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Mulberry Leaf Extract Inhibits the Development of Atherosclerosis in Cholesterol-Fed Rabbits and in Cultured Aortic Vascular Smooth Muscle Cells

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ABSTRACT: This study used high-cholesterol-fed New Zealand white rabbits and aortic vascular smooth muscle cells (VSMCs) to investigate the impact of mulberry leaf extract (MLE) on the development of atherosclerosis. The results show that the major components of MLE are polyphenols, flavonoids, carbohydrates, proteins, and lipids, and the major contituents of mulberry leaf polyphenol extract (MLPE) are polyphenols and flavonoids. In addition to improvement of liver function, the atheroma burden and levels of serum cholesterol, triglycerides, and low-density lipoprotein (LDL) are also significantly reduced after MLE treatment. MLE and MLPE improved endothelial function, inhibited proliferation and migration of aortic VSMCs, and reduced atheromas in the vascular wall. In conclusion, this study demonstrates that, in addition to exerting hypolipidemic effects, MLE and MLPE can effectively inhibit proliferation and migration of aortic VSMCs, improve vascular endothelial function, and reduce atheroma burden, thereby preventing atherosclerosis.

KEYWORDS: *atherosclerosis, proliferation, migration, mulberry leaf extract*

INTRODUCTION

The endothelium is a complex endocrine and paracrine organ that plays an important role in the maintenance of vascular homeostasis. Damage to the endothelium initiates a number of processes that promote atherosclerosis, including increased endothelial permeability, platelet aggregation, leukocyte adhesion, and cytokine production. Oxidative stress, caused by smoking, diabetes mellitus, hypertension, hypercholesterolemia, and aging, can lead to endothelial dysfunction. Structural regression of atherosclerosis is preceded by improvements in endothelial function.[1,2]

Atherosclerosis, the major cause of coronary artery disease, is a low-density-lipoprotein (LDL)-related chronic inflammatory process that involves endothelial cells, monocytes, vascular smooth muscle cells (VSMCs), and many soluble mediators. Stimulation of the vascular wall by oxidative stress leads to permeation of LDL into the subendothelial space, where it becomes oxidized. Monocytes recruited by the activation of the endothelium differentiate into macrophages. The oxidized LDL particles are then taken up by macrophages, which evolve into foam cells. Cytokines and growth factors secreted by macrophages also induce the proliferation and migration of VSMCs into the subendothelial space. These processes participate in the formation of atherosclerotic plaque. Plaques that contain inflammatory cells and have a fibrous cap covering the lipid-rich core are at high risk for rupture, which can lead to acute coronary syndrome.[3,4]

The polyphenol-rich mulberry leaf has been reported to have hypoglycemic,[5,6] hypolipidemic,[7] and antioxidant properties,[8−11] and its administration may thus reduce the risk of cardiovascular disease. Our previous studies have shown that mulberry leaf extract (MLE) is rich in polyphenols and can effectively inhibit VSMC proliferation and migration. This inhibition occurs through up-regulation of p53, inhibition of cyclin-dependent kinase, and suppression of small GTPase and Akt/NF-κB signaling,[12,13] thereby inhibiting the development of atherosclerosis. In the present study, we use an animal model (cholesterol-fed New Zealand white rabbits) and aortic VSMCs to investigate the effect of polyphenol-rich MLE on the development of atherosclerosis.

MATERIALS AND METHODS

Materials. Mulberry water extracts were prepared from the fruit of *Morus alba* L. (mulberry), which was obtained from Dadu township in Taichung, Taiwan. Rat aortic smooth muscle cell line A7r5 was obtained from Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu,

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Taiwan). All used chemicals were purchased from Sigma (St. Louis, MO, USA). The anti-PCNA (proliferating cell nuclear antigen), anti-SM α-actin (a smooth muscle cell marker), and anti-CD31 (an endothelial marker) antibodies were also obtained from Sigma.

Extraction of Aqueous Fractions from Mulberry Leaves (MLE). Fresh mulberry leaves (100 g) were harvested and immediately dried at 50 °C. The dried leaves were heated in 3000 mL of deionized water. After filtration, we removed the residue. The suspension was stored at −80 °C overnight. We then used a freeze-dryer (Labconco) to evaporate the suspension into a powder. The dried powder was from the aqueous fraction of MLE. The lyophilized powder was resuspended in distilled water and filtered (0.22 μm pore size) for subsequent use in cell cultures.

Extraction of the Polyphenol Fraction from Mulberry Leaves (MLPE). The powder made from dried leaves (100 g) was extracted three to five times with 300 mL of methanol at 5 °C for 3 h; the samples were filtered after each extraction. The solvent was removed from the combined extract using a vacuum rotary evaporator. The residue was dissolved in 500 mL of water and extracted with 200 mL of hexane to remove some of the pigments. The aqueous phase was extracted three to five times with 180 mL of ethyl acetate, which was then evaporated under reduced pressure. The residue was redissolved in 250 mL of water and lyophilized to yield the powder (MLPE). The lyophilized powder was resuspended in distilled water and filtered (0.22 μm pore size) for subsequent use in cell culture.

Assay of Total Phenolic Content. The total phenolic compound content of each extract was spectrophotometrically determined in accordance with the Folin−Ciocalteu procedure by reading the absorbance at 725 nm against a methanol blank. Briefly, samples (20 μL, water added to 1.6 mL) were placed in test tubes, to which 100 μL of Folin−Ciocalteu reagent and 300 μL of sodium carbonate (20%) were then added. The contents were mixed and incubated at 40 °C for 40 min. The absorbance at 725 nm was measured. The total phenolic content was expressed as milligrams per gram of MLE for gallic acid (GA).[14]

Assay of Total Flavonoid Content. The total flavonoid content of the fractions was measured following the aluminum chloride colorimetric assay.[15] To construct a calibration curve, a series of working standard solutions was prepared by diluting the stock standard solution with ethanol (10 mg of quercetin dissolved in 80% ethanol to give a final volume of 8 mL) to obtain concentrations of 0, 25, 50, and 100 μg/mL. To 0.5 mL of test solution was added 1.5 mL of 95% alcohol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M CH_3COOK, and deionized water to adjust the volume to 2.8 mL. After 30 min at room temperature, the absorbance was measured at 415 nm. A blank solution was prepared in the same way but without the addition of the aluminum chloride reagent.

