Licorice extract and its major polyphenol glabridin protect low-density lipoprotein against lipid peroxidation: in vitro and ex vivo studies in humans and in atherosclerotic apolipoprotein E–deficient mice \(^1,2\)

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ABSTRACT Polyphenolic flavonoids are powerful antioxidants. In the present study we investigated the antioxidative activity against low-density-lipoprotein (LDL) oxidation of a not yet studied subclass of polyphenols, the isoflavans, which are present in licorice alcoholic extract. The study was performed in humans as well as in atherosclerotic apolipoprotein E–deficient mice (E\(^0\)), because their LDL is highly susceptible to oxidation. LDL oxidation was induced by incubating it with copper ions as well as with the aqueous or lipid-soluble free radical generators 2,2′-azobis-2-amidino propane hydrochloride (AAPH) and 2,2′-azobis 2,4-dimethylvaleronitrile (AMVN), respectively. The extent of LDL oxidation was determined by measuring the formation of conjugated dienes, thiobarbituric acid reactive–substances (TBARS), and lipid peroxides. By all methods in human studies, licorice ethanolic extract as well as a pure material, which was identified by gas chromatography–mass spectroscopy as the isoflavon glabridin, were shown to inhibit LDL oxidation by a mechanism involving scavenging of free radicals. In an ex vivo study, LDL isolated from the plasma of 10 normolipidemic subjects who were orally supplemented for 2 wk with 100 mg licorice/d was more resistant to oxidation than was LDL isolated before licorice supplementation. Dietary supplementation of each E\(^0\) mouse with licorice (200 \(\mu\)g/d) or pure glabridin (20 \(\mu\)g/d) for 6 wk resulted in a substantial reduction in the susceptibility of their LDL to oxidation along with a reduction in the atherosclerotic lesion area. These results could be related to the absorption and binding of glabridin to the LDL particle and subsequent protection of the LDL from oxidation by multiple modes as shown in humans and in E\(^0\) mice. *Am J Clin Nutr* 1997;66:267–75.

KEY WORDS Antioxidants, low-density-lipoprotein oxidation, atherosclerosis, polyphenolic flavonoids, mice, humans, licorice, isoflavans, glabridin, free radicals

INTRODUCTION Oxidative damage by free radicals has been implicated as the cause of diverse diseases, including cancer and cardiovascular disease (1, 2).

Oxidative modification of low-density lipoprotein (LDL) is thought to play a key role during early atherogenesis (3–5). LDL that has undergone lipid peroxidation, induced by metal ions or by cells of the arterial wall, was shown to be taken up by macrophages at an enhanced rate via the scavenger receptor (6), leading to the formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions (7). Cells of the arterial wall, including endothelial cells, smooth muscle cells, and macrophages can oxidize LDL in vitro in the presence of catalytic amounts of transition metal ions (6, 8, 9). We showed that macrophages that were predisposed to oxidative stress can oxidize LDL in the absence of metal ions (10). On the other hand, redox transition metal ions can oxidatively modify LDL even in the absence of arterial cells (11). LDL can also be oxidized in vitro by metal ion–independent pathways by exposure to a constant flux of aqueous free radicals produced by 2,2′-azobis-2-amidino propane hydrochloride (AAPH), or by generation of peroxyl radicals within the LDL by using the lipid-soluble radical initiator 2,2′-azobis 2,4-dimethylvaleronitrile (AMVN) (12). The resistance of LDL to oxidative modification depends on extrinsic as well as on intrinsic factors (13). Antioxidants that can prevent lipid peroxidation should be critically important in preventing LDL oxidation (14–16).

Although increased resistance of LDL to oxidation was observed after treatment with various synthetic pharmaceutical agents (17–20), an effort is being made to identify natural food products that can offer antioxidant defense against LDL oxidation. We showed previously that dietary supplementation with natural \(\beta\)-carotene protects against LDL oxidation (21, 22).

Recently, a great deal of interest has been directed toward the bioactivity of polyphenolic components of plants as dietary sources of antioxidants. The polyphenolic flavonoids are a family of related compounds that include monomeric flavanols, flavanones, anthocyanidins, flavones, flavonols, flavans, and

the phenylpropanoids or hydroxycinnamic acid derivatives. The antioxidant activity of the flavonoids is related to their chemical structure, their rates of reaction with the relevant radicals, and their partition coefficient (23, 24). We and others have shown that dietary supplementation with nutrients rich in different polyphenolic flavonoids, such as olive oil, which contains hydroxytyrosol (25, 26), or red wine, which contains catechin (flavanol) and quercetin (flavonol) (27, 28), resulted in reduced susceptibility of LDL to oxidation.

