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# Amelioration of Endothelial Dysfunction Associated with Diabetes Mellitus and Hypertension by Etoricoxib, Lornoxicam and Allopurinol

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**ABSTRACT**

Endothelial dysfunction is associated with diabetes mellitus and hypertension. Oxidative stress and augmented activity of associated enzymes like cyclooxygenase (COX) and xanthine oxidase (XO) is a major culprit. In this study, diabetes mellitus (DM) was induced by alloxan (75 mg/kg, i.v) and hypertension was induced by left renal artery (LRA) ligation in male Wistar rats. Treatment with COX inhibitors, etoricoxib (10 mg/kg, p.o), lornoxicam (4.5 mg/kg, i.p) and XO inhibitor, allopurinol (50 mg/kg, p.o) was initiated after confirming induction of diabetes and hypertension and continued for 6 weeks. Vascular reactivity to catecholamines like adrenaline (1 µg/kg), noradrenaline (1 µg/kg), and phenylephrine (1 µg/kg), was evaluated in both models wherein treated rats expressed better responses than untreated groups. In an in vitro study, relaxant response to acetylcholine (ACh) and sodium nitroprusside (SNP) was observed on isolated aortas. Relaxation to ACh was impaired in untreated groups but treated groups produced near to normal relaxant responses. In case of SNP, no significant results were obtained. Diabetes and hypertension induced significant oxidative stress reflected by increase in lipid peroxidation level and decrease in antioxidant enzymes like catalase and superoxide dismutase. Drugs treatment reversed these effects on oxidative stress indices and ameliorated endothelial dysfunction.

**Keywords:** Allopurinol, diabetes, endothelial dysfunction, etoricoxib, hypertension, lornoxicam.

## INTRODUCTION

Metabolic syndrome is a group of the most dangerous risk factors, like diabetes, obesity, hypercholesterolemia and high blood pressure. [1] According to WHO, diabetes is one of the most common chronic diseases worldwide and the fourth or fifth leading cause of death in the developed countries. Also as of 2000, it was estimated that total number of hypertensive adults to be 972 million out of which 333 million in developed countries and 639 million in developing countries and this number is predicted to rise nearly by 60% that is to 1.56 billion by 2025. [2]

The most common pathological change associated with diabetes and hypertension is endothelial dysfunction. [3-4] Reduced availability of Nitric Oxide (NO), an endothelium dependent vasodilator and raised vasoconstriction by angiotensin II is a main characteristic element of endothelial dysfunction. [5] One major factor that can lead to endothelial dysfunction is uncontrolled production of reactive oxygen species (ROS), as superoxides and its metabolites. [6] In hypertension or diabetes, ROS are produced in concentration that cannot be nullified by the antioxidant mechanisms present at cellular level leading to a state of oxidative stress. [7-8] Exaggerated production of superoxides leads to reduced bioavailability of nitric oxide (NO). [9] Augmentation of ROS and formation of peroxynitrite activates inducible form of cyclooxygenase (COX) i.e.COX-2 and inhibit the activity of prostacyclin synthase necessary for vasodilatation. [10-11] Oxidoreductase enzyme system, such as xanthine oxidase pathway, may also participate in generation of oxidative stress. [12] Also, as reported earlier, the detrimental action of xanthine oxidase is raised in both diabetes and hypertension. [13-14]

Thus we hypothesized that combinatorial treatment regimen comprising of selective COX-2 inhibitor etoricoxib, non-selective COX inhibitor lornoxicam and enzyme xanthine oxidase inhibitor allopurinol could be relevant to target endothelial dysfunction in experimental models of diabetes and hypertension.

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## MATERIALS AND METHODS

### Animals

Male Wistar rats (250-300 g) were used for the study. Animals were housed in polypropylene cages and maintained at a constant temperature of 25± 2°C with 12 h light/dark cycle and 50 ± 5% relative humidity and were fed with standard laboratory food and water *ad libitum*. Animals were

acclimatized for 1 week to laboratory conditions before study. Animals were randomly divided into different groups. Each group consisted of 5 animals. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, and the Institutional Animal Ethical Committee (IAEC) of M.V.P.S College of Pharmacy, Nasik, India, approved protocol of this study (IAEC/2011/03).

