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Antioxidative Effects of *Cichorium intybus* Root Extract on LDL (Low Density Lipoprotein) Oxidation

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The water extract of *Cichorium intybus* (WECI) showed a remarkable antioxidative effect on LDL, and inhibitory effects on the production of thiobarbituric acid reactive substance and the Degradation of fatty acids in LDL. Vitamin E and unsaturated fatty acids in LDL were protected by adding WECI from the effects of metal catalyzed LDL oxidation. From the results obtained, we conclude that LDL oxidation is inhibited *in vitro* by the addition of WECI, and that LDL is protected by WECI from oxidative attack, as shown by agarose gel electrophoresis.

Key words: Antioxidative activity, *Cichorium intybus* (Compositae), LDL oxidation

INTRODUCTION

Chicory, the dried root of *Chicorium intybus* Linne (Compositae), is used as a folk remedy treatment for gallstones, hepatitis, and jaundice, and as a tonic and an additive that enhances bitterness flavor, color and form of coffee (Lim, 1996). The antihepatotoxic effects of the root callus extracts were found to protect against carbon tetrachloride induced hepatocellular damage (Zafar and Mujahid, 1998), its water soluble extract improved lipid metabolism (Kim and Shin, 1998), chicory fructan was found to have potential colon tumor inhibitory properties (Reddy, 1999), and its methanol extract was reported to have anti-inflammatory activity (Ki *et al.*, 1999) have been. Chicory contains the bitter sesquiterpenoid lactones, lactucin and intybin (Khalil *et al.*, 1991) as well as cichoriin (coumarin glucoside).

Oxidative modification of LDL has been shown to be associated with increased atherogenicity, which includes cytotoxicity to arterial wall cells and the stimulation of homostatic and thrombotic processes, and the secretion of cytokines and growth factors from cells of the arterial wall (Sato and Shimasaki, 1990; Luc and Fruchart, 1991). An important feature of oxidized LDL is its enhanced uptake by macrophages, which results in cellular cholesterol accumulation and foam cell formation, the hallmark of an early atherosclerotic lesion (Fielding, 1992; Caslake *et al.*, 1992). LDL oxidation can be achieved *in vitro* by incubating cells of the arterial wall, including endothelial cells, smooth muscle cells, and macrophages with lipoprotein (Steinbrecher *et al.*, 1989; Palinski and Rosenfeld, 1989).

The copper-mediated oxidation of LDL has properties similar to the endothelial cell-mediated oxidation of LDL that differs in several ways from native LDL in this respect (Esterbauer *et al.*, 1990). Cranberry extract was also found to inhibit low density lipoportein oxidation (Ted *et al.*, 1998)

In this paper, the antioxidative effects of water, chloroform and hexane extracts from Chicory root were compared with respect to LDL oxidation, and changes in the lipid components of LDL with or without the addition of the above extracts.

MATERIALS AND METHODS

Materials

2-thiobarbituric acid (TBA), trypsin inhibitor, aprotinin, 2,4,6-trinitrobenzene sulfonicacid (TNBS) and 1,1,3,3-tetramethoxypropane (TMP) were purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Barbital sodium was purchased from Junsei Chemical Co. (Tokyo, Japan).

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Trichloroacetic acid (TCA) was obtained from Janssen Chimica (Geel, Belgium).

Extraction of *Cichorium intybus* root

The root of *Cichorium intybus* was purchased from the *ZinBuRyung culture garden, KangWon-Do* and authenticated by Dr. Tae Hee Kim, College of Pharmacy, University of Sookmyung Women's University. The voucher specimen (SPH99010) was deposited in the authors laboratory. Dried roots of C. intybus (1 kg) were extracted with water, chloroform and hexane under reflux for 5 h. Filtration of the extract obtained and its evaporation under reduced pressure yielded water (175.6 g), chloroform (98.2 g) and hexane (83.5 g) extracts.

