Original Research

Evaluation of the Antioxidant Property of NO Donors against Vascular Damage Induced by Cyclosporine along with High Fat Diet

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Abstract

Cyclosporine (CsA) is the most effective and widely used first line immunosuppressant in solid organ transplantation which is a potent vasoconstrictor and can induce vascular damage. Endogenous L-Arginine (L-Arg) and L – Citrulline (L-Cit) are physiological amino acids in most living systems which act through NO-cGMP pathway and result vasodilation. Hence, the antioxidant property of NO donors L-Arg and L-Cit were evaluated against the vascular damage induced by cyclosporine A, along with HFD that serve as a model to induce Atherosclerosis followed by the vascular damage caused by CsA in C57BL/6J mice for 90 days. Antioxidant profiles (SOD, catalase, GSH, GSH Px and TBARS) were assessed in aorta, kidney and liver tissues at the end of experiment. L-Arginine and L-Citrulline administration resulted significant improvements in all the antioxidant parameters. The effects of L-Arginine were comparable to L-Citrulline, while the combination of L-Arginine and L-Citrulline was found superior in this study. Hence, with this present study, it could be concluded that, the ability of nitric oxide boosting substances, including L-Arg and L-Cit are having antioxidant effect and could reverse the progression of vascular disorders induced by cyclosporine along with HFD.

Key words: Antioxidant Profile, Cyclosporine, L-arginine, L-citrulline, Nitric Oxide

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Introduction

Cyclosporine (CsA) was introduced into clinical transplantation as an immunosuppressive agent three decades ago. It prevents allograft rejection and lead to significant improvement in the patient survival (de Mattos *et al*., 2000). CsA therapy is limited by severe side-effects such as nephrotoxicity, hypertension, hyperlipidemia, diabetes-induction and neurotoxicity (Kahan, 1989). During the past few years, NO and

NOS have become an important research topic in cellular and molecular biology. Endogenous L-Cit and L-Arg are physiological amino acids in most living systems (Curis *et al*., 2005). NO is a gaseous molecule synthesised from L-Arg by the enzyme NOS (Stuehr and Griffith, 1992). It acts as a neurotransmitter and is a component of the signaling pathways that operate between cerebral blood vessels, neurons and glial cells. Nitric oxide is generated by the oxidation of the amino acid L-Arg under the catalytic activity of the NOSs. This reaction requires NADPH and O2 as co-substrates and yields NO and L-Cit as end products (Moncada and Higgs, 1993). Tsao *et al*., (1994) have shown that the effect of arginine supplementation is associated with an increased synthesis of NO by the vascular endothelium.

Hence, we conducted a study to evaluate the beneficial effects of L-arginine and/or L-citrulline in vascular damage induced by cyclosporine along with high fat diet in C57BL/6J mice in 90 days.

Materials and Methods

Animals

An experimental study was conducted to evaluate the protective effect of nitric oxide donors L-Arginine and L-Citrulline alone and in combination against the vascular damage induced by cyclosporine along with high fat diet. Study was performed in C57BL/6J mice as this was the most susceptible animal model towards high fat diet induced hyperlipidemia. 36 healthy male C57BL/6J mice were purchased from National Institute of Nutrition, Hyderabad and were acclimatized for a period of 15 days. The protocol adopted in this experimental study were approved by the Institutional Animal Ethics Committee (IAEC - Approval No. CPCSEA II/06/2016, dated 07.04.16). Animals were divided into 6 groups with 6 animals each and group I was kept as normal control with standard diet. Remaining groups were fed with HFD (lard fat 15%, Cholesterol 1.25% and cholic acid 1%) along with Cyclosporine A @ 20mg/Kg in olive oil p.o. to induce vascular damage. Group II kept as positive control. Group III and IV were treated with L-Arginine and L-Citrulline @ 2.5% in drinking water respectively. Group V was given the combination of L-Arginine and L-Citrulline @ the rate of 1.25% each. Group VI animals were treated with simvastatin @10 mg/Kg BW. On day 90, the mice were sacrificed on day 90 (Sub –Chronic toxicity study manifestation period) as per standard guidelines and aorta, kidney, liver were collected and homogenized for the assay of SOD, Catalase, GSH, GSH-$P_{X,}$ and TBARS.

Antioxidant Defense Profile

Estimation of Protein (Lowry *et al*., 1951)

25 µl of homogenate was made up to 1.0 ml with distilled water. To this, 5ml of freshly prepared alkaline copper sulphate solution (a mixture of 50 ml of 2% sodium carbonate in 0.1 N sodium hydroxide and 1.0 ml of copper sulphate in 1% potassium sodium tartarate) was added and kept for 10 min at room temperature. 0.5 ml of Folin-ciocalteu reagent was added and allowed to stand at dark for 30

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min. The resultant blue color was read at 660 nm. Bovine serum albumin was used as standard.

