Effects of dietary phytoestrogen on global myocardial ischemia-reperfusion injury in isolated female rat hearts

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Phytoestrogens are natural nonsteroidal plant-derived compounds. The major classes of phytoestrogens of current interest are isoflavones. Soy products are rich sources for isoflavones. The primary soy-derived isoflavones are genistein, daidzein, and glycitein. Their structures are similar to the structure of estrogen, and they bind to estrogen receptors (20).

A diet containing soy proteins was found to lower plasma concentration of total cholesterol, low-density lipoprotein cholesterol, and very-low-density cholesterol and increase plasma concentrations of high-density lipoprotein cholesterol in young women (36). Female macaques fed a soy protein diet for 6 mo demonstrated enhanced coronary dilatation in response to acetylcholine. Those fed a similar soy protein diet, but with isoflavones removed by ethanol extraction, demonstrated constriction in response to acetylcholine (14). Genistein and daidzein inhibited mitogen-induced proliferation, migration, and extracellular matrix synthesis in cultured human aortic smooth muscle cells (7). Genistein was shown to inhibit intimal thickening and replication in carotid arteries after denudation injury in rats in vivo and to inhibit smooth muscle cell proliferation and migration in vitro (24). Intravenous administration of 1 mg/kg genistein to rats 5 min after the occlusion of the left main coronary artery lowered myocardial necrosis, decreased serum creatine kinase activity, increased myocardial contractility, and decreased the occurrence of ventricular arrhythmias (6). It is unknown whether dietary phytoestrogen has any effect on myocardial ischemia-reperfusion injury.

The experiments reported here were designed to 1) demonstrate if dietary phytoestrogen has any protective activity in global, cardioplegia-protected ischemia, followed by reperfusion, and, if so, 2) to gain information about the potential mechanisms for the cardioprotective effects.

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MATERIALS AND METHODS

Experimental animals. All experiments involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign and were conducted in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Fifty female Sprague-Dawley rats (3 mo old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and fed standard rat chow for 3 mo. At an age of 6 mo, 10 rats were prepared as sham-operated controls (sham). All other rats (n = 40) were ovariectomized (Ovx) and divided into four groups. The anesthesia, Ovx, and sham surgery procedures have been previously described (38). Briefly, the rats were anesthetized with ketamine (20 µg/g ip) and xylazine (0.5 µg/g ip). On each side, the skin was prepared for aseptic surgery and a lateral paralumbar incision was made. The ovary on that side was isolated and removed with the oviduct. Sham animals had the ovaries isolated but left intact. The incisions were closed with stainless steel wound clips that were removed in 7–10 days. Each group was fed one of the diets described in Table 1. The phytoestrogen isoflavone content in the diet containing soybean protein and soybean protein plus soj extract is summarized in Table 2. The Ovx control rats (n = 10) and sham rats were fed a diet supplemented with 200 g/kg of casein, i.e., no dietary phytoestrogen. The low-phytoestrogen (LPE) diet group (n = 10) received 200 g/kg soybean proteins instead of the casein, and the high-phytoestrogen diet groups [HPE, n = 10, and HPE + ICI-187,780 (ICI), n = 10] were both fed 200 g/kg soy protein diets plus 34.4 g/kg high-isoﬂavone soy protein extract. The HPE + ICI group was additionally treated with a weekly subcutaneous injection of ICI in castor oil vehicle at a dose of 10 mg/kg. The dose of ICI was selected based on results of a study by other faculty in the same department. This study (26) showed maximum testosterone levels in rats from the same dose. Rats were fed the same department. This study (26) showed maximum testosterone levels in rats from the same dose. Rats were fed the soybean protein diet supplemented with 200 g/kg soy protein. The same diet was fed to all groups. The dose of ICI was selected based on results of a study by other faculty in the same department. This study (26) showed maximum testosterone levels in rats from the same dose. Rats were fed the same diet as the control group. The dose of ICI was selected based on results of a study by other faculty in the same department. This study (26) showed maximum testosterone levels in rats from the same dose.

