

Anti-Fibrotic Effect of Irbesartan *via* Attenuation of Endoplasmic Reticulum stress in Isoprenaline-Induced Myocardial Fibrosis

Vandana Shamrao Nade*, Pranita Pradip Dharmadhikari, Laxman Ashok Kawale

Department of Pharmacology, M.V.P. Samaj's College of Pharmacy, Gangapur Road, Nashik, Maharashtra-422002, INDIA.

ABSTRACT

Background: Angiotensin receptor blockers are the new class of compounds used for the treatment of fibrotic diseases. **Objectives:** The purpose of this study was to investigate cardioprotective effect of irbesartan in isoproterenol induced endoplasmic reticulum stress, which results in myocardial fibrosis. **Materials and Methods:** Thirty wistar rats were assigned to study, 6 rats in a group. Myocardial fibrosis was induced by isoproterenol (5 mg/kg, s.c. for 15 days) which can cause cardiac toxicity. Irbesartan (10, 20 and 30 mg/kg, p.o.) was administered for 15 days after administration of isoproterenol. Post-treatment with irbesartan, cardiac functional measurements and the left and right ventricular weight indices were analyzed. Pathological alteration of levels of soluble collagen was determined. **Results:** The administration of irbesartan resulted in a significant ($p < 0.01$) improvement in cardiac function and reduced levels of soluble collagen. Irbesartan also showed decrease in the level of malondialdehyde and an increase in defensive antioxidant enzymes (SOD, CAT, and GSH) thus, exerts antioxidant activity. **Conclusion:** Irbesartan possesses anti-fibrotic activity and offers protection against myocardial fibrosis, due to regulation of angiotensin II activity through blockade of AT₁ receptors which might attenuate the endoplasmic reticulum stress and thus inhibit myocardial fibrosis *via* inhibition of oxidative stress. The regulation of antioxidant defensive enzymes may be involved in anti-fibrotic effect of irbesartan.

Key words: Endoplasmic reticulum stress, Myocardial fibrosis, Irbesartan, Isoproterenol, oxidative stress, Soluble collagen.

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INTRODUCTION

Endoplasmic reticulum (ER) stress, a condition characterized by an accumulation of unfolded proteins in the endoplasmic reticulum lumen which are responsible for variety of human disease conditions.^[1] However, the continuous accumulation of misfolded proteins is responsible for cellular dysfunction and myocardial cell death. Myocardial fibrosis is a condition which leads to the irreversible cell death of myocardial cells. As a result, myocardial fibrosis can further progress to several myocardial disease condition like myocardial infarction, heart failure etc. Several studies have correlated high levels of ER stress with myocardial damage.^[2] Administration of isoproterenol (ISO) in experimental animals is a well-established model for *in-vivo* study of a myocardial fibrosis. The catecholamine-induced myocardial fibrosis is multifactorial.^[3] Catecholamine-induced myocardial fibrosis is due to the several factors

like intracellular Ca⁺⁺ overloading of cardiac myocytes with their mitochondria. Which further generate an oxidative stress and oxidative free radical hence responsible for the opening of the mitochondrial inner membrane permeability transition pores.^[4] Myocardial fibrosis is responsible for alteration of level of soluble collagen. There is an imbalance

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*Correspondence : Dr.Vandana S. Nade,

Department of Pharmacology, M.V.P.S. College of Pharmacy, Gangapur Road, Nashik, 422002, INDIA.

Phone no: +91 253 2577250;

Fax: +91 253 2580250; Mob: +919922234934

E-mail: kawalevl@rediffmail.com

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between synthesis and degradation of extracellular matrix (ECM) proteins finally results in excessive accumulation of fibrillar collagen. There are three different types of collagen shows accumulation due the generation of myocardial fibrosis. Type I collagen showed a high level as compared to the others. Type I collagen is responsible for stiffening of ventricles which further alters myocardial autorhythmic activity and contraction. Thus the regulation of collagen synthesis and its deposition during the myocardial fibrosis can be attenuated *via* inhibition of generation of oxidative free radicals and thus myocardial cell death.^[5]

Generation of oxidative free radicals act as signalling pathway in between adjacent cells during proliferation, differentiation, and apoptosis. Therefore, cardiac fibrosis is tightly related with endoplasmic stress capacity and an imbalance between synthesis of extracellular matrix proteins and generation of oxidative free radicals which ultimately responsible for myocardial cell death.^[6]