The flavanone content was determined using pinocembrine as a standard for the 2,4-dinitrophenylhydrazine (2,4-DNP) method.[16] To construct a calibration curve, a series of working standard solutions was prepared by diluting the stock standard solution (2 mg/mL) with methanol to obtain concentrations of 500, 1000, and 2000 μg/mL. A mixture of 1 mL of test solution, 2 mL of 2,4-DNP reagent (1 g of 2,4-DNP dissolved in 2 mL of 96% sulfuric acid and adjusted to 100 mL with methanol), and 2 mL of methanol was heated for 50 min at 50 °C. After cooling, the mixture was added to 5 mL of 10% potassium hydroxide and incubated for 2 min at room temperature. A 1 mL aliquot of the solution was mixed with 5 mL of methanol and centrifuged at 270*g* for 10 min. The absorbance of the supernatant was measured at 494 nm. A blank solution was prepared in the same way using 1 mL of methanol instead of the test solution.

Assay of Total Polysaccharide Content. We used the phenol−sulfuric acid method[17] to measure the polysaccharide content of the samples. The MLE was diluted in deionized water, and the dissolved extracts were filtered through a 0.22 μm filter (MILLEX-HA) before treatment. Briefly, 100 mg/mL of MLE, 0.5 mL of phenol (5%), and 2.5 mL of H_2SO_4 (95.5%) were heated for 20 min at 100 °C. After cooling to room temperature, the absorbance at 490 nm was used to determine the amount of carbohydrate in the sample. Standard solutions with different concentrations of glucose (0, 10, 20, 30, 40, and 50 μg/mL) were used as standards.

Protein Content Assay. The protein content of the samples was measured using the Bradford protein assay.[18] The MLE was diluted in deionized water, and the dissolved extracts were filtered through a 0.22 μm filter (MILLEX-HA) before treatment. Briefly, 30 μL of MLE and 1 mL of Coomassie Brilliant Blue were mixed and then incubated at room temperature for 1 min. The absorbance was then measured at 595 nm. Standard solutions with different concentrations of bovine serum albumin (BSA) (0, 200, 400, 800, and 1600 μg/mL) were used as standards.

Lipid Content Assay. Lipid content was measured using the acid hydrolysis method (rapid method).[19] The MLEs (1 g) were mixed with hydrochloric acid (20 mL) in a conical flask and then heated in a water bath at 75 °C for about 50 min. The mixture was then cooled to room temperature, and 10 mL of ethanol and 20 mL of ethyl ether were added to the mixture in a separatory funnel. The separatory funnel was vigorously shaken to ensure the complete mixing of the two liquid phases. The liquid phases were then allowed to separate for at least 20 min until the layers were clearly separated. The lower solvent was collected in a new beaker, and the upper solvent (ethyl ether) was collected in a conical flask. Ethyl ether (20 mL) was added to the lower solvent and poured into a separatory funnel, which was shaken vigorously to ensure the complete mixing of the two liquid phases. The above process was repeated several times. The collected ethyl ether was evaporated under vacuum and then dried in an oven. The weight of the flask was measured and used as the content of lipid after the flask tare weight was subtracted.

Determination of Polyphenol Compounds in MLE by High-Performance Liquid Chromatography (HPLC) Assay. The composition of MLE was determined by HPLC analysis using a Hewlett-Packard Vectra 436/33N system with a diode array detector. The HPLC method employed a 5 μm RP-18 column (4.6 × 150 mm i.d.). The MLE was filtered through a 0.22 μm filter disk, and 25 mg/mL of MLE was then injected into the column. Chromatography was monitored at 260 nm, and UV spectra were analyzed to confirm peak purity. Mobile phase solvent A was acetic acid/water (2:98, v/v), and solvent B was 0.5% acetic acid in water/acetonitrile (50:50, v/v). The flow rate was 1 mL/min. The gradient for the separation was 100% solvent A at 0 min, 70% solvent A and 30% solvent B at 5 min, 65% solvent A and 35% solvent B at 50 min, 60% solvent A and 40% solvent B at 55 min, and 0% solvent A and 100% solvent B at 60 min, followed by a 5 min postrun with HPLC grade water. Then, phenolic acids were detected.

Animals and Diets. Twenty-four male New Zealand white rabbits (Animal Center of Chung Shan Medical University) weighing 2500 g were randomly divided into four experimental groups. The rabbits were individually housed in metal cages in an air-conditioned room (22 ± 2 °C, 55 ± 5% humidity) under a 12 h light/12 h dark cycle with free access to food and water. Water was allowed ad libitum, and 150 g/day of food was provided. Groups were as follows: group A, normal control group (Purina Lab Diet 5031); group B, high-cholesterol diet (HCD) (95.7% Purina Lab Diet 5031 + 3% lard oil + 0.5% cholesterol); group C, 1% MLE group (HCD + 1% MLE); group D, 2% MLE group (HCD + 2% MLE). Experimental rabbits were fed for 25 weeks on a diet containing 95.7% standard Purina Chow (Purina Mills, Inc.), 3% lard oil, and 0.5% cholesterol (HCD) to provoke the atherosclerotic process. At the same time, two of the groups were treated with oral feeding of MLE at doses of 1 and 2%. The MLE dose was selected on the basis of the suitable nutritional dose for humans in the daily diet. All handling of the animals during the 25 week feeding period was done according to the guidelines of the Institute Animal Care and Use Committee of Chung Shan Medical University (IACUC, CSMC) for the care and use of laboratory animals. At the end of 25 weeks, the rabbits were sacrificed by exsanguination after deep anesthesia with sodium pentothal (120 mg/kg) via the marginal ear vein. Serum was stored at −80 °C until analysis of serum lipids and measurement of serum variables. To protect the endothelial lining, the aortic arch and thoracic aortas were

treated carefully during removal and cleaning from the adherant soft tissue.