Table 1. Diet composition list of the phytoestrogen-free (control) diet, LPE diet, and HPE diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>LPE</th>
<th>HPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soybean protein</td>
<td>0</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Alcohol extract of isolated soybean protein</td>
<td>0</td>
<td>0</td>
<td>34.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>356.12</td>
<td>356.12</td>
<td>356.12</td>
</tr>
<tr>
<td>Corn starch</td>
<td>300</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
<td>11.5</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin mix, AIN 76A</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Diet compositions were measured in grams per kilogram. LPE, low phytoestrogen; HPE, high phytoestrogen.

Table 2. Content of isoflavones in the LPE diet and HPE diet

<table>
<thead>
<tr>
<th>Isoflavone Content, mg/d diet</th>
<th>LPE</th>
<th>HPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>All forms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein-containing compounds</td>
<td>0.37</td>
<td>0.78</td>
</tr>
<tr>
<td>Daidzein-containing compounds</td>
<td>0.18</td>
<td>0.40</td>
</tr>
<tr>
<td>Glycitein-containing compounds</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Total</td>
<td>0.58</td>
<td>1.23</td>
</tr>
<tr>
<td>Aglycone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>0.21</td>
<td>0.45</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.10</td>
<td>0.23</td>
</tr>
<tr>
<td>Glycitein</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>Total</td>
<td>0.33</td>
<td>0.72</td>
</tr>
</tbody>
</table>

mmol/l NaHCO₃ and 2 U/ml heparin; pH 7.4 at 4°C. The same cardioplegic solution was infused retrograde into the coronary arteries through an aortic catheter with the use of a speed-controlled roller pump at an infusion rate of 0.3 ml/min for 5 min. A balloon-tipped catheter was inserted into the left ventricle (LV) via the left atrium and secured. Infusion of the cardioplegic solution was then stopped, and the heart was immersed in the same cold cardioplegic solution for a total ischemia time of 30 min. The heart was mounted in a Langendorff-type isolated heart perfusion system with a water jacket-warmed organ chamber, oxygenator, and reservoir. The hearts were subjected to 2 h of retrograde coronary artery reperfusion with Krebs-Henseleit bicarbonate buffer (Sigma) oxygenated with 95% O₂-5% CO₂, pH 7.4 and 37°C, at a constant pressure of 120 cmH₂O. All hearts were converted to sinus rhythm spontaneously and were allowed to beat at their own rhythm, i.e., not paced. LV pressure (LVP) was continuously measured for the duration of reperfusion using a Digi-MED Heart Performance Analyzer-7 (Micro-Med). Heart rate, maximum LVP, end-diastolic LVP, and the first derivative of LVP (dP/dt) were continuously recorded using a computer installed with Digi-MED System software (MicroMed). The end-diastolic LVP was set so as to not exceed 10 mmHg by adjusting the volume in the balloon at the beginning of the perfusion period. The balloon volume was then kept constant throughout the 120-min reperfusion time. Coronary flow rate (CFR), coronary nitrite concentration, and Ca²⁺ concentrations in both coronary inflow and effluent were measured during the 120 min of reperfusion as previously described (36). Briefly, coronary flow rate (in ml·min⁻¹·100 g⁻¹) was measured by collecting the coronary effluent volume for a total of 120 min. This volume was divided by the time and normalized by the wet weight of the heart (g), measured at the beginning of the experiment. Nitrite production (nmol/g) was estimated as the product of coronary nitrite concentration, which was measured using the Griess reaction (12), and coronary effluent volume normalized by heart wet weight (g), which was not significantly different among the experimental groups. Myocardial Ca²⁺ accumulation (µmol/g) was estimated from the difference in Ca²⁺ concentration (µmol/ml) and was measured using inductively coupled plasma atomic emission spectrometry between perfusate and effluent. This was normalized by coronary effluent volume (ml) and the heart wet weight (g).

