Phytoestrogen genistein supplementation increases eNOS and decreases caveolin-1 expression in ovariectomized rat hearts

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Abstract: This study examined whether genistein influences the production of nitric oxide (NO) and expression of endothelial nitric oxide synthase (eNOS) and the modulators of eNOS activity in ovariectomized (OVX) rat hearts. Female mature Sprague-Dawley rats were subjected to bilateral ovariectomy, OVX rats were randomly divided into four groups: 17β-estradiol (0.1 mg/kg, s.c. daily) was used as the positive control; low dose of genistein (0.5 mg/kg, s.c. daily); high dose of genistein (5.0 mg/kg, s.c. daily) and model. Sham operations as controls, the treatment lasted 6 weeks. Blood pressure, heart rate, plasma estradiol, heart and uterine weights were measured. Nitrite production in the myocardium was determined by nitrate reductase method. Protein level of eNOS, caveolin-1 and calmodulin was determined by Western blot. The results showed that nitrite production and eNOS protein in homogenized ventricular tissue was attenuated by approximately 53% and 67% in OVX rats compared with those in sham rats, respectively. Genistein increased nitrite production in rat heart in a dose-dependent manner, genistein at the dose of 5 mg /kg·d –1 resumed nitrite production to a level similar to that in sham operated rats. Administration of genistein also increased eNOS protein expression in OVX rats myocardium with a concomitant decrease in the expression of caveolin-1, an endogenous eNOS inhibitory protein. Another eNOS stimulatory protein, similar to that in sham operated rats. Administration of genistein also increased eNOS protein expression in OVX rats myocardium with a concomitant decrease in the expression of caveolin-1, an endogenous eNOS inhibitory protein. Another eNOS stimulatory protein, calmodulin, was unchanged in these treatments. These effects were also observed in rats treated with 17β-estradiol. Genistein at the dose of 5.0 mg/kg·d –1 augmented uterine weight but this side effect in reproductive system was less than that of 17β-estradiol. These results suggest that genistein supplementation and estrogen replacement therapy directly increase eNOS functional activity and NO production in the hearts of the OVX rats, but genistein has less side effects on the reproductive system than 17β-estradiol.

Key words: genistein; nitric oxide; endothelial nitric oxide synthase; caveolins

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Population-based observational studies revealed that the incidence of cardiovascular events is higher in postmenopausal women than that in premenopausal women, but the increased incidence was cut in half after taking estrogen or phytoestrogen replacement therapy in postmenopausal women\cite{1,2}. Estrogen and phytoestrogen have important protective effects on the cardiovascular system that are mediated, to a large extent, by an enhancement in nitric oxide (NO) production by the endothelial isofrom of NO synthase (eNOS) due to increases in both eNOS expression and level of activation \cite{3,4}.

eNOS, originally identified in large vessel endothelium, is also expressed in cardiac myocytes. eNOS is quantitatively associated with caveolin, the structural protein of caveolae, which serves to inhibit eNOS. Cell stimulation with Ca\textsuperscript{2+} mobilizing agonists promotes calmodulin (CaM) binding to eNOS and caveolin dissociation from the enzyme, rendering the enzyme active \cite{5}. The isoform caveolin (caveolin-1, Cav-1) expressed in myocytes, therefore, modulates the catalytic activity of cardiac eNOS and hence regulates NO production and its biological effects \cite{6}.

Estrogen is able to increase NO production through up-regulation of the activity of eNOS. Estrogen replacement therapy, however, has adverse effects on the reproductive system and a risk of venous thromosis that limit their therapeutic use. Genistein, a dietary-derived isoflavonoid bearing an isoflavonoid structure, has many protective effects of 17β-estradiol (E\textsubscript{2}) on the cardiovascular system, such as reversing endothelial dysfunction in ovariectomized rats, improving the activity of eNOS, reducing infarct size in an experimental model of myocardial ischaemia-reperfusion injury \cite{7,8}. However, it is unknown whether genistein influences the production of NO and expression of eNOS in hearts. In this study, we used genistein and E\textsubscript{2} supplementation to the ovariectomized rats to investigate their effects on NO production and the eNOS expression, and their effects on the posttranslational allosteric modulators of eNOS, caveolin-1 and calmodulin, in hearts.

1 MATERIALS AND METHODS

1.1 Animals preparation and experimental protocol
Female mature Sprague-Dawley rats (200–220 g, Laboratory Animal Center, Central South University, China) were subjected to bilateral ovariectomy (OVX). Sham operated animals (sham) were used as controls. All rats were housed in standard conditions, light controlled cycle (06:00–18:00) and were given free access to soybean-free chow and drinking water. Experiments were consistent with the Guide for the Care and Use of Laboratory Animals (NIH Publication NO85-23, revised 1996).

