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Automated measurement method for the determination of vitamin E in plasma lipoprotein classes

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In a subendothelial space of atherosclerotic arteries, apolipoprotein B-containing lipoproteins are accumulated and oxidized, and the oxidized lipoproteins promote macrophage foam cell formation. Therefore, the analysis of vitamin E, a major antioxidant in lipoproteins, is important for understanding atherosclerotic pathogenesis. A new method for the automated measurement of vitamin-E (γ - and α -tocopherols) in plasma HDL, LDL, and VLDL was established by using anion-exchange-chromatography for separation of lipoproteins, reverse-phase-chromatography for separation of γ - and α -tocopherols in each of lipoproteins, and fluorescent detection. The within-day assay and between-day assay coefficients of variation for lipoprotein tocopherol levels were 4.73–12.84% and 7.00–14.73%, respectively. The γ - and α -tocopherol/cholesterol ratios of VLDL were higher in healthy plasma than in plasma of untreated patients with dyslipidemia, but the ratios of LDL and HDL were not different. This new estimated method can provide the reliable data of lipoprotein vitamin-E and would be useful for the clinical settings.

Atherosclerosis, that is a chronic inflammatory vascular disease, is the underlying cause of cardiovascular diseases. In the subendothelial space of atherosclerotic vascular lesion, apolipoprotein B (apoB)-containing lipoproteins, including low-density lipoprotein (LDL), are accumulated, and oxidized^{1–5}. Monocytes attach to endothelial cells by way of cell adhesion molecules and chemokines induced by inflammatory cytokines³. The monocytes migrate into the subendothelial space, and differentiate to macrophages. Oxidized LDL (OxLDL) can promote the foam cell formation of macrophages^{1–3,6}. The accumulated foam cells in the subendothelial space contribute to early and advanced atherosclerosis lesions¹.

Vitamin-E, γ - and α -tocopherol in human lipoproteins, are the main lipid-soluble antioxidant. It was previously reported that VLDL and LDL were protected against oxidation through the increased vitamin-E levels in lipoproteins after intake of vitamin-E supplementation^{7–10}. Therefore, Vitamin-E supplementation is thought to prevent LDL oxidation and foam cell formation of macrophages in subendothelial space^{10,11}. In fact, the progression of atherosclerosis in apoE-deficient mice was reduced by supplementation with vitamin-E¹². In some of human trials vitamin-E supplementation reduced the risk of coronary artery disease (CAD), but, in the other trials the supplementation did not reduce or increased the CAD risk¹³. In these human trials the vitamin-E levels in lipoprotein classes were seldom measured, because the classical measurement method for vitamin-E of lipoprotein classes, which consists of ultracentrifugation, extraction with n-hexane, and reverse-phase-chromatography, is greatly complicated^{14,15}.

Here we report a new automated method for the determination of vitamin-E concentrations in plasma HDL, LDL, and VLDL, and the new method can be applied for estimating those levels in plasma lipoproteins from healthy subjects and untreated patients with dyslipidemia.

Results

Strategy of new automated analysis method. Our previous paper shows that lipoprotein classes can be separated by an anion-exchange-chromatography which contains a column packed with diethylaminoethyl-ligand nonporous polymer-based gel and a step gradient of sodium perchlorate concentration^{16,17}. As reported previously, γ - and α -tocopherol concentrations in plasma lipoprotein classes can be measured by using an ultracentrifugation, an extraction with n-hexane and a reversed-phase-chromatography with fluorescence detection¹⁴. A new automated measurement method is composed of an anion-exchange-chromatography for

separation of plasma lipoprotein classes (HDL, LDL and VLDL) and a reversed-phase-chromatography with fluorescence detection for determination of γ - and α -tocopherol concentrations in the separated lipoprotein classes (Fig. 1). Plasma sample (7 μ L) is injected by auto sampler, and then three lipoprotein classes (HDL, LDL and VLDL) are separated on the column packed with diethylaminoethyl-ligand nonporous polymer-based gel (Column1) with two eluents with and without sodium perchlorate, as indicated by Eluent2 and Eluent1, respectively (Fig. 1). The separated lipoprotein classes eluted from the Column1 are mixed with a dilution solution which contains ethanol and detergent (TritonX-100) to apply vitamin-E samples free from lipoprotein particles. Subsequently, the samples are injected into a reversed-phase precolumn (Column2) using a valve (MV1: Mortar valve 1) and a sample loop (2.5 mL), and the vitamin-E components in the samples are trapped in Column2 with the eluent containing 30% ethanol (Eluent3). The Column2 is connected to a reversed-phase column (Column3) by using a valve (MV2: Mortar valve 2). The vitamin-E fractions trapped in Column2 are eluted with the eluent containing 83.5% ethanol (Eluent4), and are injected into a reversed-phase column (Column3). Then, the vitamin-E fractions are separated in Column3 with Eluent4, and fluorescently detected. It takes 55 min to complete the assay of one sample.

