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Anti-Atherosclerotic Effects Mediated by the Combination of Probucol and Amygdalin in Apolipoprotein E-Knockout Mice Fed with a High Fat Diet

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Abstract: To ameliorate atherosclerosis progression, researchers studied the combined therapy of amygdalin and probucol, a cholesterol-lowering drug in ApoE knockout mice. About 8 weeks old male ApoE knockout mice were fed on a High Fat Diet (HFD) and received amgydalin treatment alone, probucol treatment alone or combined therapy in the present study. Triglyceride (TG), Total Cholesterol (TC) and Low Density Lipoprotein (LDL) cholesterol levels were measure at the end of treatment. Aortic lesion area, plaque area and plaque coverage percentage of aortic sinus were also quantified. Meanwhile, inflammation related proteins as Matrix Metalloproteinase-2 (MMP-2) and Matrix Metalloproteinase-9 (MMP-9) were analyzed. Studies on blood lipid revealed that combined therapy significantly decreased both total cholesterol and LDL cholesterol levels. Moreover, significantly decreased blood lipids levels were accompanied with reduced plaque areas and plaque coverage percentages indicating the anti-atherosclerotic function of combined therapy is through reduction of cholesterol levels. Furthermore, observations on the mRNA levels and expression of MMP-2 and -9 suggested that combined therapy induces decreased expression of MMP-2 and -9 in aortic lesions. The studies indicated that the combination of amygdalin and probucol was more effective in retarding atherosclerotic lesion progression than the administration of each drug alone.

Key words: Amgydalin, probucol, Matrix Metalloproteinase-2 (MMP-2), Matrix Metalloproteinase-9 (MMP-9), China

INTRODUCTION

Atherosclerosis is an inflammatory disease of the arterial wall. Hypercholesterolemia, smoking, male gender, hypertension, diabetes and age were traditionally considered as risk factors. However, recent research with accumulating evidence suggests that the innate and adaptive immune responses play a critical role in the pathogenesis of atherosclerosis (Binder *et al*., 2002; Hansson, 2005). In this multifactorial process, endothelial cells, macrophages, smooth muscle cells and lymphocytes are involved and interacted. As a result of their interaction, atherosclerotic plaque is formed that influences on organ perfusion and ultimately results in cerebrovascular events and acute coronary syndromes. The complex interplay between inflammatory cells, cytokines and degrading enzymes may lead to atherosclerotic lesion progression and unstable plaque formation or even plaque rupture eventually (Davies and Thomas, 1985). Probucol has been reported to possess antioxidant activities. Ku *et al*. (1990) suggested that inhibition of IL-1 secretion from macrophages by probucol contributes to its therapeutic effects in atherosclerosis and may also result in beneficial activity in some chronic inflammatory disease. Furthermore, some studies reported that the anti-atherosclerotic effect of Probucol is by decreasing the expression of Monocyte Chemotactic Protein-1 (MCP-1) (Chang *et al*., 1995; Nakamura *et al*., 2002) and inhibiting monocyte infiltration *in vivo* (Nakamura *et al*., 2002). By inhibiting monocyte infiltration and macrophage foam cell accumulation in the vessel wall (Choy *et al*., 2005), probucol could conceivably decrease arterial Matrix Metalloproteinase (MMP) activity (Nakamura *et al*., 2002) and thus affect arterial remodeling. Amygdalin (vitamin B17 also called Laetrile) is extracted from Semen Persicae, the seed of

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Prunus persica (L.) *Batsch*. Amygdalin is also abundant in the seeds of apricots, almonds, peaches and other rosaceous plants. Besides the antitumor activity (Chang *et al*., 2006; Laster Jr. and Schabel Jr., 1975; Milazzo *et al*., 2007), amygdalin has also been used for the treatment of asthma, bronchitis, emphysema, leprosy and diabetes (Heikkila and Cabbat, 1980; Baroni *et al*., 2005; Hwang *et al*., 2008). Semen Persicae is a major component of Xuefu Zhuyu decoction which could relief the symptom of atherosclerosis according to Traditional Chinese Medicine (TCM) theory and clinical practice. Because amygdalin is the major component of Xuefu Zhuyu decoction, researchers focused the study on whether amygdalin has anti-atherosclerotic effect *in vivo*. The previous study has shown that amgydalin alone has anti-atherosclerotic effect *in vivo* (Jiagang *et al*., 2011). However, their combined effect on atherosclerosis is unclear. Therefore, in the present study, the aim of this study was to evaluate the combined effect of amygdalin with probucol on atherosclerotic lesions in ApoE deficient mice. Researchers show that combined therpay markedly retards the progression of atherosclerosis and this anti-atherosclerotic activity of combined therapy appears to be due to inhibition of macrophage accumulation and decreasing MMP-2 and -9 in the affected vessel wall.