After 120 min of reperfusion, mitochondrial function was estimated by the conversion of 3-(4,5-dimethylthiazol-2-yl)-
2,5-diphenyltetrazolium bromide (MTT) to an insoluble formazan dye (20). A 1-mm-thick section of right ventricle and LV was incubated with 1 ml of Dulbecco’s modified Eagle’s medium without phenol red plus 1 ml of 0.5 mg/ml MTT solution for 24 h at 37°C. The MTT media solution was then gently aspirated, and the formazan dye was extracted from the tissue with 0.5 ml of isopropanol and 0.5 ml of dimethyl sulfoxide. The absorbance at 570 nm was measured and corrected for tissue wet weight (g).

Myocardial histomorphology was evaluated using hematoxylin-eosin (H-E)- and hematoxylin-basic fuchsin-picric acid (HBFP)-stained tissue sections. Four digital images of each sample were randomly taken for morphometric analysis using NIH Image software. The contrast, threshold, and magnification of all images were identical. The percentage of myocardium with a positive HBFP stain was calculated. The percentage of interstitial space in the myocardial tissue was determined on H-E-stained sections. The myocardial ultrastructure was studied using ultrathin (0.1 μm) sections, which were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined using a Hitachi 600 transmission electron microscope. Four negative films per sample were randomly taken whenever possible from areas where longitudinal myofibrils were observed so that the influence of mitochondrial orientation on mitochondrial size could be minimized. The films were then scanned to obtain digital images, which were analyzed using NIH Image software. The mitochondrial cross-sectional area was measured. The number of fragmented mitochondria, the number of mitochondria with amorphous matrix densities or granular densities, and the total number of mitochondria studied in each group were counted.

**Measurement of plasma isoflavones.** At the time the heart was isolated, blood plasma was collected and stored at −20°C until used for measuring isoflavones. The concentration of genistein and daidzein in plasma were measured using a high-performance liquid chromatograph and mass spectrometry (HPLC-MS) method (4). The isoflavone conjugates in plasma were hydrolyzed by using β-glucuronidase-sulfatase (Helix pomatia type H-2, Sigma). A 0.5-ml sample of plasma was incubated with 609.5 units of β-glucuronidase and 25 units of sulfatase and 20 μl of 10 mM ammonium buffer at 37°C for 16 h. The isoflavone extracts were then extracted according to the method reported by Lundh et al. (22). The overall recovery efficiency was >80% for both genistein and daidzein (92% for enzyme digestion and 90% for extraction). Biochanin A was used as the internal standard in this assay procedure. The isoflavone extracts were redissolved in 30% acetonitrile in 10 mM ammonium acetate buffer and measured using a Waters 2690 HPLC-MS. HPLC separation was performed on a Waters Xterra MS C18 reverse-phase HPLC column with 3.5-μm particle size and 125-A pore size under isocratic conditions (40% acetonitrile in 10 mM ammonium acetate) at a flow rate of 0.3 ml/min. The single-ion resolution scan function was used for MS analysis after electrospray-negative ionization. The measuring mass charge ratio was set at 269.2 (genistein) and 253.2 (daidzein), respectively. The peak of interest on the chromatogram was identified according to the chromatogram of genistein and daidzein standards, respectively. The integration of peak areas was carried out using the MassLynx software, provided by the mass spectrometer manufacturer. The peak areas were compared with a series of known concentrations of genistein and daidzein standards to estimate the plasma genistein and daidzein concentrations.

**Measurement of plasma estradiol concentration.** Plasma (1 ml) was used for measuring 17β-estradiol concentration using a radioimmunoassay kit, as previously described (38).

**Statistical analysis.** All data are presented as means ± SE and were first analyzed using a two-way analysis of variance (ANOVA) for repeated measures or a single-factor ANOVA as appropriate. If significant differences were observed, a Bonferroni t-test was applied to compare differences between groups. Statistical analyses were done by running appropriate SAS procedures (SAS Institute; Cary, NC). All proportions were compared using a χ² test. The α-level was set at 0.05 and adjustment was made to control experiment type I error where appropriate.