Chromatograms. As shown in Fig. 2Ae, the lipoprotein fractions of HDL, LDL, and VLDL, and bottom fraction obtained by ultracentrifugation of one plasma sample were analyzed by the new measurement method, indicating the identification of six peaks in the chromatogram. In the chromatogram of HDL, LDL, and VLDL fraction, two peak were detected between 15–20 min, 30–35 min, and 45–50 min, respectively (Fig. 2Aa–c). In the chromatogram of the bottom fraction, peaks were not detected (Fig. 2Ad). Furthermore, the plasma samples added with γ - or α -tocopherol were analyzed to identify the peak of these tocopherols. Figures 2Ba and 2Bb show the chromatograms of the plasma samples added with γ - or α -tocopherol, respectively, and Figure 2Bc means the chromatogram of the original plasma. Therefore, the earlier peak (peak 1) and the

later peak (peak2) in each lipoprotein fraction indicate the signals of γ - and α -tocopherol, respectively (Fig. 2B).

Performance tests. One plasma (Subject3, male, aged 32 years, with TC = 4.78 mmol/L and TG = 3.61 mmol/L), two plasmas (Subject4, male, aged 53 years, with TC = 4.55 mmol/L, TG = 0.95 mmol/L; Subject5, male, aged 56 years, with TC = 5.02 mmol/L, TG = 1.86 mmol/L), and another plasma (Subject6, male, aged 45 years, with TC = 5.43 mmol/L, TG = 1.83 mmol/L) were subjected to linearity, precision, and recovery tests, respectively. Dilution linear relationships were found between the peak heights of tocopherols of lipoprotein classes and sample dilution ratios in a range of up to 5 times (Supplementary Fig. S1). The values of within-day assay and between-day assay coefficients of variation for the tocopherol concentrations in lipoprotein classes were 4.73–12.84% and 7.00–14.73%, respectively (Supplementary Table S1). For recovery test, HDL, LDL, VLDL fraction obtained from the original plasma by ultracentrifugation were added to the plasma, and these samples were analyzed by the new analysis method. The recovery rates of γ - and α -tocopherol were 92–109 and 90–114%, respectively (Supplementary Table S2). Additionally, γ - and α -tocopherol levels in 10 plasmas were compared with the sum of those levels in three lipoprotein classes obtained by the new analysis method. The recovery rates of γ - and α -tocopherol were $85 \pm 8\%$ and $93 \pm 5\%$, respectively (Supplementary Table S3). The lower limits of detection for γ - and α -tocopherol were 0.21 and 0.55 μ mol/L, respectively, at a signal-to-noise ratio of 3.

These results indicate that this new estimated method can provide the reliable data of γ - and α -tocopherol concentrations in HDL, LDL, and VLDL.

Correlation with classical method. A classical method for measurement of γ - and α -tocopherol in lipoprotein classes is composed of an ultracentrifugation, an extraction with n-hexane, and a reverse-phase-chromatography. The values of γ - and α -tocopherol in lipoprotein classes of 30 plasma samples obtained by the new estimated method were significantly correlated with those values

Figure 1 | Schematic diagram. An anion-exchange chromatography is composed of a column (Column1), two pump (Pump1 and 2), two eluents (Eluent1 and 2), and an auto sampler. The flow rate of the mixed eluent is 0.25 mL/min. The eluents (Eluent1 and 2) contained 0 and 500 mmol/L sodium perchlorate, respectively. The concentrations of sodium perchlorate for elution of HDL, LDL, and VLDL from the column (Column1) are 95, 130, and 165 mmol/L, respectively. The HDL, LDL, and VLDL fractions were mixed with a dilution solution (70% ethanol + 1% TritonX-100). The flow rate of Pump3 is 0.25 mL/min. The samples mixed with the dilution solution are injected into Column2 by changing MV1 from OFF-position to ON-position. A reversed-phase chromatography is composed of two column (Column2 and 3) which packed with octadecyl-ligand porous silica-based gel, 2 pumps (Pump4 and 5), two eluents (Eluent3 and 4), two mortar valves (MV1 and 2), and fluorescent detector. The eluents (Eluent1 and 2) contain 30.0 and 83.5% ethanol, respectively. Each flow rate of Pump4 and 5 is 1.5 mL/min.

