Ameliorative effect of daidzein: A caveolin-1 inhibitor in vascular endothelium dysfunction induced by ovariectomy

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Estrogen deficiency was produced in female Sprague-Dawley rats by surgical removal of both the ovaries and these animals were used 4 weeks later. Endothelium-dependent and endothelium-independent relaxations due to acetylcholine and sodium nitroprusside were observed respectively, in isolated rat thoracic aortic ring preparation. Extent of lipid peroxidation was measured by estimating serum TBARS. Integrity of vascular endothelium was assessed using hematoxylin and eosin staining. Generation of nitric oxide was measured indirectly, by estimating serum and urinary nitrite/nitrate concentration. Ovariectomy produced significant vascular endothelial dysfunction, measured in terms of reduced acetylcholine-induced endothelium-dependent vasorelaxation, serum and urinary nitrite/nitrate concentration and impairment of integrity of vascular endothelium. Administration of daidzein (0.2 mgkg−1day−1, sc 0.4 mgkg−1day−1, sc and 0.8 mgkg−1day−1, sc) and Atorvastatin (30 mgkg−1day−1, po Positive Control) for one week markedly improved vascular endothelial dysfunction due to increase in nitric oxide bioavailability perhaps by inhibiting caveolin-1 and activation of PI3K-AKT pathway.

Keywords: Caveolin, Estrogen, Vascular endothelium dysfunction, Ovariectomy.

Vascular endothelial dysfunction is characterized by shift of endothelial actions towards reduced vasodilation, proinflammatory and prothrombic properties. The modulation of vascular L-arginine/nitric oxide synthetase system is hallmark of vascular endothelial dysfunction. Various pathological disorders such as hypertension, coronary artery disease, atherosclerosis, stroke, hyperhomocysteinemia, diabetes mellitus have been noted to decrease the formation and bioavailability of nitric oxide which contribute markedly to the development of vascular endothelial dysfunction.

Oestrogen is an important vasoprotective molecule, with marked effects on vasculature, mediated through increased bioavailability of nitric oxide and eNOS is the principle enzyme to generate nitric oxide from L-arginine in vascular wall. Oestrogen deficiency, associated with menopause, is the major cause of vascular endothelial dysfunction. Oestrogen regulates eNOS activity either genomically or non-genomically. Oestrogen upregulates eNOS and downregulates its inhibitory protein caveolin-1.

Oestrogen modulation of eNOS expression is mediated through estrogen receptors α (ER α) and β (ER β) which are expressed on endothelial cells. Short term exposure to oestrogen, enhances the release of nitric oxide from vascular endothelium without altering eNOS expression. Oestrogen is also reported to activate mitogen activated protein kinase and phosphoinositide-3 kinase-AKT pathway which also cause the activation of eNOS.

Caveolin is a transmembrane protein present in small invaginations of the plasma membrane called caveolae. Caveolins comprise of three isoforms i.e., caveolin-1, -2, and -3. Caveolin-1 and caveolin-2 are widely expressed in various tissues. Caveolin-1 is a specific marker of caveolae and is up-regulated by oxidized LDL, oestrogen deficiency and hyperglycemia. It serves as cholesterol binding protein and help cholesterol to move from endoplasmic reticulum through golgi apparatus to plasma membrane of endothelial cells. Caveolin-1 binding suppresses the activity of eNOS. Alterations in caveolin/eNOS interaction influence various mechanisms of diseases such as atherosclerosis, diabetes, cirrhosis and oestrogen deficiency.

Daidzein, a caveolin-1 inhibitor, has been reported to increase the activity of eNOS. Ovariectomy has been reported to upregulate the expression of...
caveolin-1. Hence, we speculate that up-regulation of caveolin-1 activity may produce vascular endothelial dysfunction. Thus, present study was designed to investigate the effect of daidzein on ovariectomy-induced vascular endothelial dysfunction.

Materials and Methods

Young, female albino rats of Sprague-Dawley strain weighing between 250-300 g were employed in the present study. They were housed in an animal house, in group of three, in polypropylene cage with husk bedding and were exposed to natural photoperiod. The rats were fed on standard chow diet (Kisan Feeds Ltd., Mumbai, India) and were provided water ad libitum. The study protocol was approved by Institutional Animal Ethics Committee.