**Drugs and chemicals**

Lornoxicam (Themis laboratories, Thane, India), etoricoxib (Khandelwal laboratories, Thane, India), allopurinol (Titan laboratories, Mahad, India), alloxan monohydrate (Sigma-Aldrich, USA), acetylcholine (ACh), sodium nitropruside (SNP), nitrobluetetrazolium chloride (NBT) (HiMedia Laboratories Pvt. Ltd. Mumbai, India), thiobarbituric acid (TBA), adrenaline (Adr), nor-adrenaline (NA) (Research-Lab Fine Chem Industries, Mumbai, India), ketamine HCL (Neon laboratories Ltd. Mumbai, India), xylazine HCL (Indian Immunologicals Ltd. India), phenylephrine (PE) were used. Glucose (Pathozyme Diagnostics) was measured using the mentioned biochemical kit. The drugs were freshly prepared in suspension of 0.5% carboxymethyl cellulose (CMC) in distilled water. All chemicals used were of analytical grade and purchased from standard manufacturers.

**Experimental Design**

**Alloxan-induced diabetic rats**

Alloxan monohydrate was used to induce type-1 diabetes in rats. Single intravenous dose of 75 mg/kg was administered in different treatment groups. Blood sugar level was checked for 4 days after alloxan treatment. Animals with blood sugar level above 220 mg/dl were considered diabetic. Diabetes was completely induced in all animals at the end of $4^{th}$ day. Animals were given treatment in different groups as follows: Group I (control): Vehicle (0.5 % CMC, 0.5 ml/100g, p.o.), Group II: Alloxan (75 mg/kg, i.v.), Group III: Alloxan (75 mg/kg, i.v.) + lornoxicam (4.5 mg/kg, i.p.), Group IV: Alloxan (75 mg/kg, i.v.) + etoricoxib (10 mg/kg, p.o.), Group V: Alloxan (75 mg/kg, i.v.) + allopurinol (50 mg/kg, p.o.), Group VI: Alloxan (75 mg/kg, i.v.) + etoricoxib (10 mg/kg, p.o.) + allopurinol (50 mg/kg, p.o.), Group VII: Alloxan (75 mg/kg, i.v.) + lornoxicam (4.5 mg/kg, i.p.) + allopurinol (50 mg/kg, p.o.). Treatment was continued for a period of 6 weeks.

**Left renal artery (LRA) ligation-induced hypertension in rats**

Hypertension was produced by left renal artery (LRA) ligation. Rats were anesthetized by ketamine and xylazine (75 mg/kg and 15 mg/kg i.p. resp.). A 3-cm retroperitoneal flank incision was done to animal. The left kidney was exposed and the renal artery was carefully dissected free of the renal vein. The renal artery was then partially occluded by 4-0 sterile surgical silk. The incision was closed by careful suturing of the muscle layer with 4-0 silk using a noncutting needle. Systolic blood pressure was measured after every two week by the tail cuff technique in the conscious rat. Rats with systolic blood pressure values ≥ 140 mmHg were considered hypertensive. Animals were given treatment in different groups as follows: Group I (Control): Vehicle (0.5 % CMC, 0.5 ml/100g, p.o.), Group II: Left renal artery (LRA) ligation, Group III: LRA ligation + lornoxicam (4.5 mg/kg, i.p.), Group IV: LRA ligation + etoricoxib (10 mg/kg, p.o.), Group V: LRA ligation + allopurinol (50 mg/kg, p.o.), Group VI: LRA ligation + etoricoxib (10 mg/kg, p.o.) + allopurinol (50 mg/kg, p.o.), Group VII: LRA ligation+ lornoxicam (4.5 mg/kg, i.p.) + allopurinol (50 mg/kg, p.o.). Treatment was continued for a period of 6 weeks.