Licorice roots, which consist of the dried unpeeled roots and stolons of Glycyrrhiza glabra, are widely used in Asia as a sweetener or a spice (29). Licorice root has also been used medicinally for >3000 yr, and a wide range of therapeutic uses have been ascribed to it (30). Several pharmacologic activities, such as antibacterial and antiviral (30), antiinflammatory (31), and antiallergic and antihepatotoxic (32) effects have been attributed to the licorice compound glycyrrhizin and its aglycone, glycyrrhetic acid. Licorice root also contains flavonoids from the flavan and chalcone subclasses, which have lipophilic characteristics and biological functions, including antioxidative properties (33, 34). The purpose of the present study was to examine for the first time the in vitro and ex vivo effects of polyphenols from the isoflavon subclass that are present in licorice extract on the resistance of LDL to oxidation and to identify specific pure constituents responsible for this effect. Because it is not now clear which condition of LDL oxidation is relevant to the in vivo situation, we studied the antioxidative properties of licorice on metal ion–dependent and on free radical–induced lipid peroxidation in LDL in humans and in apolipoprotein E–deficient transgenic (E0) mice because their LDL is highly susceptible to oxidation (35).

METHODS

Materials

Lanox (licorice antioxidant) alcoholic extract free of glycyrrhizinic acid was provided by Fertilizers & Chemicals Ltd, Haifa, Israel. Butylated hydroxytoluene (BHT), EDTA, and 1,1-diphenyl-2-pirclyhydrayl (DPPH) were purchased from Sigma (St Louis, MO). AAPH and AMVN were from Polysciences (Warington, PA). The term licorice in this paper will refer to the above-mentioned extract.

Human studies

Subjects

Ten healthy men aged 20–35 y who were nonsmokers taking no medication were included in the study. All subjects received licorice (0.1 g/d) in a softgel capsule (RP Scherer, St Petersburg, FL) for 2 wk. Ten normal subjects who received placebo capsules without licorice served as a placebo control group. The placebo capsules contained the inert gelatinous material that is normally included in softgel capsules.

All the subjects included in this study were students or laboratory staff of the Faculty of Medicine. The subjects’ compliance with the licorice supplement was satisfactory, as assessed by daily contact.

The plasma cholesterol concentration did not change significantly during the study [4.34 ± 0.62 mmol/L (168 ± 24 mg/dL) and 4.42 ± 0.59 mmol/L (171 ± 23 mg/dL) before and after 2 wk of licorice supplementation, respectively]. Similarly, no significant change was found in the LDL-cholesterol concentration [2.6 ± 0.67 mmol/L (100 ± 26 mg/dL) and 2.6 ± 0.46 mmol/L (101 ± 18 mg/dL) before and at the end of the study, respectively]. No significant change could be found in other characteristics such as blood count, coagulation tests, or renal or liver function tests. The subjects’ mean body mass index (BMI; in kg/m2) was 23 ± 1.5 and did not change during the study. All subjects consumed their habitual diet during the study.

The study was approved by the Helsinki Committee of the Rambam Medical Center, Israel Ministry of Health (no. 513). Blood samples were drawn after a 12-h fast, before study entry, and at 1 and 2 wk of licorice administration.

LDL isolation

For the in vitro studies, LDL was isolated from different plasma samples derived from healthy normolipidemic volunteers. For the ex vivo studies, plasma was collected from the subjects who participated in the study, before study entry (baseline), and at 1 and 2 wk of licorice or placebo administration.

In a preliminary experiment, we isolated the LDL from plasma samples immediately after plasma separation and stored the LDL samples at 4°C for 2 wk until all three samples were collected. No significant differences could be measured in the oxidative state of the LDLs, as measured by the thiobarbituric acid–reactive substance (TBARS) assay (1.47 ± 0.09, 1.33 ± 0.05, and 1.39 ± 0.05 nmol malondialdehyde (MDA)/mg LDL in LDL separated before and after 1 or 2 wk of licorice supplementation, respectively). We also found that storage of plasma at 4°C in the presence of 1 mmol EDTA/L and under nitrogen for a period of 2 wk did not significantly affect the subsequent susceptibility of LDL to oxidation.

The content of MDA equivalents in LDL obtained before and after 1 or 2 wk of licorice supplementation was 1.25 ± 0.06, 1.37 ± 0.05, and 1.56 ± 0.07 mmol/mg LDL, respectively. Thus, all plasma samples were kept unfrozen at 4°C in the presence of 1 mmol EDTA/L and under nitrogen for a maximum period of 2 wk until all three samples from each individual were collected. Then, at one time, LDL was separated from all plasma samples of each individual and oxidized to eliminate variations that occur with the assays.