Ovariectomised rats—Rats were anaesthetized with chloral hydrate (250 mgKg−1, ip). Incision was made on left and right dorsal side of flanks. Ovaries along with uterus were pulled out and suture was applied at the end of uterus and beginning of ovary. Ovaries on both sides were removed. The uteri on both sides were pushed back and incisions were sutured in layers. Antibiotic powder (neosporin) was applied on wounds and animals were allowed to recover for four weeks

Isolated rat aortic ring preparation—The rats were sacrificed by cervical dislocation followed by decapitation. The thoracic aorta was exposed and carefully dissected out. The aorta was placed in ice cold aerated Krebs-Henseleit solution (NaCl, 118 mM; KCl, 4.7 mM; CaCl2, 2.5 mM; MgSO4·H2O, 1.2 mM; NaHCO3, 25.0 mM; KH2PO4, 1.2 mM; Glucose, 11.1 mM) and connective tissue was removed. It was cut into 3-4 mm wide rings and one ring was mounted in organ bath of 10 ml capacity, containing Krebs-Henseleit solution maintained at 37°C, pH 7.4 and bubbled with carbogen (95% oxygen and 5% carbon dioxide). The ring was allowed to equilibrate for 90 min under tension of 1.5 g and preparation was washed with Krebs-Henseleit solution once in every 15 min. After 90 min equilibration period, (80 mM) KCl was added to the organ bath to record contraction. KCl treatment was repeated thrice to sensitize the isolated aortic ring preparation. Endothelium dependent relaxation to graded doses of acetylcholine (10−8-10−4M) were recorded in phenylephrine (3×10−6M) precontracted isolated aortic ring preparation using force transducer (FT-3147) of two channel physiograph recorder (INCO, Ambala, India). Similarly, endothelium independent relaxation (Carried out in dark) to sodium nitroprusside (10−3-10−4M) in phenylephrine (3×10−6M) precontracted isolated aortic ring preparation was recorded.

Removal of vascular endothelium—Vascular endothelium of aorta was removed using blunt forceps. Removal of endothelium was confirmed by absence of relaxation to acetycholine (1×10−4M) in phenylephrine (3×10−6M) precontracted preparations.

Estimation of serum and urinary nitrite/ nitrate concentration—Estimation of nitrite and nitrate was carried out to assess the generation of nitric oxide. Each rat was individually placed in metabolic cage and its urine was collected for 24 h. Animals were denied water during 24 h of study. Blood samples from rats were collected from retro-orbital sinus and allowed to clot. The eppendorf tubes were kept at 4°C for 30 min and centrifuged at 1500 g for 10 min to obtain serum. Nitrate was first reduced to nitrite by copper-cadmium alloy. Nitrite thus formed was treated with sulphanilamide, a diazotizing agent, in acidic media to form transient diazonium salt. This intermediate was allowed to react with coupling reagent, M-naphthyl-ethylendiamine (NED) to form stable pink colour azo compound. Pink colour so developed was measured at 545 nm using spectrophotometer (DU 640B Spectrophotometer, Beckman Coulter Inc. CA, USA).

Estimation of serum malondaldehyde (MDA) concentration—MDA concentration was measured by estimating thiobarbituric acid reactive substances (TBARS) . TBARS assay forms a colour product. The primary TBARS chromogen is a reaction product of thiobarbituric acid and malonalodihyde (MDA), which is generated during the analytical procedure from the decomposition of lipid hydroperoxides in the sample. Absorbance of the product was noted spectrophotometrically (DU 640 B Spectrophotometer, Beckman Coulter Inc. CA, USA) at 532 nm against prepared blank solution. A standard curve using 1, 1, 3, 3-tetraethoxypropane was plotted to calculate the concentration of TBARS.

