

## Chronic administration of phytoestrogen “daidzein” to ameliorate mean arterial pressure and vascular function in N-G-nitro-L-arginine methyl ester hypertensive rats

Shahid Prawez<sup>1\*</sup>, Azad A. Ahanger<sup>2</sup>, Thakur U. Singh<sup>3</sup>,  
Santosh K. Mishra<sup>3</sup>, Souvendra N. Sarkar<sup>3</sup>, and Shafiqur Rahman<sup>4</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India

<sup>2</sup>Sher-e-Kashmir University of Agricultural Sciences and Technology-Kashmir, Shuhama, Alustang, Srinagar, India

<sup>3</sup>Division of Pharmacology and Toxicology, Indian Veterinary Research Institute, Izatnagar (UP), India

<sup>4</sup>Division of Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu, FVSc&AH, RS Pura, Jammu, J&K, India

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### ABSTRACT

The ‘silent killer’, hypertension, leads to heart disease, stroke, kidney failure and premature death. The phytoestrogen daidzein has been associated with vaso-protective action similar to oestrogen, with minimal side effects. To explore the vaso-protective activity of daidzein and also its effect on mean arterial pressure (MAP), daidzein was chronically administered in N-G-nitro-L-arginine methyl ester (L-NAME)-hypertensive male Wistar rats for 6 weeks. The male Wistar rats were divided into three groups, namely group-A (control), group-B (L-NAME-treated) and group-C (L-NAME+daidzein treated). After completion of 42 days (6 weeks) of daidzein treatment, MAP and vascular activity were observed in all the groups. Daidzein treatment of L-NAME hypertensive rats (group-C) for 6 weeks significantly decreased the MAP (144 mm Hg) as compared to untreated-L-NAME-hypertensive rats/group-B (173.2 mm Hg), indicating the blood-pressure lowering property of daidzein. Also daidzein significantly increased acetylcholine-induced maximal relaxations of the thoracic aorta isolated from daidzein-treated ( $E_{max} = 72.55\%$ ) in comparison to untreated-L-NAME-hypertensive rats ( $E_{max} = 39.33\%$ ). The results of the present study suggest that chronic administration of daidzein (0.5 mg/kg/day, s.c.) helps to lower blood pressure, as indicated by a decrease in MAP, and also shows vaso-protective action, as indicated by the improvement in ACh-induced relaxation.

**Key words:** hypertension, daidzein, blood pressure, vascular function, rats

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\*Corresponding author:

Shahid Prawez, Department of Pharmacology and Toxicology, Faculty of Veterinary and Animal Sciences, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005 (UP), India, Phone: +91 7067 7003; E-mail: Shahidprawez@gmail.com

### Introduction

We are in an era of a rapidly changing environment, and the factors that determine human health are rapid urbanization, ageing and globalization, which have resulted in the emergence of an unhealthy lifestyle. In recent times, a shifting of diseases has occurred from infectious to non-communicable diseases, such as cardio-vascular, diabetes, cancer and chronic lung disease, and these have now become the leading causes of mortality. Hypertension is one of the most important cardiovascular diseases that kills about nine million people every year. The disease proceeds to death without prior intimation, and is therefore called the “silent and invisible killer”. The global consequences of hypertension are heart disease, stroke, kidney failure, premature death and disability (BROWN et al., 2007; PIERDOMENICO et al., 2009; ROY et al., 2008; SCUTERI and FERRUCCI, 2003; SIMKO, 2007). Life style and heredity (MENDELSONH, 2002) also play a vital role in determining the occurrence of disease hypertension.