Figure 2 | Chromatograms of HDL, LDL, VLDL, bottom fraction and plasmas spiked with γ - and α -tocopherols. (A). The chromatograms a, b, c, and d are the fractions of HDL ($1.063 < \text{density} < 1.210 \text{ g/mL}$), LDL ($1.019 < \text{density} < 1.063 \text{ g/mL}$), VLDL ($\text{density} < 1.019 \text{ g/mL}$, and flotation rate 200–400 in a solution of 1.745 mol/L sodium chloride), and bottom fraction ($1.210 \text{ g/mL} < \text{density}$). The chromatogram e is plasma (Subject1, male, age 46, total cholesterol = 5.22 mmol/L , triglyceride = 0.86 mmol/L). (B). The chromatograms a, b, c are plasma samples spiked with γ - and α -tocopherol and original plasma (Subject1), respectively. The samples for b or c are mixture (1 : 1) of the original plasma and γ - or α -tocopherol solution, 0.06 mmol/L , respectively.

measured by the classical method (Fig. 3). The satisfactory correlations between the data of the two different methods support the usefulness of the new estimated method for determination of two tocopherol levels in HDL, LDL, and VLDL.

Additionally, the levels of γ - and α -tocopherol/cholesterol ratio in LDL were compared with the lag-time of LDL oxidation with $2 \mu\text{mol/L}$ CuSO_4 . The lag-time of LDL oxidation was significantly correlated with γ - and α -tocopherol/cholesterol ratio in LDL [$r = 0.305$ ($p < 0.05$) and $r = 0.330$ ($p < 0.05$), respectively] (Supplementary Fig. S2).

Estimation of γ - and α -tocopherol levels in volunteer subjects.

Table 1 shows the characteristic data and tocopherol levels obtained by the new estimated method in 69 volunteer subjects without drug treatment. Healthy subjects ($n = 38$) and dyslipidemic patients ($n = 31$) were grouped following the Guidelines 2012 of Japanese Atherosclerosis Society. The dyslipidemic patients contained 4 patients with hypertension diagnosed by the criteria of the Guidelines 2009, Japanese Society of Hypertension. The γ - and α -tocopherol/cholesterol ratios of VLDL were higher in the healthy subjects than in the dyslipidemic patients, but the γ - and α -tocopherol/cholesterol ratios of LDL and HDL were not different between them.

Discussion

OxLDL is generated from native LDL accumulated in the subendothelial space of arterial wall, and can cause the foam cell formation

of macrophages and atherosclerotic plaques^{1–3}. The LDL oxidation can be induced in vitro by incubation with cultured vascular cells or copper ion as a catalyst, and measured by continuously monitoring conjugated-diene formation^{18,19}. The susceptibility to LDL oxidation can be indicated by lag-time as the intercept of the tangent of the slope in the monitored curve^{18,19}. Vitamin-E supplementation was previously reported to increase the vitamin-E content of LDL, and significantly make the lag-time of LDL oxidation longer compared with baseline LDL⁹. Meanwhile, OxLDL is not one homogenous entity but contains many different types of chemical and immunogenic modifications of lipids and apoB. OxLDL assays developed as cardiovascular biomarker employ different monoclonal antibodies, mAb-DLH3, mAb-E06, mAb-4E6, and mAb-1H11, of which epitopes are oxidized phosphatidylcholine, oxidized phosphorylcholine, aldehyde-apoB obtained by incubation with copper ion, or malondialdehyde-apoB, respectively^{20–22}. OxLDL levels measured with mAb-4E6-based assay in stable angina patients were significantly higher than in control subjects, but OxLDL levels measured with mAb-1H11-based assay in stable angina patients were similar to those in control subjects²². The oxidized LDL level is important as a useful biomarker for CAD, but there would be several differences and limitations because of a variety of immunoassays.