Histological study—Aorta preserved in (10%) formalin was immersed in series of alcohol and xylene than kept overnight in molten paraffin wax maintained at 60°C. Paraffin block of the tissue sample was prepared and 10 parallel cefalocaudol thoracic aorta artery of 4 mm thickness cross-sliced incisions were performed every 1 mm using microtome. The sections were fixed on a slide smeared...
and stained with haematoxylin for 3 minutes and excess haematoxylin was washed with water. The slide was then counter stained with 1% solution of eosin for 3 minutes, followed by washing with water for one minute. The aortic sections were examined for presence of intact, partially intact and denuded lining.

**Experimental design**—Animals were randomly divided into ten groups comprising 6 rats in each group.

Group I (Sham control): rats were kept for five weeks for age-matched studies. No treatment was given to these rats.

Group II (Ovariectomised Control): Rats were subjected to ovariectomy and maintained for four weeks.

Group III [Daidzein vehicle (DMSO) treated control]: Rats were administered DMSO for one week.

Group IV [Atorvastatin vehicle (Carboxymethyl cellulose (0.5%) treated control]: Rats were administered 0.5% sodium carboxymethylcellulose for one week.

Group V (Daidzein Treatment): Rats were administered Daidzein (0.8 mg kg<sup>-1</sup> day<sup>-1</sup>, sc) for one week.

Group VI (Atorvastatin Treatment; n=6): Rats were administered atorvastatin (30 mg kg<sup>-1</sup> day<sup>-1</sup>, po) for one week.

Group VII [Daidzein (low dose) treated ovariectomised]: Ovariectomised rats were administered daidzein (0.2 mg kg<sup>-1</sup> day<sup>-1</sup>, sc) for one week starting from the fourth week after ovariectomy.

Group VIII [Daidzein (medium dose) Treated Ovariectomised]: Ovariectomised rats were administered daidzein (0.4 mg kg<sup>-1</sup> day<sup>-1</sup>, sc) for one week starting from the fourth week after ovariectomy.

Group IX [Daidzein (high dose) Treated Ovariectomised]: Ovariectomised rats were administered daidzein (0.8 mg kg<sup>-1</sup> day<sup>-1</sup>, sc) for one week starting from the fourth week after ovariectomy.

Group X (Atorvastatin Treated Ovariectomised): Ovariectomised rats were administered atorvastatin (30 mg kg<sup>-1</sup> day<sup>-1</sup>, po) for one week starting from the fourth week after ovariectomy.

**Drugs and Chemicals**—Daidzein (Tocris-Cookson, UK) was suspended in dimethyl sulfoxide (DMSO). Atorvastatin (Dr. Reddy’s Laboratories, Hyderabad, India) was suspended in 0.5% sodium carboxymethylcellulose. All other chemicals were of analar quality. All drug solutions were freshly prepared before use.

**Statistical Analysis**—All values were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using Graph Pad3 Prism Software. Data for isolated aortic ring preparation was statistically analysed using one way ANOVA followed by Newman-Keul’s test. Data for serum levels of nitrite/nitrate, TBARS and urinary nitrite/nitrate were statistically analysed using one way ANOVA followed by Tukey’s Multiple Range test. P<0.05 was considered to be statistically significant.

**Results**

**Effect of pharmacological interventions on acetylcholine-induced endothelium dependent and sodium nitroprusside-induced endothelium independent relaxation**—Acetylcholine (ACh) produced endothelium dependent relaxation in phenylepherine (PE) precontracted isolated rat aortic ring preparation (Fig. 1A). The administration of daidzein (Gr IX) or atorvastatin (Gr X) in normal rats did not produce any marked effect on acetylcholine-induced endothelium dependent relaxation (Fig. 1A)
and sodium nitroprusside induced endothelium independent relaxation (Fig. 1B). Ovariectomy markedly attenuated acetylcholine induced endothelium dependent relaxation. The administration of daidzein (Gr VII - Gr IX) or atorvastatin significantly blocked the decrease in endothelium dependent relaxation of acetylcholine due to ovariectomy (Fig. 2A).

Acetylcholine produced contraction instead of relaxation in endothelium-denuded preparation of isolated rat aortic ring moreover, sodium nitroprusside induced endothelium-independent relaxation was not modulated by treatment of daidzein (Gr IX) or atorvastatin (Gr X) (Fig. 2B). Administration of daidzein (Gr IX) or atorvastatin (Gr X) in ovariectomised rats did not produce any noticeable change in sodium nitroprusside-induced endothelium-independent relaxation (Fig. 2B).

