Con group, all rats were orally administered with 500,000 IU/kg/day vitamin D₂ for 4 consecutive days followed by HCD in the HCD group or the HCD combined with orally administration of ACT (30) in the HCD+ACT (30) group or ACT (60) in the HCD+ACT (60) group for 16 weeks. The rats in Con group were administration with a standard chow. All rats were weighed

every other week. Blood samples were collected from eyes of fasting rats (12 h) under light anesthesia by ether before vitamin D₂ treatment and 4, 8 and 16 weeks after treatment with HCD. Their sera were separated for determination of lipid files. At the end of the 16th week, blood was collected from the abdominal aorta after the fasting rats were anesthetized with 30 mg/kg pentobarbital sodium, the serum was separated, and it was stored at -70°C. The thoracic abdominal aorta was isolated and put into ice-cold phosphate buffered saline. After removing the connective tissue carefully, it was stored at -70°C. The aorta arch was removed and fixed in 10% formalin.

4. Vessel levels of cholesterol

The aortic arch was fixed in 10% formalin followed by dehydration and embedding in paraffin. Six micrometer-thick sections were cut. The accumulations of vascular cholesterol were measured using kit. Briefly, the thoracic aorta was freeze-dried to a constant weight using a freeze drier. After weighing, the cholesterol was subsequently extracted at 50°C for 20 min with chloroform-methanol (2:1) and the extracts were used for the determination of TC. The precipitation was dissolved in HNO₃ and then dried in an oven and re-dissolved with the blank solution (27 nM/ l KCl, 27 µM/ l LaCl₃ in de-ionized water). Cholesterol content was expressed by mg/g of dry tissue.

5. Serum levels of lipids

Serum was diluted with 150 mM/ l NaCl, 1 mM/ l EDTA (pH 7.4), so that the OD measurement and lipid concentrations were brought into the normal range. Serum concentration of TC was assayed enzymatically by using commercial kits and those of HDL-C and LDL-C were determined by precipitation with phosphotungstic acid/magnesium chloride or with heparin/sodium citrate, respectively.

6. Serum OxLDL content

OxLDL content in serum was assayed by competitive ELISA method following the manufacture's instruction. Briefly, 50 µl of standards, control and samples containing OxLDL were added to wells of microplate coated with high affinity anti-rat OxLDL antibodies and then 50 µl of biotin conjugate reagent was added. The microplate was incubated at 37°C for 60 min. After washing the wells 5 times using washing solution in automatic washer to remove the unbound components from the microplate, 50 µl of enzyme conjugate reagent was added to the wells and the microplate was incubated at 37°C for 30 min. After 5 more washings, 50 µl of

color A and color B reagents were added followed by incubation at 37°C for 15 min. Then an acidic stop solution was added and the absorbance (OD) of yellow solution was read at 450 nm within 20 min using microplate reader. A dose reverse response curve of OD vs. concentrations was generated using the values obtained from standard. The oxLDL level of the rat serum was determined directly from this curve.

7. Serum MDA content and Cu/Zn SOD activity

Serum MDA content was measured as thiobarbituric acid reactive substances (TBARS), as described previously. In short, to the serum was added 10% (w/v) trichoroacetic acid and 2-thiobarbituric acid followed by incubation at 95°C for 1 h. After centrifugation, TBARS in the supernatant were determined at 532 nm. The concentrations of TBARS were calculated using TEP as a reference standard. Cu/Zn SOD activity in serum was determined, as described previously⁸⁾.

Results

1. Vessel levels of cholesterol

Vascular TC content represents the degree of vascular aging. TC content of thoracic aorta in HCD group (12.3±1.37 mg/g dry tissue) was significantly higher than those in Con group (5.2±0.64 mg/g dry tissue). Both ACT (30) and ACT (60) significantly reduced TC content compared with HCD(Fig. 1).

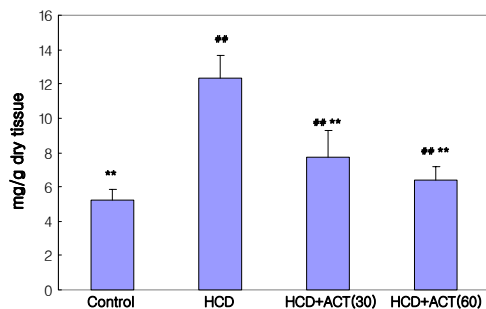


Fig. 1. Vessel levels of cholesterol. Rats were treated and levels of cholesterol in thoracic aorta were measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01 compared to the Control group; ***: P<0.01 compared to the HCD group).