OVX was performed as described in other studies \cite{9}. Briefly, the rats were anesthetized using pentobarbital sodium (35 mg/kg, i.p.). The lower part of the back was shaved and a single 1.5 to 2 cm incision was made in the skin to expose the back muscles. A small 1 to 2 cm incision was made in the muscles overlying the ovaries on both sides, and the ovaries were isolated, tied off with sterile suture, and removed. The muscles and the skin were sutured separately, and the rats were allowed to recover for 3 weeks before the time of the experiment. Sham operation rats were performed by exposing the ovaries without isolation.

Three weeks after surgery, the OVX rats were randomly assigned to four treatment groups, 12 rats each group. The first group received the vehicle as model (OVX, 100 µl sesame oil, s.c. daily); the second group was given with a low dose of genistein (L-GEN, 0.5 mg/kg in 100 µl sesame oil, s.c. daily); the third group received a high dose of genistein (H-GEN, 5.0 mg/kg in 100 µl sesame oil, s.c. daily); the forth group received 17β-estradiol (E\textsubscript{2}, 0.1 mg/kg in 100 µl sesame oil, s.c. daily) was used as the positive control; sham operation rats were treated with vehicle as control (sham, 100 µl sesame oil, s.c. daily). The treatment lasted for 6 weeks and all rats were given soybean-free diet during the treatment.

1.2 Arteria systolic pressure, heart rate, body weight and uterine assay
Systolic arterial blood pressure and heart rate (HR) were measured by the tail cuff method at baseline conditions. Body weight was also monitored at the same time points every three days. At the end of experiment uterus and the hearts were removed immediately and were subsequently weighed.

1.3 Blood E\textsubscript{2} concentration and nitrite production in
the myocardium
At the end of experiment, blood samples were collected from the carotid artery, plasma was obtained by centrifuging at 3,000×g. Plasma E2 concentration was measured by using the radioimmunoassay kit (Juding Biological Engineering Company, Tianjin). Nitrite production in the myocardium was determined by nitrate reductase method. Briefly, at the end of experiment, the rats were anesthetized using pentobarbital sodium (35 mg/kg, i.p.), the right carotid artery was cannulated and a PE-50 tube was inserted into the left ventricle. The heart was perfused with physiological saline and was taken out, the great vessel, atria and right ventricular free wall were removed, ventricular tissue samples were homogenized in a lysis buffer comprised of 25 mmol/L Hepes (pH=7.2), 140 mmol/L NaCl, 5.4 mmol/L KCl at 0 ºC. After centrifugation at 10,000×g for 5 min, reduction of nitrate to nitrite with the method of nitrate reductase kits (Jiancheng Biological Engineering, Nanjing), the amount of nitrite was corrected by protein amount which was measured by the Bradford method (Bio-Rad).

1.4 Western blot analysis
Western blot analysis was conducted as described elsewhere[6]. Briefly, ventricular tissue samples were homogenized in a lysis buffer (0.5 ml/100 mg tissue) comprised of 50 mmol/L Tris (pH 7.5), 0.1 mmol/L EDTA, 2 µmol/L leupeptin, 1 mmol/L phenylmethylsulfonylfluoride, 1% (V/V) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate at 0 ºC, after centrifugation (12,000×g for 5 min) protein was quantified in the supernatant using Bradford assay. For Western blot analysis of eNOS, caveolin-1 and calmodulin, protein was separated through 8%, 12% SDS polyacrylamide gel and electrotransferred to PVDF membranes, unbound sites were blocked 2 h at room temperature with 5% (W/V) nonfat milk in Tris-buffered saline containing 20 mmol/L Tris-HCl (pH 7.6), 140 mmol/L NaCl, and 0.1% (W/V) Tween-20. The membranes were incubated with the specified primary antibody [anti-eNOS, anti-caveolin dilution at 1:1,000, and anti-calmodulin dilution at 1:800 (Santa Cruz Biotechnology)] in TBS buffer containing 5% nonfat dry milk overnight at 4 ºC. After 4 washes, the blots were incubated with secondary antibodies linked to horseradish-peroxidase labeled anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature. The blots were developed in a chemiluminescence system (Santa Cruz Biotechnology) and then visualized by exposure to Kodak X-ray film. The accuracy of protein loading on the gel was verified by re-probing with mouse monoclonal β-actin antibody (Neomaker Company). Densitometry was analyzed using the Alphalmager 2200 (Alpha Innotech).

1.5 Statistical analysis
Data are presented as mean ± SEM. Statistical analysis was performed with SPSS for Windows. Statistical comparison was determined by analysis of variance. P<0.05 was considered as being significantly different.