LDL was separated from plasma by discontinuous density-gradient ultracentrifugation (36) and dialyzed against saline with EDTA (1 mmol/L). Before the oxidation study, LDL was diluted in phosphate-buffered saline (PBS) to 200 mg protein/L and dialyzed overnight against PBS at 4°C to remove the EDTA. LDL protein concentration was determined with the Folin phenol reagent (37).

Mouse study

Sixty E0 transgenic mice aged 6 wk were divided into three groups. 20 mice in each, and fed the following via their drinking water: 1) placebo, 2% alcohol in water (control group); 2) 200 μg licorice/d in 2% alcoholized water; or 3) 20 μg purified gabridin/d in 2% alcoholized water.

LDL was isolated from blood samples drawn from the E0 mice before and 6 wk after licorice or gabridin administration by discontinuous density-gradient ultracentrifugation (36). For
the in vitro studies, LDL was isolated from plasma of untreated E\(^3\) mice at the age of 6 wk.

**LDL oxidation**

Oxidation of LDL was carried out in a shaking water bath at 37°C, under air, and in plastic tubes 1 cm in diameter. For metal ion–dependent oxidation, LDL was incubated for 4 h at 37°C with freshly prepared copper sulfate (5 \(\mu\)mol/L). For metal ion–independent oxidation, LDL was incubated for 4 h at 37°C with AAPH (1 mmol/L) or AMVN (1 mmol/L), which are water or lipid-soluble azo compounds that thermally decompose to produce peroxyl radicals at constant rates within the water or the lipid phase, respectively (13), in the presence of 0.1 mmol EDTA/L to chelate adventitious metal ions and prevent them from contributing to the radicals initiator–induced oxidation. Oxidation was terminated by refrigeration at 4°C, and 0.1 mmol EDTA/L was also added to chelate the copper ions. LDL oxidation was determined by measuring the amount of TBARS (38) and the amount of lipid peroxides, which were determined with a commercially available kit (cholesterol color reagent, CHOD iodide method; Diagnostica Merck, Darmstadt, Germany) (39). This assay is based on the oxidative activity of lipid peroxides that convert iodide to iodoine and is measured spectrophotometrically. LDL-associated antioxidants, such as \(\alpha\)-tocopherol (vitamin E), cannot possibly affect iodide oxidation because it was shown previously (40) to be able to donate an electron and not to abstract an electron from the iodide, which is needed to convert iodide to iodoine. The assay was performed with 100 \(\mu\)L lipoprotein solution (200 mg protein/L), which was mixed on a vortex mixer with 1 mL color reagent. After the addition of 10 \(\mu\)mol EDTA/L (to stop any further oxidation), the sample was allowed to stand for 30 min in the dark, followed by determination of absorbance at 365 nm against a blank (the color reagent alone).

Formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm (41). Incubations were carried out in the spectrophotometer cuvette at 37°C in a thermostated six-cell holder in a spectrophotometer (Ultraspex 3000; Pharmacia, LKB, Biochrom Ltd, Cambridge, United Kingdom). The reference cell contained 5 mmol CuSO\(_4\)/L in PBS, or 1 mmol AAPH/L together with 0.1 mmol EDTA/L in PBS, for metal ion–dependent or metal ion–independent oxidation, respectively. The initial background of the different samples ranged between 0.1 and 0.2 absorbance as recorded at 234 nm. However, after initial absorbance was recorded, the spectrophotometer was set to zero against the blank, and the increase in absorbance during LDL oxidation was recorded every 10 min.

**Free radical–scavenging capacity**

The free radical–scavenging capacity of licorice was analyzed by the DPPH assay. Aliquots (30 \(\mu\)L) of the licorice were mixed at the indicated concentration with 3 mL 0.1 mmol DPPH/L (in ethanol) in a cuvette. The time course of the change in the absorbance at 517 nm was then monitored kinetically (42).

**Isolation and identification of pure antioxidants from licorice extract**

Five grams powdered alcoholic extract of licorice was extracted in 50 mL methylene chloride. The extract was further fractionated by using liquid-liquid extraction. Two main fractions were obtained after evaporation of solvents, which were designated F-I and F-II. The evaluation of the antioxidant activity of each fraction was made by using a \(\beta\)-carotene–linoleate system described previously (43). Briefly, 1 mg \(\beta\)-carotene was dissolved in 10 mL chloroform. Then 1.5 mL of this solution was added to a boiling flask that contained 20 mg linoleic acid and 200 mg Tween-20. After chloroform evaporation, 50 mL distilled water was slowly added to the flask to form an emulsion by vigorous agitation. The reaction mixture contained 5 mL \(\beta\)-carotene–linoleic acid emulsion and 0.2 mL ethanolic antioxidant solution.