The prevalence of hypertension is of major concern to males or post-menopausal females. With advancements in science it has become clear that oestrogen improves endothelial function (MENDELSONH, 2002; KOH, 2002). Various publications have shown the vaso-protective effect of oestrogen by diverse mechanisms (SCUTERI and FERRUCCI, 2003). Oestrogen improves endothelial function by increasing eNOS-levels (MENDELSONH, 2002; KOH, 2002) but may participate in the development of breast and endometrial cancer in women, and undesirable reproductive function in men. To circumvent these drawbacks, oestrogen alternatives have been sought, resulting in the development of plant derived phytoestrogens. Phytoestrogens are classified as isoflavones, coumestans, and lignans, that are widely distributed in soyabeans, oil seeds and vegetables (PEETERS et al., 2003). Structurally similar to oestradiol, soy contains phytoestrogens, such as genistein, daidzein, biochanin-A (DOUGLAS et al., 2006). Soy protein is phytoestrogen rich (LISSIN and COOKE, 2000) and populations that consume soy products had a lower incidence of cardiovascular disease (ANTHONY et al., 1996; NESTLE 2003). The phytoestrogen daidzein was included in the present study because of the low incidence of side effects and also because it possesses oestrogen-like vaso-protective action (PEETERS et al., 2003; NESTLE, 2003; LICHTENSTEIN, 1998; NASCIMENTO et al., 1999; PARK et al., 2005; PENG et al., 1996; WOODMAN and MISSEN, 2004). Daidzein mimics the ability of oestradiol and improves endothelium-dependent-relaxation in ovarioectomized rats (SQUADRITO et al., 2000). Also it does not significantly affect reproductive tissues in males (MITCHELL et al., 2001) and interestingly it does not show the carcinogenic potential of oestrogen (WOODMAN and MISSEN, 2004). Few scientific reports are available on daidzein that explore its vasoprotective potency and importantly its effect on blood pressure, especially in an L-NAME-hypertensive model. To investigate the activity of daidzein on the thoracic aorta and blood pressure, daidzein was chronically administered to L-NAME-hypertensive-male-Wistar-rats.

### Materials and methods

*Animals and experiment layout.* The L-NAME model of hypertension is a well-established experimental model of hypertension. Healthy adult male-Wistar-rats, weighing 150-200 g, were housed in a well-ventilated animal house, with an alternate 12 hour light and dark-cycle. The experiment was conducted as per the Institutional Animal Ethics Committee guidelines. After acclimatization, the male-Wistar-rats were divided into three groups, namely: the control/group-A, the L-NAME-hypertensive/group-B and the daidzein-treated L-NAME-hypertensive/group-C rats. The group-A rats were given drinking water *ad libitum* orally and normal-saline subcutaneously daily for 42 days. The rats of group-B were treated with L-NAME after it was dissolved in drinking water at a dosage of 50 mg/kg daily orally for 42 days. Also normal saline was administered subcutaneously for the same 42 day period in group-B rats. The group-C rats were chronically treated using L-NAME-hydrochloride at the same dose and route as group-B, and additionally daidzein was administered after it was dissolved in normal-saline at a dosage of 0.5 mg/kg/day subcutaneously for 42 days.

*Pressure measurement and isometric-tension-recording.* After 6-weeks (42-days) of the study, the rats were anaesthetized with pentobarbital sodium (60 mg/kg, intra-peritoneally), in order to measure mean arterial pressure (MAP) by an invasive-method using a transducer (Model MLT0380/D, AD Instrument). The MAP of the rats was measured after exposing the carotid artery of the anaesthetized rats. The exposed carotid artery was nicked under a microscope and cannulised. To read arterial pressure, the carotid artery was connected to the AD Instrument transducer and attached to a personal computer using the software program Lab-Chart v6.1.3 (Powerlab, AD Instruments, Australia). After completion of blood pressure monitoring of the rats in all the experimental groups, the thoracic aorta was isolated and kept in cold Modified-Krebs-Henseleit solution (MKHS, 4 °C) for further study of vascular activity in an organ bath (KANDASAMY et al., 2011). Aortic-rings of 3-4 mm length were mounted between two hooks of 37-gauge stainless steel wire under a resting tension of 1.5 g in a thermostatically controlled (37 °C ± 0.1 °C) organ-bath (UGO-Basile, Italy) of 10-mL capacity, containing MKHS supplying medical gas in bubbled form (74 % N<sub>2</sub>+21 % O<sub>2</sub>+5 % CO<sub>2</sub>). After an hour equilibration period, the aortic rings were contracted with high potassium-chloride (80 mM)-depolarizing solution to check the tissue viability, and they were also used as a reference to compare phenylephrine contraction. After attainment of the maximum-contraction-plateau produced by potassium chloride, the effect was washed with MKHS. Once the baseline was reached, the arterial rings were again contracted with vasoconstrictor-phenylephrine with an increment of 0.5 log units to see the cumulative effect. To evaluate the effect of chronically administered daidzein on vascular function, aortic rings from all the groups were relaxed using acetylcholine (ACh)/sodium-nitroprusside (SNP).