**Vascular reactivity to catecholamines**

After the completion of treatment schedule, rats from each group of both experimental models were anesthetized with ketamine and xylazine (75 mg/kg and 15 mg/kg i.p., respectively). Right jugular vein was cannulated with fine polyethylene catheter for the administration of drugs. BP was recorded from left common carotid artery using pressure transducer by direct method on Power lab data acquisition system (AD Instruments). Heparinized saline (100 IU/ml) was filled in transducer and the fine catheter cannulated to the carotid artery to prevent clotting. After 30 minutes of stabilization, mean change in BP in response to NA (1µg/kg), Adr (1µg/kg), and PE (1µg/kg) was recorded. [15]

**ACh and SNP induced vasorelaxation on isolated rat aorta**

Relaxant responses to ACh and SNP were studied on separate experimental set up after completing above studies. Rats from both experimental models were sacrificed by cervical dislocation. Midline abdominal incision was made and the entire descending thoracic aorta from arch down to the diaphragm was isolated. Isolated aorta was placed in Kreb's solution (composition in mM: NaCl 118.4; KCl 4.7; $CaCl_2$ 2.5; $KH_2PO_4$ 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25, glucose 11) at a temperature of 37 ± 0.5°C and aeration with 95% $O_2$ and 5% $CO_2$. Connective tissue and adhering fat was removed from aorta. Rings of 3 mm length were prepared and mounted in an organ bath containing 15 ml of Kreb's solution. Contractions were recorded by suspending the rings between two stainless-steel hooks, one of which was attached to the end of a bathing tube and the other to a force transducer (Power Lab, AD Instruments). Care was taken to ensure that the endothelial layer was not damaged during preparation of the aortic rings. One hook was fixed to a micrometric manipulator allowing adjustments in resting tension of the rings and the other was connected to a force displacement transducer for the measurement of isometric force. The resting tension of 1 g was applied to the preparation and equilibrated in a 15 ml bathing solution for 90 to 120 minutes before the experiment with change of solution every 15 minutes. After equilibration, the rings were exposed to 0.1 to 1µM of PE. When the contractile response to PE was plateaued, ACh (1 nM to 10µM) and SNP (1 nM to 10µM) were added in a cumulative fashion and vasorelaxation was recorded in their respective experimental setup. [15-16]

**Biochemical estimation**

**Preparation of tissue homogenate**

Aorta of rats isolated from different experimental models was subjected to these studies. Aorta was immediately washed in ice-cold saline and weighed. A 10% (w/v) tissue homogenate was prepared in ice-cold 0.1 M phosphate buffer (pH 7.4). The post nuclear fraction for catalase assay was obtained by centrifugation (Remi – C, – 30 Remi Industries Ltd. Mumbai, India) of the homogenate at 1000 rpm for 20 min, at 4°C; for other enzyme assays, centrifugation was at 12,000 rpm for 60 min at 4°C.Bio-Spectrophotometer (Elico, B-200) was used for subsequent assay. [17]

