Indian Journal of Experimental Biology
Vol. 50, January 2012, pp. 28-34

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

Saurabh Sharma, Manjeet Singh and Pyare Lal Sharma*

Cardiovascular Division, Department of Pharmacology, ISF College of Pharmacy, Moga 142 001, India

Received 17 February 2011; revised 6 September 2011

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 $mgkg^{-1}day^{-1}$, sc 0.4 $mgkg^{-1}day^{-1}$, sc and 0.8 $mgkg^{-1}day^{-1}$, sc) and Atorvastatin (30 $mgkg^{-1}day^{-1}$, 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[1]. The modulation of vascular L-arginine/nitric oxide synthetase system is hallmark of vascular endothelial dysfunction[2]. Various pathological disorders such as hypertension[3-4], coronary artery disease[5], atherosclerosis[6], stroke[7], hyperhomocysteinemia[8] diabetes mellitus[9-10] 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[11] and eNOS is the principle enzyme to generate nitric oxide from L-arginine in vascular wall[12]. Oestrogen deficiency, associated with menopause, is the major cause of vascular endothelial dysfunction[13]. Oestrogen regulates eNOS activity either genomically[11] or non-genomically[12]. Oestrogen upregulates eNOS and downregulates its inhibitory protein caveolin-1[14-15]. Oestrogen modulation of eNOS expression is mediated through estrogen receptors α (ER α) and β (ER β) which are expressed on endothelial cells[16-17]. Short term exposure to oestrogen, enhances the release of nitric oxide from vascular endothelium without altering eNOS expression[18-19]. Oestrogen is also reported to activate mitogen activated protein kinase and phosphoinositide-3 kinase-AKT pathway which also cause the activation of eNOS[20].

Caveolin is a transmembrane protein present in small invaginations of the plasma membrane called caveolae[21]. Caveolins comprise of three isoforms i.e., caveolin-1,-2, and -3. Caveolin-1 and caveolin-2 are widely expressed in various tissues[22]. Caveolin-1 is a specific marker of caveolae and is up-regulated by oxdidized LDL, oestrogen deficiency and hyperglycemia[23]. It serves as cholesterol binding protein and help cholesterol to move from endoplasmic reticulum through golgi apparatus to plasma membrane of endothelial cells[24]. Caveloin-1 binding suppresses the activity of eNOS[25]. Alterations in caveolin/eNOS interaction influence various mechanisms of diseases such as atherosclerosis[6], diabetes[26], cirrhosis[27] and oestrogen deficiency[28].

Daidzein, a caveolin-1 inhibitor, has been reported to increase the activity of eNOS[29-30]. Ovariectomy has been reported to upregulate the expression of

*Correspondent author
Mobile: 91 9888036775
E-mail: ssm.research@gmail.com

caveolin-1[28]. 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[31].

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 m*M*; KCl, 4.7 m*M*; $CaCl_2$, 2.5 m*M*; $MgSO_4.H_2O$, 1.2 m*M*; $NaHCO_3$, 25.0 m*M*; KH_2PO_4, 1.2 m*M*; Glucose, 11.1 m*M*) 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, *p*H 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 m*M*) 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 acelylcholine (10^{-8}-10^{-4}*M*) were recorded in phenylephrine (3×10^{-6}*M*) 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 nitropruside (10^{-8}-10^{-4}*M*) in phenylephrine (3×10^{-6}*M*) precontracted isolated aortic ring preparation was recorded[32].

Removal of vascular endothelium—Vascular endothelium of aorta was removed using blunt forceps[33]. Removal of endothelium was confirmed by absence of relaxation to acetylcholine ($1x10^{-4}$*M*) in phenylephrine (3×10^{-6}*M*) precontracted preparations.

Estimation of serum and urinary nitrite/ nitrate concentration—Estimation of nitrite and nitrate[34] 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 eppendorff 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-ethylenediamine (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)[35] . TBARS *assay* forms a colour product. The primary TBARS chromogen is a reaction product of thioborbituric 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[36]. 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 $mgkg^{-1}day^{-1}$, sc) for one week.