**RESULTS**

The body weights of the animals were (in g) sham, 322.5 ± 37.2; Ovx, 378.4 ± 25.8; LPE, 334.3 ± 30.7; HPE, 361.8 ± 24.3; and HPE + ICI, 367.0 ± 21.6. The wet heart weights before reperfusion were (in g) sham, 1.63 ± 0.15; Ovx, 1.58 ± 0.15; LPE, 1.31 ± 0.9; HPE, 1.49 ± 0.13; and HPE + ICI, 1.52 ± 0.16. The heart weight-to-body weight ratios were (in %) sham, 0.51 ± 0.07; Ovx, 0.42 ± 0.05; LPE, 0.39 ± 0.03; HPE, 0.41 ± 0.13; and HPE + ICI, 0.415 ± 0.05. A representative number of hearts from each group were also weighed after reperfusion but in no instance was the weight after reperfusion >0.1–0.02 g than the prereperfusion weight.

**Plasma genistein and daidzein concentration.** Plasma genistein concentration was 226.02 ± 79.67 ng/ml in those rats fed the HPE diet (HPE and HPE + ICI groups). The average plasma genistein concentration was 20.71 ± 2.33 ng/ml in the LPE group, which was significantly lower than that of the HPE and the HPE + ICI groups (P < 0.01). The concentration of plasma genistein averaged 5.17 ± 0.51 ng/ml in the phytoestrogen-free diet rats (sham and Ovx groups), which was significantly lower than that in the HPE and the HPE + ICI groups (P < 0.0001) and that of the LPE group (P < 0.01). The plasma daidzein concentration was 147.85 ± 50.8 ng/ml in the HPE and the HPE + ICI groups, which was significantly higher than that of the LPE group (18.98 ± 1.9 ng/ml, P < 0.001) and than that of the sham and the Ovx groups (8.1 ± 0.98 ng/ml, P < 0.01).

**Plasma 17β-estradiol concentration.** Plasma 17β-estradiol concentration averaged 60 ± 6 pg/ml in the sham group. The average plasma estradiol concentration of Ovx, HPE, LPE, and HPE + ICI groups was 27 ± 1 pg/ml, which was significantly lower than that of the sham group (P < 0.0001).

**LV dP/dt.** The average LV dP/dt of the sham group during the 120 min of recording was 2,658.9 ± 67.6, which was significantly higher than that of the Ovx group (1,756.3 ± 129.2 mmHg/s). HPE significantly improved LV function with an average LV dP/dt of 2,724.6 ± 118.2 mmHg/s, which was significantly higher than that of the Ovx group but not significantly different than that of the sham group (Fig. 1). The estrogen receptor blocker ICI abolished the effect of HPE on LV dP/dt (1,873.4 ± 148.9), significantly lower
than that of the HPE group and the sham group but not significantly different from that of the Ovx group (Fig. 1). The LPE group LV dP/dt was not significantly different than that of either the Ovx group or the HPE + ICI group (Fig. 1).

**Coronary flow rate.** The CFR in the sham group averaged $9.07 \pm 0.85 \text{ ml/min}^{-1} \cdot 100 \text{ g}^{-1}$. The HPE group had a CFR of $10.85 \pm 0.48 \text{ ml/min}^{-1} \cdot 100 \text{ g}^{-1}$ and the LPE group averaged slightly less. There were no statistically significant differences between the sham, HPE, and LPE groups, but all three had significantly higher flows than the HPE + ICI group ($8.12 \pm 0.69 \text{ ml/min}^{-1} \cdot 100 \text{ g}^{-1}$) and the Ovx group ($6.98 \pm 0.70 \text{ ml/min}^{-1} \cdot 100 \text{ g}^{-1}$) (Fig. 2).