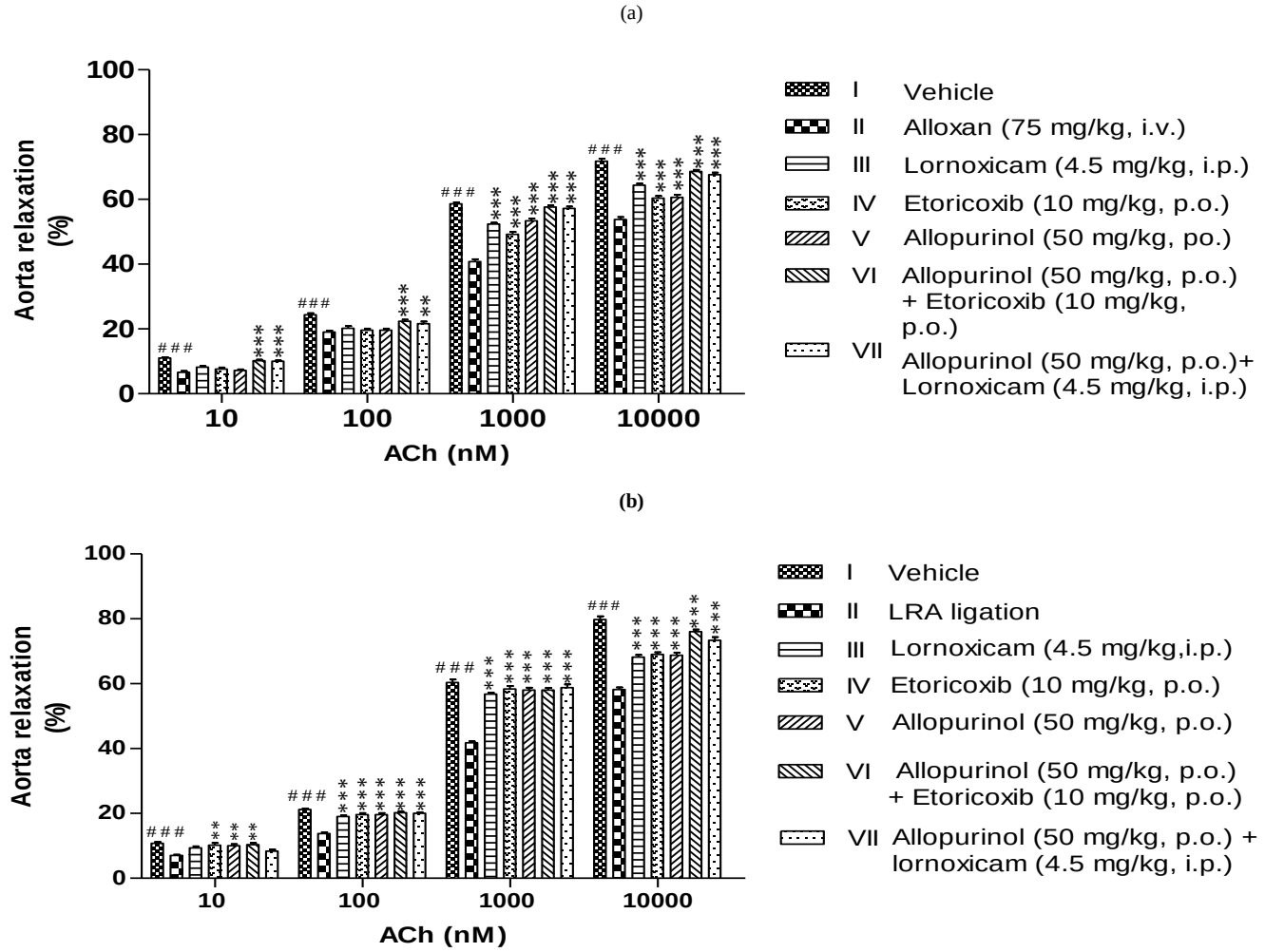


**Fig. 1: Effect of allopurinol, etoricoxib and lornoxicam on acetylcholine-induced relaxation of rat aorta pre-contracted with phenylephrine in (a) alloxan-induced diabetes (b) LRA-ligation induced hypertension.**

Each column represents mean ± S.E.M. (n=5); $^{\#}$Group I compared to Group II. $^{*}$Group III, IV, V, VI and VII compared to Group II.
$^{\#\#\#}$, $^{***}$p < 0.001(One-way ANOVA followed by Dunnett's test)

**Table 1: Effect of etoricoxib, lornoxicam and allopurinol on vascular reactivity to Adr, NA and PE in a) Alloxan-induced diabetic rats b) LRA ligation-induced hypertensive rats**