*Statistical analysis.* Vascular activity (contraction & relaxation) in the form of  $E_{max}$  (maximal response) and  $EC_{50}/pD_2$  (the concentration producing 50 % of the maximal response) were analyzed by nonlinear regression analysis (sigmoidal dose response with variable-slope) using Graph Pad Prism version 4.00 (San Diego, California, USA). Data were analyzed using the Student-Newman-Keuls method for multiple group analysis. Concentration-dependent agonist response data were analyzed by two-way ANOVA, followed by the Bonferroni post-hoc test. Differences in values were considered statistically significant at  $P<0.05$  (SNEDECOR and COCHRAN, 1989).

## Results

*Effects of chronic-daidzein treatment on Mean Arterial Pressure of L-NAME hypertensive rats.* Chronic daidzein treatment significantly ( $P<0.05$ ) decreased the MAP ( $144.50 \pm 7.15$  mm Hg,  $n = 6$ ) of L-NAME-hypertensive Group C rats in comparison to untreated-L-NAME-hypertensive rats of group-B ( $173.20 \pm 4.95$  mm Hg,  $n = 10$ ). Although daidzein ameliorated the MAP of group-C rats, the arterial pressure was still higher (Fig. 1) than the control rats of group-A ( $117.20 \pm 3.10$  mm Hg,  $n = 8$ ).

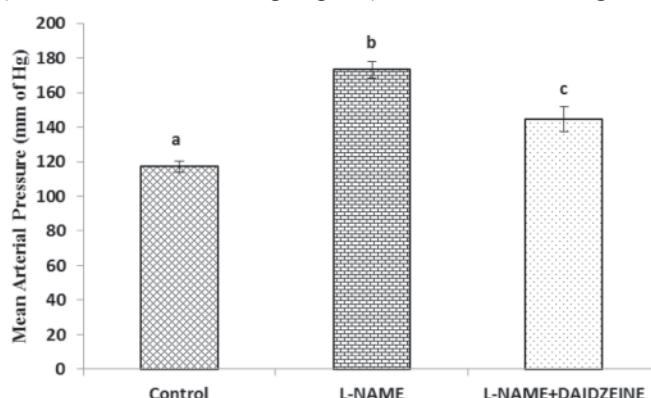


Fig. 1. Effects of chronic daidzein-treatment (0.5 mg/kg s.c.) on mean arterial pressure of L-NAME hypertensive rats.

Different alphabet/superscript signifies a significant variance between different groups at  $P<0.05$

*Effects of chronic-daidzein treatment on phenylephrine-induced contraction of thoracic-aorta isolated from daidzein-treated-L-NAME hypertensive rats.* Phenylephrine (1 nM-10  $\mu$ M) was added cumulatively, with an increment of 0.5 log unit, to produce contraction of aortic rings isolated from different groups of rats (Fig. 2 and 3). A significant ( $P<0.05$ ) increase in maximal contraction to phenylephrine was found in aortic-rings of L-NAME-hypertensive rats of group-B ( $E_{max} = 1.05 \pm 0.02$  g,  $n = 9$ ) in comparison to

control rats ( $E_{max} = 0.90 \pm 0.03$  g, n = 8). In daidzein-treated L-NAME-hypertensive rats of group-C, no change in potency or maximal contraction response to phenylephrine ( $pD_2 = 6.92 \pm 0.07$ , n = 9;  $E_{max} = 1.07 \pm 0.03$  g, n = 9) was found in comparison to L-NAME-hypertensive rats of group-B ( $pD_2 = 6.95 \pm 0.06$ , n = 9;  $E_{max} = 1.05 \pm 0.02$  g, n = 9).

