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Evaluation of xanthine oxidase inhibitor febuxostat in myocardial fibrosis

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Abstract
Xanthine oxidase inhibitors are new class of compounds for the treatment of fibrotic diseases. In the present investigation, the effect of Xanthine Oxidase Inhibitor, Febuxostat was studied in experimental model of myocardial fibrosis. Myocardial fibrosis was induced by administration of isoproterenol (5 mg/kg i.p for 15 days). Febuxostat (5, 10, 15 mg/kg, p.o) was admimistered for 15 days in Isoproterenol treated groups of animals. The most widely accepted mechanism of myocardial toxicity is through generation of reactive oxygen species (ROS), which causes mitochondrial cell death. The cardiac functional measurements and the left and right ventricular weight indices (LVWI and RVWI respectively) were analyzed. The administration of Febuxostat resulted in significant improvement in cardiac function, decrease in cardiac weight indices, and reduced fibrous tissue proliferation with normalization of electrocardiographic measurement. Febuxostat treated group of animals also showed decrease in level of malondialdehyde with increase in level of defensive antioxidant enzymes (CAT, SOD, GSH). Thus Febuxostat exerts antioxidant activity against Isoproterenol induced myocardial fibrosis. The results obtained in this study provide evidence for the usefulness of Febuxostat as a cardio protective agent.

Keywords: febuxostat, xanthine oxidase inhibitor, myocardial fibrosis, heart failure

Introduction
Myocardial fibrosis is defined by an abnormal thickening of heart valves due to inappropriate proliferation of cardiac fibroblasts but more commonly refers to the excess deposition of extracellular matrix in the cardiac muscle. Myocardial fibrosis occurs when fibroblasts are activated to my fibroblasts and produce elevated amounts of ECM proteins that form scar tissue and alter normal degradation of ECM. Both processes lead to a buildup of collagen, which impacts both systolic and diastolic function. Historically, cardiac fibrosis has not been a focus for treatment; however, it is now believed that therapy directed at cardiac fibrosis could reduce the progression of heart failure and other cardiovascular diseases. Heart failure is a complex clinical syndrome in which structural or functional abnormalities impair the heart's ability to fill with or pump blood. Affecting nearly 6 million people in the United States, heart failure is the leading reason for hospitalization in patients aged 65 years and older, as well as a major cause of impaired quality of life and chronic disability. The enzyme Xanthine oxidase produces O2 as a by-product of the terminal steps of purine catabolism and recent studies suggest that it contributes to oxidative stress in cardiac hypertrophy. Thus Xanthine oxidase plays important role in generation of Reactive Oxygen Species (ROS). Xanthine Oxidase expression and activity are increased in experimental models of cardiac hypertrophy as well as in human end-stage cardiac hypertrophy. Takashi S. *et al*. studied effect of Xanthine Oxidase Inhibitor in experimental atherosclerosis in mice. Studies showed enhanced XO expression in macrophages in atherosclerotic plaque and in aortic endothelial cells in ApoE2/2 mice, and that febuxostat, a highly potent XO inhibitor, suppressed plaque formation, reduced arterial ROS levels and improved endothelial dysfunction in ApoE2/2 mice without affecting plasma cholesterol levels. In-vitro, febuxostat inhibited cholesterol crystal-induced ROS formation and inflammatory cytokine release in murine macrophages. Therefore, in this research work an attempt has been made to study the cardio protective effect of Febuxostat in Isoproterenol-induced myocardial fibrosis which may highlight their protective role in ameliorating myocardial fibrosis.

Objectives
- To study the effect of Febuxostat in Isoproterenol- induced myocardial fibrosis in experimental wistar rats.
- To induce myocardial fibrosis by administration of Isoproterenol (5mg/kg i.p)
- To confirm the progression of cardiac fibrosis by diagnosing and analysing the electrocardiogram patterns in rats.
- To evaluate the effect of Febuxostat as a single drug by targeting antioxidant pathway. (Heart weight, Heart weight to body weight ratio, Left Ventricular Weight Index and Right Ventricular Weight Index)
- To evaluate histologically the effect of Febuxostat on myocardium of rats using H&E staining.
- To assess the effect of Febuxostat on rat heart antioxidant enzymes system.

