ORIGINAL ARTICLE

Effect of L/N-type Calcium Channel Blocker (Cilnidipine) on Oxidative Stress in Nitric Oxide-deficient Hypertensive Rats

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Abstract:

Background: The sympathetic nervous system plays a major role on the renal function through the vasoactive system and the renin-angiotensin aldosterone system. Even though interest in the renal protective effects of sympathetic blocker has been increased, there are not much data to clarify this efficiency in nitric oxide deficient hypertensive rats. *Aim and Objectives:* To find out the effect of cilnidipine, L/N-type calcium channel blocker on oxidative stress of kidney in Nitric Oxide Synthase (NOS) inhibited experimental hypertensive rats. *Material and Methods:* Male Albino Wistar rats (n-24) were randomly allocated into four groups: Group 1 control received vehicle; Group 2 received Cilnidipine; Group 3 received N^G-nitro-L-Arginine Methyl Ester (L-NAME) hydrochloride; Group 4 received L-NAME and Cilnidipine; All drugs are given orally for 4 weeks. Blood pressure was measured before and after intervention and twice during intervention for all the rats. On 29th day, blood was collected and animals were sacrificed and kidneys were collected. Serum and kidney tissue Malondialdehyde (MDA) levels are estimated. *Results:* The results demonstrate that there is a significant increase in Mean Arterial Pressure (MAP) in L-NAME treated rats compared to control group. Treatment with cilnidipine significantly decreases the MAP in Group 4 rats. We also demonstrated the significant elevated serum and kidney tissue MDA levels in L-NAME treated rats. Treatment with Cilnidipine reduced serum and kidney tissue MDA levels in Group 4 rats as compared to Group 3 rats. *Conclusion:* The results demonstrate that cilnidipine has suppressive effects against progressive renal injury as evidenced by decrease oxidative stress indicator MDA levels in NO deficient hypertensive rats. This effect is explained by the L/N type calcium channel inhibition of Cilnidipine, the L-type calcium channel blocking action lowers blood pressure and N-type calcium channel blocking action leads to suppression of the sympathetic nerve activity and renin-angiotensin aldosterone system.

Keywords: Nitric Oxide Deficient Hypertension, Oxidative Stress, Mean Arterial Pressure, Malon-dialdehyde

Introduction:

Kidney is the major target organ for hypertensive complications, therefore major aims of anti-hypertensive therapy should be to reduce the progression of hypertensive renal damage[1]. 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 and plays an important role in vascular resistance and growth. L-arginine analogues such as N^G-nitro-L-Arginine Methyl Ester (L-NAME) hydrochloride administration inhibits nitric oxide synthase activity and hence reduce nitric oxide biosynthesis, leading to hypertension [2]. Accumulation of superoxide anion in biological tissues can occur in the

condition of NO deficiency that can lead to alterations in organ function [3]. NO acts as an endogenous antioxidative agent by reacting with superoxide anion generated in the living tissues, thus it provides a protective function against the action of superoxide anion in many organs including kidney [4].

Cilnidipine, a dihydropyridine L/N type calcium channel blocker [5]. N-type calcium channels are predominantly distributed in the sympathetic nervous system, control neurotransmitter release from the nerve endings of sympathetic neurons [6]. N type calcium channel inhibitory actions of cilnidipine increase the possibility that cilnidipine may have a greater renoprotective effect than L-type calcium channel blockers, because glomerular efferent arterioles do not have L-type calcium channels [7]. Although cilnidipine is expected to suppress the renal injury by suppression of sympathetic nerve activity. Renal protective profile of cilnidipine is not much assessed in animal model of hypertension [8]. Since anti-hypertensive actions of cilnidipine has not much studied in animal model with renal injury, the present study was designed to clarify the renal protective effects of antisympathetic agent, cilnidipine in NO deficient hypertensive rats.

Material and Methods:

Experimental Animals:

Twenty four adult male Albino Wistar rats (*Rattus norvegicus*) weighing 180-250 g, brought from the animal house of BLDE (Deemed to be University). The animals were kept in a environmentally controlled room with a 12-h light/dark cycle and given standard rodent chow and tap water ad libitum. The rats were acclimated to handling. The animals were adapted to the laboratory conditions for a week before the onset of the experiment.