Irbesartan is a class of compound which belongs to the angiotensin receptor (AT₁) blockers. AT₁ receptor blockers are approved clinically in treatment of hypertension and heart failure. Studies have shown that AT₁ receptor blockers show endothelium-dependent vasomotor responses. The use of AT₁ receptor blockers may be beneficial in retarding the progression of myocardial fibrosis because of their ability to prevent oxidative free radical. Hence it is possible to speculate that inhibitors of the renin-angiotensin system by AT₁ receptor blockers may improve endothelial function by regulation of antioxidant-sensitive mechanisms in the vasculature.^[7] In the present study, we aimed to determine whether the regulation of angiotensin II activity *via* blockade of AT₁ receptors might attenuate the endoplasmic reticulum stress and thus inhibit myocardial fibrosis *via* inhibition of oxidative free radical.

MATERIALS AND METHODS

Animals

Male wistar rats (230-250 g) were used for the study. Animals were housed in polypropylene cages and maintained at a constant temperature of 25 ± 2°C with 12:12 h L/D cycle and 50 ± 5% relative humidity and were fed with standard laboratory food and water *ad libitum*. Animals were acclimatized to laboratory conditions before study. 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/Dec.2014/1B).

Drugs and chemicals

Isoproterenol hydrochloride (Samarth Life Sciences Pvt. Ltd., India.), Irbesartan (Research lab, Mumbai), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (Alfa Aesar, A Johnson Matthey Company, Chennai, India.), Nitroblue tetrazolium Chloride (NBT) (Alfa Aesar, A Johnson Matthey Company, Chennai, India), Direct red 80 (Sigma-Aldrich Co. USA.), Green fast FCF (Sigma-Aldrich Co. USA). All the chemicals used were of analytical grade and purchased from standard manufacturers.

Experimental design

Animals were randomly assigned into 5 groups (n=6). Group I Control—(Vehicle for ISO 10% w/v saline solution s.c.), Group II—Isoproterenol (5 mg/kg, s.c.) for 15 days, Group III—Irbesartan (10 mg/kg, p.o)+Isoproterenol (5 mg/kg, s.c.), Group IV—Irbesartan (20 mg/kg, p.o)+Isoproterenol (5 mg/kg, s.c.), Group V—Irbesartan (30 mg/kg, p.o)+Isoproterenol (5 mg/kg, s.c.). Irbesartan (10, 20 and 30 mg/kg, p.o.) was administered for 15 days after 60 min of isoproterenol treatment.

Induction of myocardial fibrosis

Myocardial fibrosis was induced by administration of isoproterenol (ISO). Rats received subcutaneous injection of isoproterenol (5 mg/kg/day) for 15 days. The dose of ISO had a rapid and strong cardiac damage *via* oxidative free radical generation. A group of control rats received saline injection (10% w/v).^[8]

Measurement of ECG and heart rate

Rats were anesthetized on alternate days for 15 days of treatment period with 30 mg/kg of sodium pentobarbital i.p. during experimental period. The leads were connected to the dermal layer of both front paws and hind legs to Powerlab data acquisition system (AD Instrument, Australia) for the measurement of ECG and Heart rate (HR).^[9]

Left Ventricular (LVWI) and Right Ventricular Weight Indices (RVWI)

Animals were euthanized after end of study period of 15 days; heart was rapidly excised and rinsed in cold normal saline. The left and right ventricles were separated and weighed, and the left and right ventricular weight indices were calculated as the left and right ventricular free wall

mass (mg) divided by body mass (g) respectively.^[8]

Biochemical estimation

Dissection and homogenization

On the 15th day, animals were killed by cervical dislocation. The heart was removed, rinsed with isotonic saline and weighted. A 10% (w/v) tissue homogenate was prepared in saline solution. The supernatant was obtained by centrifugation (Remi-C-30, Remi Industries Ltd. Mumbai, India) of the homogenate. A micro plate reader was used for subsequent assay method.

Hydroxyproline-Sirius red assay

Collagen was measured with a Hydroxyproline and Sirius Red assay (reagents from Sigma; 0.1% each of Fast Green FCF and Direct Red 80 in Picric Acid). A tissue supernatant (100 μ l) from each sample was mixed with 900 μ l of sirius red dye on a rotator at room temperature for 30 min. The mixture was then centrifuged at 14,000 rpm for 10 min, after which the supernatant was poured out carefully without disturbing the pellet. The pellet was resuspended in 500 μ l of 0.5 N NaOH, and shaken gently for 10 min. The sample (100 μ l) absorbance was read at 550 nm on microplate reader (Nano Spectra Max 250). A standard curve was prepared from hydroxyproline, and was used to calculate the collagen content in each set of experiments.^[10,11] (Figure 1).