**Nitrite production.** The sham group averaged $2.77 \pm 0.44 \text{ nmol/min}^{-1} \cdot 100 \text{ g}^{-1}$ nitrite during reperfusion and the HPE group averaged $2.21 \pm 0.16 \text{ nmol/min}^{-1} \cdot 100 \text{ g}^{-1}$ (Fig. 3). The nitrite production of the Ovx group was $1.05 \pm 0.11 \text{ nmol/min}^{-1} \cdot 100 \text{ g}^{-1}$, which was significantly less than that of the sham and HPE groups. The HPE + ICI group only produced an average $0.84 \pm 0.04 \text{ nmol/min}^{-1} \cdot 100 \text{ g}^{-1}$, which was significantly less than that of either the sham group or the HPE group but not the Ovx group. The LPE group was not different than either the Ovx or the HPE + ICI group.

**Myocardial 
Ca$^{2+}$ accumulation.** Over the 120 min of reperfusion, there were no significant differences in the amount of Ca$^{2+}$ between the perfusate and the effluent in the sham and HPE + ICI groups. The HPE group demonstrated an apparent Ca$^{2+}$ export, i.e., negative accumulation. There was a significant accumulation of Ca$^{2+}$ in the hearts of the Ovx and LPE groups compared with the other three groups (Fig. 4).

**Myocardial MTT extraction.** After ischemia-reperfusion, myocardial MTT reduction in the Ovx, HPE + ICI, and LPE groups was significantly lower than that in the sham and HPE groups. The latter were not significantly different from each other (Fig. 5). The HPE + ICI group had the lowest myocardial MTT reduction but was not significantly lower than that in the Ovx and LPE groups.

**Myocardial histology.** Representative slides from animals from each group are shown in Figs. 6 and 7. After ischemia-reperfusion, prominent interstitial edema was present on H-E-stained tissue sections from the Ovx (Fig. 6B), the HPE + ICI (Fig. 6D), and the LPE (Fig. 6E) samples, whereas only slight edema was demonstrated in the sham (Fig. 6A) and the HPE samples (Fig. 6C). The amount of edema in the HPE + ICI samples was not different from that of the Ovx samples, and the HPE group was not different from the Ovx group.
Myocardial ultrastructure. Representative transmission electron microscopic results from each group of rats are shown in Fig. 8. The myofibrils were intact in the sham samples. The mitochondria were abnormal in appearance, but most of them contained well-defined cristae. No significant intracellular edema was seen. In the Ovx group (Fig. 8B), the clear sarcoplasmic spaces probably indicate intracellular edema and/or loss of normal structures. The mitochondria are obviously abnormal, swollen, and abnormal in shape, and none contain clearly defined cristae. Most demonstrate amorphous matrix densities or granular densities, which may represent aggregation of proteins (such as denature enzymes) and/or deposition of Ca$^{2+}$ and phosphate (15, 19, 33). Some mitochondria are fragmented and others have their matrix cleared out, resulting in what appears to be vacuoles. The HPE samples (Fig. 8C) are not obviously different from the sham samples, i.e., they show mildly abnormal mitochondria and intact myofibrils. The HPE + ICI samples (Fig. 8D) demonstrate intracellular edema, swollen, fragmented, and vacuolated mitochondria, and mitochondria with amorphous matrix inclusions and/or granular inclusions. Some mitochondria are without clearly defined cristae. These changes are very similar to those seen in the Ovx samples. The LPE samples (Fig. 8E) also showed discontinuation of myofibrils, mild intracellular edema, vacuolated mitochondria with amorphous matrix inclusions and/or granular inclusions. Most of the mitochondria in these samples did not contain sharply defined cristae. The observed changes in the mitochondria were quantitatively indicated by the results of image analysis conducted on samples from all animals (Fig. 9). The cross-sectional areas of the mitochondria of the sham and HPE groups were the same, but both were significantly smaller than those of the Ovx and HPE + ICI groups (Fig. 9A). There was no significant difference in cross-sectional area between the LPE group and any of the other four. The percentage of mitochondria with granules in the sham and HPE groups was significantly lower than that of the Ovx, HPE + ICI, and LPE groups (Fig. 9B). The percentage of fragmented mitochondria in the sham, HPE and LPE groups was significantly lower than that of the Ovx and the HPE + ICI groups.