Materials and Methods
Experimental Animals
The animals were procured from National Institute of Biosciences, Pune. CPCSEA Reg. No.: 1091/PO/07/abc. Rats of Wistar strain (150-200g) of either sex were used for the

study. Animals were housed in polypropylene cages and maintained under the standard laboratory environmental conditions; temperature: 25 C and 50+-5 % RH with free access to food and water ad libitum. Animals were acclimatized to laboratory conditions before the test. All the experiments were carried out during the light period (8:00-16:00 h)

The studies were carried out in accordance with the guidelines given by Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institutional Animal Ethical Committee of MVP's College of Pharmacy, Nashik-02 approved the protocol of study (IAEC/FEB/2016/01)

Chemicals and Drugs Used

Chemicals and drugs used are mentioned in Table.

Table 1

Name of Chemical	Manufacturer/ Source
Nitroblue Tetrazolium Chloride (NBT)	Alfa Aesar, A Johnson Mathey Company, Chennai, India)
2-Thiobarbituric Acid (TBA)	Research Lab, Fine Chem, Mumbai, India
Ethylenediaminetetraacetic acid (EDTA)	Research Lab, Fine Chem, Mumbai, India
Sterile Saline Solution	Fresenius Kabi Pvt. Ltd., India
Picric Acid	Modern Industies, Nashik, India
Potassium Chloride	Fine Chem Industry, Mumbai, Ondia
Isoproterenol	Samarth Life Sciences Pvt Ltd, India
Febuxostat	Shreeji Pharma International, Vadiwadi,Vadodara, Gujarat

Method

In humans, cardiac fibrosis and heart failure is characterized by activation of the sympathetic nervous system leading to high circulating noradrenaline concentrations. Thus noradrenaline or a catecholamine mimicking its action like isoproterenol would only be relevant to study Cardiac fibrosis in experimental animals. Cardiac remodeling is seen in isoproterenol (ISO) infused rats with severe myocardial fibrosis accompanied by myocardial injury and β-adrenoceptor mediated apoptosis has also been demonstrated in cardiomyocytes. Increased Oxidative stress resulting from an increased cardiac generation of Reactive Oxygen Species (ROS) is implicated in the progression of cardiac fibrosis and heart failure in ISO mediated hypertensive model.

Experiment protocol

Experiment protocol is mentioned in Table.

Table 2

Groups	Treatment
I	Vehicle: Saline solution
II	ISO(5mg/kg/day, i.p) for 15 days
III	ISO (5 mg/kg/day, i.p) + Febuxostat (5 mg/kg, p.o)
IV	ISO (5 mg/kg/day, i.p) + Febuxostat (10 mg/kg, p.o)
V	ISO (5 mg/kg/ day, i.p) + Febuxostat (15mg/kg, p.o)

Procedure

1. Animals were divided in five groups as given above. Each group having 6 animals weighing 150-200g and allowed free access to standard laboratory diet and drinking water.
2. Body weight of each rat were measured before and at the end of treatment.
3. Intra peritoneal dose of Isoproterenol 5mg/kg was started accordingly and continued for 15 days.
4. Drug treatment in different animal groups were started in groups II to V after pretreatment period.
5. Heart rate and ECG were recorded in rats to check the development of cardiac fibrosis.
6. H&E staining was carried out for examination of tissue architecture.
7. Antioxidant activity was evaluated for presence of catalase emzyme, superoxide dismutase and malonaldehyde.

Result

Febuxostat (Xanthine Oxidase Inhibitor) in Isoproterenol-induced myocardial fibrosis in experimental wistar rats results-

1) Effect on body weight

ISO (5 mg/kg) treated rats showed significant ($p < 0.0001$) decrease in body weight as compared to vehicle treated groups in 15 days of study. Febuxostat (5, 10, 15 mg/kg/day) treated rats did not show significant decline in body weight compared to ISO (5 mg/kg) treatment in 15 days. The results are shown in table 3.