Group VI (Atorvastatin Treatment; n=6): Rats were administered atorvastatin (30 $mgkg^{-1}day^{-1}$, po) for one week.

Group VII [Daidzein (low dose) treated ovariectomised]: Ovariectomised rats were administered daidzein (0.2 $mgkg^{-1}day^{-1}$, 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 $mgkg^{-1}day^{-1}$, 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 $mgkg^{-1}day^{-1}$, sc) for one week starting from the fourth week after ovariectomy.

Group X (Atorvastatin Treated Ovariectomised): Ovariectomised rats were administered atorvastatin (30 $mgkg^{-1}day^{-1}$, po) for one week starting from the fourth week after ovariectomy.

Drugs and Chemicals—Daidzein (Tocris-Cookson, UK) was suspended in dimethyl sulphoxide (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 perfomed 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 acetycholine-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)

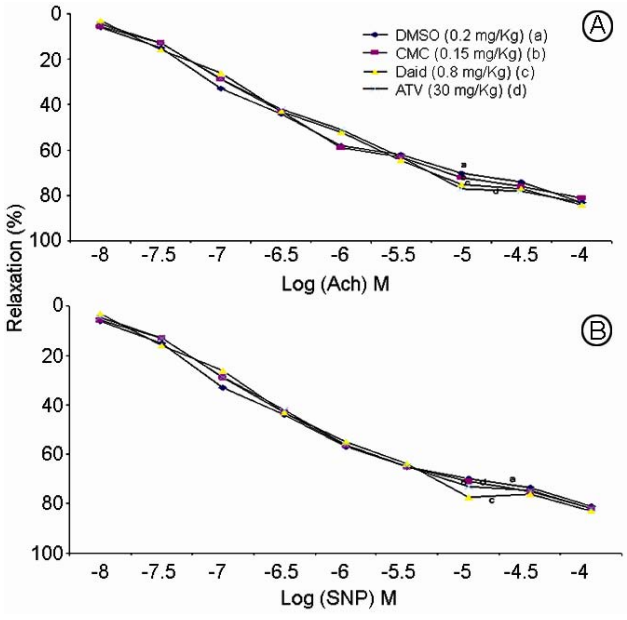

Fig. 1—(A) Effect of Pharmacological interventions on A) Acetyl choline induced endothelium dependent relaxation in normal rats .(B) Sodium nitroprusside induced endothelium independent relaxation in normal rats [Values are mean ± SEM of 6 rats in each group].

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).

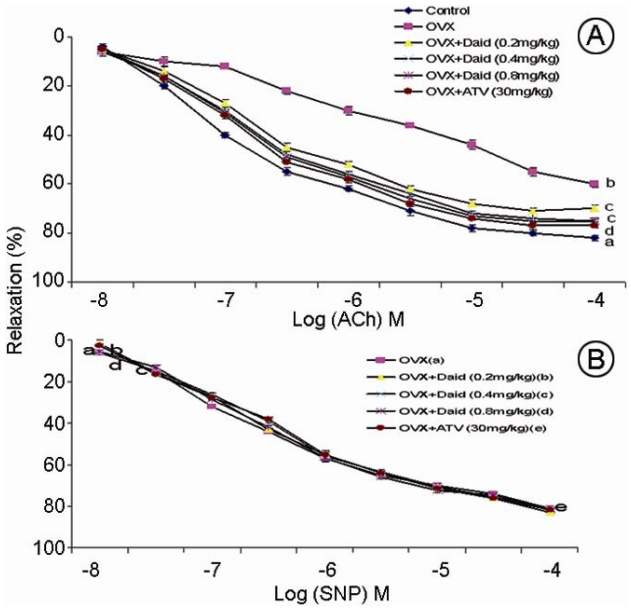

Fig. 2—Effect of Pharmacological interventions on (A) Acetylcholine induced endothelium dependent relaxation in ovariectomised rats a = P<0.05 Vs first Control value, b = P<0.05 Vs Control, c= P<0.05 Vs Ovariectomised Control. (B) Sodium nitroprusside induced endothelium independent relaxation in ovariectomised 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].