DISCUSSION

This study shows that dietary phytoestrogen increases circulating genisteen and daidzein. Increased circulating genisteen and daidzein apparently preserve myocardial structure and improve myocardial function after 30 min of global, hypothermic ischemia, followed by 120 min of normothermic crystalloid reperfusion in mature female rats. Our results also indicate that the protective effects of phytoestrogen in the HPE group are not significantly different from those of endogenous estrogen in the sham-operated group. These results point to a cardioprotective effect of the phytoestrogens genisteen and daidzein. The experimental findings in this study do not completely rule out the potential beneficial systemic effects (i.e., on plasma lipids) of dietary phytoestrogens. However, the rat is a poor model of diet-induced cardiovascular disease, so it is difficult to comment on these issues.

Body weights, heart wet weights before reperfusion, and heart weight-to-body weight ratios tended to be less in the LPE group but were not statistically different between the five groups. This result was expected because all groups of rats were fed diets with balanced total caloric intake in controlled amounts.

Only some of the protective effects of phytoestrogens were blocked by treatment with ICI, an anti-estrogen that is reported to block both the α- and β-estrogen receptors. Our results indicated that the protective effects of phytoestrogen were mediated, in part, by interacting with estrogen receptors. Soybean isoflavones are reported to have weak estrogenic activity (5). They bind to both estrogen receptor-α and estrogen receptor-β (20). They reportedly stimulated the transcriptional activity of estrogen receptor that was inhibited by ICI in cell culture experiments (25). After oral intake, genisteen labeled with $^{14}$C or its metabolites, was shown to accumulate in both reproductive and...
peripheral tissues where estrogen receptors were present (3). Both estrogen receptors have been demonstrated in the coronary vasculature (30) and the myocardium (12). We (37) previously reported that male estrogen receptor-α knockout mice developed ventricular arrhythmias and demonstrated marked myocardial damage following global ischemia-reperfusion, suggesting an important role for estrogen receptor-α during ischemia-reperfusion.

One potential mechanism of the cardioprotective effects of phytoestrogens is to maintain nitric oxide (NO) production during ischemia-reperfusion. The basal release of NO from isolated working rat hearts was reduced by ischemia-reperfusion (8). Endothelial NO synthase activity was decreased during ischemia and only partially restored during reperfusion (10). Inhibition of NO synthesis resulted in impaired postischemic recovery of function in isolated hearts (27). NO probably protects the myocardium by improving coronary flow (27) and decreasing the area of no reflow (29) under these experimental conditions. This study indicates that hearts from Ovx rats that had been supplemented with phytoestrogen produced significantly more nitrite than hearts from Ovx rats. This suggests that phytoestrogens maintained NO release. In this study, the effect of phytoestrogen on preserving myocardial systolic function, represented by increased dP/dt max, did not totally depend on the effect of phytoestrogen on improving coronary flow, i.e., myocardial perfusion, suggesting that flow-mediated mechanisms alone are not sufficient to provide the protection observed. The differences in nitrite production between the LPE group and the Ovx group were not paralleled by those of coronary flow rate in this study, further suggesting that NO may exert protective effects through mechanisms beyond improving coronary flow.

Treatment with ICI in these studies blocked the effect of the phytoestrogen extract on NO release. This suggests phytoestrogens act by interacting with estrogen receptors in maintaining normal NO levels. This may be important because an estrogen response element and activator protein 1, both estrogen receptor-
binding motifs, are present in the NO synthase gene (28). In a previous study, subcutaneous administration of genistein at a daily dose of 0.2 mg/kg to Ovx rats for 4 wk increased the activity of Ca\textsuperscript{2+}-dependent NO synthase to the same extent as 17\beta-estradiol (34). Both endothelial NO synthase and inducible NO synthase were expressed in endothelial cells, vascular smooth muscle cells, and cardiac myocytes (28). Phytoestrogens may maintain NO production by enhancing the activity and the expression of NO synthase by interacting with estrogen receptors. This, however, does not rule out other possible mechanisms through which phytoestrogens may also regulate NO production.