Catalase activity (CAT)

Method of Luck was used to estimate the catalase activity at 240 nm where the breakdown of H₂O₂ was measured. The measurement of absorbance at 240 nm was carried out by using assay mixture of 3 ml of H₂O₂ in phosphate buffer (0.0125 M H₂O₂) and 0.05 ml of supernatant of

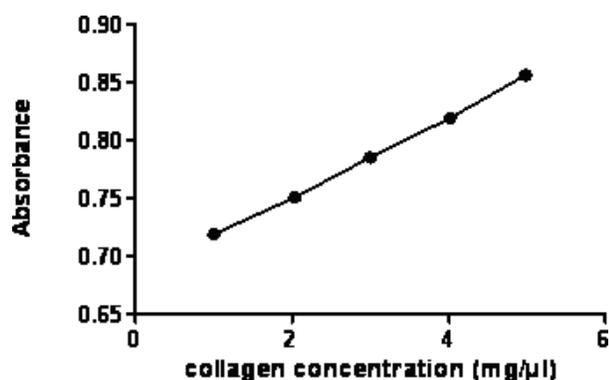


Figure 1: The concentration dependence of the absorbance of collagen solution of control group in ringer solution at 650 nm (Hydroxyproline assay)

heart tissue homogenate. Millimolar extension coefficient of H₂O₂ (0.07) was used to calculate catalase activity. The catalase activity was expressed as micro moles of H₂O₂ decomposed per minute per milligram of protein.^[12]

Estimation of reduced glutathione (GSH)

Ellman's (1959) method of estimation of reduced glutathione (GSH) was carried out by using the heart tissue homogenate. A 0.75 ml sample of homogenate was allowed to precipitate with 0.75 ml of 4% sulphosalicylic acid and centrifuged at 1,200 g for 15 min at 4°C. The assay mixture was consisting of 0.5 ml of supernatant and 4.5 ml of 0.01 M DTNB in 0.1 M phosphate buffer with pH at 8.0. The absorbance was recorded at 412 nm and the amount of GSH was expressed as micro moles of GSH per milligram of proteins.^[13]

Superoxide dismutase activity (SOD)

Method of Kono (1978) was used to assess the superoxide dismutase activity. The reduction of nitroblue tetrazolium chloride (NBT) was inhibited by the superoxide dismutase and absorbance was measured at 560 nm spectrophotometrically. Hydroxylamine hydrochloride was used to initiate the reaction by adding it to the reaction mixture containing NBT and fraction of homogenate. Results were reported as percentage inhibition of reduction of NBT.^[14]

Lipid peroxidation assay (LPO)

The quantitative measurement of malondialdehyde in heart tissue homogenate was done by the lipid peroxidation method of Wills (1966). The reaction mixture was consisting of 0.2 ml of 8% SLS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA) and 0.1 ml of tissue homogenate. The 4.0 ml of distilled water was used to make up the final volume, then heated at 95°C for 60 min on water bath and cooled under tap water. The mixture was further subjected to 5 ml mixture of n-butanol: Pyridine (15:1 by volume) with vigorous shaking. The amount of malondialdehyde (MDA) formed was measured by reaction with thiobarbituric acid at 532 nm and expressed as nanomoles of MDA per milligram of protein, using the molar extension coefficient of chromophore ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).^[15]

Statistical analysis

The results are expressed as mean \pm SEM. Data were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test by keeping the significance level as $p < 0.05$.

RESULTS

ECG parameters

ST segment evaluation

ISO administered group of animals showed significant ST segment elevation as compared to control group. The ST segment elevation represents conduction block and the consequent loss of myocardial cell membrane function. Treatment with irbesartan (10, 20, 30 mg/kg) showed significant cardioprotective effect by showing decline in ST segment elevation of ECG as compared to ISO group (Table 1).

Segment PR, QRS, QT and RR evaluation

The data of the experimental study such as PR segment, QRS complex, QT interval and R-R interval are shown in Table 1. ISO treated group showed decline of PR, QRS and RR segment and prolongation of QT segment as compared to control group. The treatment with irbesartan showed restoration of PR, QRS, QT, and RR segment as compared to ISO group. Irbesartan showed cardioprotection by restoration of changes in ECG segments as compared to ISO group (Table 1).

Effect on heart rate (HR)

ISO administration induced rise in heart rate as compared to control group. Treatment with irbesartan (10, 20, and 30 mg/kg) significantly restored HR in comparison to ISO group (Table 2).