Table 3

Groups Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Vehicle (n=6)	161	161	161	161	162	162	162	163	163	163	163	164	164	164	164
ISO (5 mg/kg) for 15 days	159	159	158	158	157	156	156	155	155	155	154	153	152	151	151
ISO+FEB(5 mg/kg)	159	159	159	158	158	158	158	157	157	157	157	157	157	157	157
ISO+FEB(10 mg/kg)	160	160	160	160	160	159	159	159	159	159	158	158	158	157	157
ISO+FEB(15 mg/kg)	157	157	157	160	156	156	156	156	156	155	155	156	156	155	155

2) Effect on heart rate

ISO (5 mg/kg) treated rats showed significant ($p < 0.0001$) increase in heart rate compared to vehicle treated group during treatment schedule. Febuxostat (5, 10, 15 mg/kg/day) pretreated ISO (5 mg/kg) showed significant ($p < 0.0001$) decrease in heart rate compared to ISO (5 mg/kg) treated rats during treatment schedule. The results are shown in Table 4.

Table 4

Treatment Groups	Heart Rate Measurement (Beats/min)
Vehicle (n=6)	381± 0.2582
Isoproterenol (5 mg/kg) for 15 days (n=6)	425 ± 0.3651
Isoproterenol + Febuxostat (5 mg/kg) (n=6)	392 ± 0.3651
Isoproterenol + Febuxostat (10 mg/kg) (n=6)	391 ± 0.3651
Isoproterenol + Febuxostat (15 mg/kg) (n=6)	391 ± 0.3651

3) Effect on electrocardiographic recording

a) Vehicle

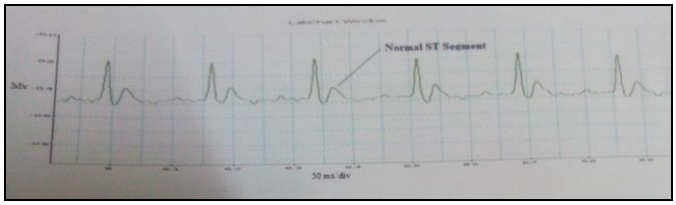

Fig 1

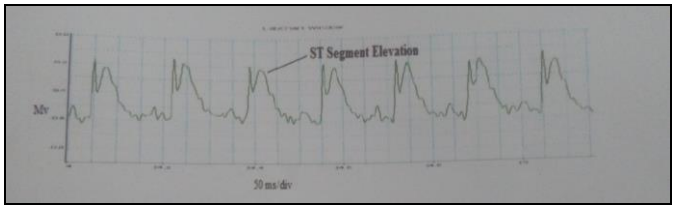

Fig 2

b) Isoproterenol

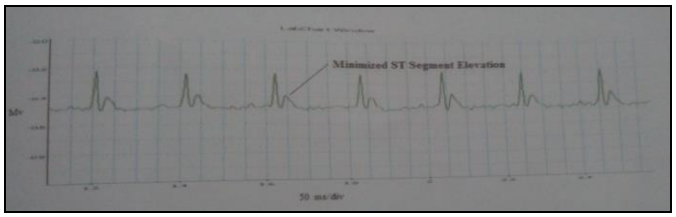

Fig 3

c) Isoproterenol + Febuxostat

A. Electrocardiographic pattern of vehicle treated rat's heart showed normal ST-segments.
B. Electrocardiographic pattern of ISO (5 mg/kg) induced cardiac dysfunction heart showed elevated ST- segments
C. Electrocardiographic pattern of Febuxostat (pretreated ISO 5 mg/kg) showed rat's heart showing minimized ST-segment elevation.

4) Effect on Heart Weight

ISO (5 mg/kg) treated rats showed significant ($p<0.0001$) increase in heart weight compared to vehicle treated rats. Febuxostat post-treated ISO (5 mg/kg) rats showed significant ($p< 0.0001$) decrease in heart weight compared to ISO (5 mg/kg) treated rats. The results are shown in Table 5.