The tubes were placed in a water bath at 50°C and oxidation was determined as \(\beta\)-carotene consumption by measuring the absorbance at 460 nm at 15-min intervals. Thus, the main activity was found in fraction F-I, which was then chromatographed on aluminium oxide by using a gradient of methylene chloride and then a mixture of methylene chloride and methanol as eluants.

The fractions collected were run on silica gel thin-layer chromatography plates developed in toluene:ether (4:1, by vol), and those having a main spot at R\(_0\) 0.18 were combined and rechromatographed on silica gel plates by using the same eluant. As a result, seven potent constituents were isolated and identified by using ultraviolet, proton magnetic resonance, Fourier transform infrared, and mass spectrometric analyses (34, 44). Four constituents were found to be isoflavan derivatives, and among them, glabridin was found to be the major component (11.6% by wt) in comparison with only 0.2–2% of the others.

**Gas chromatography–mass spectrometric analysis of glabridin in LDL**

The quantity of the trimethylsilyl ether derivatives of glabridin in LDL was measured on a Hewlett-Packard model 5890 series II gas chromatograph (GC) (Valbron, Germany) with a 30-m HP-5MS (5% phenyl methylpolysiloxane) capillary column, with an internal diameter of 0.25 mm and film thickness of 0.25 \(\mu\)m. The GC was coupled to a model 5972 mass selective detector (MS) in the electron impact mode with HP G1034B software. The conditions of the analysis were as follows: splitless for the first 1 min and then, in split mode with a 1 to 15 ratio; the oven was programmed from 150 to 290°C at a rate of 10°C/min and then held at 290°C for 20 min; the helium flow was 33.7 cm/s, and the flow in column was 0.8 mL/min with a constant pressure of 7.2 × 10\(^5\) Pa. The injector temperature was 250°C and the interface was 280°C. The electron energy was 70eV.

**Preparation of glabridin trimethylsilyl ether derivatives**

The standard solution was prepared as follows: glabridin (3 \(\mu\)mol) was dissolved in 200 \(\mu\)L pyridine, and 100 \(\mu\)L trimethylsilyl chloride was then added. The solution was stirred for 15 min at room temperature and used as a standard after dilution with dichloromethane for the GC-MS, in a selected ion-monitoring mode. The ions selected had a mass-to-charge ratio...
(m/z) of 468 (M', glabridin ditrimethylsilyl ether), m/z 453 (M'-15), and m/z 454 (M' +1-15). The analysis was linear over a range of 0–30 ng/μL injected sample.

The dry extracts from the LDL samples were treated with 100 μL pyridine and 50 μL trimethylsilyl chloride. After they were stirred for 15 min at room temperature, 2 μL from the aliquots was injected into the GC-MS.

Analysis of aortic atherosclerotic lesions

At the end of the experimental period the mice were killed. The heart and entire aorta were rapidly removed and immersion fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.1 mol/L) with 0.01% Ca at room temperature. The aortic arch was dissected free from the surrounding fatty tissue and the first 4 mm of the ascending aorta (beginning with the aortic valves) was removed and cut transversely with razor blades into four blocks of ~1 mm each. The samples were kept in the fixative at room temperature overnight. The samples were then rinsed and stored in sodium cacodylate buffer (0.1 mol/L) containing 7.5% wt:vol sucrose before treatment with an un-buffered 1% aqueous solution of osmium tetroxide for 4 h. This was followed by a sodium cacodylate buffer rinse and dehydration in ethanol solutions with graded ethanol concentration (70%, 95%, and 100%) before being treated with propylene oxide and embedded in epoxy resin (Eponeate 12; Pelco International, Reading, CA). Transverse sections (1 μm) were cut for light microscopy. The prolonged osmium treatment stains the intraluminal, intramural, and intracellular lipid a dense black color. The sizes of the lesion areas were determined by using a computerized quantitative image-analysis system (Olympus Cue-2; Lake Success, NY) with morphometric software.

The system consists of a Zeiss Universal R photomicroscope (×10 objective; Carl Zeiss, Oberkochen, Germany) fitted with a Panasonic WV-CD50 camera (Metsushita, Corp. Tokyo), with the video image seen on a Sony 14-inch (35.6-cm; Sony Corp. Tokyo) color monitor and an IBM-compatible personal computer. Measurements were made in standardized “windows” with an area of 176 758 μm².

