Effects of Genistein on Oxidative Injury in Endothelial Cells

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Summary The aim of this study was to test the hypothesis that genistein protects vascular endothelial cells against the pro-atherosclerotic stressor, oxidized low-density lipoprotein (ox-LDL), by inducing antioxidant enzymes and preventing apoptosis. Human umbilical cord-derived endothelial cells (ECV 304) were incubated with genistein (10–100 μmol/L), the radical scavenging antioxidant vitamin E (α-tocopherol, 50 μmol/L), or vehicle for 24 h and then were incubated with ox-LDL for an additional 24 h. Subsequently, antioxidant enzyme activities, lipid peroxidation, adhesion to monocytes, cell morphology, viability and apoptotic index were assessed. Ox-LDL decreased superoxide dismutase and glutathione peroxidase activities in endothelial cells and caused lipid peroxidation, adhesion to monocytes, morphological injury and apoptosis (p<0.05). These effects were prevented by vitamin E and dose-dependently by genistein (p<0.05). Further, this effect of genistein is associated with maintenance of antioxidant enzyme activities and inhibition of lipid peroxidation.

Key Words genistein, vascular endothelial cell, apoptosis, antioxidation, in vitro

The Food and Drug Administration in the United States recently approved a health claim for soy, because laboratory investigations, clinical trials and epidemiological data indicate that a high consumption of soy is associated with a lower incidence of coronary artery disease (1, 2). A dose-dependent, inverse relationship between soy food intake and risk of coronary heart disease has been reported in 40- to 70-y-old women in China, although population-based studies of this nature cannot prove causality and may be confounded by other dietary influences (3). Substantial evidence indicates that it is the isoflavone fraction, especially the isoflavone genistein, that confers the beneficial cardiovascular effects of soy (4).

Genistein may improve vascular function but the mechanism of this effect is unclear. Many studies have confirmed that genistein is an antioxidant but reports are contradictory about whether it is a strong or weak antioxidant compared, for example, to quercetin or vitamin E (5). Moreover genistein, like vitamin E, can increase lipid peroxidation under certain circumstances (6).

Instead of acting principally as an antioxidant that reacts with and detoxifies harmful oxidants directly, genistein may protect against coronary artery disease by activating intracellular signaling pathways that upregulate antioxidant gene expression (7). For example, genistein (50 μM) increases expression of glutathione peroxidase mRNA and enzyme activity in EA-hy926 cells (8). Genistein also prevents the effects of ox-LDL on protein expression, nuclear fragmentation and disintegration of plasma membrane induced by the pro-atherosclerotic stressor, ox-LDL (5 μg/mL), in the EA-hy926 cell line (9). Therefore, the present study was designed to test the hypothesis that genistein protects vascular endothelial cells by inducing antioxidant enzymes and inhibiting apoptosis.

MATERIALS AND METHODS

Cells and reagents. Human umbilical cord-derived endothelial cells (ECV 304) were provided by Tianjin Huanhu Hospital (Tianjin, China). The low density lipoprotein was obtained from the Institute of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). Genistein (Gen), vitamin E (VE, α-tocopherol) and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) was produced by Gibco BRL (Grand Island, NY, USA). The fetal bovine serum (FBS) was produced by HyClone Laboratories (Logan, UT). The test kits for the determination of malondialdehyde (MDA), superoxide dismutase (SOD) activity, glutathione peroxidase (GSH-Px) activity and lactate dehydrogenase (LDH) were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).
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Endothelial cell culture. After anabiosis of the frozen endothelial cells, they were grown to confluence in DMEM supplemented with 10% fetal bovine serum with 100 units of penicillin and 0.1 mg/mL streptomycin, at 37˚C in a humidified atmosphere with 5% CO2. The identity of endothelial cells was confirmed by the determination of factor VIII expression (10).

Preparation of genistein and vitamin E. Vitamin E and genistein were dissolved in dimethyl sulfoxide (DMSO) and diluted 1:1,000 in culture medium. Controls were treated with the vehicle (0.1% DMSO).

