ORIGINAL ARTICLE

β-sitosterol on heart rate variability in L-NAME induced hypertensive rats

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Abstract

Background: β-sitosterol is a bioactive compound extracted from *Mucuna pruriens* and found to be effective in protecting against cerebrovascular diseases. *Aim and Objectives:* The present study is aimed to assess the effect of β-sitosterol on heart rate variability in L-NAME induced hypertensive rats. *Material and Methods:* The study involved laboratory-bred 24 adult male Wistar rats (*Rattus norvegicus*) randomly allocated into four groups. Group 1: Control (n=6), Group 2: L-NAME (n=6), Group 3: β-sitosterol (n=6) and Group 4: L-NAME+β-sitosterol (n=6) for 28 days. All the experimental animals were subjected to Heart Rate Variability (HRV) analysis at the beginning as well as at the end of 28 days of before and after L-NAME treatment and simultaneous supplementation of β-sitosterol. Animals were subjected for assessment of other hemodynamic parameters such as heart rate and blood pressure. Oxidative stress parameters like serum Malondialdehyde (MDA) and Nitric Oxide (NO) were also assessed. *Results:* The present study demonstrated alteration in HRV with increased LF (sympathetic function) and HF (parasympathetic function) ratio in Group 2 L-NAME treated hypertensive rats. In Group 4 (β-sitosterol supplemented), hypertensive rats showed remarkable reduction in the values of LF and HF ratio and other vascular parameters as compared to Group 2. Increased level of serum MDA and decrease level of serum NO in Group 2 with concomitant decreased level of MDA and increased level of NO were also observed in Group 4 rats. *Conclusion:* This study clearly indicates an alteration in cardiovascular physiology with altered sympathovagal balance in L-NAME treated hypertensive rats. Administration of β-sitosterol in hypertensive rats was found to be beneficial against L-NAME induced hypertension.

Keywords: Heart Rate Variability analysis, oxidative stress, L-NAME induced hypertensive rat model, β-sitosterol.

Introduction

β-sitosterol is a bioactive phytosterol which is naturally present in plant cell membranes with chemical structure similar to the mammalian cell-derived cholesterol. They are abundantly present in lipid-rich plant foods such as nuts, seed, legumes and olive oil. β-sitosterol is a potent micronutrient and it is present widespread in higher plants. Animals obtain these phytosterol through their diet [1].

Heart Rate Variability (HRV) is "beat-to-beat" oscillation of the heart rate around its mean value and represents physiological phenomenon determined predominantly by the balance between Autonomic sympathetic and parasympathetic Nervous System (ANS). Therefore, the measurement of the HRV is a non-invasive technique that can be used to investigate the dynamic balance between sympathetic and vagal activity [2].

In vivo, vasodilators and vasoconstrictors modulate the endothelial function. It is established that Nitric Oxide (NO) produced in vascular endothelial cells has a potent vasodilator effect. It plays an important role in vascular resistance and growth. L-arginine analogues such as N-nitro-L-Arginine

Methyl Ester (L-NAME) hydrochloride administration inhibit Nitric Oxide Synthase (NOS) activities [3]. Hence, they will reduce NO biosynthesis leading to hypertension. In conditions of NO deficiency, there will be accumulation of superoxide anion in biological tissues which can lead to alterations in organ function. NO acts as an endogenous anti-oxidative agent. It reacts with superoxide anions generated in the living tissues and provides protection from their deleterious effects on many organs including heart, kidney etc. [4]. There were several adverse reports of therapeutic use of plant based bioactive compounds as antihypertensive agents hence more studies are necessary for greater understanding of their safety and efficacy to prevent possible risks [5]. As antihypertensive effects of β-sitosterol have been least studied, the present study was undertaken to assess it's effects on heart rate variability in L-NAME induced hypertensive rats with special reference to oxidative stress.