| Treatment groups | Alloxan-induced diabetic rats | | | LRA ligation-induced hypertensive rats | | |
|---|---|---|---|---|---|---|
| | Rise in blood pressure (mm/Hg) | | | Rise in blood pressure (mm/Hg) | | |
| | Adr (1 µg/kg) | NA (1 µg/kg) | PE (1 µg/kg) | Adr(1 µg/kg) | NA (1 µg/kg) | PE (1 µg/kg) |
| I (Control) | 159.6 ± 0.92 | 157.2 ± 1.02 | 160.2 ± 0.37 | 162.8 ± 0.73 | 159.4 ± 0.87 | 160.4 ± 1.03 |
| II | 180 ± 1.22$^{\#\#\#}$ | 182.6 ± 1.07$^{\#\#\#}$ | 185.2 ± 1.15$^{\#\#\#}$ | 187.8 ± 1.71$^{\#\#\#}$ | 189.2 ± 0.73$^{\#\#\#}$ | 185.2 ± 0.86$^{\#\#\#}$ |
| III | 160.4 ± 0.68$^{***}$ | 159.6 ± 0.92$^{***}$ | 163 ± 1.41$^{***}$ | 168.6 ± 1.02$^{***}$ | 171.4 ± 1.36$^{***}$ | 163.6 ± 0.67$^{***}$ |
| IV | 159 ± 0.70$^{***}$ | 159 ± 0.70$^{***}$ | 157.2 ± 1.15$^{***}$ | 166.6 ± 0.81$^{***}$ | 166.8 ± 0.96$^{***}$ | 162.4 ± 1.03$^{***}$ |
| V | 159.2 ± 0.73$^{***}$ | 159.6 ± 0.90$^{***}$ | 158.2 ± 0.80$^{***}$ | 164.2 ± 1.02$^{***}$ | 167.4 ± 0.87$^{***}$ | 164.2 ± 0.73$^{***}$ |
| VI | 158.4 ± 0.92$^{***}$ | 159.4 ± 0.51$^{***}$ | 160 ± 0.70$^{***}$ | 161.2 ± 0.66$^{***}$ | 161.2 ± 0.66$^{***}$ | 161.8 ± 0.86$^{***}$ |
| VII | 163.4 ± 1.56$^{***}$ | 159.6 ± 0.50$^{***}$ | 161.4 ± 0.51$^{***}$ | 157. 8 ± 1.02$^{***}$ | 162.4 ± 0.51$^{***}$ | 161 ± 0.70$^{***}$ |

All values are expressed as mean ± S.E.M. (n=5).
$^{\#\#\#}$, $^{***}$ p < 0.001(One-way ANOVA followed by Dunnett's test), $^{*}$Group III, IV, V, VI and VII compared to Group II, $^{\#}$Group II compared to Group I.

**Table 2: Protective effect COX inhibitors and allopurinol on biochemical alterations in isolated aortas a) alloxan-induced diabetic rats and b) LRA-ligation induced hypertensive rats**

| Treatment (Groups) | Alloxan-induced diabetic rats | | | LRA-ligation induced hypertensive rats | | |
|---|---|---|---|---|---|---|
| | CAT (µMole of $H_2O_2$ decomposed/mg protein/min) | SOD (% inhibition of reduction of NBT) | LPO (nMole of MDA/mg protein) | CAT (µMole of $H_2O_2$ decomposed/mg protein/min) | SOD (% inhibition of reduction of NBT) | LPO (nMole of MDA/mg protein) |
| I (Control) | 82.4 ± 0.81 | 80.6 ± 0.98 | 6.6 ± 0.25 | 83.2 ± 0.37 | 80.2 ± 0.67 | 7 ± 0.316 |
| II | 47.2 ± 0.38$^{\#\#\#}$ | 60 ± 0.70$^{\#\#\#}$ | 14 ± 0.32$^{\#\#\#}$ | 56.6 ± 0.4$^{\#\#\#}$ | 57.8 ± 0.66$^{\#\#\#}$ | 14.2 ± 0.37$^{\#\#\#}$ |
| III | 65.6 ± 0.51$^{***}$ | 68.4 ± 0.51$^{***}$ | 10 ± 0.32$^{***}$ | 67 ± 0.7$^{***}$ | 66.6 ± 0.4$^{***}$ | 10.2 ± 0.37$^{***}$ |
| IV | 63.8 ± 0.37$^{***}$ | 61. 2 ± 0.58$^{ns}$ | 10.6 ± 0.24$^{***}$ | 64 ± 0.31$^{***}$ | 61.2 ± 0.37$^{*}$ | 11 ± 0.31$^{***}$ |
| V | 61.8 ± 0.37$^{***}$ | 61.8 ± 0.66$^{ns}$ | 12.6 ± 0.4$^{*}$ | 60.8 ± 0.37$^{*}$ | 58.8 ± 0.37$^{ns}$ | 11.4 ± 0.51$^{***}$ |
| VI | 81.4 ± 0.51$^{***}$ | 74.4 ± 0.51$^{***}$ | 9 ± 0.31$^{***}$ | 79.4 ± 0.51$^{***}$ | 73 ± 0.44$^{***}$ | 8.8 ± 0.37$^{***}$ |
| VII | 75.6 ± 0.51$^{***}$ | 78.8 ± 0.37$^{***}$ | 10.6 ± 0.24$^{***}$ | 76.4 ± 0.51$^{***}$ | 67 ± 0.44$^{***}$ | 10.2 ± 0.37$^{***}$ |