Superoxide Dismutase (SOD) (Madesh and Balasubramanian, 1998)

This reaction involves generation of superoxide by pyrogallol autooxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [3-(4-5 dimethyl thiazol 2-xl) 2, 5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm. The sample, control and blank were incubated for 5 min at room temperature. The reaction is terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazan formed. The colour evolved is stable for many hours and is expressed as SOD Units (one unit of SOD is the amount in mg of protein required to inhibit the MTT reduction by 50%). The absorbance was read at 570 nm against distilled water (blank).

Table 1: Reagents for SOD estimation

Reagents	Sample			Control Blank (Duplicate)		
PBS		0.65 mL	0.65 mL	0. 65 mL		
MTT		30 µL		30 µL		30 µL
Homogenate	10 µL		-		-	
Pyrogallol		75 µL		75 µL		75 µL
The sample, control and blank were incubated for 5 min at room temperature						
DMSO		0.75		0.75		0.75
Homogenate	-		10 µL		-	

Catalase (Asru, 1972)

0.1mL of homogenate was added to assay mixture containing 0.4 mL of 0.2 M H_2O_2 and 0.5 mL of 0.01 M phosphate buffer (pH 7), and mixed well. 2mL of dichromate acetic acid solution was blown into this exactly after 60 sec and kept in boiling water bath for 10 min. The absorbance of green colored chromic acetate was measured at 570 nm against blank containing 0.4 mL of 0.2 M H_2O_2 and 0.5 mL of 0.01 M phosphate buffer (pH 7).

Reduced Glutathione (GSH) (Moron *et al.*, 1979)

The method is based on reaction of reduced glutathione (GSH) with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) to give a compound that absorbs light at 412 nm. 100 µL of 25% trichloroacetic acid was added to 400 µL of homogenate, centrifuged, collected supernatant and was used as sample. To 2.0 mL of 0.6 mM DTNB in 0.2 M sodium phosphate (pH 8), added 0.1mL of sample and 0.9 mL of 0.2 M phosphate buffer and read the absorbance at 412 nm against a reagent blank. The standards (0.05-5 mg/mL) were also treated in the same way.

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Glutathione Peroxidase (GSH Px) (Paglia and Valentine, 1967)

0.10 mL homogenate was added to 2mL 0.1M phosphate buffer, followed by 0.10 mL reduced glutathione and 0.10 mL H_2O_2. Tubes were incubated at 25^0C for 5 min, following which 0.1 mL NADPH was added and the enzyme activity was monitored at 60 sec intervals for 5 min at 320 nm wavelength.

Thiobarbituric Acid Reacting Substances (TBARS) (Balasubramanian *et al.*, 1988)

1g of tissue sample with 10 ml of 0.2 M TrisHCl buffer (pH 7.2) was taken in a tissue homogenizer to get a 10% homogenate. 500µl of supernatant from the homogenate, 1ml of 10% trichloroacetic acid and 1ml of 0.67% thiobarbituric acid were taken in a tightly stoppered tube. The tube was heated to boiling temperature for 45 min. After cooling the tube, the contents were centrifuged. The supernatant was read at 535nm against blank. The concentration of test samples was obtained using molar extinction coefficient of MDA. Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red compound absorbing light maximally at 535 nm.

Result

SOD and Catalase

The activity of SOD and catalase (U/mg of protein) in the heart, kidney and liver revealed a significant ($p<0.05$) decrease in group II when compared to all the other groups on day 90. Among the treated groups (III, IV, V and VI), group VI revealed a significant ($p<0.05$) increase (6.72 ± 0.14, 13.74 ± 0.27 and 7.92 ± 0.14, and 49.87 ± 0.63, 40.26 ± 0.94 and 79.00 ± 1.14, respectively in heart, kidney and liver).