Effect of Irbesartan on LVWI and RVWI in ISO-induced myocardial fibrosis

LVWI and RVWI are significantly higher in ISO treated group as compared to control group. Treatment with Irbesartan (10, 20 and 30 mg/kg) significantly decreased LVWI and RVWI as compared to the ISO treated group (Table 3).

Hydroxyproline assay

The standard curve of absorbance vs. concentration of hydroxyproline standard showed in Figure 1. The least squares linear regression analysis on the data points were used to determine line equation. For the collagen sample in Figure 1 the equation of the line is

$$A = 0.03429c + 0.6828$$

$$\text{Absorbance} = 0.03429 \times [\text{hydroxyproline}] + 0.6828$$

ISO administered group of animals showed an increase in myocardial tissue soluble collagen as compared to control group due to increase in collagen deposition. Treatment with irbesartan (10, 20 and 30 mg/kg) showed significant decrease in soluble collagen level (Table 4).

Effects on change in SOD and CAT levels

Group of animals with isoproterenol administration were reported cardiotoxicity when compared to control group, indicating significant decrease in levels of SOD and CAT enzymes. Group of animals subjected to treatment with irbesartan (10, 20 and 30 mg/kg) showed significant rise in levels of SOD and CAT enzymes as compared to animals treated with ISO (Table 5).

Effects on change in GSH levels

The amount of GSH was decreased significantly in isoproterenol treated group as compared to control group of animals. On the other hand, animals subjected to irbesartan (10, 20 and 30 mg/kg) showed significantly elevated levels of GSH (Table 5).

Effects on change in MDA (lipid peroxidation)

Isoproterenol treated group of animals were compared to control group of animals which reported significant increased in level of MDA. Administration of irbesartan at doses of (10, 20 and 30 mg/kg) in treatment group of animal's significantly lower levels of MDA as compared to ISO treated group (Table 5).

DISCUSSION

ISO, a non-selective beta-adrenergic agonist leads to cardiac fibrosis *in vivo*, and the dose used was in according to those described before. In the ER stress signaling cascade, irbesartan is responsible for the decrease in the pro-oxidative environment. ER is the major site of calcium storage and protein folding. It has unique oxidizing folding environment due to predominate disulfide bond formation during the process of protein folding. Alteration in oxidative environment of ER due to generation of ROS causes the generation of ER stress. Our findings demonstrated that treatment with irbesartan decreased levels of lipid peroxidation, monocyte binding and superoxide production and significantly decreased the activation of apoptotic pathway and thus cell death *via* reactive oxygen species generation. Angiotensin II binds to AT₁ receptor and exerts the peripheral and central effects on blood pressure (BP) and cell growth, and

Table 1: Effect of irbesartan on electrocardiographic measurements in ISO-induced myocardial fibrosis

Groups	ST segment elevation (mv)	PR segment (ms)	QRS complex (ms)	QT interval (ms)	RR interval (ms)
Control	0.177 ± 0.0034	31 ± 0.0041	433 ± 0.002	67 ± 0.02	160 ± 2.42
Isoproterenol (5 mg/kg)	0.429 ± 0.006	30 ± 0.00039	420 ± 0.002	64 ± 0.03	164 ± 2.33
Irbesartan (10 mg/kg) + ISO	0.290 ± 0.0031*	31 ± 0.003	429 ± 0.002	65 ± 0.021	162 ± 2.482
Irbesartan (20 mg/kg) + ISO	0.169 ± 0.0030*	31 ± 0.003	430 ± 0.0021	66 ± 0.22	163 ± 2.36
Irbesartan (30 mg/kg) + ISO	0.167 ± 0.0032*	31 ± 0.003	431 ± 0.0021	67 ± 0.32	162 ± 2.18

Each value represents mean ± S.E.M. (n=5).

*p< 0.01 vs. Group II (One way ANOVA followed by Dunnett's test).

Table 2: Effect of irbesartan on heart rate in ISO-induced myocardial fibrosis

Groups	Heart rate (Beats/min)
Control	380.43 ± 7.50
Isoproterenol	412 ± 5.60
Irbesartan (10 mg/kg)+ISO	378 ± 7.45
Irbesartan (20 mg/kg)+ISO	371 ± 7.43
Irbesartan (30 mg/kg)+ISO	376 ± 7.64

Each value represents mean ± S.E.M. (n=5).