Serum Biochemical Assays. The serum sample was collected using EDTA tubes and centrifuged at 3000 rpm for 10 min at 4 °C. Concentrations of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), triglycerides, total cholesterol, LDL-cholesterol (LDL-c), and HDL-cholesterol (HDL-c) were measured by enzymatic colorimetric methods using commercial kits (HUMAN, Germany).

Evaluation of Atherosclerotic Lesions. Thoracic aortas were rapidly dissected, opened longitudinally, and stained with Oil Red O. Photographs of the inner surface were taken and copied onto graph paper with magnification (×2), and athermanous plaques were delineated. The number of small squares surrounded by the line on the graph paper was counted, and the percent of the area with athermanous plaque was calculated. Aortic arches were rapidly dissected out and kept at −80 °C or kept in 10% neutral buffered formalin. For the pathological analysis, paraffin-embedded tissue sections of the aortic arch were stained with hematoxylin and eosin (H&E). Experienced pathologists evaluated the presence of fatty streaks, calcifications, and VSMCs in the prepared samples. Lesions were scored using a 4-point semiquantitative scale (−, absent; +, mild; ++, moderate; +++, severe for each type of damage).[20,21]

Immunohistochemical Stain. Commercial monoclonal anti-PCNA (a proliferation marker), anti-SM α-actin (a smooth muscle cell marker), and anti-CD31 (an endothelial marker) were used for target detection in paraffin-embedded tissues from all rabbits.

Migration Assay. A7r5 aortic VSMCs were respectively treated with 0.5−2.0 mg/mL of MLE and 0.2−0.6 mg/mL of MPLE for 24 h. Cells were washed with PBS and trypsinized. After calculating the viability ratio with trypan blue, we seeded live cells at a density of 5×10^4 in the upper chamber of a 48-well Boyden chamber. The lower chamber contained culture medium with 20% FBS. The chamber was incubated at 37 °C for 6 h. The cells migrating to or invading the lower surface of the membrane were fixed with methanol for 10 min and stained with Giemsa for 1 h. The number of cells was quantified by counting five randomly chosen microscopic fields (100× magnification).

Invasion Assay. A7r5 aortic VSMCs were respectively treated with different concentrations of MLE (0.5−2.0 mg/mL) and MPLE (0.2−0.6 mg/mL) for 24 h. After calculating the viability ratio using trypan blue, we seeded live cells at a density of 5×10^4 in the upper chamber of a 48-well Boyden chamber. For the invasion assay, 100 μg/cm^2 of Matrigel (25 mg/mL, BD. Biosciences, Bedford, MA) was precoated onto 8 μm pore size polycarbonate membrane filters. The lower chamber contained culture medium with 20% FBS. The chamber was incubated at 37 °C for 6 h. The cells migrating to or invading the lower surface of the membrane were fixed with methanol for 10 min and stained with Giemsa for 1 h. The number of cells was quantified by counting five randomly chosen microscopic fields (200× magnification).

Statistical Analysis. Results are reported as the mean (standard deviation of three independent experiments), and statistical comparisons were evaluated using one-way analysis of variance (ANOVA). $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Components of MLE and MLPE. Table 1 shows the major components of MLE, including polyphenols, flavonoids, carbohydrates, proteins, and lipids. As we know, carbohydrates and proteins are not reported to be bioactive to inhibit atherosclerotic processes. Many studies have found that certain bioactive lipids may be beneficial for cardiovascular disease.[22] A diet high in monounsaturated and polyunsaturated fatty acids, especially long-chain omega-3 fatty acids, and low in saturated and trans-fatty acids was reported to reduce the risk of developing coronary artery disease and ischemic stroke.[23,24] The major components of MLPE are polyphenols and flavonoids. Previous studies have shown that flavonoids have antioxidant[25−27] and lipid-lowering effects[28] and can reduce the risk of death from coronary artery disease.[29,30] Polyphenols are reported to have many biochemical activities. As shown in Table 2 and Figure 1, the major phenolic compounds in MLPE include protocatechuic acid (3.71%), gallocatechin (1.11%), gallocatechin gallate (10.06%), caffeic acid (2.12%), rutin (5.42%), quercetin (11.70%), and naringenin (9.00%). Quercetin reportedly has inhibitory effects on xanthine oxidase, cyclooxygenase, and lipoxygenase, thereby reducing oxidative injury and the formation of inflammatory metabolites.[31,32] Gallocatechin has also been reported to have antioxidative capacity and inhibitory effects on xanthine oxidase, thus preventing oxidative injury.[33,34] Our previous study showed that gallocatechin gallate, quercetin, and naringenin exert antiatherosclerotic effects via inhibition of LDL oxidation and foam cell formation.[35] Protocatechuic acid, a major component of anthocyanins, was also reported to have significant antioxidative, anticarcinogenic, and antiplatelet effects.[36−38] Rutin was also previously reported to inhibit LDL oxidation.[39] Caffeic acid was reported to exert potent protective effects in endothelial cells, preventing LDL oxidation and subsequent LDL-induced apoptosis.[40]

Table 1. Components of MLE and MLPE

component	MLE (%)	MLPE (%)
polyphenol (Folin−Ciocalteu)	10.20 ± 0.05	27.61 ± 0.09
flavonoid (Jia method)	3.9 ± 0.09	21.67 ± 0.13
carbohydrate	25.43 ± 3.56	
protein	2.43 ± 0.26	
lipid	8.40 ± 1.63	

Table 2. Composition of the Phenolic Compounds of MLPE by HPLC Analysis

peak	retention time (min)	assigned identity[a]	recovery (%)
2	14.43	PCA	3.71 ± 1.13
4	23.03	GC	1.11 ± 1.21
5	26.21	GCG	10.06 ± 2.48
6	27.58	CA	2.12 ± 0.88
7	32.49	R	5.42 ± 1.36
8	50.27	Q	11.70 ± 1.19
9	54.87	N	9.00 ± 1.72

[a]PCA, protocatechuic acid; GC, gallocatechin; CA, caffeic acid; GCG, gallocatechin gallate; R, rutin; Q, quercetin; N, naringenin.