All values are expressed as mean ± S.E.M. (n = 5).
$^{ns}$Non-significant, $^{\#\#\#}$,$^{***}$ p<0.001(One-way ANOVA followed by Dunnett's test), $^{*}$Group III, IV, V, VI and VII compared to Group II. $^{\#}$ Group II compared to Group I.

**Catalase Activity**

Catalase (CAT) activity was assayed by the method of Luck, 1971; wherein the breakdown of $H_2O_2$ is measured at 240 nm. Briefly, the assay mixture consist of 3 ml of $H_2O_2$-phosphate buffer (pH 7) and 0.05 ml of supernatant of tissue homogenate (10 %), the change in absorbance was recorded after 1 min at 240 nm. Enzyme activity was calculated using the millimolar extinction coefficient of $H_2O_2$ (0.071). The results were expressed as micromole of $H_2O_2$ decomposed per minute per milligram of protein. [18]

**Superoxide dismutase activity**

Superoxide dismutase (SOD) activity was assayed according to the method of Kono, 1978 wherein the reduction of nitrobluetetrazolium (NBT) was inhibited by the SOD and was measured spectrophotometrically at 560 nm. Briefly, the reaction was initiated by the addition of the hydroxylamine hydrochloride to the reaction mixture containing NBT and the postnuclear fraction of the homogenate (10%). The result was expressed as unit per milligram of protein, with one unit of enzyme defined as the amount of SOD required to inhibit the rate of reaction by 50 %. [19]

**Lipid peroxidation assay**

The quantitative measurement of lipid peroxidation (LPO) in rat aorta was done by the method of Wills, 1966. The reaction was initiated by addition of 0.2 ml of 8% SLS, 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA) to 0.1 ml of tissue homogenate. Finally, the volume was made to 4.0 ml by adding distilled water. It was then heated at 95°C for 1 hour on water bath and cooled to room temperature. Then, 5 ml mixture of n-butanol: Pyridine (15:1 by volume) was added to it and further it was shaken vigorously for 10 minutes. The amount of malondialdehyde (MDA) formed was measured by reaction withthiobarbituric acid at 532 nm. The results were expressed as nanomoles of MDA per milligram of protein, using the molar extension coefficient of chromophore ($1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$). [20]

**Statistical Analysis**

Results are expressed as mean ± SEM, and the statistical analysis of data was done using one-way analysis of variance (ANOVA).

## RESULTS

**Vascular reactivity to catecholamines**

The vehicle groups of both diabetic and hypertensive animals showed a normal vascular response to catecholamines (Adr, 1µg/kg; NA, 1µg/kg; and PE, 1µg/kg), whereas alloxan-treated group and LRA ligation-treated group showed a significant ($p$<0.001) exaggeration in mean change in BP to catecholamines as compared to the vehicle group. Chronic treatment with COX inhibitors, allopurinol and their combination showed a significant ($p$<0.001) fall in mean change in BP to the catecholamines (Table 1).

**ACh and SNP-induced vasorelaxation on isolated rat aorta**

The aorta from vehicle group showed normal relaxant response to cumulative doses of ACh and SNP (1 nM to 10µM). This signifies normal endothelial function. However, the alloxan-treated group in diabetic study and LRA ligation-treated group in hypertensive study showed a significant ($p$<0.001) impairment of relaxation to ACh. Aortas of rats treated with COX inhibitors, allopurinol, and their combination showed significant ($p$<0.001) improvement in relaxation to ACh (Figure 1). However, the relaxant response to cumulative doses of SNP were not significantly variable in any animal group of either diabetic or hypertensive model (Result not shown).