Table 5

Treatment Groups	Heart Weight (g)
Vehicle (n=6)	0.94± 0.0031
Isoproterenol (5 mg/kg) for 15 days (n=6)	1.45 ± 0.0058
Isoproterenol+ Febuxostat (5 mg/kg) (n=6)	1.00 ± 0.0026
Isoproterenol+ Febuxostat (10 mg/kg) (n=6)	1.01 ± 0.0021
Isoproterenol+ Febuxostat (15 mg/kg) (n=6)	1.02 ± 0.0040

5) Heart weight to body weight ratio

ISO (5 mg/kg) treated rats showed significant ($p<0.0001$) increase in the HW/BW compared to vehicle treated group. Febuxostat (5, 10, 15 mg/kg) post treated ISO (5 mg/kg) rats showed significant ($p<0.0001$) decrease in HW/BW compared to ISO (5 mg/kg) treated rats. The results are shown in Table 6.

Table 6

Treatment Groups	Heart Weight/ Body Weight (g)
Vehicle (n=6)	0.0047
Isoproterenol (5 mg/kg) for 15 days(n=6)	0.0061
Isoproterenol+ Febuxostat(5 mg/kg) (n=6)	0.0043
Isoproterenol+ Febuxostat (10 mg/kg) (n=6)	0.0044
Isoproterenol+ Febuxostat (15 mg/kg) (n=6)	0.00455

6) Effect on right ventricle (RVWI) and left ventricle weight indices (LVWI)

ISO (5 mg/kg) treated rats showed significant ($p<0.0001$) increase in the LVWI as well as RVWI compared to vehicle treated groups. Febuxostat (5, 10, 15 mg/kg) post treated DOX (2.5 mg/kg rats showed significant ($p<0.0001$) decrease in both LVWI and RVWI compared to ISO (5 mg/kg) treated rats. The results are shown in Table 7.

Table 7

Treatment Groups	LVWI	RVWI
Vehicle (n=6)	0.002639	0.002519
Isoproterenol (5 mg/kg) for 15 days (n=6)	0.004957	0.003803
Isoproterenol+ Febuxostat (5 mg/kg) (n=6)	0.003254	0.002936
Isoproterenol+ Febuxostat (10 mg/kg) (n=6)	0.003175	0.002975
Isoproterenol+ Febuxostat (15 mg/kg) (n=6)	0.003027	0.003085

7) Effect on biochemical parameters in rat heart

Levels of MDA were significantly increased in Isoproterenol treated group, as compared to vehicle treated group. Febuxostat (5, 10, 15 mg/kg) significantly lowered levels od LPO as compared to Isoproterenol treated group. Significant ($p<0.0001$) decrease in levels of CAT and SOD enzymes were observed after Isoproterenol administration as compared to vehicle treated group, indicating induction of cardiotoxicity in rats. Treatment with Febuxostat (5, 10, 15 mg/kg) showed significant ($p<0.0001$) rise in levels of CAT and SOD as compared to rats treated with Isoproterenol. The content of GSH was depleted significantly ($p<0.0001$) in Isoproterenol treated group as compared to vehicle treated group. Febuxostat (5, 10, 15 mg/kg) treated groups showed significant ($p<0.0001$) elevated cardiac GSH levels. The results are shown in Table 8.

Table 8

Treatment Groups	Catalase activity (µ mole of H202 decomposed/mg Protein/min	SOD level (% inhinition of reduction of NBT)	GSH levels (µ mole of GSH/mg protein)	Lipid peroxidation (µ mole of MDA/mg protein)
Vehicle (n=6)	11.12 ± 0.04773	81.88 ± 0.2600	9.400 ± 0.0258	15 ± 0.2582
Isoproterenol (5 mg/kg) for 15 days (n=6)	7.503 ± 0.1476	60.07 ± 0.1838	7.950 ± 0.0224	26.19 ± 0.3073
Isoproterenol+ Febuxostat (5 mg/kg) (n=6)	6.863 ± 0.1229	67.55 ± 0.2861	7.933 ± 0.0452	20.33 ± 0.4256
Isoproterenol+ Febuxostat (10 mg/kg) (n=6)	7.787 ± 0.06667	70.38 ± 0.3270	8.500 ± 0.0758	20.83 ± 0.3573
Isoproterenol+ Febuxostat (15 mg/kg) (n=6)	8.250 ± 0.09916	72.22 ± 0.6892	8.650 ± 0.0224	21.67 ± 0.4216