2 RESULTS
2.1 Effects of genistein and 17β-estradiol supplementation on ovariectomy
The effects of genistein treatment on body weight, heart weight, heart-to-body ratio, uterine weight, blood pressure (BP), heart rate (HR) and plasma concentrations of estradiol are presented in Table 1. Genistein treatment did not alter the decrease in plasma estradiol levels (<3.5 pmol/L) produced by bilateral ovariectomy. BP and HR values in OVX rats treated with genistein were similar to those in vehicle-treated controls. OVX animals gained an average

Table 1. Effects of genistein and E2 treatment on body weight, uterine weight, heart weight, heart-to-body ratio (HW/BW), blood pressure (BP), heart rate (HR) and serum concentrations of estradiol

<table>
<thead>
<tr>
<th></th>
<th>OVX</th>
<th>L-GEN</th>
<th>H-GEN</th>
<th>E2</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ Body weight (g)</td>
<td>105±11</td>
<td>99±8</td>
<td>102±6</td>
<td>67±9*</td>
<td>71±7*</td>
</tr>
<tr>
<td>Uterus (mg)</td>
<td>108±6</td>
<td>112±10</td>
<td>252±9*</td>
<td>637±35***,**</td>
<td>494±97*,#</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>995±48</td>
<td>976±59</td>
<td>985±43</td>
<td>933±32*</td>
<td>925±45*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>3.16±0.09</td>
<td>3.17±0.06</td>
<td>3.18±0.08</td>
<td>3.33±0.09*</td>
<td>3.31±0.07*</td>
</tr>
<tr>
<td>BP (systolic mmHg)</td>
<td>132±8</td>
<td>124±7</td>
<td>121±6</td>
<td>125±10</td>
<td>122±9</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>371±12</td>
<td>365±13</td>
<td>369±9</td>
<td>361±16</td>
<td>364±10</td>
</tr>
<tr>
<td>Estradiol (pmol/L)</td>
<td>&lt;3.5</td>
<td>&lt;3.5</td>
<td>&lt;3.5</td>
<td>132±18*</td>
<td>36±11*</td>
</tr>
</tbody>
</table>

mean ± SEM. Δ Body weight, the increase value of body weight during the whole experiment; OVX, ovariectomized treated with vehicle; L-GEN, treated with 0.5 mg/kg·d–1 genistein. H-GEN, treated with 5 mg/kg·d–1 genistein; Sham, sham operation rats treated with vehicle. *P<0.05 vs OVX, **P<0.05 vs sham rats, ***P<0.05 vs H-GEN. n=12.
of (105±11) g, which was greater than that in sham-operated controls [(71±7) g, P<0.05]. The total heart weight in 17β-estradiol treated and sham groups was lower than that in OVX group, 17β-estradiol treatment resulted in a higher heart-to-body weight ratio than that in vehicle controls. Genistein did not change the gain of body weight and total heart weight compared with OVX group. In addition, genistein at dose of 0.5 mg/kg·d−1 did not augment uterus weight, compared with OVX treated with vehicle, dose of 5 mg/kg·d−1 augmented significantly uterus weight [(252±9) mg, P<0.05]. Rats treated with 17β-estradiol had greater uterus weight than that of the OVX treated with high dose of genistein [(637±35) mg, P<0.01].

2.2 Nitrite production in the myocardium
Nitrite production in the homogenized ventricular tissue was studied at the end of experiment. The effects of genistein and estradiol supplementation were shown in Fig. 1. Ovariectomy markedly reduced nitrite production in homogenized ventricular tissue. Genistein supplementation increased nitrite production in a dose-dependent manner. Genistein at the dose of 5 mg/kg·d−1 and E2 treatment could restore the reduced nitrite production caused by OVX to the level similar to sham operated rats. Nitrite production in the myocardium showed no significant difference between the sham operated rats and E2, H-GEN treated rats.

Fig. 1. Effects of genistein and E2 supplementation on nitrite production in the homogenized ventricular tissue. The amount of nitrite was corrected by protein amount which was measured by the Bradford method. mean ± SEM. * P<0.05 vs H-GEN, ‡ P< 0.05 vs sham. n=12.

2.3 eNOS, caveolin-1 and calmodulin protein level in each group
As shown in a representative Western blot by densitometry analysis (Fig.2A), a significant reduction in the ventricular eNOS protein was detected in OVX rats compared with that in sham operated rats. Genistein supplementation increased the eNOS protein expression compared with that of the OVX group and displayed a dose-dependent effect. 5 mg/kg·d−1 genistein could restore the reduced eNOS protein expression caused by ovariectomy to the level similar to that in the sham operated controls. On the other hand, when compared with caveolin-1 expression in the drug treatment groups (Fig.2B), the result was reversed. Genistein decreased caveolin-1 expression and also displayed a dose-dependent effect. As shown in Fig.2B, calmodulin expression in heart tissues was not different among the five groups of rats.