**Biochemical effects**

**Effect on CAT and SOD**

Tissue homogenate of aorta of vehicle-treated group showed normal levels of CAT and SOD. Alloxan-treated group and LRA-treated group showed significant ($p$<0.001) decrease in SOD and CAT levels as compared to vehicle-treated group indicating induction of endothelial dysfunction. Treatment with COX inhibitors, allopurinol, and their combination showed significant ($p$<0.01) increase in SOD and CAT levels compared to rats treated with alloxan in diabetic study and LRA ligation in hypertensive study (Table 2).

**Effect on LPO levels**

MDA levels were significantly ($p$<0.001) higher in rats treated with alloxan and hypertensive animals, as compared to vehicle group; while treatment with COX inhibitors and allopurinol significantly ($p$<0.01) lowered LPO levels when compared to alloxan-treated rats and LRA ligation-treated rats (Table 2).

## DISCUSSION

The results of the present work indicated beneficial effects of COX inhibitors; lornoxicam and etoricoxib and xanthine oxidase inhibitor allopurinol in endothelial dysfunction associated with diabetes mellitus and hypertension.

In the present study, the pressor response to various catecholamines likes Adr, NA and PE was significantly elevated in alloxan treated diabetic and LRA ligation treated hypertensive rats as compared to their vehicle groups. As reported earlier the elevation in pressor response is a consequence of rise in oxidative and shear stress in subjects due to hypertension and diabetes mellitus, where COX-2 and xanthine oxidase is over expressed in endothelial cells. [21- 22] Treatment with COX inhibitors and allopurinol significantly normalized pressor response to Adr, NA and PE in both animal models.

In hypertension it is observed that isolated arteries respond abnormally to many external stimuli and endothelium cellular structure is especially prone to dysfunction. [23] Impaired release of the endothelial vasodilator nitric oxide (NO) has been demonstrated repeatedly in hypertension. [24] Also, it is reported that an impaired endothelium-mediated relaxation occurs in diet induced hyperinsulinemia. [25] It is established that acetylcholine and sodium nitroprusside induces endothelium-derived NO release in blood vessels causing vessels to dilate. [26-27] In present study, there was a normal relaxant response to cumulative doses of ACh and SNP on isolated aortic rings pre-contracted with PE in vehicle group whereas, relaxant response to cumulative doses of ACh was significantly blunted on aortas isolated from alloxan treated diabetic rats and LRA ligation-treated hypertensive rats. Treatment with allopurinol, etoricoxib and lornoxicam significantly improved the relaxant response to ACh on aortas isolated from diabetic as well as hypertensive rats indicating the amelioration of endothelial dysfunction. [28] However, similar to other previous studies, no difference in response to SNP was observed in any treatment group and relaxation was similar in all animals irrespective of treatment schedule in this study. [29] In diabetes mellitus, there is an increase in $NADH/NAD^+$ ratio due to impaired oxidation of

NADH to $NAD^+$. Imbalanced NADH/$NAD^+$ ratio cause formation of free radicals thus oxidative stress raises. [30] Similarly in hypertension angiotensin II activates NADPH/NADH oxidase of the vascular smooth muscle cells, resulting in release of reactive oxygen species. [31] As described previously, Oxidative stress is one of the major reasons of endothelial dysfunction in metabolic syndrome. [32] In current study, untreated diabetic and hypertensive rats displayed reduction in antioxidant enzymes such as SOD and CAT whereas LPO was elevated indicating oxidative stress. Treatment with COX inhibitors and allopurinol significantly attenuated the reductions in antioxidant enzymes and elevation of LPO suggesting a possible antioxidant mechanism as reported earlier. [33- 34]

From the results obtained in this study, we conclude that management of endothelial dysfunction associated with metabolic syndrome like diabetes mellitus and hypertension is possible while targeting cyclooxygenase and xanthine oxidase enzyme systems. The combination of COX inhibitors like etoricoxib or lornoxicam with allopurinol is clearly beneficial. Yet, the selection of selective COX-2 inhibitor; etoricoxib or non-selective COX inhibitor; lornoxicam with allopurinol remains questionable and further study is desired.

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