2. Serum levels of lipids

Serum levels of TC and LDL-C in the HCD group increased while HDL-C decreased in a time-dependent manner, implying that HCD gradually induced hypercholesterolemia in rats. At the end of the experiment, levels of TC, LDL-C in the HCD group were about 5 and 29 times higher, respectively, than those of control group. No significant differences in the serum TC, LDL-C, HDL-C were detected between ACT groups

and the HCD group, suggesting complementation of ACT to rat did not affect serum lipids(Fig. 2-4).

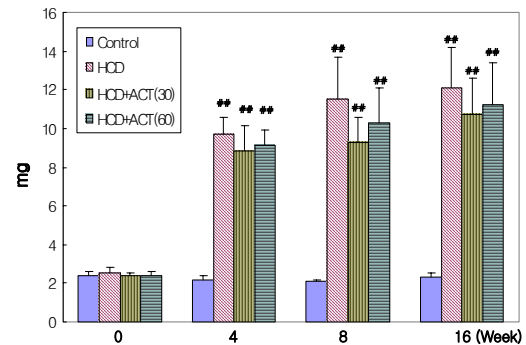


Fig. 2. Serum levels of TC. Rats were treated and serum levels of lipids were measured as described in Materials and methods. (mean±S.D., n=10. ##: P<0.01 compared to the Con group).

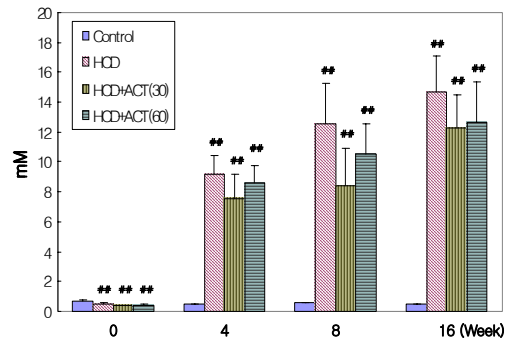


Fig. 3. Serum level of LDL-C. Rats were treated and serum levels of lipids were measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01 compared to the Con group).

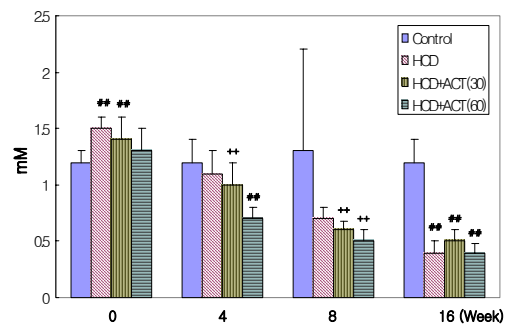


Fig. 4. Serum level of HDL-C. Rats were treated and serum levels of lipids were measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01, ++ : p<0.05 compared to the Control group).

3. Contents of OxLDL and MDA and Cu/Zn SOD activity in serum

Because OxLDL is mainly formed by oxidized modification of LDL under the vessel endothelium and released into the blood, the serum OxLDL concentration indirectly represents its production in vessel endothelium. Serum MDA content partly reflects the amount of serum

OxLDL and the oxidation state, while Cu/Zn SOD activity represents the anti-oxidation state in the body. The results showed that serum levels of OxLDL and MDA increased while Cu/Zn SOD activity decreased in the HCD group compared with those in the Con group, suggesting an increased production of OxLDL under vessel endothelia and an imbalance between oxidation state and anti-oxidation state in the body. Both ACT(30) and ACT(60) treatments decreased OxLDL content, reduced MDA content, and increased Cu/Zn activity compared with HCD treatments(Fig. 5-7).

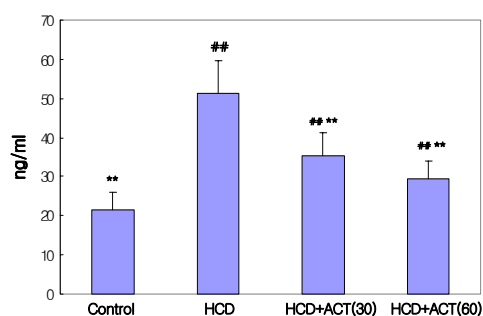


Fig. 5. Serum levels of OxLDL. Rats were treated and the serum level of OxLDL was measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01 compared to the Control group; **: P<0.01 compared to the HCD group).