Table 2: Least square means of CAT and SOD

	n	CAT (U/mg Protein)			SOD (U/mg Protein)		
		Heart	Kidney	Liver	Heart	Kidney	Liver
Group I	6	$51.27^{a} \pm 0.63$	$41.10^{a} \pm 0.94$	$81.91^{a} \pm 1.14$	$6.91^{a} \pm 0.14$	$14.56^{a} \pm 0.27$	$8.38^{a} \pm 0.14$
Group II	6	$25.82^{d} \pm 0.63$	$21.42^{d} \pm 0.94$	$46.01^{d} \pm 1.14$	$3.52^{d} \pm 0.14$	$6.07^{d} \pm 0.27$	$3.90^{d} \pm 0.14$
Group III	6	$42.68^{c} \pm 0.63$	$34.79^{b} \pm 0.94$	$75.20^{b} \pm 1.14$	$5.46^{c} \pm 0.14$	$11.51^{c} \pm 0.27$	$6.61^{c} \pm 0.14$
Group IV	6	$44.00^{c} \pm 0.63$	$35.63^{b} \pm 0.94$	$74.45^{b} \pm 1.14$	$5.55^{bc} \pm 0.14$	$11.72^{c} \pm 0.27$	$6.73^{c} \pm 0.14$
Group V	6	$46.56^{b} \pm 0.63$	$35.66^{b} \pm 0.94$	$74.36^{b} \pm 1.14$	$5.90^{b} \pm 0.14$	$11.90^{c} \pm 0.27$	$6.96^{c} \pm 0.14$
Group VI	6	$49.87^{a} \pm 0.63$	$40.26^{a} \pm 0.94$	$79.00^{a} \pm 1.14$	$6.72^{a} \pm 0.14$	$13.74^{b} \pm 0.27$	$7.92^{b} \pm 0.14$

Means with similar superscripts within the column do not differ significantly

GSH and GSH-Px

The concentration of GSH (n mol/mg protein) and the activity of GSH-Px (U/mg protein) in the heart, kidney and liver revealed a significant ($p<0.05$) decrease in group II as compared to all other groups. Among

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the treated groups (III, IV, V and VI), group VI revealed a significant ($p<0.05$) increase (5.86 ± 0.07, 1.33 ± 0.06 and 23.00 ± 0.57 and 8.10 ± 0.08, 10.13 ± 0.16 and 9.80 ± 0.15, respectively in heart, kidney and liver).

Table 3: Least square means for GSH Px and GSH

	n	GSH Px(U/mg Protein)			GSH (nmol/mg Protein)		
		Heart	Kidney	Liver	Heart	Kidney	Liver
Group I	6	8.00^{a} ± 0.08	10.7^{a} ± 0.16	10.15^{a} ± 0.15	6.11^{a} ± 0.07	1.52^{a} ± 0.06	26.02^{a} ± 0.57
Group II	6	4.76^{c} ± 0.08	6.78^{d} ± 0.16	6.81^{d} ± 0.15	3.12^{d} ± 0.07	0.62^{d} ± 0.06	15.09^{d} ± 0.57
Group III	6	6.45^{b} ± 0.08	7.57^{c} ± 0.16	7.62^{c} ± 0.15	4.87^{c} ± 0.07	0.91^{c} ± 0.06	20.92^{c} ± 0.57
Group IV	6	6.46^{b} ± 0.08	7.61^{c} ± 0.16	7.55^{c} ± 0.15	4.88^{c} ± 0.07	0.92^{c} ± 0.06	21.29^{bc} ± 0.57
Group V	6	6.69^{b} ± 0.08	7.80^{c} ± 0.16	8.14^{b} ± 0.15	5.03^{c} ± 0.07	0.93^{c} ± 0.06	21.49^{bc} ± 0.57
Group VI	6	8.10^{a} ± 0.08	10.13^{b} ± 0.16	9.80^{a} ± 0.15	5.86^{b} ± 0.07	1.33^{b} ± 0.06	23.00^{b} ± 0.57

Means with similar superscripts within the column do not differ significantly

TBARS

The concentration of TBARS (nM MDA/mg protein) of the heart, kidney and liver revealed a significant ($p<0.05$) increase in group II when compared to all other groups. Among the treated groups (III, IV, V and VI), group VI revealed a significant ($p<0.05$) decrease (0.91 ± 0.06, 1.83 ± 0.05 and 1.00 ± 0.03, respectively in heart, kidney and liver).

Table 4: Least squares means for TBARS

	n	TBARS(nM of MDA/mg Protein)		
		Heart	Kidney	Liver
Group I	6	0.82^{d} ± 0.06	1.81^{c} ± 0.05	0.97^{c} ± 0.03
Group II	6	3.57^{a} ± 0.06	3.95^{a} ± 0.05	3.94^{a} ± 0.03
Group III	6	1.66^{b} ± 0.06	2.36^{b} ± 0.05	2.40^{b} ± 0.03
Group IV	6	1.68^{b} ± 0.06	2.32^{b} ± 0.05	2.40^{b} ± 0.03
Group V	6	1.42^{c} ± 0.06	2.25^{b} ± 0.05	2.40^{b} ± 0.03
Group VI	6	0.91^{d}± 0.06	1.83^{c} ± 0.05	1.00^{c} ± 0.03