Effects of MLE on the Serum Biochemical Parameters of HCD-Fed Rabbits. As shown in Table 3, MLE can dose-dependently and significantly reduce the elevation of serum levels of GOT and GPT induced by HCD, thus improving liver function. Besides, the levels of serum cholesterol, triglycerides, and LDL and the ratio of LDL/HDL are significantly reduced in a dose-dependent manner after treatment of HCD-fed rabbits with MLE. Previous study has shown that flavonol glycoside in mulberry leaves can reduce oxidative stress in the liver.[41] Data from two statin clinical studies, REVERSAL (Reversal of Atherosclerosis with Aggressive Lipid Lowering) and ASTEROID (A Study To evaluate the Effect of Rosuvastatin On Intravascular ultrasound-Derived coronary atheroma burden), showed that atheroma volumes decreased when the LDL/HDL ratio was below 1.5 after statin treatment; atheroma volumes still increased if the LDL/HDL ratio was higher than 2.0, despite statin treatment. These results indicate

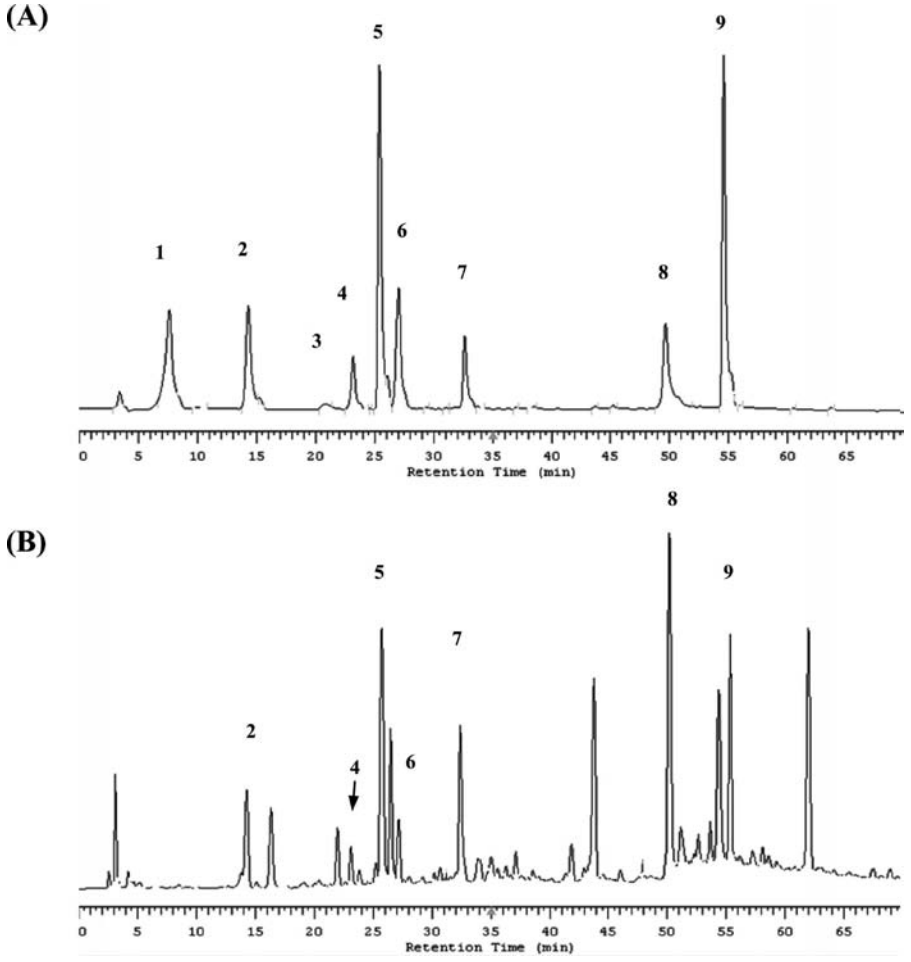

Figure 1. HPLC chromatogramd of phenolic compounds from MLPE. (A) HPLC chromatogram of nine types of standard polyphenols (1 mg/mL; 10 μL). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, gallocatechin; 5, gallocatechin gallate; 6, caffeic acid; 7, rutin; 8, quercetin; 9, naringenin. (B) HPLC chromatogram of free polyphenols from MLPE (10 mg/mL, 10 μL).