**Effect of pharmacological interventions on serum nitrite/nitrate concentration**—Administration of daidzein (Gr IX) or atorvastatin (Gr X) did not produce any marked effect on serum nitrite/nitrate concentration in normal rats. Ovariectomy markedly reduced serum nitrite/nitrate concentration. Daidzein (Gr VII-Gr IX) or atorvastatin (Gr X), significantly prevented ovariectomy induced decrease in serum nitrite/nitrate concentration (Table 1).

**Effect of pharmacological interventions on urinary nitrite/nitrate concentration**—Daidzein (Gr IX) or atorvastatin (Gr X) treatment did not show any marked effect in normal rats on urinary nitrite/nitrate concentration. Ovariectomy markedly reduced urinary nitrite/nitrate concentration. Daidzein (Gr VII-Gr IX) or atorvastatin (Gr X) treatment significantly prevented decrease in nitrite/ nitrate concentration due to ovariectomy (Table 1).

**Effect of pharmacological interventions on serum thiobarbituric acid reactive substances (TBARS)**—Administration of daidzein (Gr IX) or atorvastatin (Gr X) did not affect serum TBARS concentration in normal rats. Ovariectomy markedly increased serum TBARS concentration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrite/Nitrate (μM) concentration</th>
<th>TBARS (μM) concentration</th>
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<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Urine</td>
</tr>
<tr>
<td>DMSO(1%) in normal rats</td>
<td>11.8 ±0.69</td>
<td>5.39±0.51</td>
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<tr>
<td>CMC(0.5%) in normal rats</td>
<td>12.0 ±0.8</td>
<td>5.65±0.59</td>
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<tr>
<td>Daidzein (0.8mg/kg/day)Perse</td>
<td>11.7 ±0.95</td>
<td>5.65±0.56</td>
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<tr>
<td>Atorvastatin(30mg/Kg/day)Perse</td>
<td>11.87±0.65</td>
<td>5.59±0.38</td>
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<td></td>
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<tr>
<td>Sham Control</td>
<td>11.9±0.85</td>
<td>5.7±0.45</td>
</tr>
<tr>
<td>Ovariectomised(OVX)</td>
<td>5.1 ±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.9±0.62&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OXV+Daid(0.2mg/kg/day)</td>
<td>7.4 ±0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.6±0.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OXV+Daid(0.4mg/kg/day)</td>
<td>8.5 ±0.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5±0.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OXV+Daid(0.8mg/kg/day)</td>
<td>8.6 ±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.6±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OXV+Atorvastatin (30mg/kg/day)</td>
<td>9 ±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

OVX represents ovariectomised control. OXV + Daid represents daidzein treatment in ovariectomised rats. OVX+ATV represents atorvastatin treatment in ovariectomised rats. <sup>a</sup>-<sup>b</sup> = P<0.05 vs Control, <sup>ab</sup> = P<0.05 vs Ovariectomised control.

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Fig. 2—Effect of Pharmacological interventions on (A) Acetylcholine induced endothelium dependent relaxation in ovariectomised rats a = P<0.05 Vs first Control value, b = P<0.05 Vs Control, c= P<0.05 Vs Ovariectomised Control. (B) Sodium nitroprusside induced endothelium independent relaxation in ovariectomised rats Control represents sham control. OVX represents ovariectomised control. OVX + Daid represents daidzein treatment in ovariectomised rats. OVX+ATV represents atorvastatin treatment in ovariectomised rats [Values are mean ± SEM of 6 rats in each group].
TBARS concentration. However, daidzein (Gr VII-IX) or atorvastatin (Gr X) treatment significantly prevented ovariectomy induced increase in serum TBARS (Table 1).