Statistics

Student’s paired t test was performed for all statistical analyses. Results are given as means ± SDs for the in vitro studies and as means ± SEMs for the in vivo studies. For the in vitro experiments presented in Figures 1A, 2A, and 3A, only one experiment, representative of three different studies performed, is shown. The degree of variation between the three experiments ranged between 7% and 9%. The computer software program STATEASE (version 1.00; Data Plus Systems Inc, New York) was used for computation.

RESULTS

In vitro studies with human LDL

To study the effect of licorice alcoholic extract on the oxidation of LDL, the lipoprotein was subjected to metal ion-dependent oxidation by incubation with 5 μmol CuSO₄/L or to metal ion-independent oxidation by exposure to 1 mmol AAPH/L and the lipid-soluble radical initiator AMVN. BHT, a synthetic phenolic compound that acts as a potent chain-break-

![Figure 1](https://academic.oup.com/aajn/article-abstract/66/2/267/4655670/fig1){:width=0.8}

**FIGURE 1.** Effect of licorice ethanolic extract on copper ion-induced LDL oxidation. A: Kinetic analysis—low density lipoprotein (LDL; 200 mg protein/L) was incubated for 4 h at 37 °C with 5 μmol CuSO₄/L in the absence (control) or presence of 1.5 or 3.0 mg licorice/L or 3.0 mg butylated hydroxytoluene (BHT)/L. The change in absorbance at 234 nm was continuously monitored and recorded. One experiment representative of three separate experiments is shown. Oxidation was determined as thiobarbituric acid-reactive substances (TBARS; B) and as lipid peroxide (C) content in LDL. i ± SD; n = 9 for three separate experiments performed in triplicate.
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The absence (TBARS; B) and as lipid peroxide (C) content in LDL. \(\bar{x} \pm SD; n = 9\) for three separate experiments performed in triplicate.

We also studied the ability of licorice to protect LDL from oxidation by free radicals. In the control experiment, in the absence of antioxidants, the rate of the propagation phase was only 10-fold higher than the rate of the lag phase; this was probably due to the small concentration of AAPH used (1 mmol/L). Figure 2A shows that licorice inhibited the onset of LDL oxidation by the water-soluble radical generator AAPH (1 mmol/L) for 260 min, even at a concentration as low as 1.5 mg/L. As shown for copper ion–induced LDL oxidation, licorice also inhibited AAPH-induced LDL oxidation in a dose-dependent manner similar to that of BHT (Figure 2, B and C).

The IC\(50\) that inhibited formation of MDA equivalents (Figure 2B) and of lipid peroxides (Figure 2C) induced by 1 mmol AAPH/L during a 4-h incubation at 37 °C was 0.3 and 1.2 mg/L, respectively, six and two times lower, respectively, than the IC\(50\) that was required in the copper sulfate–induced LDL oxidation system (Figure 1). Licorice was also found to be an effective antioxidant when peroxyl radicals were generated within the LDL. Oxidation of LDL by incubation with the lipid-soluble radical initiator AMVN (1 mmol/L) was markedly inhibited by licorice in a dose-dependent manner (Table 1), with IC\(50\) values of 0.3 and 1.5 mg/L for the inhibition of LDL-associated TBARS and lipid peroxide formation, respectively (Table 1).

In an effort to identify pure constituents in licorice with antioxidative characteristics, the licorice extract was fractionated on a silica gel column. This procedure has lead, so far, to the isolation of seven pure materials; the major one identified was glabridin (Figure 3, inset). A 300-mg/L stock solution was prepared by dissolving 3 mg glabridin in 200 μL ethanol. The volume was brought up to 10 mL with water. Incubation of LDL (200 mg protein/L) with 1 mmol AAPH/L in the presence of 3 mg glabridin/L (which is equivalent to 9.2 μmol/L) resulted in a 100-min prolongation of the lag phase and an 87% and 70% reduction in the formation of TBARS and lipid peroxides in LDL, respectively (Figure 3), similar to the effect of the crude licorice extract.

To study the mechanism by which licorice or glabridin inhibits LDL lipid peroxidation, we analyzed the free radical–scavenging capacity of both licorice and glabridin by the DPPH assay. The addition of licorice (10 mg/L) or glabridin (10 mg/L) to DPPH induced a rapid decrease in the absorbance at 517 nm, which reached a plateau within 5 min, in a pattern similar to that of BHT (10 mg/L) (Figure 4). Sodium EDTA, which can inhibit metal ion–induced LDL peroxidation because of its ability to chelate metal ions, did not show any ability to scavenge free radicals (Figure 4).