Preparation of ox-LDL. Human LDL was dialyzed against phosphate buffered saline (PBS) for 24 h at 4˚C at pH 7.4 without EDTA. LDL was oxidized by the addition of CuSO4 at a concentration of 7.5 μmol/L for 6 h at 37˚C. This reaction was stopped by the addition of 10 μmol/L EDTA. LDL samples were stored at 4˚C. The oxidative degree was confirmed by the determination of its MDA content. ox-LDL containing 1 nmol/mL MDA was added to the cell culture medium, so that the final concentration of ox-LDL in the medium was 65 μg/mL.

Experimental design. Cells were divided into 6 groups. They were the control group, oxidative damage group (ox-LDL group), oxidative damage + vitamin E group (ox-LDL+VE, 50 μmol/L VE), and oxidative damage + Gen 10, 50, 100 μmol/L groups (ox-LDL+Gen-L, M, H groups). We did not test higher concentrations because genistein is reportedly toxic to endothelial cells at concentrations of 300 μmol/L or higher (11).

To begin each experiment, the culture medium was removed and the cells were washed with PBS. Next the cells were incubated with genistein, vitamin E or vehicle for 24 h and then were incubated with ox-LDL for an additional 24 h. Subsequently, cell morphology, viability, lipid peroxidation, antioxidant enzyme activities, apoptotic index, and monocyte-endothelial cell adhesion were measured.

Cell morphology. The effects of treatments on endothelial cell morphology were observed by light microscopy.

Fig. 1. Morphological changes of endothelial cells (EC). A: Control group. EC were incubated in DMEM medium. The cells form the regular monolayer of cobblestone shape. Magnification ×200. B: Ox-LDL group. EC were incubated for 24 h in DMEM medium with ox-LDL (65 μg/mL). The cell bodies contracted and rounded up. Magnification ×200. C: Ox-LDL+VE group. EC were incubated for 24 h in DMEM medium with ox-LDL (65 μg/mL) in the presence of vitamin E (50 μmol/L). Magnification ×200. D, E, F: Ox-LDL+Gen-L, M, H groups, respectively. The cells appeared with a typical spindle-shaped morphology. EC were incubated for 24 h in DMEM medium with ox-LDL (65 μg/mL) in the presence of genistein (10, 50, 100 μmol/L). Magnification ×200.
Cell viability. Mitochondrial dehydrogenase activity was used as an index of cell viability and was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Endothelial cells were seeded onto 96-well plates (10^4 cells/well) and were grown to confluence in DMEM. After treatment with samples, MTT (0.5 mg/mL) was added to each well and the cultures were incubated for 4 h at 37°C under 5% CO₂. Cells were lysed in 10% (wt/vol) SDS in 0.01 mol/L HCl before the absorbance was measured at 595 nm.

Measurement of MDA, SOD, GSH-Px and LDH. After the end of cell culture, the treated cells were scraped and collected. The cells were deliquesced by frozen distilled water. The levels of MDA, SOD, GSH-Px and LDH were measured using assay kits according to the manufacturers’ instructions. LDH release was calculated as: [Absorbance of cellular culture fluid/(Absorbance of cellular culture fluid + Absorbance of cellular supernatant)]×100%.

Isolation and culture of monocytes. Monocytes were isolated by density-gradient centrifugation. Twenty milliliters of human peripheral vein anticoagulated blood were collected and added slowly into the tubes with leukocyte cell isolation solution. After centrifugation for 10 min at 2,000 rpm, single cells at the middle-interphase were added into another tube. Leukocytes were washed twice with PBS, suspended in DMEM media containing 10% FCS, and incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO₂. Next, the non-adherent leukocytes were rinsed off and adherent cells were washed twice with PBS. Subsequently the cell monolayers were detached using a 1:1 0.25% trypsin and 0.2% EDTA solution (v:v) and the cells were resuspended at a concentration of 10^6 cells/mL in DMEM media containing 10% FCS. Cell viability was identified by a trypan blue exclusion test. Monocytes were determined with Wright’s staining. Monocytes were used for the monocyte-endothelial cell adhesion test within the following 2 h period.