Isolation of low density lipoproteins

The blood from healthy donors was collected in sterilized bags containing 0.2 M EDTA, 2.5 % sodium azide, 2000 U/ml aprotinin and 0.3 M NaCl. Plasma was obtained by centrifugation of the fresh blood at 3000 rpm for 30 min at 4°C. Aprotinin (0.055 units/ml), 0.05% EDTA and 0.05 % NaN3 were added to the plasma to prevent coagulation and further hydrolysis. The LDL, $1.025<d<1.055$ g/ml, was isolated by sequential ultracentrifugation using a KBr gradient at 40,000 rpm for 15 h at 4°C with a Beckmam 50.2 Ti rotor, followed by one more run for 24 hrs under the same conditions (Yang *et al.*, 1989). The protein concentration of the LDL was determined using a modification of the method of Lowry (Lowry and Farr, 1951). Purified LDL was dialyzed against 0.1 M ammonium bicarbonate (NH4HCO3), buffer at pH 8.0 using a No.2 dialysis bag (Spectra/por). The purification of LDL was verified by 5-14% SDS-PAGE.

Delipidation of LDL

LDL (50 mg) was delipidated by ether/ethanol (3:1, v/v). Each extraction was carried out by adding the ether/ethanol to the sample, vortexing, placing the sample in a freezer for one hour, centrifuging at 2,000 rpm for 20 min, and finally removing the solvent under vaccum.

Gas chromatography of fatty acid

The analysis of fatty acid composition was performed using a gas chromatograph (Hitachi model 163) equipped with a flame ionization detector. A glass column (200 × 3 mm) containing GP 3% SP-2310/2% SP-2300 on 100/120 Chromosorb was used for the GLC analysis (Choi *et al.*, 1994). The temperature characteristics were: injector, 220°C : column, 130°C for 1 min. and detector, 220°C. The carrier gas flow rate was 575 ml/min (nitrogen, 39 ml/min., hydrogen, 36 ml /min., and air, 500 ml/min.

Oxidation of LDL

LDL was incubated with cupric sulfate(16 uM) at a concentration 0f 400 μg/ml protein of LDL in LDL 1 ml PBS in the presence and absence of Cichorium intybus at 37°C.

The extent of lipid peroxidation was measured by determining the quantity of thiobarturic acid reactive substances (TBARS) and expressed as MDA equivalents (Esterbauer et *al.*, 1990). 1.0 ml of 25% trichloroacetic acid (TCA) was added to each extract (100 μg/ml), followed by 1.0 ml of 1% TBA. The samples were vortexed and incubated at 95°C for 50 min., and were then centrifuged (1000 × g) for 15 min. The supernatant was analyzed by UV-spectrophotometer at 532 nm. A solution of freshly diluted 1,1,3,3-tetramethoxypropan (TMP) was used as a standard.

Agarose gel electrophoresis of LDLs

To identify the oxidized-LDLs by their increased electrophoretic mobility, agarose gel electrophoresis was carried out on 0.7% agarose gel in 0.05 M barbital buffer, pH 8.6 (Yang and Shim, 1997).

Determination Vitamin E in oxidatively modified LDL by Cu^{+2}

The amount of vitamin E in oxidized LDL was tested on a Water's HPLC system equipped with a variable wavelength detector, and a sperisorb-ODS2 column (150 × 4.6 mm) eluted with 7% dichloromethane in methanol at a flow rate of 1.0 ml/min. The eluted fractions were monitored at 292 nm with a UV detector (Jessup *et al.*, 1990).

RESULTS AND DISCUSSION

Prevention of LDL peroxidation by *Chicorium intybus* extracts

Cichorium intybus dried root was extracted with water, chloroform and hexane to determine the inhibitory effects of LDL peroxidation. Each extract was added to LDL and the susceptibility of LDL to copper mediated oxidation was compared with and without the addition of *Cichorium intybus* extracts. The level of thiobarbituric acid reactive substances (TBARS) was measured during the oxidation. Changes in the extracts oxidation patterns are shown in Fig. 1. The rate of TBARS formation was remarkably lower after the addition of the water extract than in the control. The water extract of *Cichorium intybus* showed the highest antioxidative activity on LDL oxidation, and this was followed in order by the chloroform and the hexane extracts. The decrease in the level of TBARS formed in LDL caused by the water extract over a 4 h period is probably due to its inhibitory effect upon the degradation of hydroperoxy fatty acids to alde-