Material and Methods

Experimental animals

The study involved laboratory-bred adult male Wistar rats (*Rattus norvegicus*), weighing about 180-250g (age 8-10 weeks) obtained from the animal house of Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura. The experimental animals were kept at 22-24^{0}C and exposed to 12 hours light/dark cycle with food and water being made available *ad libitum*. Utmost care was taken to avoid any laboratory induced stress in animals. The procedure was approved by Institutional Animal Ethics Committee (IAEC) and the animals were acclimatized to the laboratory conditions for one week before initiating the experimental protocol. All the experimental procedures were done in accordance with national guidelines (Committee for the Purpose and Control and Supervision of Experiments on Animals, Government of India).

Experimental groups

The acclimatised animals were randomly allocated to the following four groups: (Table 1)

Table 1: Experimental groups and dosages

Groups	Number of rats	Intervention
Control	n=6	Normal water and food.
L-NAME	n=6	L-NAME, 40 mg/kg/day orally in distilled water for 28 days
β-sitosterol	n=6	HPCM emulsion, 0.05 mg added in 5ml distilled water 1 to 2 unit/kg/day as per body weight administrated orally for 28 days.
L-NAME+β-sitosterol	n=6	L-NAME, 40 mg/kg/day orally in distilled water for 28 days.HPCM emulsion, 0.05 mg added in 5ml distilled water and β-sitosterol 0.14 to 0.26 mg/kg/day 1 to 2 unit/kg/day as per body weight administrated orally for 28 days.

Gravimetry

The body weights of all rats were recorded on day 0 of the experiment (initial body weight) and after 28 days of intervention (day 29, final body weight) (as the period of intervention was 28 days i.e. from day1-day 28) with an electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). Percentage change in body weight was calculated using the formula shown in Table 2.

Change in body weight = Final body weight-Initial body weight/Initial body weight ×100

Administration of drugs

L-NAME procured from Sumedha Research World, India. L-NAME was refrigerated at -20^0 C for further use. L-NAME daily dose (40 mg/kg/day) was calculated and given in the morning by oral gavage at once in distilled water to Group 2 and Group 4 rats for 28 days [6]. β-sitosterol was extracted from *Mucuna prueins* plant in our Laboratory of Vascular Physiology and Medicine following all the laboratory protocol [7]. Extracted β-sitosterol was stored in the refrigerator (-20^{0}C) until further use. The daily dose of β-sitosterol was prepared as follows –HPCM emulsion 0.05 mg added in 5ml distilled water and β-sitosterol 0.14 to 0.26 mg/kg/day 1 to 2 unit/kg/day as per body weight was prepared freshly every day and was administered by oral gavage once in the morning to Group 3 and Group 4 rats for 28 days [7].

Evaluation of cardiovascular parameters

Blood pressure: Blood pressure was recorded non-invasively (NIBP) using a tail-cuff sensor (BioPac 200A) after placing the animal in a re-strainer. Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) were recorded. Three values were obtained and the average of the three readings was considered for computation. Mean Arterial Pressure (MAP) was calculated using the formula (MAP = Diastolic Blood Pressure+ 1/3(Systolic blood pressure – diastolic blood pressure).

Electrocardiogram (ECG): ECG was recorded using needle subcutaneous electrodes using MP45 Biopac instrument with a PC based BSL 4.1 (Biopac Student Lab 4.1) software. All the recordings were performed in the morning hours following overnight fasting. Ten minute ECG was recorded in anaesthetized rats (Ketamine, 60 mg/kg b. wt.i.p and Xylazine, 6 mg/kg b. wt.i.p) in dorsal recumbency by inserting the needle electrodes into right (negative) and left (positive) front legs of the animal. From the recorded ECG, heart rate was calculated.

Heart Rate Variability (HRV) analysis: HRV components of low (LF, sympathetic index), high (LH, parasympathetic index), very low (VLF, injury index) frequencies and the LF/HF ratio were calculated. The recorded ECG was inspected offline for artefacts and ectopic beats that were manually deleted from the recording. RR intervals obtained from the recorded ECG were exported to Kubios software version 2.0 (developed by Department of Physics, University of Kuopio, Finland) for HRV analysis. Short term HRV analysis using 5 min ECG RR interval data was done to assess cardiac autonomic balance. Frequency-domain method of HRV analysis was used to assess the level of sympathetic activity, parasympathetic activity and sympathovagal balance.