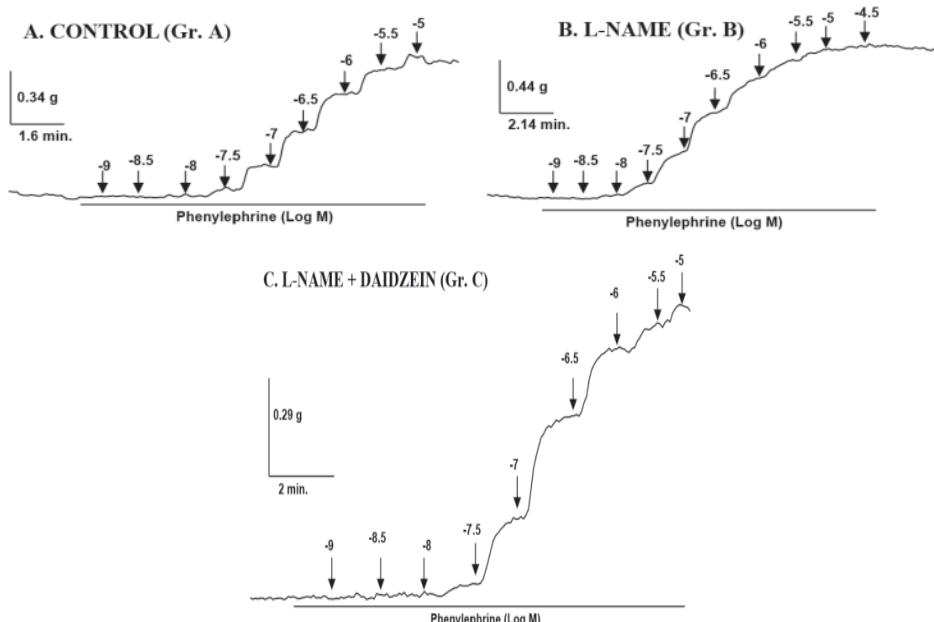


Fig. 2. Tracing showing the effect of chronic daidzein-treatment on Phenylephrine-induced contraction of thoracic-aorta isolated from L-NAME-hypertensive rats

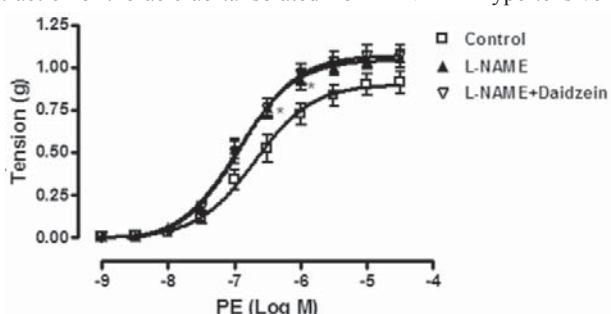


Fig. 3. Effects of chronic daidzein-treatment on Phenylephrine-induced contraction of thoracic-aorta isolated from L-NAME-hypertensive rats.

\*L-NAME treated hypertensive rats vary significantly in comparison to Control rats at  $P < 0.05$