MATERIALS AND METHODS

Animls: ApoE knockout mice on a C57BL/6J background (Plump *et al*., 1992) and their wild-type littermates were purchased from Jackson Laboratories and kept in 12 h light/dark cycle with free access to water and food which is in accordance with IVC requirement in Sichuan University. Animal handling was in accordance with Ethics Committee of Sichuan University.

Study design: Amygdalin was purchased from changsha staherb natural ingredients Co., Ltd. About 8 weeks old male mice were divided into six groups.

C57 control group: About 8 weeks old male C57BL/6J mice were given a standard laboratory diet (10% fat, 15% protein and 75% carbohydrate).

ApoE control group: About 8 weeks old male ApoE knockout mice were given High Fat Diet (HFD, containing 40% fat, 14% protein and 46% carbohydrate) for 12 weeks.

Amygdalin group: Amygdalin (1 mg kg^{-1}) was intraperitoneally injected.

Probucol group: About 1% (wt/wt) probucol were added in diet.

Combined therapy group: Atherosclerotic mice received both amgydalin daily injection and probucol in diet. All groups of mice were sacrificed after 8 weeks of drug delivery.

Assesment of aortic sinus atherosclerosis: Hearts and upper sections of the aorta were removed and fixed, embedded and sectioned. Atherosclerotic lesions were quantified by calculating the lesion size in the aortic sinus as previous described (George *et al*., 2000, 2001). Sections were stained with hematoxylin and eosin and vessel areas were measured by Image J software (NIH) from images obtained with Nikon 80i microscope. For comparisons of plaque areas between amygdalin groups and control groups, 100 and 200 μm distant sites were used.

Lipid profiles: Sera from mice were obtained at the time of killing after an overnight fast. Total Cholesterol (TC) concentrations were determined in duplicate by using a colorimetric assay (infinity cholesterol reagent, Thermo Fisher). Triglyceride (TG) concentrations were determined by using the L-type triglyceride H assay according to the manufacture's instructions (Wako chemicals). Low Density Lipoprotein-cholesterol (LDL-c) and High Density Lipoprotein-cholesterol (HDL-c) concentrations were determined by the CHOD-PAP method (Shensuo, Shanghai).

Real-time PCR analysis: Total RNA was prepared by RNeasy kit following the manufacture's instructions (Qiagen). About 2 μg of RNA was used for cDNA synthesis. β-actin cDNA from each sample was used to normalize the samples for differences in PCR efficiency. cDNA was amplified with Taq polymerase (Takara) and appropriate primers at 94°C for 40 sec, 60°C for 30 sec and 72°C for 40 sec for 30 cycles with an initial 5 min denaturation at 95°C and final 10 min extension at 72°C. The primers and probes for MMP-2 and -9 were previously described by Qin *et al*. (2008) and Kuzuya *et al*. (2006). The real-time PCR was performed on an ABI 7900 using Taqman Universal PCR maser mix (TAKARA) in triplicates. The C_T for GAPDH was used to normalize the samples.

Immunohistochemistry: Tissues were fixed in 10% formalin for 24 h at room temperature, dehydrated, embedded in paraffin and sectioned. Serial paraffin

sections at 100 and 200 μm distant of aortic sinus (n = 8 mice of each group) were used for IHC staining. Rat anti-MMP-2 antibody (Abcam) and rat anti-MMP-9 antibody (Abcam) were used. Primary antibodies were detected with corresponding secondary antibodies (Jackson Immuno research). At least 4 sections per mouse were examined for each immunostaining and appropriate negative controls were used.

Statistical analysis: Data were expressed as mean±SEM. Statistical significance was determined by one-way ANOVA with Bonferroni correction and Student's t-test. $p<0.05$ was considered to be statistically significant.