Assessment of oxidative stress

Malondialdehyde (MDA): It is a product of lipid peroxidation. Concentration of MDA is frequently used as a marker for oxidative stress. MDA concentration was estimated in the serum by the method of Buege and Aust (1978). MDA reacts with thiobarbituric acid to give a pink colour and absorbance was read at 535 nm using spectrophotometer (Schimadzu UV 800, Schimadzu Corporation, Japan)[8].

Serum nitric oxide (NO): It is a stable by product of nitric oxide; nitrate was coupled to N-naphthyl ethylene diamine and then reduced to nitrate using cadmium reduction process. A spectrophotometer was used to measure the coloured complex produced at 540 nm by modified Griess reaction method [9-10].

Statistical analysis

SPSS software (version 20.0) was used for statistical analysis. Data were presented as Mean ± SD. To analyse multiple groups, one way ANOVA was used followed by Tukey's *post hoc* test to ascertain significant intergroup differences. Value of $p < 0.05$ was considered statistically significant.

Results

Gravimetry

Table 2 shows percentage change of body weight at the onset and before sacrifice of the rats. There was significant less body weight gain in group 2 (L-NAME) rats as compared to the control as well as other groups.

Effect of β-sitosterol on systolic and diastolic blood pressures

There were significant increase in SBP and DBP in the Group 2 of L-NAME treated rats (from D7 to D29) as compared to control. But a significant decrease in both SBP and DBP have been observed in Group 4 (β-sitosterol+ L-NAME treated) rats as compared to Group 2 (L-NAME treated) rats (Table 3, Figures 1 and 2). Table 3 also shows percentage change difference of SBP and DBP before and after treatment in different groups of rat.

Table 2: Percentage change in body weight of the rats

Body weight (g)	Control (n=6)	L-NAME (n=6)	β-sitosterol (n=6)	L-NAME+ β-sitosterol (n=6)
Day 1	173.3 ± 2.88	178.6 ± 5.50	178.12 ± 3.21	179.3 ± 4.93
Day 29	278.3 ± 5.40	266.0 ± 6.19	286.6 ± 3.14	280.0 ± 4.82
Percent body weight gain	37.76 ± 7.00^{a}	32.85 ± 8.00^{b}	37.85 ± 8.78^{a}	35.96 ± 9.78^{a}

Values with different superscripts are significantly different from each other ($p < 0.05$)*

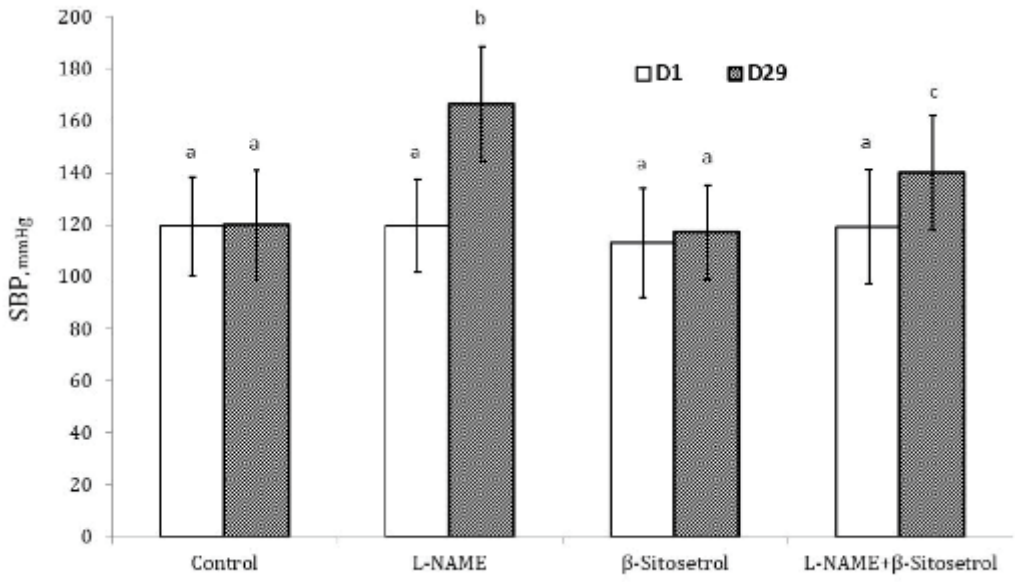

Figure 1: Effect of L-NAME induced alteration of SBP (mmHg) on β-sitosterol supplemented rats (n=6, each group) on D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