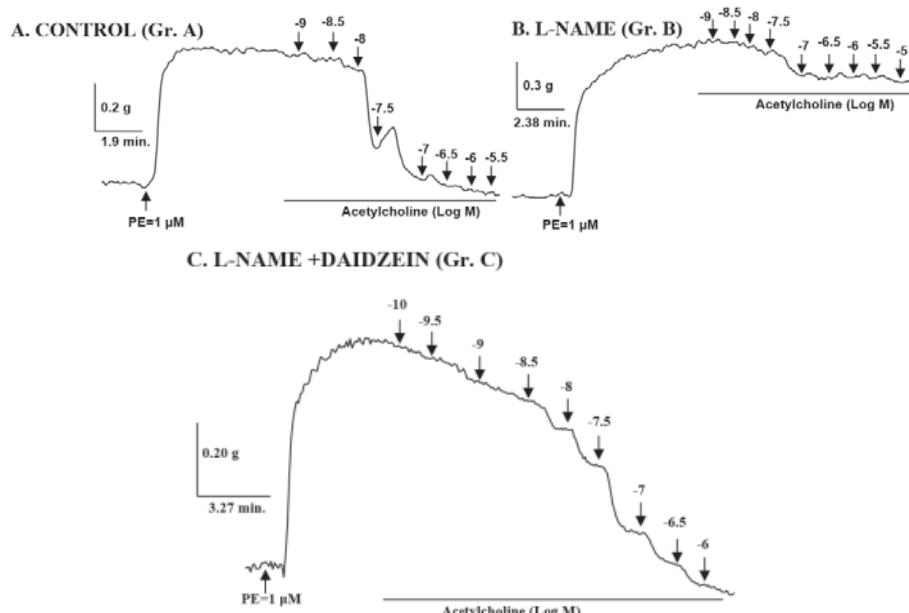


Fig. 4. Tracing showing the effect of chronic daidzein-treatment on % relaxation to ACh-elicited on phenylephrine-precontracted thoracic-aorta isolated from L-NAME-hypertensive rats

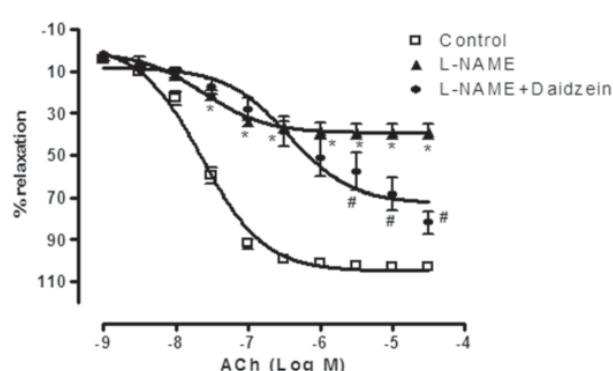


Fig. 5. Effects of chronic daidzein-treatment on % relaxation to ACh-elicited on phenylephrine-precontracted thoracic-aorta isolated from L-NAME-hypertensive rats.

\*L-NAME hypertensive rats vary significantly in comparison to Control at P<0.05

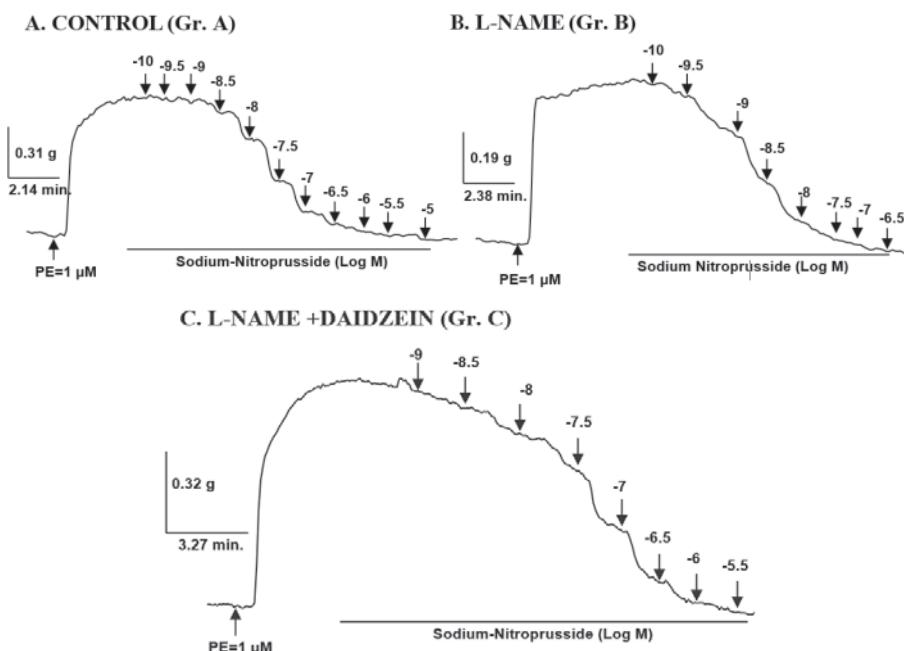


Fig. 6. Tracing showing the effect of chronic daidzein treatment on % relaxation to SNP-elicited on phenylephrine-precontracted thoracic-aorta isolated from L-NAME-hypertensive rats