Effect of Pharmacological Interventions on Vascular Endothelium Integrity—Hematoxylin eosin staining on aortic sample in ovariectomised rats (Fig. 3 B) shows denuded innermost lining and senescence of endothelium layer with atherosclerotic lesions as compared to vascular endothelium of normal rats (Fig. 3A). However, daidzein (Gr IX, Fig. 3 C) or atorvastatin (Gr X, Fig. 3D) treatments shows partially intact and intact vascular endothelium.

Discussion

The endothelium-dependent vasodilatation has been used as a parameter to assess endothelial function\(^{37}\). Thus acetylcholine-induced endothelium-dependent vasorelaxation in isolated rat aortic ring was employed in present study. Cumulative dose-response curve has been employed in this study because isolated aortic ring preparation takes long time to relax and does not demonstrate the fade phenomenon\(^{38-39}\). The isolated rat aortic ring preparation used in this study offers an advantage to avoid risk of damage to vascular endothelium\(^{40-41}\). Further, sodium nitroprusside-induced endothelium-independent vasorelaxation has been used in this study to investigate the effect of endothelium-independent vascular reactivity. Estimation of serum and urinary nitrite/nitrate concentration has been reported to be an indirect measure nitric oxide production\(^{34}\). Thus, this indirect parameter was used as an index of change in nitric oxide formation due to modulation of endothelium function. The lipid peroxidation\(^{42}\) is responsible to generate malondialdehyde (MDA) or thiobarbituric acid reactive substance (TBARS). Therefore, degree of lipid peroxidation due to generation of oxygen free radical has been observed by measuring TBARS, in present study. The integrity of vascular endothelium has been reported to be disrupted as a result of vascular injury\(^{36}\). Therefore, the integrity of vascular endothelium has been assessed using light microscopy in present study. Statins have been reported to increase generation of nitric oxide by activating eNOS\(^{43}\) and by inhibition of caveolin-1\(^{44}\). Thus, atorvastatin was used as a standard drug to improve vascular endothelial dysfunction.

![Fig. 3](image_url)—Effect of Daidzein on vascular endothelium integrity. (a) Normal Control, (b) Ovariectomised control, (c) Daidzein treatment in ovariectomised rats, (d) Atorvastatin treatment in ovariectomised rats.
Ovariectomy has been demonstrated to decrease acetylcholine-induced endothelium-dependent vasorelaxation\(^4\), decrease in production of nitric oxide\(^5\) and increase in oxidative stress\(^6\). The observed decrease in acetylcholine-induced endothelium-dependent vasorelaxation in isolated rat aortic ring preparation, increase in serum and urinary nitrite/nitrate concentration and impairment of vascular endothelial integrity due to ovariectomy is noted in present study as a consequence of vascular endothelial dysfunction.

Daidzein is reported to mimic oestrogen activity on oestrogen receptors ER\(\alpha\) and ER\(\beta\)\(^9,10\). It has been reported that daidzein has no effect on endothelium dependent vasorelaxation when administered with ER antagonist\(^11\). Ovariectomy is reported to up-regulate caveolin-1\(^12\) which is responsible for inhibition of eNOS\(^13\). Daidzein, being a caveolin-1 inhibitor, is reported to increase the activity of eNOS\(^14-16\). Thus, Daidzein induced improvement in vascular endothelium in ovariectomised rats may be due to increase in production of nitric oxide (NO), and stimulation of eNOS through inhibition of caveolin-1. Moreover, the observed sodium nitroprusside induced endothelium-independent relaxation was not modulated by daidzein supports this contention. Daidzein has been observed to attenuate ovariectomy induced increase in serum TBARS, possibly by improving the formation of nitric oxide and consequently preventing oxidative stress\(^17\). On the basis of above discussion following conclusions may be drawn: (1) Ovariectomy induced endothelial dysfunction, resulted in reduced acetylcholine-induced endothelium dependent vasorelaxation, impairment of integrity of vascular endothelium and decrease in serum and urinary nitrite/nitrate concentration, and (2) Daidzein improved endothelial dysfunction may be due to inhibition of caveolin-1, the consequent increase in production of nitric oxide and decrease in oxidative stress.

Acknowledgement
This paper is dedicated to the fond memory of Prof. Manjeet Singh who expired on 30.3.2009 while this work was in progress.

References


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