Vitamin-E is one of major lipid-soluble antioxidants of lipoproteins. Therefore, the antioxidant level of LDL in blood circulation may be clinically useful as well as the OxLDL level to estimate the risk of atherosclerosis progression. The classical method for measurement of vitamin-E in LDL class consists of ultracentrifugation,

Figure 3 | Correlation between the values of γ - and α -tocopherol in lipoprotein classes obtained by the new estimated method and by the classical method. Plasmas of 30 subjects are analyzed by the new estimated method and classical method with ultracentrifugation, extraction of n-hexane, and reverse-phase chromatography. The data of 30 subjects are as follows: male only, age = 43.2 ± 10.4 year, TC = 5.05 ± 0.67 mmol/L, TG = 1.28 ± 0.94 mmol/L.

extraction with n-hexane, and reverse-phase-chromatography, which is very complicated¹⁴. Haidari et al. showed that vitamin-E levels measured by the classical method were higher in LDL of controls than those in LDL of patients with CAD¹⁴. The present paper demonstrates the accurate and convenient automated method for measurement of vitamin-E in lipoprotein classes. By this new method, vitamin-E levels not only in LDL but also in HDL and VLDL can be measured and only 7 μ L volume of plasma is required for one assay. Vitamin-E levels obtained by this new automated method were significantly correlated with those by the classical method ($r = 0.785$ – 0.951). Carr et al. reported that the recovery rate of sum of three lipoprotein classes was $88 \pm 2\%$ in a method using gel-filtration chromatography for measurement of α -tocopherol levels in HDL, LDL, and VLDL, as compared with the plasma level²³. The recovery rate of sum of α -tocopherol in three lipoprotein classes

in our new method was showed $93 \pm 5\%$, respectively (Supplementary Table S3). However, the vitamin-E levels of LDL were significantly but modestly correlated with the LDL lag-time in copper ion-induced oxidation expressing the resistance to LDL oxidation (Supplementary Fig. S2). The previous report showed that an increased consumption of lycopene-rich vegetables elevated the lycopene levels of LDL, and provided the increased resistance to LDL oxidation²⁴. The resistance to LDL oxidation would be dependent on the other antioxidants, such as lycopene, in addition to vitamin-E.

A previous paper indicated that α -tocopherol level in VLDL of healthy male was increased by a 15% low-fat, high-carbohydrate diet feeding, and the clearance rate was decreased²⁵. The α -tocopherol levels per VLDL particle were approximately 66 mol. Moreover, it was known that the levels per LDL particle were 8–12 mol²⁵. Another paper reported that the α -tocopherol level/cholesterol ratios of LDL

Table 1 | Characteristic data and tocopherol levels in volunteer subjects

	Total (n = 69)	Healthy (n = 38)	Dyslipidemia (n = 31)	P value Healthy vs dyslipidemia
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (year)	39.4 ± 8.0	39.4 ± 9.1	39.5 ± 6.6	NS
BMI (kg/m ²)	22.9 ± 2.6	21.7 ± 2.1	24.4 ± 2.4	P < 0.00001
Systemic blood pressure (mmHg)	110.9 ± 12.1	108.0 ± 10.9	114.6 ± 114.6	P < 0.05
Diastolic blood pressure (mmHg)	71.6 ± 8.9	68.8 ± 7.2	75.0 ± 9.7	P < 0.001
Total cholesterol (mmol/L)	5.06 ± 0.83	4.55 ± 0.59	5.70 ± 0.70	P < 0.00001
Triglyceride (mmol/L)	1.37 ± 1.79	0.68 ± 0.25	2.21 ± 2.42	P < 0.00001
HDL cholesterol (mmol/L)	1.49 ± 0.37	1.64 ± 0.34	1.31 ± 0.34	P < 0.0005
LDL cholesterol (mmol/L)	3.30 ± 0.90	0.87 ± 0.53	3.93 ± 0.87	P < 0.00001
VLDL cholesterol (mmol/L)	0.44 ± 0.61	0.19 ± 0.09	0.74 ± 0.81	P < 0.00001
γ-tocopherol in HDL (mmol/mol-cholesterol)	0.62 ± 0.19	0.59 ± 0.16	0.65 ± 0.23	NS
α-tocopherol in HDL (mmol/mol-cholesterol)	5.20 ± 0.84	5.20 ± 0.83	5.20 ± 0.87	NS
γ-tocopherol in LDL (mmol/mol-cholesterol)	0.32 ± 0.11	0.32 ± 0.09	0.32 ± 0.13	NS
α-tocopherol in LDL (mmol/mol-cholesterol)	3.23 ± 0.60	3.25 ± 0.62	3.20 ± 0.60	NS
γ-tocopherol in VLDL (mmol/mol-cholesterol)	1.74 ± 0.84	2.18 ± 0.79	1.20 ± 0.53	P < 0.00001
α-tocopherol in VLDL (mmol/mol-cholesterol)	12.74 ± 3.88	13.83 ± 4.49	11.39 ± 2.40	P < 0.05
γ/α-tocopherol in HDL (mol/mol)	0.116 ± 0.034	0.110 ± 0.030	0.122 ± 0.038	NS
γ/α-tocopherol in LDL (mol/mol)	0.097 ± 0.029	0.095 ± 0.025	0.098 ± 0.034	NS
γ/α-tocopherol in VLDL (mol/mol)	0.133 ± 0.055	0.158 ± 0.052	0.103 ± 0.041	P < 0.00005