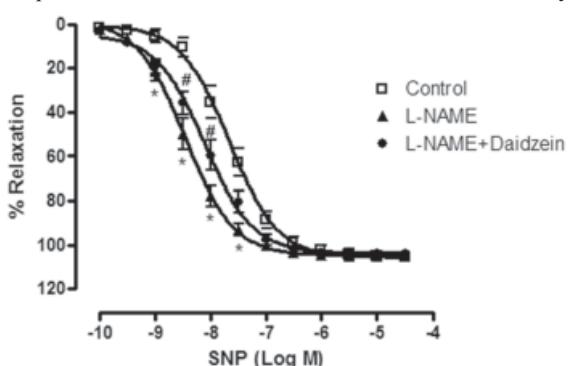


Fig. 7. Effects of chronic daidzein-treatment on % relaxation to SNP-elicited on phenylephrine-precontracted thoracic aorta isolated from L-NAME hypertensive rats.

\*L-NAME hypertensive rats vary significantly in comparison to Control at  $P<0.05$

#L-NAME+Daidzein group rats vary significantly in comparison to L-NAME hypertensive rats at  $P<0.05$

*Effects of chronic-daidzein treatment on % relaxation to ACh elicited on phenylephrine-pre-contracted thoracic-aorta isolated from L-NAME hypertensive rats.* ACh (1 nM-10  $\mu$ M) was added cumulatively to produce concentration-dependent relaxation of phenylephrine-pre-contracted aortic-rings, isolated from different groups (Fig. 4 and 5). A significant ( $P<0.05$ ) decrease in maximal relaxation to ACh was found in the aortic rings isolated from L-NAME-hypertensive rats of group-B ( $E_{max} = 39.33 \pm 1.33 \%$ ,  $n = 8$ ) in comparison to control rats ( $E_{max} = 104.9 \pm 1.07 \%$ ,  $n = 8$ ). Daidzein treatment significantly increased the ACh-induced maximal-relaxations of the aortic-rings of daidzein-treated-L-NAME-hypertensive rats of group-C ( $E_{max} = 72.55 \pm 4.0 \%$ ,  $n = 8$ ) in comparison to L-NAME rats of group-B ( $E_{max} = 39.33 \pm 1.23 \%$ ,  $n = 8$ ).

*Effects of chronic-daidzein treatment on % relaxation to SNP elicited on phenylephrine-pre-contracted thoracic-aorta isolated from L-NAME hypertensive rats.* Sodium nitroprusside (0.1 nM-10  $\mu$ M) was added cumulatively to evaluate the changes in relaxation of phenylephrine-pre-contracted aortic-rings isolated from different groups (Fig. 6 and 7). A significant ( $P<0.05$ ) increase of vasodilatory potency of SNP ( $pD_2 = 8.46 \pm 0.05$ ,  $n = 6$ ) was found in the aortic rings of L-NAME-hypertensive rats of group-B in comparison to control rats ( $pD_2 = 7.67 \pm 0.05$ ,  $n = 5$ ). Daidzein treatment significantly decreased the vasodilator potency of SNP ( $pD_2 = 8.11 \pm 0.05$ ,  $n = 8$ ) in the aortic-rings of group-C rats in comparison to L-NAME-hypertensive rats of group-B ( $pD_2 = 8.46 \pm 0.05$ ;  $n = 6$ ). However no change in maximal relaxation response to SNP was found between group-A, B and C (Control,  $E_{max} = 105.1 \pm 1.85 \%$ ,  $n = 5$ ; L-NAME,  $E_{max} = 104.8 \pm 1.36 \%$ ,  $n = 6$  and L-NAME+daidzein,  $E_{max} = 103.70 \pm 1.32 \%$ ,  $n = 8$ ).