ACh 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

Table 1—Effect of pharmacological interventions on serum and urinary nitrite and TBARS concentration in ovariectomised rats. [Values are mean ± SE of 6 rats in each group]

Treatment Groups	Nitrite/Nitrate (μM) concentration		TBARS (μM) concentration
	Serum	Urine	Serum
DMSO(1%) in normal rats	11.8±0.69	5.39±0.51	3.3±0.19
CMC(0.5%) in normal rats	12.0±0.8	5.65±0.59	3.39±0.082
Daidzein (0.8mg/kg/day)Perse	11.7±0.95	5.65±0.56	3.41±0.26
Atorvastatin(30mg/Kg/day)Perse	11.87±0.65	5.59±0.38	3.48±0.041
Sham Control	11.9±0.85	5.7±0.45	3.4±0.06
Ovariectomised(OVX)	5.1 ±0.49[a]	2.9±0.62[a]	8.7±0.34[a]
OVX+Daid(0.2mg/kg/day)	7.4 ±0.71[b]	3.6±0.61[b]	5±0.71[b]
OVX+Daid(0.4mg/kg/day)	8.5 ±0.84[b]	4.5±0.45[b]	5.9±0.37[b]
OVX+Daid(0.8mg/kg/day)	8.6 ±0.58[b]	4.6±0.49[b]	5.1±0.12[b]
OVX+Atorvastatin (30mg/kg/day)	9 + 0.19[b]	4.9±0.18[b]	4.7±0.31[b]

OVX represents ovariectomised control. OVX + Daid represents daidzein treatment in ovariectomised rats. OVX+ATV represents atorvastatin treatment in ovariectomised rats. [a]= P<0.05 vs Control, [b]= P <0.05 vs Ovariectomised control.

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.