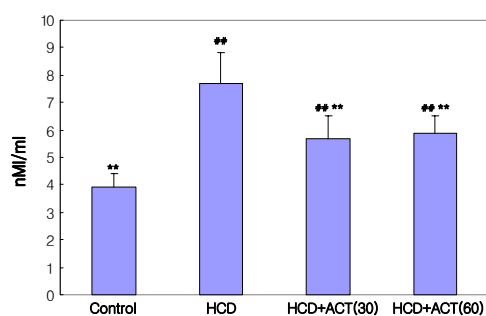


Fig. 6. Serum level of MDA. Rats were treated and the serum levels of MDA were measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01 compared to the Control group; **: P<0.05 compared to the HCD group).

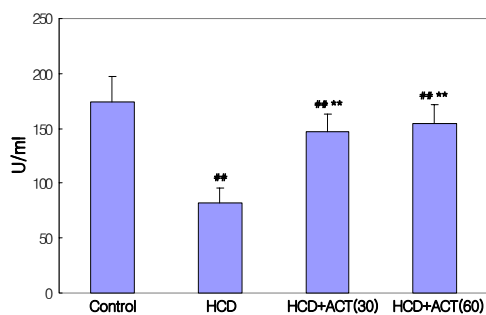


Fig. 7. Serum level of Cu/Zn SOD activity. Rats were treated and the serum level of Cu/Zn SOD activity were measured as described in Materials and Methods. (mean±S.D., n=10. ##: P<0.01, ++ : p<0.05 compared to the Control group; **: P<0.01 compared to the HCD group).

Discussion

In the present study, we have successfully established a vascular aging model in rats by orally administrating excessive vitamin D₂ (500,000 IU/kg/day) for 4 days followed by feeding HCD containing cholic acid and thiourcil for 16 weeks. The role of vitamin D₂ in vascular aging formation is to cause endothelial injury, leading to increased permeability of endothelium. The mechanism of action of cholic acid is two fold: an increase in cholesterol absorption and a concomitant suppression of cholesterol 7 α -hydroxylase activity that results in decreased cholesterol excretion. Thiouracil induces clinical hypothyroidism with decreased LDL-receptor activity and hypercholesterolemia⁹. On this animal model, we have also found that ACT, which is widely used for prevention and treatment of cardiovascular diseases in Korea, significantly attenuated the vascular aging pathological changes in rats.

ACT, commonly known as Dokhwal, has long been used as a traditional oriental medicine for angina pectoris and cancer. The aqueous extracts of ACT have long been used as a traditional oriental herb medicine in the treatment of cardiovascular diseases, chronic hepatitis, and cancer¹. Several active ingredients have been identified that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage and reducing atherosclerosis¹⁰.

Biochemically, vessel cholesterol contents in vascular aging rats were significantly increased compared with those in normal rats. The vessel levels of cholesterol in vascular aging rats were much higher than those in normal rats. These results suggested that vascular aging rats could be a good model for studying anti-vascular aging drugs. ACT was shown to dose-dependently inhibit the vascular aging formation in rats by reducing deposition of cholesterol in vessel. The results suggest that the extracts of ACT might be beneficial to vascular aging patients related with cardiovascular disease. Indeed, the quality of ACT dripping pills widely used in Korea for prevention and treatment of cardiovascular diseases is controlled based on the content of ACT. Nevertheless, the mechanism by which ACT exerted the anti-vascular aging effect remains unknown. The hypothesis of lipid metabolism disorders proposed that hypercholesterolemia is an important risk factor for vascular aging and this theory is supported by the fact that cholesterol lowering drugs such as statins are widely used and very effective for reducing serum cholesterol level, leading to attenuation of vascular aging. Thus, we firstly observed the effect of ACT on the serum cholesterol level including TC, LDL-C and HDL-C in rats. The results showed that ACT had

no influences on the serum cholesterol level, suggesting that other actions rather than a cholesterol lowering effect of ACT were attributed to its inhibition of vascular aging. Many studies using statins in low dosage without cholesterol lowering effect found that it was still effective for treatment of vascular aging in patients or animal models^{11,12}, suggesting that alternative actions of statins on vascular aging exist beyond cholesterol lowering.