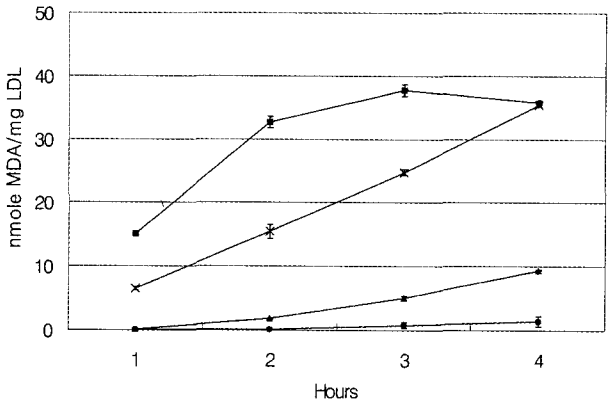

Fig. 1. TBARS formation in LDL from several *Cichorium* extracts *intybus*. Each extract (100 μg/ml) was added to LDL. Data points represent mean. Key; ■ No Extract, × Hexane Extract, $CHCl_3$ Extract, ● Water Extract

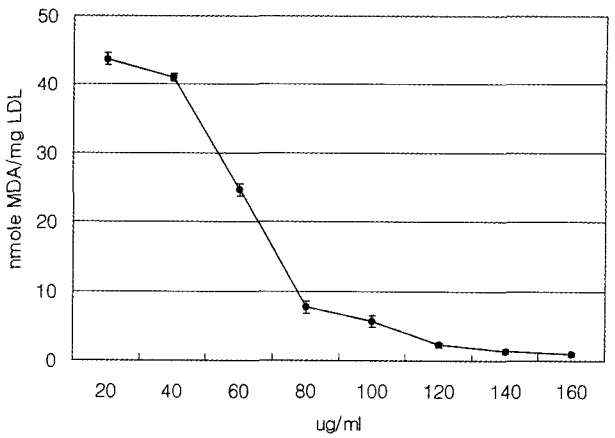

Fig. 2. Inhibition of TBARS formation in LDL on increasing the amount of *Cichorium intybus* water extract. Data are presented as the means.

hydes (Esterbauer *et al.*, 1989, Jurgens *et al.*, 1987).

Various lines of research provide strong but inconclusive evidence that LDL may become oxidized *in vivo*, and that oxidized LDL is involved in the formation of early atherosclerotic lesions. The early atherosclerotic lesion is characterized by a massive accumulation aggregation of cells filled with lipid droplets, which consist of cholesterol and cholesterol esters (Esterbauer *et al.*, 1989; Jurgens *et al.*, 1987). Moreover, because of their foamy appearance such cells are called foam cells (Brown, 1992). In culture, macrophages take upnative LDL only slowly, and only if incubated over long periods at high LDL concentrations. However, macrophages do not accumulate cholesterol esters and transform to lipid-laden cells. It is currently believed that post secretory modifications of LDL may render the lipoprotein more atherogenic. Studies have suggested that oxidatively modified LDL may represent one such modified form of LDL (Fogelman *et al.*, 1988).

Cell-mediated modification of LDL can be reproduced by incubating the lipoprotein with redox-active metal ions such as Cu^{+2}. This leads to similar changes in the physicochemical properties of LDL, which include increased density, decreased content of esterified cholesterol, and the fragmentation of apolipoprotein B-100 (apo B-100) (Steinbrecher *et al.*, 1987, 1989).

Fig. 2 shows the quantity of water extract of *Chicorium intybus* (WECI) to prevent the formation of malondialdehyde (MDA). Our results indicate that 100 μg/ml of WECI was enough to inhibit LDL oxidation. Therefore, this level of WECI was only chosen and used for the following experiments.

The incubation of LDL with WECI (100 μg/ml) prior to 4 h of Cu+2 mediated oxidation, resulted in a remarkable decrease in the level of TBARS compared to the control (Fig. 3).