Table 3. Effects of MLE on the Serum Biochemical Parameters of Rabbits Induced by a High-Cholesterol Diet[a]

variable[b]	ND	HCD	HCD + MLE 1%	HCD + MLE 2%
GOT (U/L)	27.43 ± 7.82	323.69 ± 27.38[c]	99.80 ± 41.22[d]	74.20 ± 11.70[e]
GPT (U/L)	18.00 ± 0.79	72.25 ± 6.70[c]	41.00 ± 6.32[d]	22.83 ± 5.53[e]
cholesterol (mg/dL)	84.90 ± 56.23	652.34 ± 90.64[c]	548.70 ± 50.62[e]	459.17 ± 56.23[e]
triglycerides (mg/dL)	34.00 ± 9.00	74.00 ± 7.00[c]	49.50 ± 4.00[e]	43.00 ± 3.00[e]
LDL-c (mg/dL)	57.31 ± 3.88	547.00 ± 90.74[c]	430.50 ± 90.56[d]	305.66 ± 76.17[e]
HDL-c (mg/dL)	27.43 ± 5.37	78.83 ± 15.32	98.16 ± 9.45	117.43 ± 23.52
LDL-c/HDL-c	1.21 ± 0.13	7.41 ± 2.09[c]	4.35 ± 0.57[d]	2.55 ± 0.80[e]
BUN (mg/dL)	16.1 ± 4.70	30.10 ± 17.20	21.90 ± 3.30	27.70 ± 10.80
CRE (mg/dL)	1.70 ± 0.30	1.50 ± 0.40	1.00 ± 0.20	1.30 ± 0.10

[a]Each value is expressed as the mean ± SD (n = 6/group). Duration of the experiment = 25 weeks. Results were statistically analyzed with Student's t test. [b]GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BUN, blood urea nitrogen; CRE, creatinine. [c]$p < 0.05$ compared with the ND group. [d]$p < 0.05$ compared with the HCD group. [e]$p < 0.01$ compared with the HCD group.

that reduction of the LDL/HDL ratio, not just the LDL level alone, is important for reducing the atheroma burden[42,43] Our present data show that in addition to improving liver function, MLE can effectively reduce serum LDL, triglycerides, and the LDL/HDL ratio, thus improving atherosclerosis.

MLE Reduces the Atheroma Burden in HCD-Fed Rabbits. As shown in Figure 2, the plaque volume in HCD-fed rabbits was significantly reduced, in a dose-dependent manner, after treatment with MLE (panels A and B). Histological analysis showed the same results (panel C). When administered in doses up to 3 g/day, MLE has been reported to exhibit potential hypoglycemic and hypolipidemic effects in patients with type II diabetes.[10] A previous study showed that MLE has hypolipidemic effects but did not test whether this effect led to a direct decrease in atheroma volume.[44] Our study has proven that, in addition to reducing serum lipid levels, MLE can reduce the volume of atheromas in high-cholesterol-fed rabbits, thus improving atherosclerosis.

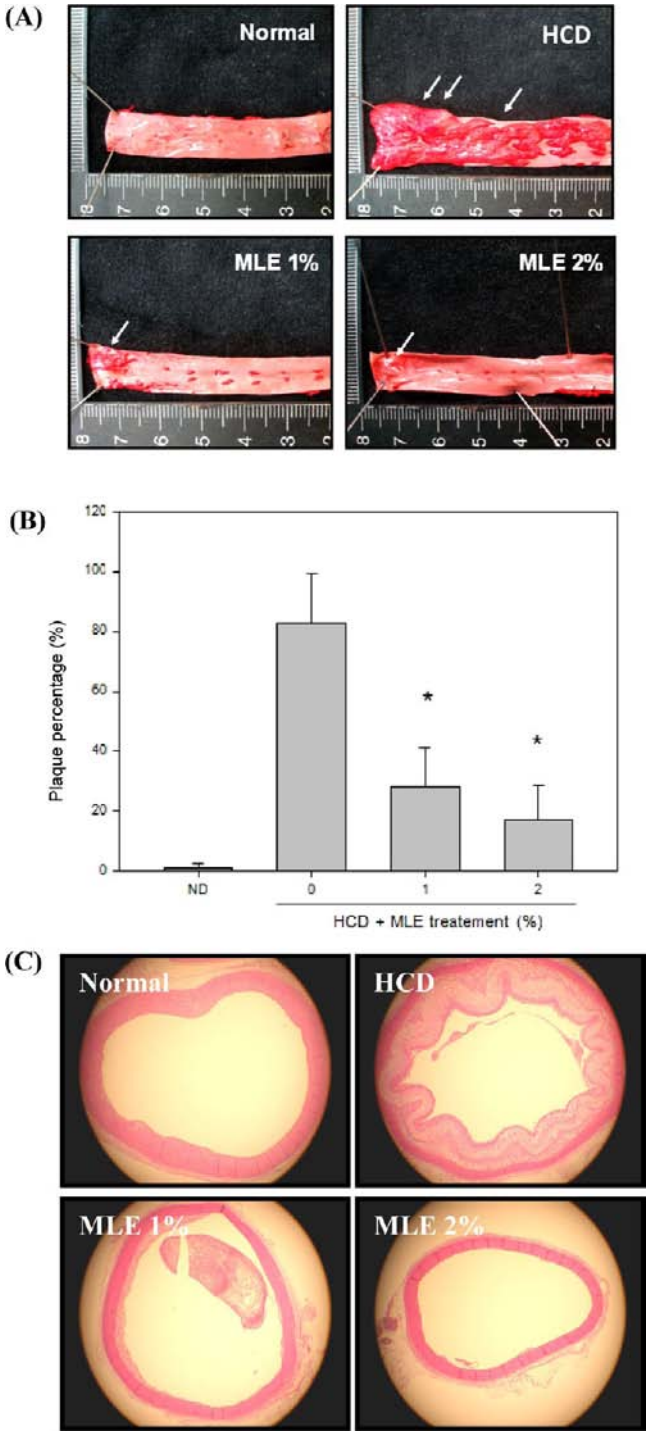

Figure 2. MLE reduces atheroma burden in HCD-fed rabbits. (A) Representative aortas from rabbits fed HCD, HCD + MLE 1%, or HCD + MLE 2% were dissected from the origin at the aortic valve to the bifurcation of the internal iliac arteries and stained with Oil Red O. (B) The percent aortic area positively staining with Oil Red O was calculated. Values are shown as the mean ± SD. (∗)$p < 0.01$, compared with HCD group. 0, HCD without MLE; 1, HCD with 1% MLE; 2, HCD with 2% MLE. (C) Histological photomicrographs of aortic segments from atherosclerotic rabbits fed HCD. The plaque volume in HCD-fed rabbits was significantly reduced, in a dose-dependent manner, after treatment with MLE. Normal, normal control group; HCD, high-cholesterol diet; MLE 1%, high-cholesterol diet with 1% MLE; MLE 2%, high-cholesterol diet with 2% MLE.