Preserving mitochondrial structure and function may be another mechanism of cardioprotection by phytoestrogens. It was reported (16) that mitochondria isolated from dog myocardium after 60 min of ischemia had only 3–4% of the capacity to completely oxidize pyruvate to carbon dioxide and water. The activity of electron transfer complex I (NADH-Q reductase) was decreased in ischemic cardiac muscle (32). Structural changes observed in dysfunctional mitochondria from the Jennings et al. (16) study included relatively clear matrices, rare and loosely packed cristae, and dense mitochondrial granules. A reduction in energy production by mitochondria was shown to result in decreased recovery of cardiac function after reperfusion (31). 17\beta-Estradiol reportedly stabilized mitochondrial function, i.e., prevented decreases in transmembrane potential and energy charge-redox state, in neurons exposed to apoptotic agents (24). Zheng and Ramirez (39) have demonstrated that the oligomycin-sensitive-conferring protein, a subunit of the F\textsubscript{0}F\textsubscript{1} mitochondrial ATP synthase-ATPase system, is apparently responsible for maintaining Ca\textsuperscript{2+} homeostasis in the mitochondria. Loss of estrogen may block this function and result in Ca\textsuperscript{2+} accumulation within the mitochondria.

Fig. 7. Hematoxylin, basic fuchsin, and picric acid-stained myocardial samples and results of image analysis. Scale bar, 50 \textmu m. A: sham; most of myocytes are not stained (viable) and only a few were positively stained (intensive dark areas), i.e., injured. B: Ovx; most of the myocytes are injured, the extent of which is much more than that of the sham group. C: HPE; few myocytes are injured, the extent of which are not different from that of the sham group but much smaller than that of the Ovx group. D: HPE + ICI; most of myocytes are injured to an extent not different from that of the Ovx group but much more than those of the sham and the HPE groups; interstitial edema is clearly shown. E: LPE; most of myocytes are injured, the extent of which is not different than those of the Ovx and the HPE + ICI groups. F: the percentage of positively stained myocardium (%), means \pm SE. *Significantly different from the Ovx group. **Significantly different from the HPE + ICI and LPE groups.
previous study (38), estrogen was shown to improve myocardial function by preserving mitochondrial structure and mitochondrial function in female rat hearts. In the present study, dietary phytoestrogen significantly inhibited mitochondrial swelling, reduced the number of mitochondria that were either fragmented, fragmented with granules, or unfragmented with granules, and improved myocardial MTT reduction. Treatment with ICI significantly attenuated the effect of phytoestrogens on mitochondrial function. This suggests that phytoestrogens may inhibit Ca\(^{2+}\) entry through mechanisms independent of classical estrogen receptors, possibly related to the oligomycin-sensitive-conferring protein mechanism. Other studies have suggested an action of genistein, independent of estrogen receptor, in inhibiting Ca\(^{2+}\) entry. Genistein reportedly (9) antagonized Ca\(^{2+}\) entry in isolated rabbit coronary artery rings and inhibited L-type Ca\(^{2+}\) currents in guinea pig ventricular myocytes through an action not affected by ICI. Genistein was reported to inhibit L-type Ca\(^{2+}\) channel currents in rat ventricular myocytes by acting as a tyrosine kinase inhibitor. The possible mechanism was reported (18) to be influencing the phosphorylation level and thus decreasing the open probability of the channel.

To our knowledge, this study is the first indication that dietary phytoestrogens have a protective role during global, hypothermic, cardioplegia-protected ischemia, followed by normothermic reperfusion in rats. Our experimental results suggest that the protective effects of dietary phytoestrogens are the following: 1) main-
taining NO release through classical estrogen receptor-mediated mechanisms, 2) preserving mitochondrial structure and function through the function of classical estrogen receptor, and 3) attenuating myocardial Ca\(^{2+}\) accumulation through mechanisms independent of classical estrogen receptor.

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