Monocyte-endothelial cell adhesion assay. Endothelial cell monolayers were washed with PBS and incubated for 1 h in DMEM with monocytes (10^6 cells/mL) at 37°C in a humidified atmosphere containing 5% CO₂. Then the culture medium was discarded, the monolayers were washed to remove non-adherent monocytes, and the endothelial cells and adherent monocytes were solubilized for subsequent protein analysis. Monocyte adhesion was determined by calculating the difference between the micrograms of protein in wells containing endothelial cells and monocytes and the micrograms of protein in wells containing endothelial cells alone, and then dividing by the micrograms of protein present in 1 mL of the initial monocyte suspension. The percent adhesion of monocytes (MC) to endothelial cells (EC) was calculated according to the following equation: MC-EC (%)=[protein of (EC+MC)−protein of EC]/protein of EC×100% (12).

Determination of apoptosis. To identify apoptotic cells, an in situ cell death detection kit was used. The levels of apoptosis were evaluated by TUNEL assay.

Table 1. Effects of ox-LDL, vitamin E and genistein on cell viability measured by MTT reduction and LDH release in endothelial cell cultures.

<table>
<thead>
<tr>
<th>Group</th>
<th>MTT (arbitrary units)</th>
<th>LDH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.80±0.05ab</td>
<td>33.70±7.37a</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>1.51±0.04b</td>
<td>55.80±7.12b</td>
</tr>
<tr>
<td>ox-LDL+VE</td>
<td>1.69±0.05a</td>
<td>36.13±7.34a</td>
</tr>
<tr>
<td>ox-LDL+Gen-L</td>
<td>1.66±0.06a</td>
<td>45.35±7.83ab</td>
</tr>
<tr>
<td>ox-LDL+Gen-M</td>
<td>1.70±0.05a</td>
<td>39.13±7.88a</td>
</tr>
<tr>
<td>ox-LDL+Gen-H</td>
<td>1.74±0.06a</td>
<td>34.93±8.76a</td>
</tr>
</tbody>
</table>

*p<0.05 compared with ox-LDL group; *p<0.05 compared with ox-LDL+VE group. Values are the mean±SD of six samples. Comparison of any two group mean values in ox-LDL+Gen-L, ox-LDL+Gen-M and ox-LDL+Gen-H groups was statistically significant (p<0.01) in MTT and LDH release. There was no statistically significant difference between ox-LDL+VE and ox-LDL+Gen-L, ox-LDL+Gen-M, or ox-LDL+Gen-H groups in MTT (p>0.05). There was a statistically significant difference between ox-LDL+VE and ox-LDL+Gen-L groups (p<0.05) in LDH release but there was no statistically significant difference between ox-LDL+VE and the ox-LDL+Gen-M or ox-LDL+Gen-H groups (p>0.05).

Table 2. Effects of ox-LDL, vitamin E and genistein on the content of MDA and activities of SOD and GSH-Px in endothelial cell cultures.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/mg protein)</th>
<th>SOD (NU/mg protein)</th>
<th>GSH-PX (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>32.95±6.33ab</td>
<td>34.08±6.39ab</td>
<td>54.08±10.20ab</td>
</tr>
<tr>
<td>ox-LDL</td>
<td>50.08±6.75b</td>
<td>13.17±5.39b</td>
<td>14.66±6.86b</td>
</tr>
<tr>
<td>ox-LDL+VE</td>
<td>38.75±5.82a</td>
<td>28.81±7.55a</td>
<td>41.16±11.47a</td>
</tr>
<tr>
<td>ox-LDL+Gen-L</td>
<td>39.42±5.63a</td>
<td>23.54±6.82ab</td>
<td>26.00±11.10ab</td>
</tr>
<tr>
<td>ox-LDL+Gen-M</td>
<td>36.09±4.74a</td>
<td>27.55±7.21a</td>
<td>39.19±9.42a</td>
</tr>
<tr>
<td>ox-LDL+Gen-H</td>
<td>33.73±4.38ab</td>
<td>32.15±7.01ab</td>
<td>51.31±13.40ab</td>
</tr>
</tbody>
</table>