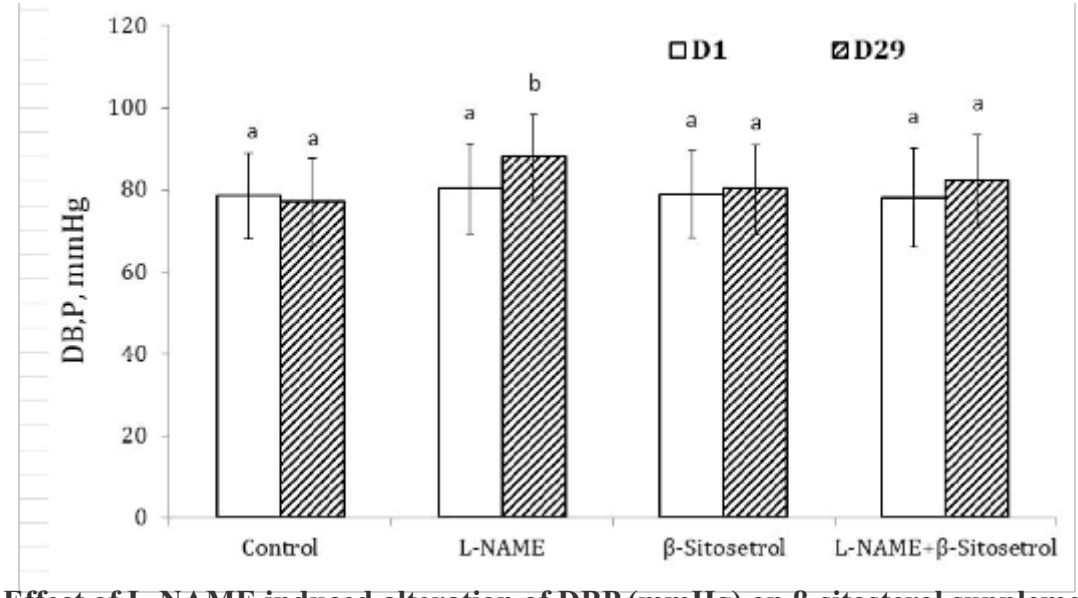

Figure 2: Effect of L-NAME induced alteration of DBP (mmHg) on β-sitosterol supplemented rats (n=6, each group) on D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

Table 3: Percentage change difference of SBP and DBP before and after treatment in different groups of rats

Parameters	Groups (n=6 /group)	Before treatment (D1)	After treatment (D29)	Percentage change (D1 Vs D29)
SBP (mmHg)	**Controls**	119.50 ± 18.50 [a]	120.00 ± 21.00 [a]	(+) 0.42
	L-NAME	119.66 ± 20.00 [a]	166.50 ± 24.25 [b]	(+) 39.14
	β-sitosterol	113.00 ± 16.75 [a]	117.00 ± 14.20 [a]	(+) 3.53
	L-NAME + β-sitosterol	119.16 ± 18.00 [a]	140.00 ± 15.25 [c]	(+) 17.48
DBP (mmHg)	**Controls**	78.50 ± 9.75 [a]	77.00 ± 10.00 [a]	(-) 1.91
	L-NAME	80.33 ± 9.56 [a]	88.00 ± 9.56 [b]	(+) 9.55
	β-sitosterol	79.00 ± 8.75 [a]	80.00 ± 10.25 [a]	(+) 1.26
	L-NAME + β-sitosterol	78.00 ± 9.00 [a]	82.00 ± 9.75 [a]	(+) 5.13

SBP-systolic blood pressure; DBP-diastolic blood pressure; D1,day one (before treatment), D29, day 29 (after treatment). Values with different superscripts are significantly different from each other ($p<0.05$).*

Effect of β-sitosterol on Mean Arterial Pressure (MAP)

Administration of L-NAME (40 mg/kg/day) induced a progressive increase in MAP. We found progressive increase in MAP with L-NAME treated groups as compared to Control Group (from D7 to D29). We also observed a slow increase till D14 and subsequently decrease in MAP in Group 4 (β-sitosterol + L-NAME treated) rats as compared to L-NAME treated Group 2 rats (Figure 3). Results also reflect the impact of β-sitosterol alone on MAP during pre-established hypertension from D1 to D7,D14,D21 and D29.