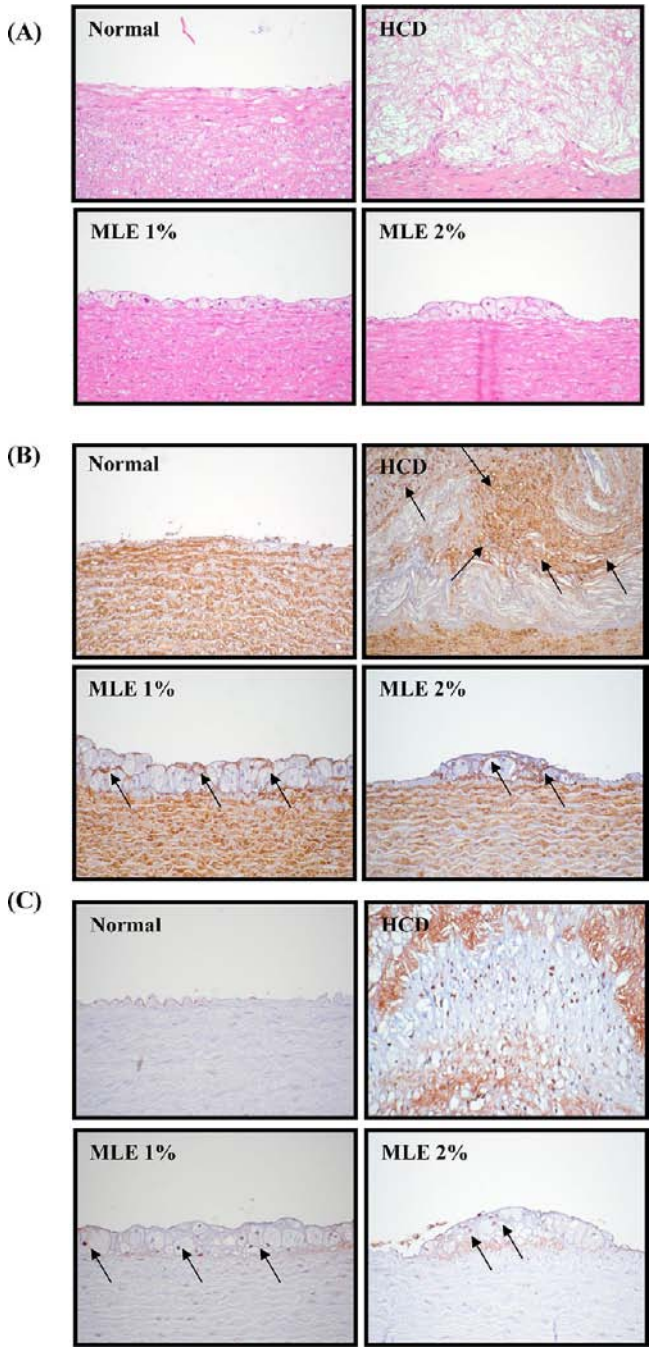

Figure 3. Histological photomicrographs of aortic segments from atherosclerotic HCD-fed rabbits. (A) Hematoxylin and eosin (H&E) stain reveals subintimal deposits of foam cells, extracellular lipids, and thickening of the aortic arch in atherosclerotic HCD-fed rabbits. The subintimal deposition of extracellular lipids and foam cells in HCD-fed rabbits is improved after treatment with MLE. (B) Immunohistochemistry using antibodies against smooth muscle cell α-actin reveals smooth muscle cells located in the aortic segments in atherosclerotic HCD-fed rabbits. (C) Immunohistochemistry using antibodies against proliferating cell nuclear antigen (PCNA) reveals proliferation of cells in the aortic segments of atherosclerotic HCD-fed rabbits (200×). The proliferation and migration of VSMCs are inhibited by MLE in a dose-dependent manner.

Intravascular ultrasound (IVUS) would be a useful way to evaluate the clinical results of MLE treatment on coronary

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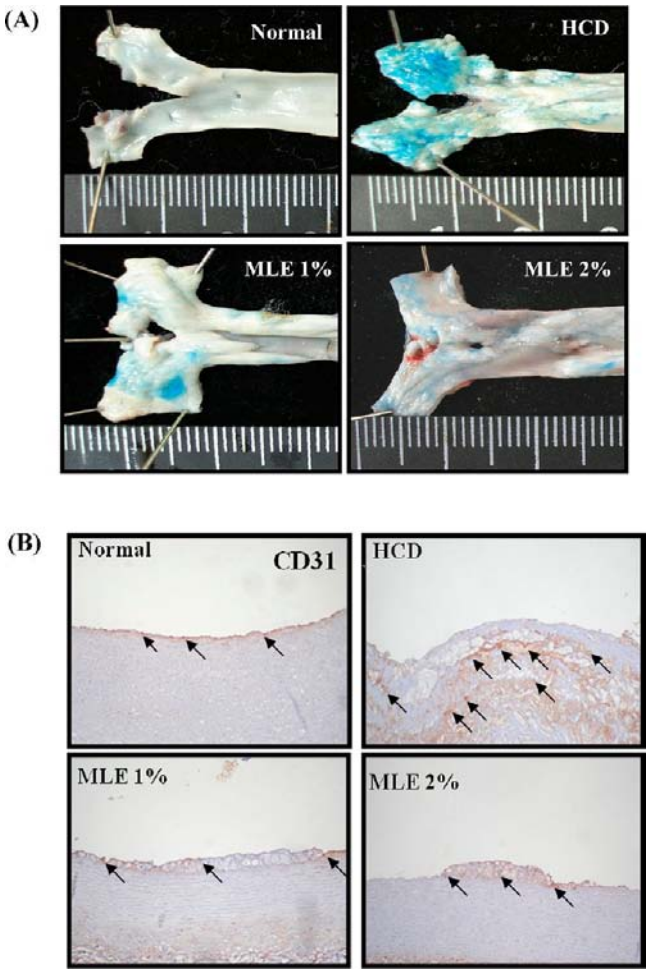