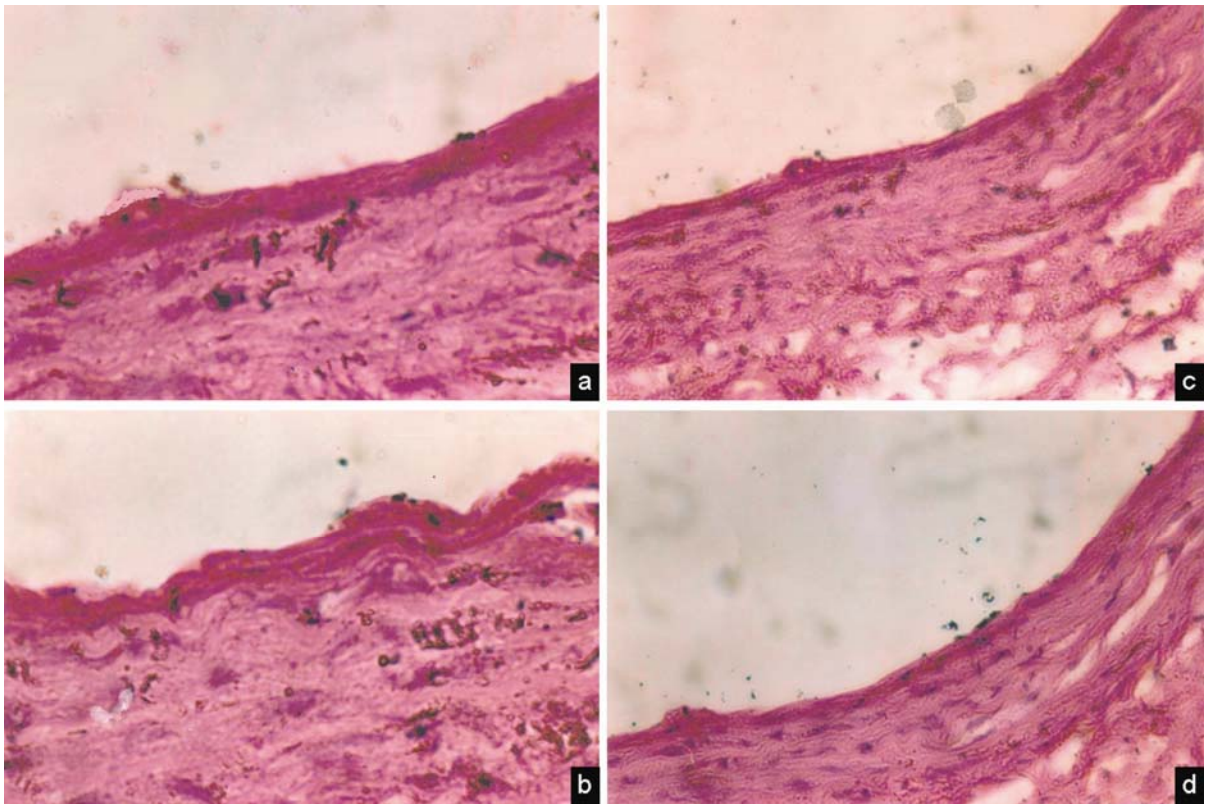

Fig. 3—Effect of Daidzein on vascular endothelium integrity. (a) Normal Control, (b) Ovariectomised control, (c) Daidzein treatment in ovariectomised rats, (d) Atorvastatin treatment in ovariectomised rats.

Ovariectomy has been demonstrated to decrease acetylcholine-induced endothelium-dependent vasorelaxation[45], decrease in production of nitric oxide[46] and increase in oxidative stress[47]. 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 α and ER β[29-30]. It has been reported that daidzein has no effect on endothelium dependent vasorelaxation when administered with ER antagonist[30]. Ovariectomy is reported to up-regulate caveolin-1[28] which is responsible for inhibition of eNOS (Woodman *et al.*, 2004). Daidzein, being a caveolin-1 inhibitor, is reported to increase the activity of eNOS[29-30]. 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 nitropruside 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[46] .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

1 Endemann H D & Ernesto L S, Endothelial dysfunction *J Am Soc Nephrol*, 15(2004) 1983.

2 Esper R J, Roberto A N , Jorge O V, Antonio P , José L C & Rogelio A M, Endothelial dysfunction: A comprehensive appraisal *Cardiovasc Diabetol*, 5 (2006) 4.

3 Taddie S, Virdis A, Ghiadoni L, Sudano I & Salvetti A Endothelial dysfunction in hypertension *J Cardiovasc Pharm* 38 (2001) S11

4 Sainani G S & Maru V G, Role of endothelial dysfunction in essential hypertension, *JAPI*, 52 (2004) 966.

5 Caramori P R A & Zago A J, Endothelial dysfunction & coronary artery disease *Arg Bras Cardiol*, 75 (2000) 173.

6 Spieker L E, Luscher T F & Noll G, Current strategies & perspectives for correcting endothelial dysfunction in atherosclerosis, *J Cardiovasc Pharm*, 38, (2001) S35.

7 Haorah J, Ramirez S H, Schall K, Smith D, Pandya R, Persidsky Y, Oxidative stress activates protein tyrosine kinase & matrix metalloproteinases leading to blood-brain barrier dysfunction, *Journal of Neurochemistry* 101(2) (2007) 566.

8 Faraci F M & Lentz S R, Hyperhomocysteinemia, oxidative stress & cerebral vascular dysfunction, *Stroke*, 35 (2004) 345.

9 De Vriese A S, Verbeuren T J, Vande V J, Lameire N H & Vanhoutte P M, Endothelial dysfunction in diabetes, *Br J Phamacol*, 130 (2000) 963.

10 Nakagami H, Kaneda Y, Ogihara T & Morishita R, Endothelial dysfunction in hyperglycemia as a trigger of atheroscelerosis, *Curr Diabet Rev*, I (2005) 59.