Means with similar superscripts within the column do not differ significantly

Discussion

CsA causes vasoconstriction by its direct action on the arterioles (Lanese *et al*., 1994). The vasoconstriction is reported to be due to CsA action in blocking mitochondrial calcium release. These changes could lead to regional hypoxia -reoxygenation injury and production of reactive oxygen free radicals (Wolf *et al*., 1994). NO has been reported to exert various physiological roles due to its ability to induce vasodilatation. By the time, numbers of other physiological roles of NO have been demonstrated that include its role in immune system, nervous system, inflammation and blood flow. It has been comprehensively reported that NO possesses indirect effects at low concentration but the direct actions will be shown at higher concentration. Cooke *et al.* (1992) reported that L-Arg supplementation to hypercholesterolemic rabbits partially restored

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endothelium-dependent vasorelaxation and also reduced the extent of atherosclerosis. Cooke and Tsao (1997) suggested that dietary supplementation of arginine inhibits atherogenesis by enhancing the synthesis of NO. EDRF has been identified as NO (Ignarro, 1987). L- Arg reduces Cu^{2+} oxidation of LDL in vitro (Aji, 1997) suggesting that it has antioxidant properties. Bode-Boger *et al.* (1996) have suggested a likely mechanism for the enhancement of NO synthesis by arginine supplementation. L-Cit is known to enhance the bioavailability of L-Arg, the endothelial substrate for the production of NO, and ultimately to increase endogenous NO production (Schwedhelm *et al*., 2008). Unlike other amino acids, L-Cit possesses a highly specific metabolism that bypasses splanchnic (internal organ) extraction. Because L-Cit is not used by the intestine or taken up by the liver, it is made available throughout the body rapidly after ingestion and thus may act directly (Bahri *et al*., 2013). L-Cit is a non-protein amino acid that is produced predominantly in the intestines (Betue *et al*., 2013). L-Cit supplementation not only increases L-Arg synthesis, but also inhibits cytosolic arginase I, a competitor of eNOS for the use of L-Arg in the vasculature and hence L-Cit protects from kidney damage in type 1 diabetes (Romero *et al*., 2013).

In the present study, the antioxidant enzymes such as SOD, CAT, GSH and GSH-Px of heart, kidney and liver were showing significant decrease in group II when compared to other groups. Arginine and/or citrulline treated groups (group III, IV and V) also had shown a significant elevation when compared to group II. De-Nigris *et al.* (2003) reported that the common feature of inflammation and atherosclerosis is oxidative stress that can lead not only to cell membrane injury but also the destruction of NO. Thus, the natural antioxidant properties of NO are lost and oxidative stress continues unabated. Our results are consistent with the findings of Hayashi *et al.* (2005) which demonstrated the fatty diet induced atherosclerosis and oxidative stress were reversed upon oral administration of L-arginine and L-citrulline, these observations suggest that NO is the active species in reducing the markers for oxidative stress and the progression of atherosclerosis. There is evidence that L-arginine is a versatile amino acid in animal and human cells, serving as a precursor for the synthesis of proteins, NO, urea, proline, glutamine, creatinine, polyamines and other molecules involved in regulating cellular homeostasis (Mendez and Balderas, 2001). Mantha (1999) reported that there was an increase in the activities of catalase and GSH-Px in the aorta of cholesterol-fed rabbits but the activity of SOD remained unchanged in rats treated with arginine.

The TBARS level of aorta got significantly increased in group II when compared to L- Arginine and/or L-citrulline treated groups (group III, IV and V). A decrease in antioxidant reserve would increase the chances of lipid peroxidation in the aortic tissue and hence development of atherosclerosis (Prasad *et al.*, 1994). Our findings were consistent with the findings of El Kirsh *et al.* (2011) who reported that increased formation of MDA in the aorta of hypercholesterolemic rabbit. L- Arginine and/or L- citrulline treatment did not decrease the aortic MDA levels in spite of improvement in the antioxidant reserve. The nonspecific assay for the measurement of MDA levels could have allowed for this discrepancy.

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Conclusion

From this study it was concluded that the degree of vascular damage induced by Cyclosporin (CsA) with high fat diet is significantly attenuated by the administration of nitric oxide donors L-Arginine and L-Citrulline. The protective effect of the amino acid supplements in vascular system could be well explained with the vasodilatory, antioxidant and regulatory properties of NO. The effects of L-Arginine were comparable to L-Citrulline, while the combination of L-Arginine and L-Citrulline was found superior in this study. Hence, with this present study, it could be demonstrated that, at least in mice, the ability of nitric oxide boosting substances, including L-Arg and L-Cit to ameliorate the biochemical changes and reverse the progression of vascular disorders induced by cyclosporine along with HFD.

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