Effect of β-sitosterol on HRV analysis

The alteration in HRV was characterized by increased LF (sympathetic function) and HF (parasympathetic function) ratio in Group 2 (L-NAME treated) hypertensive rats. In Group 4 (β-sitosterol + L-NAME) rats showed remarkable reduction in LF and HF ratio as compared to Group 2 rats (Figure 4).

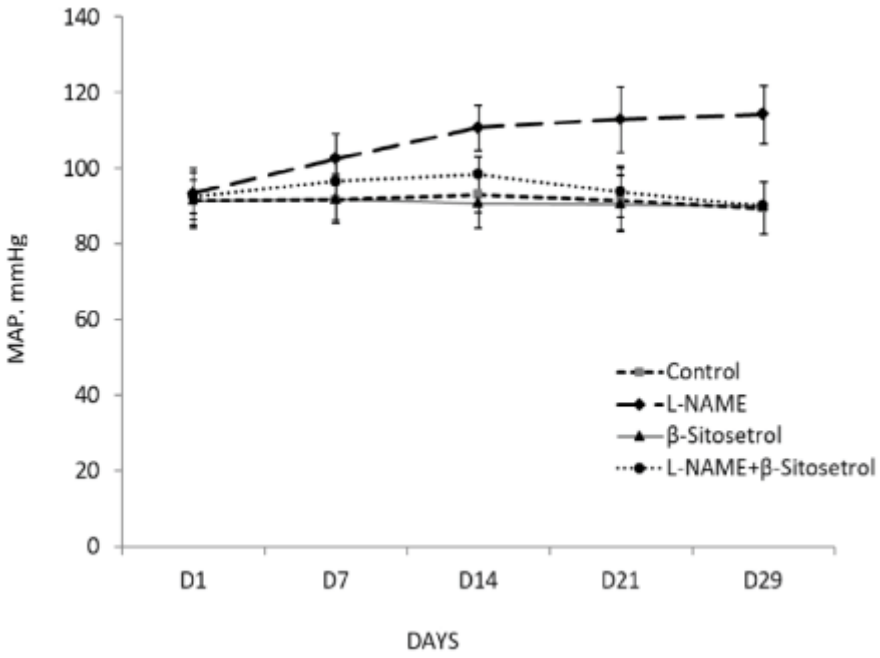

Figure 3: Comparison of MAP (mmHg) among four groups on D1, D7, D14 and D29 in L-NAME Induced and β-sitosterol supplemented rats (n= 6 in each group)