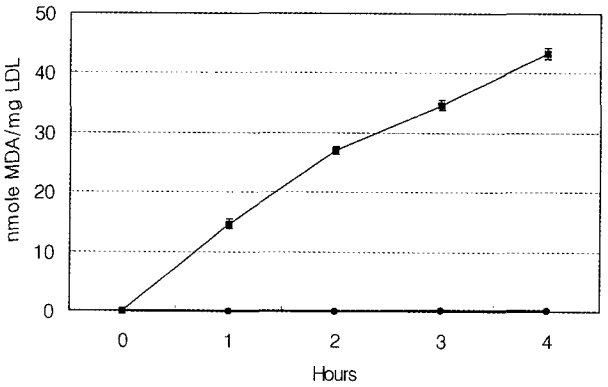

Fig. 3. Inhibition of TBARS formation in LDL on increasing the amount of *Cichorium intybus* water extract The amount of *Cichorium intybus* water extract added was 100 μg/ml. Each point of data shown represents the mean. Key; ■ LDL +Cu, ● LDL+Cu+Ex

Agarose gel electrophoresis pattern of oxidized-LDL

The effect of WECI on the 0.7% agarose gel electrophoresis pattern of oxidized LDL with or without addition was examined. The electrophoretic mobilities of LDL in the absence of WECI showed increased mobility as the oxidation time increased (0-8 h) compared to native LDL (0 h). It was reported (Esterbauer *et al.*, 1990) that the oxidation of LDL by the copper ion results in a consistent decrease in histidine (32%), lysine (15%), and proline (10 %) residues as well as smaller decreases in methionine (6 %), while aspartic acid levels increased (6%). Therefore, oxidized LDL shows greater electrophoretic mobility than native LDL. However, in the presence of WECI, the electrophoretic mobility of oxidized LDL decreased remarkably (0-8 h), although it showed a slightly higher mobility than native LDL.

From the above results, it may be deduced that the amino acid composition of apo B was changed during LDL oxidation, and that the negative charges on the

molecular components of LDL were higher when LDL was not treated with WECI. Moreover, it was revealed that the addition of WECI to LDL showed significant antioxidative effects upon LDL oxidation and that it protected the positively charged amino acids, such as the lysine of apo B-100.

Changes of fatty acid composition of LDL

The fatty acids of LDL were analyzed by gas chromatography using an F.I. detector. Fatty acid compositions were compared for LDL with and without WECI, to determine how WECI might protect unsaturated fatty acids in LDL. The major fatty acids in LDL were palmitic (16:0), oleic (18:1), and linoleic acid (18:2). Without the addition of WECI, LDL oxidation by copper caused a drastic reduction in the unsaturated fatty acid content as oxidation progressed. In particular, the decomposition of linoleic acid was remarkable, from 2041 to 44 nmol/mg LDL, after 6 h of oxidation, while under identical circumstances the addition of WECI retarded its decomposition to 1290 nmol/mg LDL (Table I). These results indicate that the addition of WECI protected saturated and unsaturated fatty acids from copper mediated LDL oxidation. The level of unsaturated fatty acids, which are more vulnerable to lipid peroxidation, were more significantly reduced than the levels of saturated fatty acids on oxidation. Polyunsaturated fatty acids (PUFA), although less closely associated with coronary heart disease than saturated fatty acids, are actually more susceptible to oxidation (Esterbauer *et al.*, 1990, Steinbrecher *et al.*, 1989). At present, dietary PUFA is recommended to reduce heart disease, but current research upon the roles of polyunsaturates and monounsaturates in atherogensis suggests that their comparative roles warrant a re-investigation.

Protection of vitamin E in LDL

Changes of vitamin E in LDL were also compared during copper mediated oxidation and protective effects of WECI on vitamin E were evaluated (Fig. 7). After 8 h of oxidation the amount of vitamin E decreased from 29 to 12.8 nmole/mg in LDL without WECI and but this was reduced to 21.8 nmol/mg LDL with WECI, (Table II). The addition of WECI (100 μg/ml) thus protected Vitamin E in

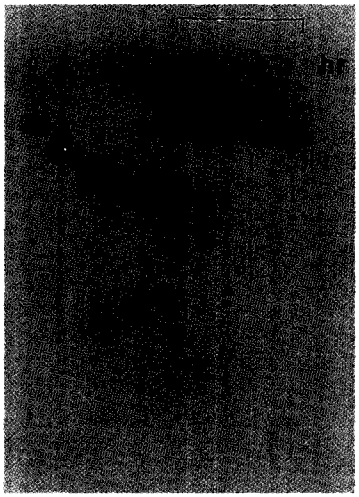

Fig. 4. Copper mediated peroxidation of LDL in the presence or absence of the water extract of *Cichorium intybus*. The five gel patterns on the left show LDL (Lane 0) and oxidized LDL pattern (Lane 2-8) and patterns on the right show the mobility pattern obtained after adding the *Cichorium intybus* water extract (at 100 μg/ml).