RESULTS

Decreased blood lipod levels induced by combined therapy: Treatment with probucol and treatment with amygdalin did not result in any statistically significant differences in body weight among the four groups of mice. Nor did mice have elevated body weight with the combined therapy. However, plasma total cholesterol concentrations were significantly decreased in the probucol-treated ApoE knockout mice and amgydalin-treated ApoE knockout mice (Fig. 1). With the prolonged dietary of HFD in ApoE knockout mice, atherosclerosis developed which is accompanied with changes in blood lipid levels. TG increased 7-8 folds compared with normal control fed with normal diet while the increase of TC was about 9-10 fold. In order to assess the anti-atherosclerotic effect of combined therapy, TG levels and TC levels were compared among probucol-treated alone group, amygdalin-treated alone groups and control groups.

Although, mice treated with amygdalin alone or probucol alone had decreased TG and TC levels ($p<0.05$ compared with control mice), mice treated with both amygdalin and probucol had significantly decreased TG and TC levels compared with amygdalin treated alone or probucol treated alone (for TC, $p<0.05$ compared with amygdalin alone or probucol alone for TG, $p<0.05$ compared with amygdalin alone and $p<0.01$ compared with probucol alone).

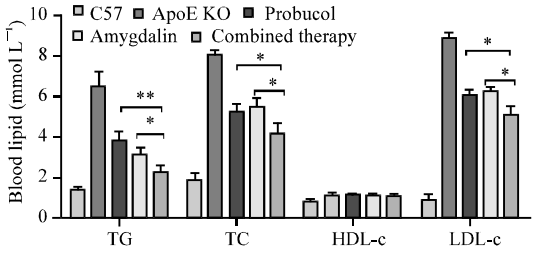

Fig. 1: Blood lipid (TG, TC, HDL-c and LDL-c) of different groups of mice (n = 8 in each group) (C57, C57/B6 mice and ApoE KO, ApoE knockout mice)

Meanwhile, combined therapy could also decrease LDL-c levels ($p<0.05$). But the treatment of any of these two drugs did not induce decreased HDL-c level ($p>0.05$).

Combined therapy decreases plaque area: In order to further investigate the pathological changes of combined therapy, researchers analyzed vessel areas, lumen areas and plaque areas after the treatment (Fig. 2). With the progression of atherosclerosis, the diameters of aortic sinus increased with time (Fig. 2a), combined with decreased lumen areas (Fig. 2b) and increased plaque areas (Fig. 2c). Observations on lumen areas and plaque areas revealed that both amygdalin treatment alone and probucol treatment alone could alleviate the atherosclerosis situation. However, mice administered combined therapy of both amygdalin and probucol exhibited a significantly reduced extent of atherosclerosis as evident by smaller aortic sinus plaques, less enlarged aortic sinus and the expanded lumen areas, compared with those of in ApoE knockout control mice treated with either amygdalin alone or probucol alone ($p<0.05$, Fig. 2b and c). This finding was validated by analysis of atherosclerosis surface coverage areas, showing decreased lesions in ApoE knockout mice received combined therapy ($p<0.05$, Fig. 2d).

Combined therapy decreases MMP-2 and -9 expression: Accumulative evidence suggests that in human or animal models of atherosclerosis, varying matrix Metalloproteinases (MMPs) have been demonstrated to increase in atherosclerotic lesions including MMP-1, -2, -3, -7, -9, -12, -13 and MT-MMPs (Newby, 2005; Galis and Khatri, 2002; Kuzuya and Iguchi, 2003) MMPs have been believed to play a critical role in the development and progression of atherosclerosis. It is therefore, assessed the activities of MMP-2 and -9 in carotid arteries of ApoE knockout control mice and mice received drug therapy (amygdalin alone, probucol alone or combined therapy). Immunohistochemical analysis revealed that expression of MMP-2 and -9 was increased in ApoE knockout mice (fed with HFD and combined therapy significantly inhibited the expression of MMP-2 (Fig. 3a) and -9 (Fig. 3b). Meanwhile, MMP-2 and -9 mRNA expression was significantly decreased in atherosclerotic plaque lesions in mice received combined therapy (Fig. 3c) suggesting combined therapy appeared to decrease the expression of MMP-2 and -9.