11 Levin R E, Integration of the extra-nuclear & nuclear actions of estrogen, *Mol Endocrin*, 19 (2005) 1951.

12 Chambliss K L & Shaul P W, Estrogen modulation of endothelial nitric oxide synthase, *Endocr Rev*, 23(5) (2002) 665.

13 Raghvendra K D, Bruno I, Matthias B & Edwin K J, Vascular consequences of menopause & hormone therapy: Importance of timing of treatment & type of estrogen, *Cardiovasc Res*, 66 (2005) 295.

14 Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R & Saruta T, Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells, *FEBS Lett*, 360 (1995) 291.

15 Jeffrey B M, Olivier F, David S & Thomas M, Reciprocol regulation of endothelial nitric oxide synthatase by Ca^{2+} Calmodulin & caveolin *J Bio Chem*, 272(25) (1997) 15583.

16 Rubanyi G M, Freay A D, Kauser K, Sukovich D, Burton G, Lubahn D B, Couse J F, Curtis S W & Korach K S, Vascular estrogen receptors & endothelium-derived nitric oxide production in the mouse aorta Gender difference & effect of estrogen receptor gene disruption, *J Clin Invest* , 99 (1997) 2429.

17 Raz&i M, Pedram A, Greene G L & Levin E R, Cell membrane & nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER & ERß expressed in Chinese hamster ovary cells *Mol Endocrinol*, 13 (1999) 307.

18 Chen Y Z & Qiu J, Pleiotropic signaling pathways in rapid, nongenomic action of glucocorticoid, *Mol Cell Biol Res Commun*, 2 (1999) 145.

19 Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, K Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N, Taniguchi N & Murata Y, Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells *J Biol Chem*, 276 (2001) 3459.

20 Simoncini T, Hafezi-Moghadam, A, Brazil D P, Ley K, Chin W W & Liao J K, Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase, *Nature*, 407 (2000) 538.

21 Gratton J P, Fontana J, O'Connor D S, Garcia-Cardena G, Mccabe T J & Sessa W C, Reconstitution of endothelial nitric oxide synthase, Hsp90 & caveolin-1 complex in vitro: evidence that Hsp90 facilitates calmodulin stimulated displacement of eNOS from caveolin-1 *J Biol Chem*, 275 (2000) 22268.

22 Smart E J, Graf G A, McNiven MA, Sessa W C, Engelman J A, Scherer P W, Okamoto T & Lisanti M P, Caveolins, liquid-ordered domains & signal transduction, *Mol Cell Biol*, 19 (1999) 7289.

23 Sharma S, Singh M & Sharma PL, Beneficial effect of insulin in hyperhomocysteinemia & diabetes mellitus-induced vascular endothelium dysfunction: role of phosphoinositide dependent kinase & protein kinase B, *Mol Cell Biochem*, 348 (2011) 21.

24 Fulton D, Gratton J P & Sessa W C, Post-translational control of endothelial nitric oxide synthase: Why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther*, 299 (2001) 818.

25 Minshall R D, Tiruppathi C, Vogel S M & Malik A B, Vesicle formation & trafficking in endothelial cells & regulation of endothelial barrier function, *Histochem Cell Biol*, 117 (2002) 105.

26 Elçioglu K H, Kabasakal L, Cetinel S, Conturk G, Sezen S F & Ayanoglu-Dulger G Changes in caveolin-1 expression & vasoreactivity in the aorta & corpus cavernosum of fructose & streptozotocin-induced diabetic rats *Eur J Pharmacol* 642(2010) 113.

27 Xu B, Zhu GH, Weng JF, Cai WS, Xia JT & Li SH The roles of caveolin-1 & endothelial nitric oxide synthase in the development of portal hypertension in rats with liver cirrhosis *Zhonghua Gan Zang Bing Za Zhi* 16 (2008) 184.