Figure 4. MLE reduces aortic endothelial injury in HCD-fed rabbits. (A) Evans blue stain indicates the endothelial injury site. The aortic segment of HCD-fed rabbits shows strong positive Evans blue staining. The aortic segment of rabbits in the MLE group shows weakly positive Evans blue staining. (B) Immunohistochemical evaluation of CD31 expression in cryostatic sections reveals weak positive staining of endothelial cells in an area far from the branches in this HCD-induced atherosclerotic rabbit. The endothelial layers of the aorta were injured by a high-cholesterol diet in rabbits. After MLE treatment, the endothelial injury improved in a dose-dependent manner.

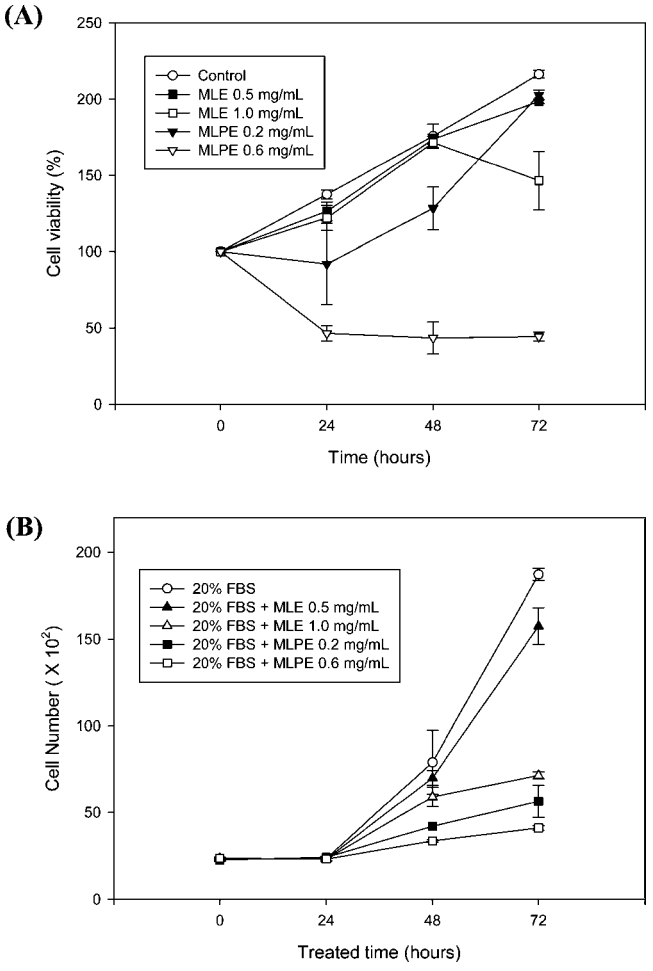

Figure 5. MLE and MLPE reduced proliferation of A7r5 VSMCs as determined by cell counting. (A) Effect of MLE and MLPE on A7r5 VSMCs by MTT assay. A7r5 VSMCs were treated with different concentrations of MLE or MLPE. The culture medium was changed daily. At each indicated time point, cell viability was analyzed by MTT assay. (B) A7r5 VSMCs were seeded into 24-well plates at a density of 2×10^3 cells/well. After 24 h, cells were treated with 20% FBS plus different concentrations of MLE or MLPE, and the culture medium was changed daily. At each indicated time point, the number of viable cells was determined by the trypan-blue exclusion test. Data are the mean ± SD of three independent studies.

atheroma in patients with coronary artery disease in future studies.

Histological Photomicrographs of Aortic Segments from Atherosclerotic HCD-Fed Rabbits. Figure 3 shows that the subintimal deposition of extracellular lipids and foam cells in HCD-fed rabbits is improved after treatment with MLE (A). As shown in Figure 3B,C, VSMCs proliferated and migrated into plaque in the HCD-fed rabbits. MLE treatment inhibited the proliferation and migration of VSMCs in a dose-dependent manner. Our previous studies have shown that the polyphenol-rich MLE can inhibit the proliferation and migration of VSMCs in vitro.[12,13] Our present study further demonstrates that MLE can effectively inhibit the proliferation and migration of VSMCs in vivo, thus improving atherosclerosis.

MLE Reduced Aortic Endothelial Injury in HCD-Fed Rabbits. As shown in Figure 4, the endothelial layers of the aorta were injured by a high-cholesterol diet in rabbits. After MLE treatment, the endothelial injury improved in a dose-dependent manner. Because endothelial dysfunction is the initial step of atherosclerosis, improvements in endothelial function will prevent atherosclerosis.[1,2] Our study has demonstrated that MLE can effectively improve endothelial function in HCD-fed rabbits, thus improving atherosclerosis.