![FIGURE 2](https://academic.oup.com/ajcn/article-abstract/66/2/267/4655670/figure2)

**FIGURE 2.** Effect of licorice alcoholic extract on 2,2'-azobis-[2-amidino propane hydrochloride (AAPH)-induced LDL oxidation (1 mmol/L). A: Kinetic analysis. See Figure 1 for the experimental procedure. Oxidation was determined as thiobarbituric acid-reactive substances (TBARS; B) and as lipid peroxide (C) content in LDL. \(\bar{x} \pm SD; n = 9\) for three separate experiments performed in triplicate.

![FIGURE 3](https://academic.oup.com/ajcn/article-abstract/66/2/267/4655670/figure3)

**FIGURE 3.** Kinetic analysis of the antioxidative and radical scavenging effects of licorice alcoholic extract on the copper (II) sulfate (CuSO\(4\))–induced LDL oxidation system (100 μM CuSO\(4\)).

The data are presented as mean ± SD of three separate experiments performed in triplicate.  

### TABLE 1

<table>
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<th>Licorice concentration (mg/L)</th>
<th>LDL oxidation</th>
<th>Lipid peroxides</th>
<th>MDA/LDL protein</th>
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\(\bar{x} \pm SD\) of three separate experiments performed in triplicate; \(n = 9\).

1 LDL (200 mg protein/L) was incubated with 1 mmol AMVN/L for 4 h at 37 °C in the absence (control) or presence of the indicated licorice concentration.

2 TBARS, thiobarbituric acid–reactive substances; MDA, malondialdehyde.

Significantly different from control, \(P < 0.01\).
FIGURE 3. Effect of glabridin on 2,2′-azobis-(2-amidino propane hydrochloride (AAPH)-induced LDL oxidation. LDL (200 mg protein/L) was incubated for 4 h at 37 °C with 1 mmol AAPH/L in the presence of 3.0 mg licorice alcoholic extract/L or 3.0 mg glabridin/L. Oxidation was determined as thiobarbituric acid-reactive substances (TBARS; A) and as lipid peroxide (B) content in LDL. \( \bar{x} \pm SD; n = 9 \) for three separate experiments performed in triplicate. The inset in (A) represents the molecular structure of glabridin (molecular weight = 324).

Ex vivo studies in humans

The protective effect of licorice against LDL oxidation was tested ex vivo after oral administration of licorice (encapsulated in a softgel) at a concentration of 100 mg/d for 2 wk. This quantity is equivalent to a concentration ranging between 1 and 10 mg licorice/L used in the in vitro studies. In a control experiment, copper ion–mediated LDL oxidation was markedly inhibited by licorice (1.5 mg/L) that was derived from these capsules (from 24.8 ± 12 in the absence of licorice to 1.4 ± 0.5 nmol MDA equivalents/mg LDL in the presence of licorice).

Oral supplementation with licorice (100 mg/d) resulted in a gradual reduction with time in the susceptibility of LDL to copper ion–induced lipid peroxidation as measured by a 70- and 110-min prolongation of the lag phase and by a 33% and 44% reduction in lipid peroxide formation after 1 and 2 wk of licorice supplementation, respectively (Figure 5A). Similarly, the susceptibility of these LDL preparations to AAPH-induced lipid peroxidation also decreased in a gradual manner (Figure 5B), with a significant \( P < 0.01 \) reduction in lipid peroxides, by 36%, that was observed after 2 wk of licorice consumption (Figure 5B), and an 80-min prolongation of the lag phase. In contrast, the susceptibility of LDL from control subjects who did not receive licorice (the placebo group) to copper ions or to AAPH-induced oxidation was not significantly different when the LDL isolated at baseline or 2 wk later. LDL-associated lipid peroxides for copper sulfate–induced oxidation were 210 ± 19 and 219 ± 20 nmol/mg LDL at baseline and 2 wk after licorice consumption (Figure 5B), with a significant \( \bar{x} \pm SD; n = 10 \), and significantly different from time 0, \( *P < 0.01 \).