*p<0.05 compared with ox-LDL group; *p<0.05 compared with ox-LDL+VE group. Values are the mean±SD of six samples. Comparison of any two group mean values in ox-LDL+Gen-L, ox-LDL+Gen-M and ox-LDL+Gen-H groups was statistically significant (p<0.01) in MDA content, SOD and GSP-PX activities. There were statistically significant differences between ox-LDL+VE and ox-LDL+Gen-L, or ox-LDL+Gen-H groups in MDA content, SOD and GSP-PX activities (p<0.05).
concentrations of genistein (10, 50, 100 mol/L) and vitamin E (50 mol/L) were randomly counted per slide and the apoptotic index (AI) was counted. AI = the number of apoptotic cells/total number of cells $\times 1,000$ ($\%$).

Statistical analysis. The results are presented as mean±standard deviation (SD) for n number of experiments. Differences between means were evaluated by a one-way ANOVA using GraphPad Prism version 3.0 followed by Tukey’s multiple range test. $p<0.05$ was considered statistically significant.

RESULTS

Effects of ox-LDL, vitamin E and genistein on endothelial cell morphology

Cell morphology to some degree reflects cell function and therefore the morphology of ox-LDL-injured endothelial cells in the presence of vitamin E and different concentrations of genistein (10, 50, 100 μmol/L) was observed. Under light microscopy, control endothelial cells appeared polygonal or spindle-shaped when subconfluent and had a “cobblestone” mosaic appearance after reaching confluence (Fig. 1A). Incubation of endothelial cells with ox-LDL (65 μg/mL) led to morphological changes. Most endothelial cells showed contraction and rounding up (Fig. 1B). Compared to the ox-LDL group, the endothelial cells in the ox-LDL+Gen-L group became narrower, with clear cell boundaries, and a few cells became spindle-shaped (Fig. 1D). However, higher concentrations of genistein (50, 100 μmol/L) and vitamin E (50 μmol/L) largely prevented the morphological changes caused by ox-LDL; the cells displayed the typical conversion to a spindle-shaped morphology (Fig. 1C, E and F). These results indicate that genistein dose-dependently prevents the morphological changes induced by ox-LDL.

Effects of ox-LDL, vitamin E and genistein on endothelial cell function

Incubation of endothelial cells with ox-LDL decreased cell viability as indicated by inhibited MTT reduction and augmented LDH release, compared to the vehicle control ($p<0.05$) (Table 1). ox-LDL also decreased the activities of SOD and GSH-Px ($p<0.05$) (Table 2). This was accompanied by increased lipid peroxidation, as indicated by elevated MDA content (Table 2), as well as by increased monocyte-endothelial cell adhesion (Fig. 2) and endothelial cell apoptosis ($p<0.05$) (Fig. 3). All of these effects of ox-LDL were prevented by preincubation of the endothelial cells with vitamin E or genistein (Tables 1 and 2, Figs. 2 and 3). The cell viability was increased and the release percentage of LDH and MDA content were decreased ($p<0.05$) with the increasing of genistein. Simultaneously, the activities of SOD and GSH-Px in the vein endothelia cells of oxidative injury were increased ($p<0.05$) and the activities of SOD and GSH-Px in the ox-LDL+Gen-H group were significantly higher than that in ox-LDL+VE group ($p<0.05$). The functional status of cells in the ox-LDL+VE (50 μmol/L) group was similar to that in ox-LDL+Gen-M (50 μmol/L) group. Concentration-dependent, protective effects of genistein in cells exposed to ox-LDL were observed for monocyte-endothelial cell adhesion and endothelial cell apoptosis (Figs. 2 and 3).
**DISCUSSION**