NS indicates not significant.

in CAD and control were 2.4 ± 1.0 and 2.9 ± 1.1 mmol/mol¹⁴. We estimated the ratio of γ- and α-tocopherol levels in HDL, LDL, and VLDL to cholesterol levels in each of lipoprotein classes, and those levels in total volunteers were 0.62 ± 0.19 , 5.20 ± 0.84 , 0.32 ± 0.11 , 3.23 ± 0.60 , 1.74 ± 0.84 , and 12.74 ± 3.88 mmol/mol, respectively (Table 1). We also showed that the γ- and α-tocopherol/cholesterol ratios of VLDL were higher in the healthy subjects than in the dyslipidemic patients without drug treatment (Table 1).

A lot of clinical studies for CAD prevention with intake of vitamin-E supplementation were reported, but its beneficial effect is controversial¹³. As a possible reason for such uncertain effects of vitamin-E, the combination drug, including antihypertensive drugs, i.e., angiotensin-converting-enzyme (ACE) inhibitor, beta-blocker, and angiotensinII-receptor blocker (ARB), might interfere with vitamin-E effect. These blood pressure lowering agents normalized blood pressure levels, while lag-time of LDL oxidation or vitamin-E contents in LDL were increased^{26,27}. Therefore, some of wide-used antihypertensive drugs would have the effect of antioxidant, and increase the vitamin-E levels in lipoproteins during the administration. The controversial effects of vitamin-E intake for CAD prevention would be resolved by adding data of vitamin-E levels in lipoprotein classes, measured by the new convenient method established in the present study.

In conclusion, we showed that γ- and α-tocopherols of HDL, LDL, and VLDL in human plasma can be analyzed by using anion-exchange-chromatography, reverse-phase-chromatography, and fluorescent detection. The new method was validated by examining its linearity, precision, and recovery and by the correlation of values obtained by the new method with those by a classical method composed of an ultracentrifugation, an extraction with n-hexane, and a reverse-phase-chromatography. These results suggest that this developed method is suitable for accurate and convenient evaluation of γ- and α-tocopherol levels in lipoprotein classes in clinical studies.

Methods

Subjects. The all subjects were the volunteers from employees of the Tokyo Research Center of Tosoh Corporation. At entry, written informed consent was obtained from all volunteers. The study protocol was approved by the ethical committee of the Jikei University and Tosoh Corporation.

The condition of new analysis method. An anion-exchange column (Column1: DEAE-NPR, 4.0 mmID × 15 mm in size, Tosoh Corp., Japan), two eluents (Eluent1: 50 mmol/L Tris-HCl + 1 mmol/L EDTA2Na pH7.5, Eluent2: 50 mmol/L Tris-HCl + 1 mmol/L EDTA2Na + 500 mmol/L sodium perchlorate pH7.5) were used for anion-exchange-chromatography. The step gradient patterns for separation of the lipoprotein classes were 19% Eluent2 for 0–2 min, 19–10% linear gradient of Eluent2 for 2–5 min, 10% Eluent2 for 5–15 min, 26% Eluent2 for 15–17 min, 26–10% linear gradient of Eluent2 for 17–20 min, 10% Eluent2 for 20–30 min, 33% Eluent2 for 30–32 min, 33–10% linear gradient of Eluent2 for 32–35 min, 10% Eluent2 for 35–43 min, 100% Eluent2 for 43–45 min, 100–10% linear gradient of Eluent2 for 45–52 min, 10% Eluent2 for 52–60 min.