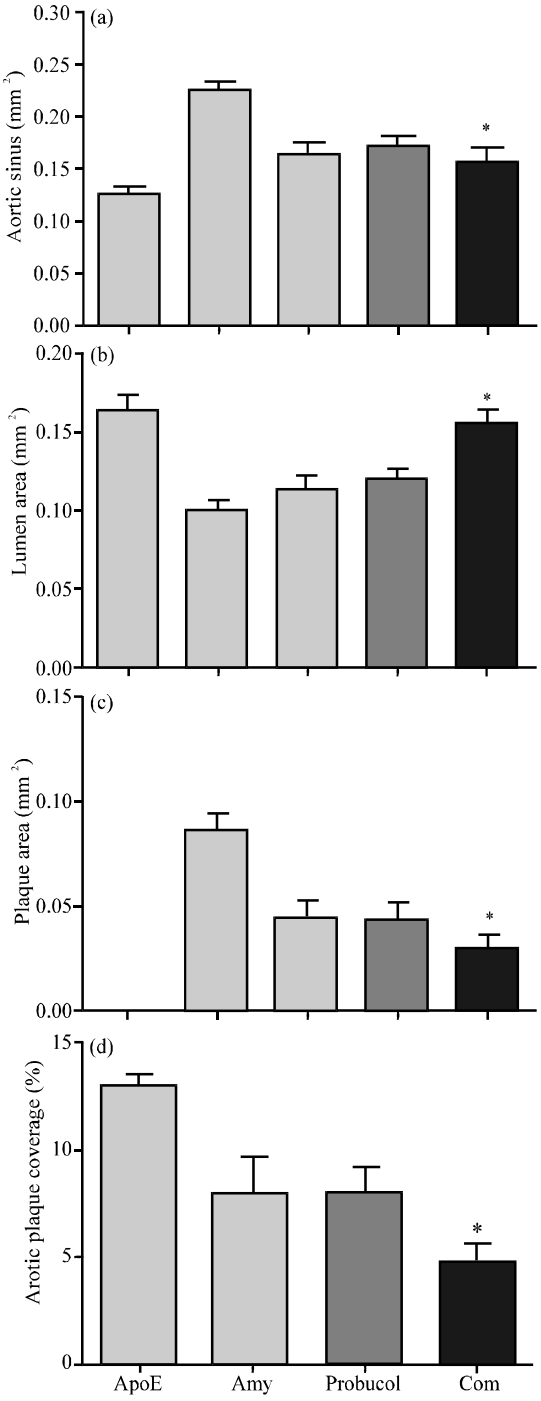

Fig. 2: Combined therapy (Com) could relief the symptom of atherosclerosis. After the administration of drugs for 8 weeks there is significantly increased vessel area (a) and lumen area (b), decreased plaque area (c) and aortic plaque coverage percentage (d). All data are calculated by one-way ANOVA ($p<0.05$ vs. amygdalin treatment alone or probucol treatment alone)

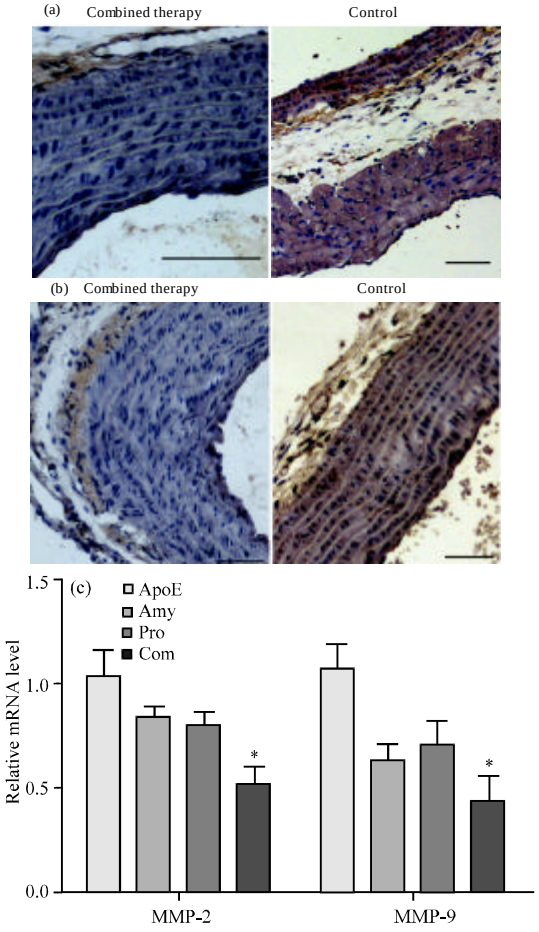

Fig. 3: Combined therapy decreases MMP-2 and -9 in carotid arteries of ApoE knockout mice fed with HFD; a) Immunohistochemistry showing presence of MMP-2 and -9 protein in aortic arches of control mouse and combined therapy decrease the expression of MMP-2 and -9 protein and b) Data of mRNA quantification were obtained from the independent aortic arches of control mice and drug delivered mice (Amy: Amgydalin; Pro: Producol; Com: Combined therapy)