Isoflavones, such as genistein, exert diverse biological actions including beneficial effects on the cardiovascular system. Epidemiological studies show that genistein intake is inversely associated with the risk of cardiovascular diseases. Accumulating evidence from cell culture and laboratory animal experiments indicates that isoflavones have the potential to prevent or delay atherosclerosis (1-3). However, the mechanisms of the genistein action on vascular protective effects are unclear. Some studies showed genistein protects against pro-inflammatory factor-induced vascular endothelial barrier dysfunction and inhibits leukocyte-endothelium interaction, thereby modulating vascular inflammation, a major event in the pathogenesis of atherosclerosis. Recent studies found that genistein exerts a novel non-genomic action by targeting important signaling molecules in vascular endothelial cells (ECs). Genistein rapidly activates endothelial nitric oxide synthase and production of nitric oxide in ECs. Further studies demonstrated that genistein directly stimulates the plasma membrane-associated adenylate cyclases, leading to activation of the cAMP signaling pathway. In addition, genistein activates peroxisome proliferator-activated receptors, ligand-activated nuclear receptors important to normal vascular function. These new findings reveal the novel roles for genistein in the regulation of vascular function and provide a basis for further investigating its therapeutic potential for inflammatory-related vascular disease (4, 14). In this study we examined whether genistein protects vascular endothelial cells against the pro-atherosclerotic stressor (ox-LDL) by inducing antioxidant enzymes and preventing apoptosis or not.

The concentration of genistein in plasma of healthy people is about 2.5 μmol/L (14). In vitro experiments, human umbilical vein endothelial cells (HUVEC) cultures were preincubated in the absence or presence of 100 μmol/L genistein for 24 h. The results showed that 100 μmol/L genistein did not damage HUVEC in vitro (15). The doses of genistein (5, 10, 25, 50, 75, 100 μmol/L) were applied to in vitro studies of macrophages and endothelial cells (16). In our experiment, EC were incubated for 24 h in DMEM medium with 5, 10, 25, 50, 75, 100 and 200 μmol/L genistein. Changes in cellular shape and SOD and GSH-PX activities were discovered. Lissin and Cooke (17) also thought genistein was not cytotoxic at concentrations up to 200 μmol/L. The concentration of α-tocopherol in plasma of healthy people is 11.5–46 μmol/L. In the effects of α-tocopherol on oxidized low-density lipoprotein (ox-LDL)-induced apoptosis in human coronary artery endothelial cells, the results showed a high concentration of α-tocopherol (50 μmol/L) was more effective as a low concentration of α-tocopherol (10 μmol/L) (18). So the α-tocopherol of 50 μmol/L was used as a positive control.

The present study showed that genistein can protect against cytotoxic effects of ox-LDL as assessed by cellular morphologic features and lactate dehydrogenase release by cultured endothelial cells. In particular, ox-LDL decreased superoxide dismutase and glutathione peroxidase activities and caused lipid peroxidation, morphological injury and apoptosis. These effects were prevented by pretreatment of the endothelial cells with vitamin E or genistein. The results showed the ox-LDL could impair the cells and cause the decrease of cell viability, and increase the release percentage of LDH and MDA content (p<0.05). Vitamin E and genistein could prevent or decrease the damage of ox-LDL to vascular endothelial cells. Further, the protective effect of genistein was found to be dose-dependent. The mechanisms are that the genistein could increase the activity of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase. Furthermore, some studies showed that genistein can inhibit superoxide anion generation by xanthine/xanthine oxidase (17) and reduce reactive oxygen species (ROS) by attenuating the expression of ROS-producing enzymes (4).

High levels of ox-LDL trigger endothelial dysfunction. Indeed, ox-LDL induces coronary artery disease, in part, by stimulating apoptosis in endothelial cells (19). Blebbing of the plasma membrane of apoptotic endothelial cells releases membrane vesicles, which contain oxidized lipids that increase adhesion of monocytes to the endothelium (20). The concentration of ox-LDL applied to cells in the present study was 65 μg/mL. This concentration is near the range of levels that circulate in patients with coronary artery disease. For instance, ox-LDL concentrations were 1.48±0.105 and 1.20±0.74 mg/dL (i.e., 14.8±10.5 and 12.0±7.4 μg/mL) for men and women, respectively, without clinical evidence of coronary artery disease and they were 3.15±1.22 and 3.03±1.12 mg/dL (i.e., 31.5±12.2 and 30.3±11.2 μg/mL) for men and women, respectively, with coronary artery disease (21). The induction of apoptosis was associated with increased caspase-3 activity and the generation of intracellular reactive oxygen species, both of which effects were attenuated by α-tocopherol (22). Genistein caused inhibition of homocysteine-mediated apoptotic cell death as indicated by inhibition of DNA fragmentation and chromatin condensation (23) and exhibited the strongest activity against hydrogen peroxide-induced apoptosis of HUVE-12 cells (24).