A reversed-phase precolumn (Column2: ODS-80Ts, 4.6 mmID × 35 mm in size, Tosoh Corp., Japan), a reversed-phase analytical column (Column3: ODS-80Ts, 4.6 mmID × 75 mm in size, Tosoh Corp., Japan), two eluents (Eluent3: 25 mmol/L sodium perchlorate + 30.0% ethanol, Eluent4: 25 mmol/L sodium perchlorate + 83.5% ethanol + 0.025 mmol/L perchlorate acid), and fluorescence detector (FS-8020, Tosoh Corp., Japan) were used for reversed-phase-chromatography.

A sample loop and Column2 were in series at ON-position of MV1. The position of MV1 is ON for 5.3–14.3 min, 20.3–29.3 min, and 35.3–59.3 min, and OFF for the other periods. Each of the broken lipoprotein samples, HDL, LDL, and VLDL was injected into Column2 for 5.3–14.3 min, 20.3–29.3 min, and 35.3–59.3 min, respectively. Column2 and 3 were in series at ON-position of MV2, and each of vitamin-E fractions in lipoprotein classes which bound on Column2 was injected into Column3. The position of MV2 is ON for 7.3–16.3 min, 22.3–31.3 min, and 37.3–61.3 min, and OFF for the other periods. Each of the vitamin-E fractions in HDL, LDL, and VLDL was injected into Column3 for 7.3–16.3 min, 22.3–31.3 min, and 37.3–61.3 min, respectively.

Ultracentrifugation. Sequential ultracentrifugation of plasma lipoproteins was performed by the method reported previously^{28,29}. Densities of chylomicrons + VLDL, HDL, LDL, and bottom fraction were set as follows: density < 1.019 g/mL, $1.063 < \text{density} < 1.210$ g/mL, $1.006 < \text{density} < 1.063$ g/mL, and $1.210 \text{ g/mL} < \text{density}$, respectively. The flotation rates of chylomicrons and VLDL were set at >400, and 20–400, respectively, in a solution of 1.745 mol/l sodium chloride ($d = 1.063$ g/mL). An SCP70H2 ultracentrifuge (Hitachi Koki Co., Japan) and an RP55T angle rotor (Hitachi Koki Co., Japan) were used.

Classical method and analysis of LDL oxidation lag-time. The classical method reported previously³⁰ was partially modified. The plasma and the fractions of HDL ($1.063 < \text{density} < 1.210$ g/mL), LDL ($1.019 < \text{density} < 1.063$ g/mL), and VLDL ($\text{density} < 1.019$ g/mL, and flotation rate 200–400 in a solution of 1.745 mol/L sodium chloride) were obtained from plasma samples by ultracentrifugation, and added with δ-tocopherol as internal standard. The organic layer obtained by extraction of n-hexane was evaporated under a stream of nitrogen, and redissolved in ethanol. The tocopherols samples were analyzed by reverse-phase-chromatography with a column (ODS-80Ts, 4.6 mmID × 150 mm in size, Tosoh Corp., Japan), an eluent (25 mmol/L sodium perchlorate + 83.5% ethanol + 0.025 mmol/L

perchlorate acid), and fluorescence detector (FS-8020, Tosoh Corp., Japan, excitation 295 nm, emission 330 nm).

The in vitro oxidation of LDL was reported previously^{21,32}. The dialyzed LDL fraction (1.019 < density < 1.063 g/mL, 0.05 g protein/L) in EDTA free phosphate buffer was added CuSO₄ solution (final concentration 2 μmol/L), and incubated at 37°C. Conjugated diene formation during LDL oxidation was monitored by changed in wavelength absorbance at 234 nm.

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Author contributions

Y.H. conceived the project, designed the experiments, analyzed the data wrote the manuscript, and prepared figures; H.Y. contributed to the project and the study design, and wrote the manuscript; H.K. and N.T. contributed to the study design and the manuscript editing; D.M. analyzed the data, and contributed to the manuscript editing.

Additional information

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