Fig. 2. A: Western blot analysis of effects of genistein and E2 supplementation on eNOS protein expression in the rat hearts in each group. β-actin was used as internal control. B: Densitometric value of caveolin-1 (Cav-1) and calmodulin (CaM) protein expression in the rat hearts. *P<0.05 vs H-GEN, ‡ P<0.05 vs sham. n=12.

3 DISCUSSION
The present study demonstrated that phytoestrogen genistein supplementation increased eNOS protein expression and NO production in the hearts of OVX rats. The study was extended to investigate the two counterbalancing allosteric modulators, caveolin-1 and calmodulin in the regulation of eNOS activity by genistein treatment. Our data show: (1) both phytoestrogen genistein treatment and E2 are able to increase the eNOS expression and NO production in the hearts of OVX rats in dose-dependent manners. (2) Caveolin-1, a negative modulator of eNOS, is increased by OVX and restores to normal level of ex-
pression after genistein and E$_2$ replacement, however, the positive modulator calmodulin is not changed by genistein and E$_2$ treatment. (3) Genistein and E$_2$ show overlapping effects on the regulation of NO, however, the side effects of genistein on the reproductive system was obviously less than that of E$_2$.

Genistein is a naturally occurring plant-derived estrogen-like compound and has been shown to mimic many of the biological activities of 17β-estradiol. Previous reports have demonstrated that genistein has, either in vitro or in experimental animals, a positive effect on cardiovascular protection, thus, raising the possibility that it may have the potential to be a cardiovascular-protective agent, with the benefit of no increased risk of cancer or less side effects in the reproductive system. More specifically, it has been reported that the protective effects of genistein supplementation on the cardiovascular apparatus may be mediated by an increased production of NO from the vasculature$^{[10,11]}$. NO has several actions that are vasoprotective, increases the activity in endothelial cells and enhances NO release.$^{[14]}$ More specifically, it has been demonstrated that genistein and E$_2$ supplementation increases eNOS and decreases caveolin-1 biological activities of 17β-estradiol. Previous reports have demonstrated that the expression of cardiac eNOS protein was similar potency to E$_2$, markedly increases eNOS protein expression and functional activity that can lead to the production of NO, a vasoactive molecule, in the hearts of OVX rats. These findings implicate that genistein has a potential protection to resist the diseases in which the regulation of NO, however, the side effects of genistein on the reproductive system was obviously less than that of E$_2$.

As a cautionary note, Western blot evaluations do not reveal whether the proteins are interacting in any way. Nevertheless, a higher ventricular expression of eNOS relative to caveolin-1 can be viewed as suggestive of a less eNOS/caveolin-1 association and a greater functional eNOS activity. Furthermore, we demonstrated that NO metabolic production, nitrite, was increased after genistein treatment. The precise molecular mechanisms of genistein and E$_2$ supplementation in the regulation of eNOS activity still remain unknown. eNOS activity is under a posttranslational regulation, recent reports have shown that the eNOS activity is influenced by a microdomain in cell membrane named caveolae$^{[15]}$. A characterized mechanism for regulating eNOS activity is its binding to the caveolar protein, caveolin-1, that association represses eNOS activity. Stimulation of eNOS occurs when Ca$^{2+}$-activated calmodulin displaces caveolin-1 from its binding site on the eNOS molecule$^{[16]}$. Therefore, the abundance of caveolin-1 is involved in the eNOS activity and NO production. It has recently been reported that the protein expression of caveolin-1 in median eminence of rats was markedly increased by estrogen depletion, the increased caveolin-1 inhibited the activity of eNOS and reduced eNOS-dependent NO production$^{[17]}$. In cerebral blood vessels, caveolin-1 and eNOS expressions altered in opposite directions with chronic estrogen depletion and repletion in female rats$^{[18,19]}$. These results imply that estrogen can, either through a genomic or non-genomic effect on modulation caveolin-1, influence the activity of eNOS. In the present study, we found that not only E$_2$ but also genistein, in addition to theirs association with higher eNOS expression in the rat cardiac myocyte, was also associated with lower ventricular caveolin-1 expression. These findings, therefore, point to the possibility that the genistein-related potentiation of eNOS-dependent NO production is due not only to an up-regulation of eNOS protein expression, but also to a combination of increased eNOS and diminished caveolin-1 expression in the heart.

In summary, we report that genistein, in a dose with similar potency to E$_2$, markedly increases eNOS protein expression and functional activity that can lead to the production of NO, a vasoactive molecule, in the hearts of OVX rats. These findings implicate that genistein has a potential protection to resist the diseases in which the release of NO is reduced in the heart. Phytoestrogen genistein supplementation increases eNOS and decreases caveolin-1 expression in ovariecromized rat hearts.

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