We studied the antioxidative action to evaluate the effect of citrus essential oil components on human LDL in vitro. Among the authentic volatile compounds tested, γ-terpinene showed the strongest antioxidative effect, and inhibited both the Cu²⁺-induced and AAPH-induced oxidation of LDL. γ-Terpinene added after 30 min (mid-lag phase) and 60 min (propagation phase) of incubation of LDL with Cu²⁺ inhibited LDL oxidation.

Key words: antioxidative effect; low-density lipoprotein; γ-terpinene

It is widely accepted that oxidative modification of plasma lipoproteins, particularly low-density lipoprotein (LDL), plays an important role in the initiation of atherosclerosis. Oxidatively modified LDL within arterial walls or in the serum is believed to be readily taken up by macrophages leading to the formation of lipid-laden foam cells. It has been reported that natural antioxidants such as α-tocopherol, ascorbate, β-carotene, lycopene and epigallocatechin gallate (EGCG) inhibited the oxidative modification of LDL in vitro.

On the other hand, in a recent study of citrus essential oils, Choi et al. have reported that the oils or their components had DPPH radical-scavenging activity. However, there are no reports concerning the prevention of oxidative modification of LDL.

We therefore studied the antioxidative effects of three citrus essential oils and their components on human LDL in vitro. Human LDL was purchased from Sigma (St. Louis, MO, USA) and stored at 4°C in the dark in a nitrogen gas-saturated vial. The LDL solution obtained was dialyzed in order to remove EDTA in a 1000-fold volume of 100 mM PBS at pH 7.4 containing 160 mM NaCl and 0.1 mg/ml of chloramphenicol at 4°C overnight, this dialyzed LDL being used within 2 hours. The protein concentration of LDL was measured by the method of Lowry et al.

LDL (0.1 mg of protein/ml) was prepared by diluting the dialyzed LDL solution with 100 mM PBS at pH 7.4 containing 160 mM NaCl, and the resulting LDL solution was incubated with 2 mM CuSO₄ or 4 mM AAPH at 37°C in the presence or absence of 1, 10, or 100 μg/ml of a sample dissolved in DMSO (final concentration < 0.5%). The mixtures each formed a complete suspension without oil separation. These experiments were conducted two or three times, and the results obtained show almost the same trend. Typical results are indicated in the figures presented. Three citrus essential oils were supplied by Wakayama Agricultural Cooperatives Momoyama fruit juice factory (Wakayama, Japan). γ-Terpinene was purchased from Tokyo Kasei Co., and authentic compounds were purchased from Tokyo Kasei Co. and Wako Pure Chemical Industries (Osaka, Japan). These compounds were used without further purification. LDL peroxidation was fluorometrically determined as thiobarbituric acid reactive substances (TBARS) and/or measured by the absorbance at 234 nm to determine conjugated diene formation.

As a preliminary study, we first examined the antioxidative effect of three citrus essential oils on the Cu²⁺-induced oxidation of LDL. After 180 min of incubation at a concentration of 100 μg/ml of oil, the TBARS formation had decreased to 27.6% (Natsudaidai essential oil), 47.8% (Hassaku), and 69.5% (Unshiu mikan) as compared to that for the Cu²⁺-induced oxidation of LDL (data not shown). Therefore, in order to clarify the antioxidative components of the essential oils, we compared the antioxidative effects of the essential oil components on both the Cu²⁺-induced and AAPH-induced oxidative modification of LDL, as it is generally not clear which of these conditions is more relevant to human LDL oxidation in vivo. Authentic compounds were used as oil components, and as shown in Fig. 1(A), γ-terpinene (2.9 nmol MDA/mg of protein) most markedly inhibited TBARS formation with Cu²⁺-induced LDL oxidation among the 21 authentic compounds examined. Terpinolene (12.1 nmol MDA/mg

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; AAPH, 2,2’-azobis(2-aminopropane)hydrochloride; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MDA, malondialdehyde
Fig. 1. Inhibitory Effects of Authentic Compounds on Cu²⁺- and AAPH-Induced TBARS Formation from LDL.
LDL (0.1 mg of protein/µl in PBS) was incubated with 2 mM CuSO₄ (panel A) or 4 mM AAPH (panel B) at 37°C for 3 h in the presence or absence of 10 µg/ml of an authentic compound.

Fig. 2. Inhibitory Effect of γ-Terpinene on Cu²⁺-Induced Conjugated Diene and TBARS Formation from LDL.
LDL (0.1 mg of protein/µl in PBS) was incubated with 2 µM CuSO₄ at 37°C in the presence (●) or absence (□) of 2 µg/ml (14.7 µM) of γ-terpinene. After 30 min of incubation, 0.2 µg/ml (1.47 µM, ○) or 2 µg/ml (▲) of γ-terpinene was added to the control (□), and after 60 min of incubation, 0.2 µg/ml (□) or 2 µg/ml (▲) of γ-terpinene was added to the control. Conjugated diene (panel A) and TBARS formation (panel B) were measured every 30 min for 5 h.

of protein) and α-terpinene (13.2 nmol MDA/mg of protein) also inhibited TBARS formation. Similar to TBARS formation from the Cu²⁺-induced oxidation of LDL, γ-terpinene (6.8 nmol MDA/mg of protein) also markedly inhibited TBARS formation from the AAPH-induced oxidation of LDL, as shown in Fig. 1(B). However, terpinolene (27.1 nmol MDA/mg of protein) and α-terpinene (29.2 nmol MDA/mg of protein) did not inhibit TBARS formation from the AAPH-induced oxidation of LDL. In contrast, although bisabolene (11.5 nmol MDA/mg of protein) inhibited TBARS formation from the AAPH-induced oxidation of LDL, it (41.8 nmol MDA/mg protein) did not inhibit TBARS formation from the Cu²⁺-induced oxidation of LDL. Choi et al. have recently reported that geraniol, terpinolene, and γ-terpinene showed marked scavenging activity against DPPH. Although there are differences between the DPPH radical scavenging activity and the antioxidative activity against Cu²⁺ or AAPH oxidation, the antioxidative effects of terpinolene and γ-terpinene are consistent with our results. However, geraniol showed no antioxidative activity against either Cu²⁺-induced or AAPH-induced oxidation of LDL.

Yamanaka et al. have reported that 5 µM caffeic acid inhibited LDL oxidation during the initiation phase, and that 0.5 µM caffeic acid accelerates LDL oxidation in the propagation phase. We therefore examined the prooxidative effect of γ-terpinene on LDL. As shown in Fig. 2(A), when 2 µg/ml (14.7 µM) of γ-terpinene was added at the middle of the lag phase (after 30 min of incubation of LDL with Cu²⁺), LDL oxidation was inhibited. Two µg/ml (14.7 µM) of γ-terpinene inhibited LDL oxidation even in the propagation phase (after 60 min of incubation). Moreover, the addition of 0.2 µg/ml (1.47 µM) of γ-terpinene in the middle of the lag phase showed little antioxidative effect, and did not
accelerate LDL oxidation in the propagation phase. As shown in Fig. 2(B), similar results were obtained in the formation of TBARS. These findings therefore suggest that γ-terpinene exerted no prooxidative effect in either the lag or propagation phase.

We found for the first time that γ-terpinene generated an antioxidative effect on both the Cu²⁺-induced and AAPH-induced oxidation of human LDL in vitro, and that γ-terpinene inhibited LDL oxidation even in the propagation phase. Therefore, the supplementation of γ-terpinene to foodstuffs and beverages may play an important role in preventing LDL oxidation. Further atherosclerotic studies involving γ-terpinene with animal models are currently in progress.

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References