Ethical Considerations:

Institutional Animal Ethics Committee clearance certificate was obtained for the study (Ref: BLDE/BPC/644/2018-2019 dated 15.12.2018). 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 experimental animals were randomly allocated to four groups as shown in Table 1.

Study Protocol: The experimental protocol followed

Table 1: Experimental Groups of Rats

Groups	No of rats	Intervention
Group 1 (Control)	6/set	Vehicle (0.5% Na CMC) orally for 28 days
Group 2 (Cilnidipine)	6/set	Cilnidipine, 2 mg/kg/day in 0.5% Na CMC orally for 28 days
Group 3 (L-NAME)	6/set	L-NAME, 40 mg/kg/day orally in distilled water for 28 days
Group 4 (L-NAME+ Cilnidipine)	6/set	L-NAME, 40 mg/kg/day orally in distilled water for 28 days, Cilnidipine 2 mg/kg/day in 0.5% Na CMC orally for 28 days

Na CMC- Sodium Carboxy Methyl Cellulose, L-NAME - N^G-nitro-L-Arginine Methyl Ester

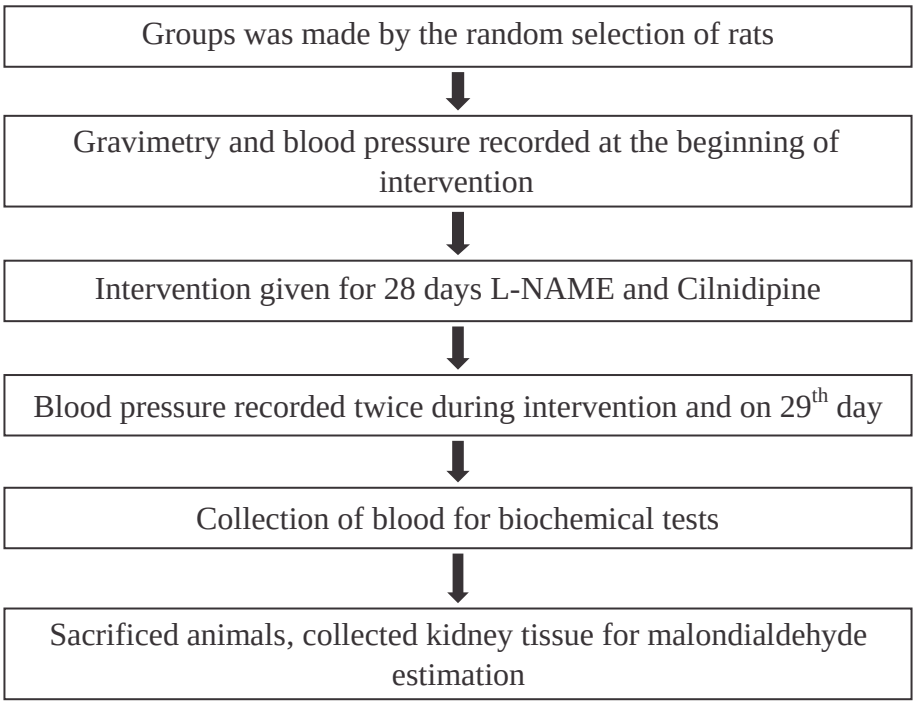

Fig. 1: Experimental Protocol

Body Weight

The body weight of all the rats was recorded on Day 1 and Day 29 using electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). The weight of all the groups of rats were matched at the beginning of experiment (Table 2).

Table 2: Changes in Body Weight of the Rats

Body weight (g) (n= 6)	Group 1 Control	Group 2 Cilnidipine	Group 3 L-NAME	Group 4 L-NAME + Cilnidipine	*P*
1st day	206.5 ± 4.5	201.5 ± 5.8	209.5 ± 9.00	211.67 ± 4.08	0.191
29th day	275.75 ± 10.11	258.0 ± 0.95[a,c]	237.5 ± 7.5[a,b,d]	263.75 ± 2.62[c]	0.000*
% body weight gain	33.66 ± 2.73	32.97 ± 3.47[a,c]	13.42 ± 2.88[a,b,d]	25.02 ± 1.73[a,c]	0.000*

*L-NAME - N^G-nitro-L-Arginine Methyl Ester. Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for comparison of multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with Group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05.*

Administration of Drug:

1. Procured L-NAME from Pro Lab Marketing PVT, Limited, New Delhi, India. L-NAME was stored in -20^{o}C refrigerator 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 3 and Group 4 rats for 28 days. [2]
2. Procured cilnidipine from Laksh Finechem Pvt. Limited, Gujarat, India. Cilnidipine was stored in the refrigerator (-4^{o}C) until further use. Cilnidipine dose for rats was calculated using the formula: Rats (mg/kg) = Human dose×0.018×5 [9]. The daily dose (2 mg/kg body weight) of cilnidipine was calculated. A suspension of cilnidipine in 0.5% Sodium Carboxy Methyl Cellulose (0.5% Na CMC) was prepared freshly every day and was administered by oral gavage once in the morning to Group 2 and Group 4 rats for 28 days.

L-NAME Induced Hypertensive Rat Model

Hypertension was induced by oral administration of L-NAME (40 mg/kg/day) in distilled water for 4 successive weeks [2].

Blood Pressure Recording

Blood Pressure (systolic and diastolic) of conscious rats was measured at the start and end of the experiment and twice during intervention. Animals were kept in the restainer for 10–20 min/day for 5 days prior to recording BP in the tail-cuff technique, and tail of the animals were warmed for 30 min for better detection of tail artery pulsations. BP was recorded using noninvasive tail cuff sensor (NIBP) Bio Pac Instrument (Bio Pac MP 100: PC windows based animal electro-physiology system) and all the parameters will be analysed by Bio Pac Student Lab 4.1 software. Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP) was measured. All the measurements were made thrice and the mean of three measurements was considered for each rat. Mean Arterial Pressure (MAP) was calculated by using formula MAP = Diastolic Blood Pressure+ 1/3 Pulse Pressure [2].

Assessment of Oxidative Stress

Malondialdehyde (MDA) is a product of lipid peroxidation. Concentration of MDA are used frequently as a marker for oxidative stress. MDA concentration was estimated in the serum and kidney tissue homogenate by the method of Buege and Aust (1978) [10].10% of tissue homogenate was prepared in 0.1M phosphate buffer using tissue homogenizer (Remimotors, Bombay, India) and supernatant was used for the assay. MDA reacts with thiobarbituric acid to give a pink colour and absorbance was read at 535 nm using spectro-photometer (Schimadzu UV 800, Schimadzu Corporation, Japan).

Statistical Analysis:

Statistical analysis was done using SPSS16.0 (SPSS Inc., Chicago, USA). The values were presented as Mean ± SD. Statistical significance of multiple groups was analysed using One-way Analysis of Variance (ANOVA) followed by Post hoc Tukey's multiple comparison test. P-value < 0.05 was considered as statistically significant.

Results:

Effect of Cilnidipine on Systolic Blood Pressure and Diastolic Blood Pressure:

Hypertensive rat model by NOS3 inhibitor L-NAME was successfully developed in our laboratory (Table 3). Significant increase in SBP

in L-NAME treated rats from 9th day when compared to control while significant decrease in SBP in cilnidipine treated rats when compared to L-NAME treated was observed. Significant increase in DBP from 9th day in L-NAME treated group. There is a decrease in DBP in cilnidipine treated group on 9th and 18th day although not significant. We observed significant decrease in DBP on 29th day (Table 3).

Effect of Cilnidipine on Mean Arterial Pressure (MAP)

There is no significant difference in baseline mean arterial pressure among groups. No significant differences was observed in MAP in the control group over the 4 week experimental period. Administration of L-NAME (40 mg/kg/day) induced a progressive increase in mean arterial pressure. We found significant increase in MAP with L-NAME treated groups when compared to control group from 9th day onwards. We observed decrease in the MAP in cilnidipine treated rats on 9th day and 18th day when compared to L-NAME treated group although not significant but we found significant decrease in MAP on 29th day (Fig. 2).

Oxidative Stress

We observed significant increase in MDA levels in serum and kidney tissue of L-NAME treated rats when compared to control group. We also observed significant decrease in MDA levels in serum of cilnidipine group. MDA levels in kidney tissue of cilnidipine treated rats also decreased though not significant (Table 4).