Secondary, Oxidative modification hypothesis of vascular aging emphasizes the pivotal role of OxLDL in initiation and progression of vascular aging. It was shown that enhanced serum levels of OxLDL are strongly predictive for coronary heart disease closely linked vascular aging¹³. OxLDL behaves differently, compared to native LDL, in physicochemical and biological properties. Because OxLDL is main carrier of cholesterol under the vessel intima and released into blood, is mainly formed by oxidized modification of LDL. So serum OxLDL concentration indirectly represents production of cholesterol in the vessel intima. Meanwhile, serum MDA content partly reflects the amount of serum OxLDL. Therefore, we secondly measured the serum levels of OxLDL and MDA in order to further investigate the anti-vascular aging mechanism of ACT. The present study showed that serum levels of both OxLDL and MDA in the HCD group were significantly increased compared with those in Con group, suggesting an increased production of OxLDL in the vessel. ACT was shown to lower serum levels of OxLDL and MDA in a dose dependent manner, implying that the production of OxLDL in vessel was significantly inhibited. The results suggested that ACT inhibited OxLDL production and then could be used as a potential anti-atherosclerotic agent in aged cells.

In summary, the present study has demonstrated that ACT significantly attenuated vascular aging formation in a rat model. Studies of mechanisms showed that inhibition of OxLDL production under intima by ACT might be responsible for those effects on vascular aging rats. These observations offer further insights into the anti-vascular aging mechanisms of antioxidants and provide a potential target for antioxidants in the prevention and treatment of vascular aging.

References

1. Chang, H.M., But, P.P.H. *Pharmacology and Applications of Chinese Materials Medica*. Singapore:World Scientific Publishing Co. pp 255-268, 605, 1986.
2. Dang, N.H., Zhang, X., Zheng, M., Son, K.H., Chang, H.W., Kim, H.P., Bae, K., Kang, S.S. Inhibitory constituents against cyclooxygenases from *Aralia cordata* Thunb. *Archives of Pharmacal Research* 28(1):28-33, 2005.
3. Yahara, S., Ishida, M., Yamasaki, K., Tanaka, O., Mihashi, S. Minor diterpenes of *Aralia cordata* Thunb: 17-hydroxy-ent-kaur-15-en-19-oic acid and grandifloric acid. *Chem Pharm Bull (Tokyo)*. 22(7):1629-1631, 1974.
4. Cai, G.J., Miao, C.Y., Xie, H.H., Lu, L.H., Su, D.F. Arterial baroreflex dysfunction promotes atherosclerosis in rats. *Atherosclerosis*. 183: 41-47, 2005.
5. Galle, J., Hansen-Hagge, T., Wanner, C., Seibold, S. Impact of oxidized low density lipoprotein on vascular cells. *Atherosclerosis*. 185: 219-226, 2006.
6. Anselmi, M., Garbin, U., Agostoni, P., Fusaro, M., Pasini, A.F., Nava, C., Keta, D., Turri, M., Zardini, P., Vassanelli, C., Lo Cascio, V., Cominacini, L. Plasma levels of oxidized-low-density lipoproteins are higher in patients with unstable angina and correlated with angiographic coronary complex plaques. *Atherosclerosis*. 185: 114-120, 2006.
7. Dominguez-Rodriguez, A., Abreu-Gonzalez, P., Garcia-Gonzalez, M., Ferrer-Hita, J., Vargas, M., Reiter, R.J. Elevated levels of oxidized low-density lipoprotein and impaired nocturnal synthesis of melatonin in patients with myocardial infarction. *Atherosclerosis*. 180: 101-105, 2005.
8. Park, W.H., Shin, S.S., Lee, Y.C., Kim, C.H. The inhibitory effects of Silso-San-Gami on atherosclerosis in KHC rabbits. *Thrombosis Res*. 113: 235-241, 2004.
9. Moghadasian, M.H. Experimental atherosclerosis: a historical overview. *Life Sci*. 70: 855-865, 2002.
10. Liu, G.T., Zhang, T.M., Wang, B.E., Wang, Y.W. Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. *Biochemical Pharmacology*. 43(2):147-152, 1992.
11. Kleemann, R., Princen, H.M., Emeis, J.J., Jukema, J.W., Fontijn, R.D., Horrevoets, A.J., Kooistra, T., Havekes, L.M. Rosuvastatin reduces atherosclerosis development beyond and independent of its plasma cholesterol-lowering effect in APOE*3-Leiden transgenic mice: evidence for antiinflammatory effects of rosuvastatin. *Circulation*. 108: 1368-1374, 2003.
12. Yeung, A.C., Tsao, P. Statin therapy: beyond cholesterol lowering and antiinflammatory effects. *Circulation*. 105: 2937-2938, 2002.
13. Meisinger, C., Baumert, J., Khuseynova, N., Loewel, H., Koenig, W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation*. 112: 651-658, 2005.