DISCUSSION

Atherosclerosis is a multifactorial disease developing over many years with symptoms becoming apparent in the late stages of the disease. Inflammation, oxidative stress and endothelial dysfunction are associated with the pathogenesis of atherosclerosis. The previous study has shown that amgydalin can inhibit the development of atherosclerosis in ApoE knockout mice (Jiagang *et al*., 2011). In particular, the anti-atherosclerotic effect of amygdalin is through induction of regulatory T cells and

probucol is a known lipophilic antioxidant with modest lipid-lowering properties. However, studies on the anti-atherosclerotic effect of combined therapy with both amygdalin and probucol are elusive. In the present study, the levels of TC, TG and LDL-c were higher in ApoE knockout mice fed with HFD compared with amygdalin-treated mice and probucol-treated mice. Herein researchers first reported that mice received combined therapy have reduced the TG, TC and LDL-c levels compared with amgydalin treatment alone or probucol treatment alone.

Furthermore, there were significant positive correlation between blood lipid concentration and the aortic plaque coverage area and percentage. In this study, there were observed significantly decreased plaque area and plaque coverage percentage in mice with combined therapy, compared with other groups including control mice, angydalin-treated mice and probucol-treated mice. These findings suggested that the elevated cholesterol levels and lipid concentration may cause an endothelial dysfunction with a consequent increase in the plaque area. However, the exacerbated situation of atherosclerosis could be partially reversed by the combined therapy of amygdalin and probucol. Studies on the significant positive correlation between the plaque areas and the levels of TG, TC, LDL-c demonstrated that the anti-oxidant probucol and immune regulatory agent of amgydalin delayed the formation of plaque at aortas in hypercholesterolemic mice and the vascular protection of both agents may partly be due to its ability of lowering TG, TC and LDL-cconcentration and therefore attenuate atherosclerosis.

Inflammation is recognized as a key process in atherogenesis and many cytokines and other factors are involved in inflammatory processes which in turn modulate cellular signaling, cell growth, differentiation and a variety of other cellular processes.

Previous observations illustrate the potential complexities of manipulating a system in which MMPs have a dual role in plaque growth by means of SMC migration, matrix deposition and instability caused by matrix destruction. Recent report by Johnson *et al.* (2005) suggested that MMP-9 deficiency increased plaque size with a increase in macrophage accumulation and a decrease in SMCs in brachiocephalic artery from apoE knockout mice. Moreover, Masafumi Kuzuya reported that MMP-2 significantly contributes to the development of atherosclerosis in apoE knockout mice (Kuzuya *et al.*, 2006). In light of the importance of inflammatory cells and MMPs in atherosclerosis, the results of immunohistochemical study and real-time PCR analysis of MMA-2 and -9 mRNA levels suggested that the inhibitory activity of combined therapy was due to its ability to inhibit the accumulation and expression of MMP-2 and -9.

Although, in this study, the mRNA levels and expression of MMP-2 and -9 were comparatively decreased in mice treated with amgydalin and probucol, mice received combined therapy showed sigfnificantly decreased levels of MMP-2 and -9 indicating the attenuation of lesion formation in the combined therapy treated ApoE knockout mice was associated with decreased expression of MMP-2 and -9 in aortic lesions.

CONCLUSION

In this study, researchers demonstrated that combined therapy with both amygdalin and probucol reduced atherosclerotic plaque lesions in apoE knockout mice. This reduction of the plaque lesions in apoE knockout mice was associated with the reduction of MMP-2 and -9 expression in the plaque lesions. These results suggested that combined therapy contributes to the retarded development of atherosclerotic plaque in apoE knockout mice and could be potential drugs in atherosclerosis prevention and therapy.

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