FIGURE 4. Free radical–scavenging capacity of licorice and glabridin. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) ethanolic solution at a final concentration of 100 μmol/L was mixed with licorice (10 mg/L), 10 mg glabridin/L, 10 mg butylated hydroxytoluene (BHT)/L, or 10 mg EDTA/L. The time course of the change in absorbance was continuously monitored at 517 nm. Results represent one experiment of three separate experiments performed.
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later, respectively, and for AAPH-induced oxidation lipid peroxides values of 340 ± 25 and 335 ± 30 nmol/mg LDL were obtained. Supplementation of LDL (1 g protein/L) with 50 mmol glabridin/L for 1 h at 37 °C, followed by extensive dialysis to remove unbound material, resulted in 75–85% binding of glabridin to LDL.

Studies in E0 mice

The E0 atherosclerotic mouse is a good model to study LDL oxidation because increased LDL oxidation was shown in these mice. Furthermore, in these mice we could study the effect of both licorice and its major polyphenol glabridin on LDL oxidizability in vivo. Because LDL from E0 mice differs in its composition from human LDL, we first tested the capacity of the licorice alcoholic extract and of the purified glabridin to inhibit copper ion–induced LDL oxidation in vitro. Incubation of LDL (100 mg protein/L) with 5 mmol CuSO4/L in the presence of licorice alcoholic extract (3 mg/L) or purified glabridin (3 mg/L) resulted in an 84% and 69% reduction in LDL oxidation measured as TBARS (Figure 6A), and by a 70- and 50-min prolongation of the lag phase, respectively. Administration per mouse of 200 μg licorice/d, or 20 μg glabridin/d, for a period of 6 wk resulted in a significant (P < 0.01) reduction in the susceptibility of the LDL of the E0 mice to copper ion–induced oxidation as measured by a 68% and 22% inhibition in TBARS formation (Figure 6B), and by a prolongation of the lag phase by 50 and 35 min, respectively. An analysis of the mice aortic arch lesions after consumption of licorice, in comparison with mice treated with placebo, was performed (Figure 7). The lesions were categorized into four grades according to their histopathology and size: 1. minimal pathologic damage with only isolated foam cells present in the Tunica intima; 2. small lesions only (areas < 3500 μm²) with limited numbers of discrete foam cells in the Tunica intima; 3. larger, more extensive lesions with well-defined fatty streaks and disruption of elastic lamina (areas ≤ 20 000 μm²); 4. advanced lesions (areas ≤ 50 000 μm²).

Light microscopy revealed histopathologic atherosclerotic lesions in the aortic arch of both groups of mice, although the incidence of the lesions was far greater in the placebo-treated mice. Sixty percent of the placebo-treated mice showed well-defined lesions including three grade 2 lesions (30%), two grade 3 lesions (20%), and one advanced grade 4 lesion reaching the dimension of 50 000 μm². In contrast, only 2 of 10 licorice-treated mice showed atherosclerotic lesions of the aortic arch, and these were grade 3 lesions with maximum dimensions of 18 000 μm². The eight remaining licorice-treated mice

![Figure 6](https://academic.oup.com/ajcn/article-abstract/66/2/267/4655670)

**FIGURE 6.** The effect of licorice alcoholic extract and of glabridin on copper ion–induced LDL oxidation in vitro and in vivo in atherosclerotic apolipoprotein E–deficient (E0) mice. A: LDL (100 mg protein/L) from nontreated E0 mice was incubated for 4 h at 37 °C with 5 μmol CuSO4/L in the absence (control) or presence of 3 mg licorice alcoholic extract/L or 3 mg glabridin/L. B: LDL (100 mg protein/L) derived from E0 mice after administration of placebo for 6 wk (control), licorice (200 μg/d), or glabridin (20 μg/d), was incubated for 4 h at 37 °C with 5 μmol CuSO4/L. Oxidation was measured by the thiobarbituric acid–reactive substances (TBARS) assay. *P < 0.01.

![Figure 7](https://academic.oup.com/ajcn/article-abstract/66/2/267/4655670)

**FIGURE 7.** Photomicrographs of a typical atherosclerotic lesion of the aortic arch in atherosclerotic apolipoprotein E–deficient (E0) mice that consumed placebo (A) or licorice (B). The sections were stained with alkaline toluidine blue. All micrographs are at the same magnification.
showed minimal histopathologic changes (grade 1 only, Figure 7).

Next, we questioned whether the antioxidant effects observed after licorice or glabridin administration could be attributed to absorption of the polyphenol into the LDL. Analysis of the LDL derived from E0 mice by GC-MS revealed that LDL derived from the mice that consumed licorice contained 9.6 ± 0.85 nmol glabridin/mg LDL, and LDL derived from the mice that consumed purified glabridin contained 1.6 nmol glabridin/mg LDL. No glabridin could be detected in LDL derived from mice that served as the placebo control group.