The present study showed that the ox-LDL could result in a significant increase of the apoptosis index (AI) in the vein endothelia cells of oxidative injury (p<0.05). The results of dose-dependent genistein showed the genistein could inhibit the cellular apoptosis decrease with the increasing of genistein (p<0.05). AI in the ox-LDL+Gen-H group was significantly lower than that in ox-LDL+VE group (p<0.05).

Atherosclerosis is initiated by monocytes binding to the endothelium and migrating into the intimal layer to develop into foam cells. The adhesiveness of endothelial cells is due to lipid-induced, oxidant-sensitive transcription of adhesion molecules and chemokines, which promote monocyte binding. This binding is reliant upon intercellular signalling and oxidant-sensitive transcrip-
tion of adhesion molecules. Phytoestrogens may further protect against atherosclerosis by interfering with these initial processes. Although scant information has been published thus far, genistein is capable of inhibiting the expression of ICAM-1 and VCAM-1 on human endothelial cells co-cultured with monocytes. Furthermore, by reducing oxidative stress, it is possible that phytoestrogens deactivate the transcriptional pathways leading to the expression of genes involved in monocyte adhesion and infiltration (17). Our study showed that genistein could decrease the monocyte-endothelial cell adhesion and was found to be dose-dependent, so it was concluded that the effects of genistein could be produced by reducing oxidative damage and inhibiting the expression of cell adhesion-related genes.

Vitamin E, a lipid-soluble vitamin, is a chain-breaking tissue antioxidant (25) that is present in all cell membranes in low concentrations, and is reported to be an anti-atherogenic agent (26). α-Tocopherol is believed to have a cytoprotective effect, which is attributed to its ability to prevent leukocyte-endothelial interactions (27–29), and to act as a scavenger of highly reactive oxygen radicals in various pathophysiological processes (30, 31). α-Tocopherol protects against apoptosis not only by scavenging reactive oxygen species, but also by inhibiting caspase activity, which means that its activity may exceed that of a mere antioxidant (22). In this study, we compared genistein to the antioxidant vitamin E. Preclinical and clinical studies have tested the efficacy of vitamin E on vascular function and the prevention of atherosclerosis (32). We observed that vitamin E pretreatment can protect vascular endothelial cells from oxidative injury during exposure to ox-LDL. The morphology and functional status of cells in the ox-LDL+VE (50 μmol/L) group was similar to that in ox-LDL+Gen-M (50 μmol/L) group. Thus, vitamin E and genistein were equally effective when tested at the concentration of 50 μmol/L. It is likely that both vitamin E and genistein oppose effects of ox-LDL on intracellular signal transduction and gene expression, and thereby prevent the inhibition of SOD and GSH-Px activities caused by ox-LDL. In turn, maintaining the antioxidant activities of SOD and GSH-Px may contribute to the inhibition of vitamin E and genistein of ox-LDL induced lipid peroxidation, monocyte-endothelial cell adhesion, and apoptosis. Hernandez-Montes et al. (8) found that genistein-induced protective effects depend primarily on the activation of glutathione peroxidase mediated by Nrf1 activation, and not on Nrf2 activation or increases in glutathione synthesis.

In conclusion, our results suggest that genistein protects endothelial cells against ox-LDL-induced apoptosis and monocyte adhesion. Further, this effect of genistein is associated with maintenance of antioxidant enzyme activities and inhibition of lipid peroxidation in endothelial cells. These effects of the isoflavone on endothelial cells likely contribute to protection against atherosclerosis and coronary artery disease.

Acknowledgments
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