Table 3: Effect of Cilnidipine on Systolic And Diastolic Blood Pressure

Groups	Measurement	1st Day	9th Day	18th Day	29th Day
Group 1 Control	SBP (mmHg)	107.32±3.19	112.685±8.19	99.78±8.05	111.01±9.5
	DBP (mmHg)	72.4±10.2	72.63±12.28	74.24±14.66	84.64±6.24
Group 2 Cilnidipine	SBP (mmHg)	104.20±6.43	111.21±4.09^{c}	111.71±6.43^{c}	114.37±4.87^{c}
	DBP (mmHg)	78.7±3.85	83.00±7.65	84.70±4.79^{c}	89.83±4.02^{c}
Group 3 L-NAME	SBP (mmHg)	103.33±3.32	124.11±3.96^{a,b,d}	142.73±13.42^{a,b}	152.24±8.38^{a,b}
	DBP (mmHg)	71.93±4.7	89.36±7.75^{a}	106.32±7.64^{a,b}	122.60±3.27^{a,b}
Group 4 L-NAME + Cilnidipine	SBP (mmHg)	110.29±4.14	113.46±6.71^{c}	128.25±4.23^{a,b,c}	130.24±4.03^{a,b,c}
	DBP (mmHg)	79.39±6.13	88.34±4.96^{a}	95.22±3.32^{a}	98.12±3.42^{a,b,c}
P	SBP (mmHg)	0.054	0.005*	0.000*	0.000*
	DBP (mmHg)	0.130	0.011*	0.000*	0.000*

*L-NAME - N^{G}-nitro-L-Arginine Methyl Ester. Values are expressed as Mean ± SD. One Way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for multiple groups Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with Group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05. SBP-Systolic blood pressure, DBP-Diastolic blood pressure*

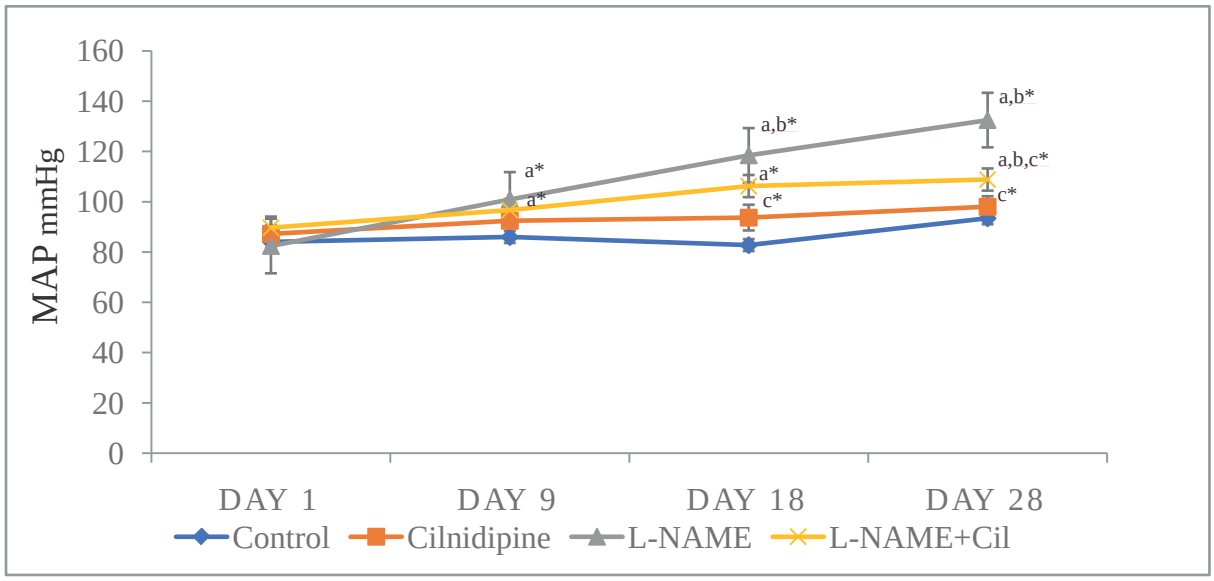

Fig. 2: Effects of Cilnidipine on Mean Arterial Blood Pressure

*Values are expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test was done for multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with group 1, 'b' denotes comparison with group 2, 'c' denotes comparison with group 3. *p<0.05. L-NAME - N^G-nitro-L-Arginine Methyl Ester, Cil- Cilnidipine, MAP-mean arterial pressure*