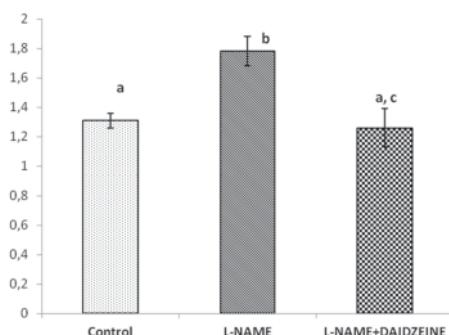


Fig. 8. Effects of chronic daidzein-treatment on high-concentration of potassium (80 mM)-induced contraction of thoracic aorta isolated from L-NAME-hypertensive rats  
Different alphabet/superscript signifies a significant variance between different groups at  $P<0.05$

*Effects of chronic-daidzein treatment on high-concentration potassium (80 mM)-induced contraction of thoracic aorta isolated from L-NAME hypertensive rats.* Fig. 8 shows the effect of chronic daidzein-treatment on high concentration potassium-induced (80 mM) contraction of aortic-rings isolated from different groups. Daidzein treatment significantly ( $P<0.05$ ) decreased the potassium-induced-contraction of the thoracic-aorta of group-C ( $1.26 \pm 0.13$  g, n = 12) in comparison to the L-NAME-hypertensive rats of group-B ( $1.78 \pm 0.10$  g, n = 9).

### Discussion

Daidzein treatment for 42 days significantly decreased the MAP of L-NAME hypertensive rats of group-C. CAO et al. (2006) reported that daidzein-sulphate at 20 and 40 mg/kg as a single or multiple oral dose, decreased the blood pressure in spontaneous hypertension rats. In the present experiment, the significant decrease in MAP of the daidzein-treated L-NAME-hypertensive rats may be because of the improvement of endothelial function mediated through the generation of nitric-oxide, as indicated by improvement in the relaxation of aortic rings to ACh (Fig. 4 and 5). Similar scientific reports have disclosed that daidzein improves the nitric oxide activity of blood vessels (MENDELSON, 2002; MISHRA et al., 2000; WOODMAN et al., 2004). The decreased MAP did not reach the level observed in the control rats. It would be of interest to determine whether increasing the dose or length of treatment with daidzein can completely prevent the effect of L-NAME.

In the present study it was observed that chronic L-NAME treatment potentiates the vasoconstrictor activity of phenylephrine. The increase in phenylephrine-induced contraction noted in L-NAME-hypertensive rats of group-B may be due to the decrease in basal nitric-oxide levels, influencing the vasodilator tone and the resulting increase in vasoconstrictor potency (DENNINGER and MARLETTA, 1999; FURCHGOTT and ZAWADZKI, 1980a; FURCHGOTT and ZAWADZKI, 1980b).

Nitric-oxide is one of the key constituents of vascular endothelium participating in relaxing the blood-vessels (VANHOUTTE and MILLER, 1985). Vascular relaxation also depends on the type of blood vessels and mediators. Decrease in NO-level/bioavailability may lead to cardiovascular diseases such as hypertension. In the present experiment, a marked increase in ACh induced vascular relaxation of the thoracic aorta was found in daidzein-treated L-NAME-hypertensive rats, as compared to L-NAME-hypertensive rats, which indicates the contribution of daidzein to improving endothelial function. WOODMAN and BOUJAOUDE, (2004) also reported a significant increase in sensitivity and maximal-response to ACh in aortic-rings of daidzein-treated rats, in comparison to vehicle-treated. Many scientific reports reveal that daidzein improves vascular function, primarily or partly through generation of nitric oxide, along with the generation of other

mediators such as EDHF, depending upon the type of blood vessels or the decrease of free radical generation, since free radicals scavenge nitric oxide (PARK et al., 2005; MISHRA et al., 2000; WOODMAN et al., 2004; CAI and HARRISON, 2000; HUANG et al., 2000; MAHN et al., 2005; TAYLOR et al., 2004; VERA et al., 2005).

A significant increase in vasodilator potency ( $pD_2$ ) of SNP was found in the thoracic aorta of L-NAME-hypertensive rats compared to the control and daidzein-treated L-NAME-hypertensive rats. The change in (L-NAME-hypertensive rat) vascular sensitivity to SNP may be due to the increase in sensitivity to SNP. An increase of vascular sensitivity to SNP may be mediated through L-NAME by inhibiting vascular nitric oxide synthesis, or it may inhibit eNOS, or development of vascular super-sensitization to SNP through sensitization of sGC to NO (ROY et al., 2008; GUPTA et al., 2008; MONCADA et al., 1992).