Table I. The effect of *Cichorium intybus* on the fatty acid contents of of LDL with respect to oxidation time

(unit: nmol/mg LDL)

Fatty Acid	Group	Oxidation Time (h)			
		0	2	4	6
Palmitic Acid (16:0)	LDL+Cu^{2+}	1335	1000	845	800
	LDL+Cu^{2+}+C.*i.*	1335	990	831	830
Palmitoleic Acid (16:1)	LDL+Cu^{2+}	84	75	48	30
	LDL+Cu^{2+}+C.*i.*	84	76	72	70
Stearic Acid (18:0)	LDL+Cu^{2+}	278	180	160	148
	LDL+Cu^{2+}+C.*i.*	278	200	198	190
Oleic Acid (18:1)	LDL+Cu^{2+}	843	658	422	363
	LDL+Cu^{2+}+C.*i.*	843	606	580	514
Linoleic Acid (18:2)	LDL+Cu^{2+}	2041	678	155	44
	LDL+Cu^{2+}+C.*i.*	2041	1950	1600	1290
Arachidonic Acid (20:4)	LDL+Cu^{2+}	210	ND	ND	ND
	LDL+Cu^{2+}+C.*i.*	210	200	200	198

C. *i*: Water extract of *Chichorium intybus*. ND: Not detected.

Table II. The effect of *Chichorium intybus* on the level of Vit.E in LDL with respect to oxidation time

(unit: nmol/mg LDL)

Group	Oxidation Time (h)				
	0	2	4	6	8
LDL+Cu^{2+}	29 ± 0.58	24 ± 1.15	17 ± 0.38	14.33 ± 0.34	13.33 ± 0.31
LDL+Cu^{2+}+C.i.	29 ± 0.58	26 ± 0.55	26 ± 0.55	23.33 ± 0.43	21.67 ± 0.38

C.i.: *Chichorium intybus* (100 μg/ml). Data are expressed as the mean.

LDL. Since vitamin E is a fat-soluble vitamin, it is present in the cellular membranes and the lipoproteins of blood. Vitamin E does not have any special binding protein, which separates it from other fat-soluble vitamins, and therefore, it is transported with the blood lipoproteins (Fielding, 1992). Moreover, lipid peroxidation was found to propagate by chain reaction when LDL was depleted of endogenous lipophilic antioxidants, such as vitamin E (Esterbauer *et al.*, 1989). Vitamin E is a chain-breaking antioxidant, which prevent the propagation step initiated by scavenging lipid peroxy radicals. Therefore, it is of considerable importance to protect vitamin E in the LDL, and the protective effect of WECI upon Vitamin E help to maintain the LDL structure and prevent atherosclerosis.

Observable characteristics change with advancing age, and their wide-ranging implications and causes are the subject of much study. Atherosclerosis is a metabolic disease, which leads to reductions in the luminal diameter of the main arteries, and the severity of its associated lesions increases as of age (Brown, 1992). The cholesterol that accumulates is derived primarily from the plasma lipoproteins (Caslake *et al.*, 1992), and among these lipoproteins, LDL appears to be the most important, since clinical epidemiologic studies have demonstrated that an elevated level of LDL is an important risk factor for coronary heat disease. Various lines of research have provided strong evidence that LDL may become oxidized *in vivo* and that oxidized LDL is a key component in the formation of early atherosclerotic lesions. It has also been reported that oxidized LDL has chemotactic properties and if present in the intimal space(**s?**) of arteries may recruit blood monocytes that can then develop into tissue macrophages (Fogelman *et al.*, 1988), take up oxidized LDL to form lipid laden foam cells (Luc and Fruchart, 1991). Therefore, inhibition of lipid peroxidation of LDL by *Cichorium intybus* is desirable to protect against some of the effects of aging and atherosclerosis in particular. Further study is required to identify its active constituents and their modes of action.

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