8) Histological staining: H&E staining study

ISO (5 mg/kg) treated rats showed hard tissue compared to vehicle treated group. Febuxostat (5, 10, 15 mg/kg/day) post treated ISO (5 mg/kg) rats showed significant change in tissue architecture (mild hard and scar tissue). The result are shown in Fig.2

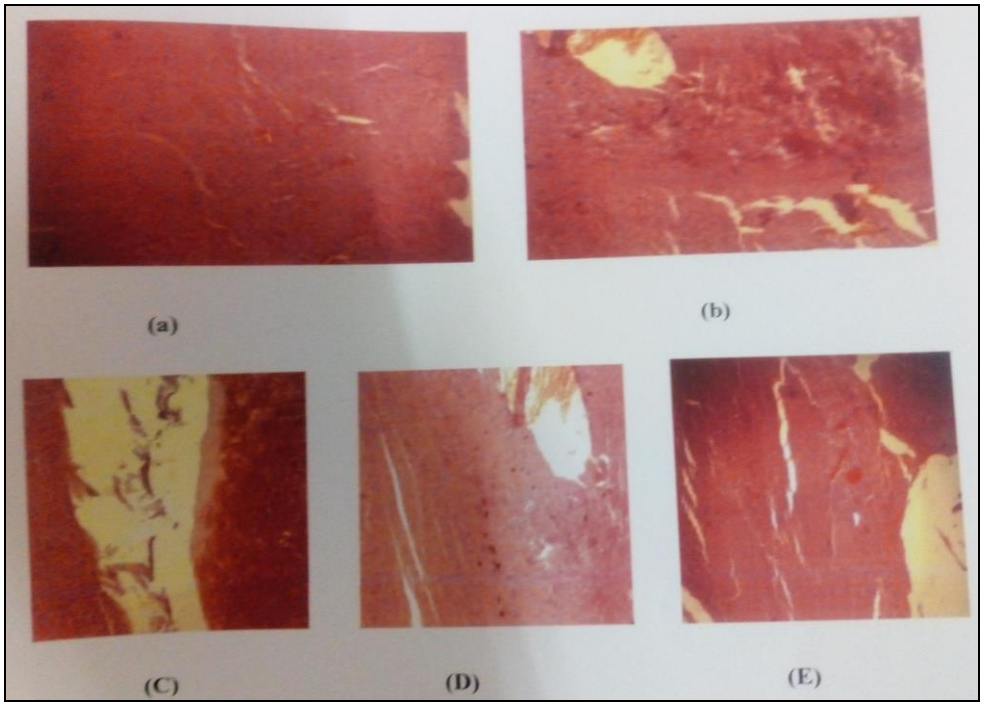

Fig 2: H&E Staining

a. Histological section of vehicle treated rat's heart showed normal tissue architecture.

b. Histological section of isoproterenol (5 mg/kg) induced rat's heart showed abnormal tissue architecture (Hard and scar tissues)

c. Histological section of Febuxostat (5 mg/kg) post treated Isoproterenol (5 mg/kg) induced rat's heart showed mild hard and scar tissues.

d. Histological section of Febuxostat (10 mg/kg) post treated Isoproterenol (5 mg/kg) induced rat's heart showed mild hard and scar tissues.

e. Histological section of Febuxostat (15 mg/kg) post treated Isoproterenol (5 mg/kg) induced rat's heart showed mild hard and scar tissues.

Conclusion

The result of present study clearly indicated the cardio protective role of Febuxostat in treatment of myocardial fibrosis as a single drug regimen. In Isoproterenol treated animals, body weight decreased over the period of 15 days of study indicating change in body weight after the administration of isoproterenol. The ISO treated rats receiving Febuxostat for 15 days did not show any decline in body weight over the period of study indicating protective effect. Moreover in Isoproterenol treated groups elevation in ST segment was observed compared to normal rats indicating myocardial fibrosis. Febuxostat successfully minimized the elevated ST-segmemt in Isoproterenol induced myocardial fibrosis indicating its cardio protective properties. Hence febuxostat is drug of choice for treatment of myocardial fibrosis.

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