A non-significant change in maximal relaxation ( $E_{max}$ ) of the aortic-rings to SNP was found in all groups of rats. Similarly, VERA et al. (2005) and WOODMAN and BOUJAOUDE, (2004) reported a non-significant change of SNP-induced relaxation of aortic-rings isolated from spontaneous-hypertensive rats and daidzein-treated control rats, respectively. There was no significant change in  $E_{max}$  value was found, perhaps because isoflavones or 17- $\beta$ -estradiol may not participate in improving nitric-oxide-induced sensitivity (VERA et al., 2005) but rather act as NO donors.

To check the tissue viability of thoracic aorta, a high potassium (80 mM)-depolarising solution was used. Daidzein treatment significantly decreases the potassium-chloride (80 mM) induced contraction of aortic-rings isolated from daidzein treated L-NAME-hypertensive rats, compared to L-NAME-hypertensive rats. Similarly, CAO et al. (2006) reported that daidzein decreases the contractile response induced by potassium chloride, and a decrease in contractile response may be accomplished by reducing extracellular calcium-dependent contraction.

The results of the present study suggest that chronic administration of L-NAME to male Wistar-rats produces significant changes in thoracic aorta vascular activity. An increase of sensitivity to phenylephrine and SNP were found, followed by suppression of ACh-induced dilatation of the thoracic aorta isolated from L-NAME hypertensive rats. An interesting finding of the study was that, with chronic administration of daidzein together with L-NAME, daidzein was able to reduce the power of L-NAME, to develop MAP to the level found in hypertensive rats treated with L-NAME alone. Also daidzein improves the ACh-induced relaxation of the thoracic aorta, indicating the ameliorative action of daidzein on vascular activity.

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**PRAWEZ, S., A. A. AHANGER, T. U. SINGH, S. K. MISHRA, S. N. SARKAR, S. RAHMAN:** Trajna primjena fitoestrogena daidzeina poboljšava srednji arterijski tlak i vaskularnu funkciju u L-NAME štakora s povećanim krvnim tlakom. *Vet. arhiv* 85, 451-464, 2015.

**SAŽETAK**

Hipertenzija kao „tihi ubojica“ dovodi do bolesti srca, moždanog udara, zatajivanja bubrega i prerane smrti. Fitoestrogen daidzein, slično kao i estrogen, djeluje zaštitno na krvne žile uz minimalne popratne učinke. S ciljem istraživanja zaštite krvnih žila, te učinka na krvni tlak (srednji arterijski tlak - SAT), daidzein je tijekom 6 tjedana bio primjenjivan Wistar štakorima s L-NAME - hipertenzijom. Štakori su bili podijeljeni u tri skupine: skupina A (kontrola), skupina B (L-NAME liječena) i skupina C (L-NAME + liječena daidzeinom). Nakon 42 dana liječenja u svim je skupinama bila analizirana funkcija krvnih žila i SAT. Liječenje štakora oboljelih od L-NAME hipertenzije (skupina C) daidzeinom dovelo je do signifikantnog sniženja srednjega arterijskog tlaka (144 mm Hg) u usporedbi s neliječenim oboljelim štakorima iz skupine B (173,2 mm Hg). Navedeno pokazuje učinke daidzeina na snižavanje krvnog tlaka. Osim toga, daidzein dovodi do signifikantnog povećanja maksimalne relaksacije torakalnog dijela aorte izazvane acetilkolinom. To pokazuju vrijednosti u skupini štakora liječenih daidzeinom ( $E_{maks} = 72,55\%$ ), u usporedbi sa skupinom neliječenih štakora ( $E_{maks} = 39,33\%$ ). Rezultati istraživanja potvrđuju da trajna primjena daidzeina (0,5 mg/kg/dan s.c.) pomaže snižavanju krvnog tlaka, na što upućuje njegova smanjena arterijska vrijednost. Osim toga, opisana primjena daidzeina djeluje zaštitno na krvne žile, jer poboljšava njihovu relaksaciju izazvanu acetilkolinom.

**Ključne riječi:** hipertenzija, daidzein, krvni tlak, funkcije krvnih žila, štakori

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