Table 4: Malondialdehyde Levels in Serum and Kidney Tissue Homogenate among Groups

Parameters	Control	Cilnidipine	L-NAME	L-NAME + Cilnidipine	*P*
MDA in serum µmoles/L	1.21 ± 0.43	1.23 ± 0.14^{c}	$1.755 \pm 0.08^{a,b}$	0.819 ± 0.11^{c}	0.001*
MDA in kidney tissue µmoles/gm	24.67 ± 0.55	24.53 ± 0.9^{c}	31.25 ± 0.54^{a}	29.95 ± 1.05	0.000*

*Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test was done for comparison in multiple groups. Superscript a, b, c, indicate significant difference between groups. 'a' denotes comparison with group 1, 'b' denotes comparison with Group 2, 'c' denotes comparison with Group 3. *p<0.05. MDA- Malondialdehyde, L-NAME - N^G-nitro-L-Arginine Methyl Ester.*

Discussion:

Cilnidipine has renoprotective effect in L-NAME-induced hypertensive rats. Chronic blockade of NO synthesis by L-NAME is a well-known model of hypertension. Although this model cannot be extrapolated to human hypertension, it provides the possibility of reducing the causes of increased blood pressure to a single factor, that is decrease in NO bio availability. Sufficient NO is needed for normal blood pressure. Thus, a failure to generate NO or an enhanced NO consumption can lead to hypertension. Deficiency of NO in the kidney might have caused vasoconstriction of the renal

artery and stimulated renin and angiotensin II production. This activation of renin angiotensin system may lead to vasoconstriction and hypertension [2]. Another mechanism of endothelial dysfunction might be NO synthase inhibition by L-NAME may have exaggerated the effect of Reactive Oxygen Species (ROS) generated by vascular NADPH oxidase [11]. Treatment with cilnidipine (Group 4) there was significant decrease in MAP observed compared to L-NAME treated rats.

In the kidney, renal sympathetic nerve activity contributes to the regulation of renal blood flow, glomerular filtration rate, electrolyte transport, and hormonal release. Sympathetic imbalance may lead to hypertension and progressive renal disease. Cilnidipine is a dihydropyridine calcium channel blocker, and it has been demonstrated to inhibit both N-type and L-type (long acting) calcium channels in various types of neurons. In one study on dogs, the increase in heart rate and plasma Norepinephrine (NE) level induced by bilateral carotid artery occlusion. This effect was blocked by cilnidipine through an inhibitory effect on sympathetic nerve overactivity. Cilnidipine has been shown to reduce NE secretion in response to renal nerve stimulation in dogs. This result was not observed by selective L-type calcium channel blocker nifedipine. Because of the N-type calcium channel blocking action of cilnidipine, there are some possibilities to suppress progressive renal injury [8].

MDA is a product of lipid peroxidation and has been used as a biomarker of oxidative stress [10]. Serum MDA levels in L-NAME treated hypertensive rats were increased when compared to control rats indicating high oxidative stress in L-NAME treated rats. It was found significant decrease in MDA level of serum of cilnidipine treated rats. We also found decrease in MDA level of kidney tissue of cilnidipine treated rats though not significant. Supporting this notion, previous studies also demonstrated that NOS inhibition enhances vascular super oxide release in rats [12] mice [13] and humans [14].

Along with L/N type calcium channel blocker, cilnidipine acts as a strong antioxidant. Cilnidipine demonstrates strongest lipophilicity and has highest antioxidant actions compare to other dihydropyridine derivatives [15]. The L/N type inhibitory actions of Cilnidipine would have a greater renoprotective effect than L-type calcium channel blockers, as there is absence of L-type calcium channels on glomerular efferent arterioles [16]. Oxidative stress can accompany hypertension in many animal studies, including Spontaneously Hypertensive Rats (SHR), angiotensin II-infused rats, renovascular hypertension and Deoxy-corticosterone Acetate (DOCA) salt hypertension.

Conclusion:

Result of our study demonstrate that the enhanced oxidative stress because of chronic NO synthase inhibition contributes to the impairment of renal function thus plays a role in the pathogenesis of NO-deficient form of hypertension. The L/N type calcium channel inhibitory actions of cilnidipine raise the possibility that cilnidipine would have a higher renoprotective effect by its strong anti-oxidant property compare to L-type calcium channel blockers.

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