28 Pelligrino DA, Ye S, Tan F, Santizo R A, Feinstein D L & Wang Q, No-dependent piol reticular dilation in the female rat: Effects of chemic estrogen depletion & repletion, *Biochem Biophys Res Commun*, 269 (2000) 165.

29 Sobey G C, Jane M W, Mirna B & Owen L W, Effect of short term phytoestrogen treatment is male rat on nitric oxide mediated responses of carotid & cerebral arteries: Comparison with 17 β estradiol, *JPET*, 310 (2004) 35.

30 Woodman LO, Melinda A M & Mirna B, Daidzein & 17 estradiol enhance nitric oxide synthatase activity associated with an increase in calmodulin & a decrease in caveolin-1, *J Cardiovasc Pharmacol*, 44 (2) (2004)

31 Catania M A, Crupi A, Firenzuoli F, Parisi A, Sturiale A, Squadrito F, Caputi A P & Calapai G, Oral administration of a soy extract improves endothelial dysfunction in ovariectomized rats *Planta Med*,68(12)(2002) 1142.

32 Mitra S & Singh M, Possible mechanism of captopril induced endothelium dependent relaxation in isolated rabbit aorta, *Mol Cell Biochem*, 183(1998) 63.

33 Auck-Schwelk W, Katusic Z S & Vanhoutte P M, Nitric oxide inactivates endothelium derived contracting factor in the rat aorta, *Hypertension*, 19 (1992) 442.

34 Sastry K V H, Moudgal R P, Mohan J, Tyagi J S & Rao G S Spectrophotometric determination of serum nitrite & nitrate by copper cadmium alloy, *Anal Biochem*, 306 (2002) 79.

35 Ohkawa H, Ohishi N & Yagi K Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal Biochem*, 95 (1979) 351.

36 Moya M, Campana V, Gavotto A, Spitale L, Simes J & Palma J, Histopathological lesions in aorta from rats with hiperfrinogenemia published by 2nd virtual congress of cardiology (1999).

37 Shah D I & Singh M , Inhibition of Protein tyrosin Phosphatase improves vascular endothelial dysfunction, *Vascular Pharmacology*,44(2006) 177.

38 Furchgott R F & Zawadski J V, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine, *Nature*, 288 (1980) 373.

39 Furcgott R F, The discovery of endothelium-dependent relaxation, *Circulation*, 87 (1993) V3.

40 Furchgott R F, The 1989 Ulf von Euler Lecture: studied on endothelium dependent vasodilatation & the endothelium-derived relaxing factor *Acta Physiol Sci*, 139 (1990) 257.

41 Iwama Y, Kato T, Muramatsu M, Asano H, Shimizu K, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, Ito T & Satake T, Correlation with blood pressure of the acetylcholine-induced endothelium-derived contracting factor in the rat aorta, *Hypertension*, 19 (1992) 326.

42 Janerw D R, Malondialdehyde & thiobarbituric acid reactivity as diagnostic indices of lipid peroxidation & peroxidative tissue injury, *Free Radic Biol Med*, 9(1990) 515.

43 Wolfrum S , Jensen K & Liao J K, Endothelium-dependent effects of statins, *Arteriosceler Thromb Vasc Biol*, 23 (2003) 729.

44 Feron O, Dessy D, Jean-Pierre Desager J L & Ballig M D Hydroxy-methylglutaryl–coenzyme a reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance, *Circulation*, 103 (2001) 113

45 Virdiss A, Lorenzo G, Stefania P, Maurizio L, Felice P L, andrea G, Simona B, Stefano T & Antoni S, Mechanisms responsible for endothelial dysfunction associated with acute estrogen deprivation in normotensive women, C*irculation*, 101 (2000) 2258.

46 Zhang X, Li H, Jin H, Ebin Z, Brodsky S & Goligorsky M S, Effects of homocysteine on endothelial nitric oxide production, *Am J Physiol Renal Physiol*, 279 (2000) F671.

47 Gryglewski R J, Palmer R M & Moncada S, Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor, *Nature*, 320 (1986) 454.