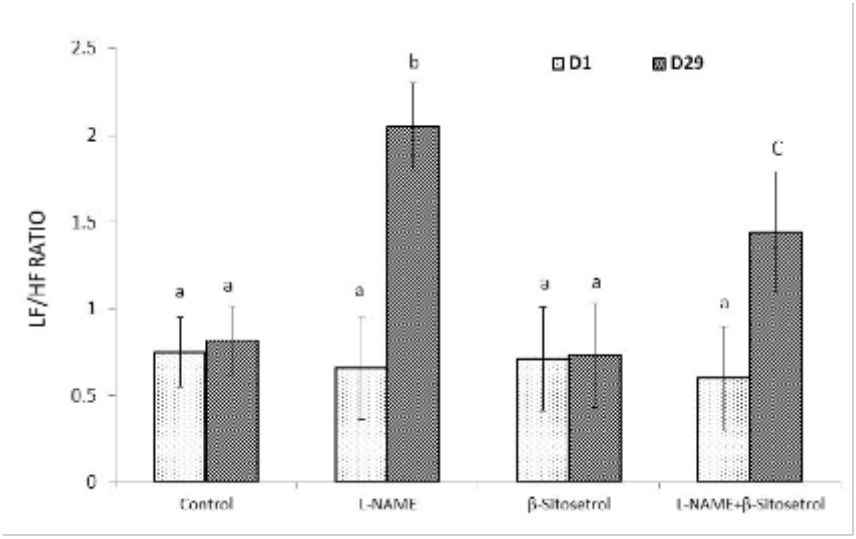

Figure 4: Effect of L-NAME induced alteration of frequency domain results of HRV analysis by LF/HF ratio on β-sitosterol supplemented rats (n=6, each group). D1, beginning of the experiment; D29, at the end of experiment and before sacrifice. Values with different superscripts are significantly different from each other ($p<0.05$*).

Table 4: Oxidative stress parameters

Parameters	Control (n=6)	L-NAME (n=6)	β-sitosterol (n=6)	L-NAME+ β-sitosterol(n=6)
MDA in serum µmoles/L	1.620.02[a]	2.610.54[b]	1.640.04[a]	1.650.03[a]
Nitric oxide in serum µmoles/L	0.520.02[a]	0.440.03[b]	0.550.06[a]	0.500.03[a]

Values with different superscripts are significantly different from each other (p<0.05).*

Oxidative stress

We observed significant increase in MDA levels in serum of Group 2 (L-NAME treated) rats when compared to respective control group 1. We also observed significant reduction in MDA levels in serum of Group 4 (β-sitosterol + L-NAME) rats (Table 3). In case of serum nitric oxide (NO) there was a significant decrease in NO levels in Group 2 (L-NAME treated) rats as compared to control. With the supplementation of β-sitosterol in Group 4 rats, the level of NO elevated significantly to near control level (Table 4).

Discussion

Decreased body weight in case of L-NAME treated rats after the end of treatment reflects rise of blood pressure induced stress phenomenon [3]. Present studies revealed that β-sitosterol- a bioactive compound of *Mucuna prueins* possesses cardio protective effects on L-NAME induced hypertensive rats. Chronic blockage of NO synthesis by L-NAME is well established method of inducing experimental hypertension in small animals [11]. Although this model cannot be extrapolated to human being, but it provides a possibility of understanding the mechanism of elevated blood pressure to a certain extent in relation to NO bioavailability. Sufficient magnitude of NO is needed for maintaining normal blood pressure. Another mechanism of endothelial dysfunction might be NO synthase inhibition by L-NAME with elevation of the Reactive Oxygen Species (ROS) via vascular NADPH oxidase [12]. When treated with β-sitosterol (Group 4), there was a significant decrease in MAP as compared to L-NAME treated rats. Our results showed decreased LF/HF ratio along with MAP in case of Group 4 (β-sitosterol+ L-NAME) rat, which might be due to regulating sympathetic overdrive during L-NAME induced hypertension by β-sitosterol supplementation. The observation of reduction of oxidative stress parameter and improvement in NO levels in our study further confirmed efficacy of β-sitosterol supplementation on cardiovascular abnormalities during L-NAME induced hypertension in rats [13-15].

β-sitosterol, by nature, is a phytosterol. Accumulating evidence indicates that cardiovascular protective effects of bioactive plant extracts are mainly via their cholesterol lowering ability, modulation of endothelial function and antioxidant capacity. More importantly, the cardiovascular protective effect of β-sitosterol, a key component of cholesterol controlling functional foods, has been related to its antioxidant and hypocholesterolemic capacity [16-17]. Further β-sitosterol possibly increases eNOS activity and decreases

NADPH oxidase in the vascular system which leads to increased level of NO production and ameliorate L-NAME induced elevated blood .pressure in experimental animals [18]. As β-sitosterol, a plant derived bioactive compound may protect against hypertension in animals, further studies are required to consider it as a possible therapeutic agent against hypertension in human.

Conclusion

The present study indicates altered cardiovascular physiology in L-NAME treated hypertensive rats but simultaneous supplementation of bioactive phytocompound β-sitosterol was found to be cardio-protective against L-NAME induced hypertension. These findings may be further extrapolated in clinical research as possible therapeutic measure against hypertension. This finding highlights the tremendous scope for using medicinal plants as possible therapeutic measure against cardiovascular diseases.

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