MLE and MLPE Inhibited Proliferation and Migration of A7r5 Aortic VSMCs. Plaques that contain a large lipid core and have a thin fibrous cap are at high risk for rupture. Plaque rupture leads to acute coronary syndrome, which has a high mortality rate. However, the risk of rupture does not appear to depend on the size of the plaque. The stimuli that induce fibrous cap formation probably induce proliferation and migration of vascular smooth muscle cells.[3] The proliferation of VSMCs also plays an important pathophysiological role in restenosis after percutaneous coronary intervention.[45−47] As shown in Figure 5, the proliferation of A7r5 VSMCs was inhibited in a dose-dependent manner by MLE and MLPE in both the 20% FBS-treated group and controls. Figure 6 shows

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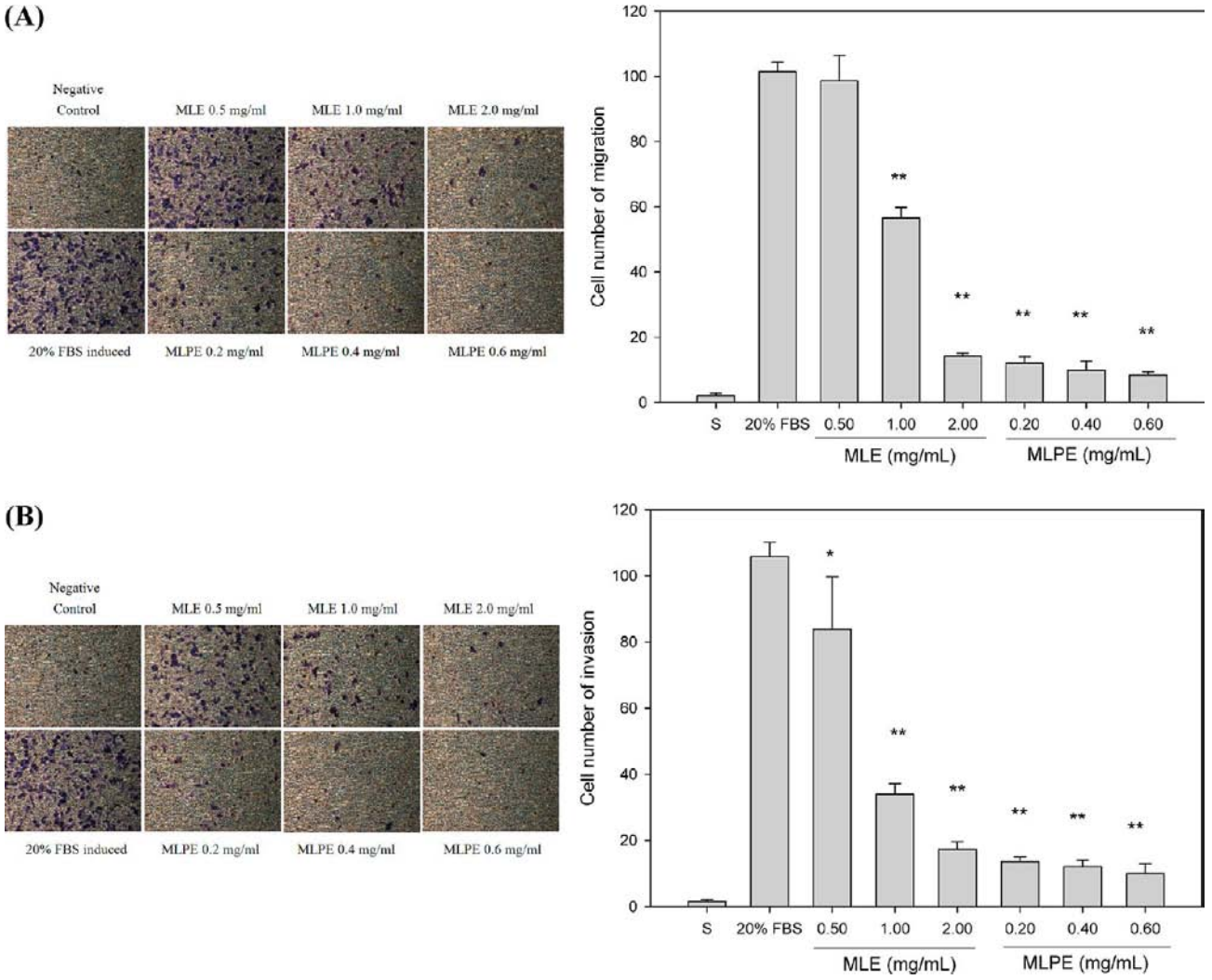

Figure 6. Effect of MLE and MLPE on migration and invasion of A7r5 VSMCs by Boyden chamber assay. A7r5 VSMCs, treated with MLE or MLPE, were plated in the upper chamber of a modified Boyden chamber containing a membrane. The number of cells on the underside of the membrane was quantitated 6 h later under a light microscope. Representative photomicrographs show migrating cells (A) and invading cells (B) assayed by Giemsa stain. Data represent the mean number of cells ± SD from three experiments for each group. (∗) $p < 0.05$, compared with 20% FBS-induced control; (∗∗) $p < 0.01$, compared with 20% FBS-induced control.

that both MLE and MLPE can inhibit migration and invasion of A7r5 VSMCs and that this inhibition is dose-dependent. However, the effect of MLPE is more powerful than that of MLE in inhibiting VSMC proliferation and migration, indicating that the antiatherosclerotic effect of MLPE is more potent than that of MLE. Our results also identify MLPE as the functional component of MLE that inhibits atherosclerosis.

In summary, this in vivo study demonstrates that, in addition to exerting hypolipidemic effects, MLE can effectively inhibit the proliferation and migration of vascular smooth muscle cells, improve vascular endothelial function, and induce atheroma regression. We identified MLPE as the component of MLE that inhibits atherosclerosis. Verification of this in vivo result with a long-term clinical study is necessary to understand the long-term effects of mulberry therapy in preventing atherosclerosis and CAD.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

CAD, coronary artery disease; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; HCD, high-cholesterol diet; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MLE, mulberry leaf extract; MLPE, mulberry leaf polyphenol extraction; MLE, 1% high-cholesterol

diet + 1% MLE; MLE, 2% high-cholesterol diet + 2% MLE; ND, normal diet; VSMC, vascular smooth muscle cell

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