DISCUSSION

This study shows for the first time that a subclass of polyphenols (isoflavans) present in licorice alcoholic extract, which was not tested before for its antioxidant activity against LDL oxidation, has the ability to protect LDL against oxidative modification under multiple types of oxidative stress both in vitro and in vivo. Most important, licorice consumption by E0 mice resulted in a considerable overall reduction in the incidence and extent of atherosclerotic lesions in the aortic arch compared with the placebo-treated mice. The alcoholic extract of licorice lacks the water-soluble constituents that are used in sweeteners and in the cigarette industry (including glycyrrhizinic acid), but contains several constituents with antioxidant activity (33, 34). Among those constituents, polyphenols such as licochalcones, glabrene, and glabridin were found in licorice (34); these phenolic antioxidants can react with and scavenge free radicals forming phenoxy radicals, followed by radical rearrangement and a coupling reaction that neutralizes the oxidative stress. We have indeed found that glabridin, which is an isoflavan, is the major polyphenol in licorice alcoholic extract and that it has antioxidant activity against LDL oxidation in vitro as well as in vivo in humans and in atherogenic E0 mice. The mechanism responsible for this effect lies in the lipophilic characteristics of the isoflavans, which enable them to bind to the LDL molecule, subsequently offering protection against oxidation through their radical-scavenging capacity. Similarly, we showed previously that consumption of natural food products rich in polyphenolic flavonoids, such as red wine (27) or olive oil (26), also results in increased resistance of LDL to oxidation. Licorice consumption by normal volunteers resulted in the protection of their LDL from oxidation. Note, however, that the potent antioxidative capacity of licorice ex vivo was lower than that shown in vitro. These results suggest that some other potent constituents are metabolically altered in the circulation, or that the active antioxidant constituents in licorice also bind to plasma proteins other than plasma LDL.

We isolated and purified glabridin, the major polyphenol in licorice, and tested its ability to reduce the susceptibility of LDL to oxidation in vivo in the E0 mice. We showed that administration of licorice alcoholic extract or pure glabridin to E0 mice at equivalent concentrations resulted in a remarkable reduction in the susceptibility of the LDL to copper-induced oxidation. However, the effect obtained after administration of pure glabridin was four times lower than that of whole licorice extract, suggesting that the whole extract contains other antioxidants besides glabridin. In addition, it is possible that the bioavailability of glabridin is greater when it is within the licorice extract than when it is administered in its pure form. Follow-up of licorice or pure glabridin administration for a longer period of time is warranted to assess the long-term effects of this polyphenol.

To study the mechanism involved in the antioxidative activity of licorice, LDL was subjected to several different modes of oxidation: copper ion–mediated oxidation; the lipid-soluble free radical generator AMVN, which produces peroxyl radicals within the LDL particle; and water-soluble AAPH, which exposes the LDL surface phospholipids to a constant flux of aqueous peroxyl radicals. Licorice alcoholic extract showed antioxidant activity against LDL oxidation in all of these systems.

The mechanism involved in the antioxidative characteristics of the licorice alcoholic extract and glabridin was shown by the DPPH assay to involve free radical scavenging. The ability of licorice to inhibit lipid peroxidation by aqueous as well as lipid-soluble agents may suggest that in vivo, licorice can exert its antioxidant activity against LDL oxidation on the surface of the lipoprotein as well as in the core of the LDL molecule. After incubation of LDL with 5 μmol CuSO4/L in the metal ion–dependent lipid peroxidation study, oxidative modification of LDL resulted from the interaction between copper ions and traces of lipid peroxides present in the LDL particle (13, 14). In this system, licorice also proved to act as a powerful antioxidant, although less efficiently than in the free radical–generating system.

This can be attributed to the fact that licorice inhibits initiation of LDL lipid peroxidation in the free radical generating systems, whereas in the presence of copper sulfate, scavenging of the lipid peroxyl radicals by licorice inhibits the propagation, not the initiation, of the LDL lipid peroxidation process.

It has been shown that licorice root contains phenolic flavonoids that can scavenge reactive oxygen species (33, 34). Phenolic substances acting as chain-breaking antioxidants were shown to react with lipid peroxyl radicals (45) and to inhibit the propagation phase of lipid peroxidation.

We conclude that some licorice constituents, one of which was isolated and identified as the isoflavonoid glabridin, are responsible for its antioxidative characteristics in LDL oxidation. Supplementation of the diet with licorice may prove beneficial in arresting accelerated atherosclerosis, secondary to its antioxidative capacity against LDL oxidation.

The technical assistance of Ludmilla Mazor with the histopathologic studies is very much appreciated.

REFERENCES