Table 4: Effect of Irbesartan on soluble collagen level in ISO-induced myocardial fibrosis

Groups	Absorbance (λ= 650 nm)	Concentration of soluble collagen (mg/μl)
Control	0.750	5.95
Isoproterenol	1.20	9.30
Irbesartan (10 mg/kg)+ISO	0.950	7.12
Irbesartan (20 mg/kg)+ISO	0.780	5.79
Irbesartan (30 mg/kg)+ISO	0.579	5.76

Each value represents mean ± S.E.M. (n=5).

Table 3: Effect of Irbesartan on Left Ventricular Weight Index (LVWI) and Right Ventricular Weight Index (RVWI) in ISO-induced myocardial fibrosis

Groups	LVWI	RVWI
Control	0.00248 ± 0.009	0.00197 ± 0.0073
Isoproterenol	0.00312 ± 0.0089	0.00210 ± 0.0085
Irbesartan (10 mg/kg) + ISO	0.00291 ± 0.0085*	0.00208 ± 0.0088*
Irbesartan (20 mg/kg) + ISO	0.00249 ± 0.0084*	0.00198 ± 0.0086*
Irbesartan (30 mg/kg) + ISO	0.00247 ± 0.0097*	0.00196 ± 0.0088*

Each value represents mean ± S.E.M. (n=5).

*p<0.05 vs. Group II (One way ANOVA followed by Dunnett's test).

finally on the renin angiotensin system in cardiovascular pathology. Drugs that block the renin-angiotensin system ultimately inhibit angiotensin II. Recent study has focused on the inhibitory activity of the angiotensin II on the AT₁ receptors. Our results showed that administration of ISO resulted in to an increase of tissue soluble collagen level, LVWI and RVWI, oxidative indices a common of the main signaling pathways of the ROS mediated myocardial cell death and alteration in renin angiotensin system. At 15 days of treatments of ISO, the protein collagen, reached its maximal levels.^[16,17]

The results of the present study demonstrated that the levels of LPO, GSH, CAT and SOD were altered by ISO. The level of LPO was significantly increased after ISO treatment indicating initiation of lipid peroxidation, whereas the levels of defensive antioxidant enzymes were significantly reduced. Tissue soluble collagen level showed an alteration during treatment scheduled, ISO treated group showed an increase in soluble collagen level. Irbesartan treated group showed significantly decrease in the level of soluble collagen. These results suggest that the increase in the oxidative stress and soluble collagen level could be linked to ER stress, since at this timing cardiac fibrosis has not yet been fully developed.

Supporting this, LVWI and RVWI were calculated after the entire scheduled of treatment. Based on literature, TGF-β expression is most likely to be protective against cardiac damage, as its cytoprotective effect has been described *via* an interaction with the redox-balancing protein SOD and CAT, thus preventing post-ischemic insults in the heart and diminishing inflammation and apoptosis.^[18,19]

On the other hand, the levels of tissue soluble collagen increased late with respect to increase in collagen synthesis. Irbesartan significantly reduced the level of soluble collagen synthesis.^[20]

Table 5: Effect of Irbesartan on oxidative stress indices

Groups	SOD (% inhibition of reduction of NBT)	CAT (μ mole of H_2O_2 decomposed/ mg protein/min)	GSH (μ mole of GSH/mg protein)	LPO (nmole of MDA/mg protein)
Control	82.58 \pm 1.0	10.88 \pm 0.4	9.18 \pm 0.03	16.4 \pm 0.062
Isoproterenol	46.52 \pm 0.9	6.52 \pm 0.03	8.94 \pm 0.05	24.2 \pm 0.023
Irbesartan (10 mg/kg) + ISO	55.12 \pm 0.9*	7.762 \pm 0.03*	8.93 \pm 0.07*	23.2 \pm 0.047*
Irbesartan (20 mg/kg) + ISO	58.04 \pm 0.7*	7.622 \pm 0.01*	9.25 \pm 0.023*	22.6 \pm 0.067*
Irbesartan (30 mg/kg) + ISO	63.02 \pm 0.5*	8.316 \pm 0.02*	10.59 \pm 0.076*	21.1 \pm 0.031*

Each value represents mean \pm S.E.M. (n=5).

*p<0.01 vs. Group II (One way ANOVA followed by Dunnett's test).

Irbesartan prevented the increase in ROS generation and alteration of autorthymicity that was responsible for cell death and apoptotic activation. This could indicate that in the presence of Irbesartan leads to a lower degree of ER stress, as evidenced by no significant elevation in ST segment on ECG recordings. Irbesartan is an angiotensin receptor inhibitor and antioxidant, which showed protective effects against fibrotic disorders, cardiac fibrosis and remodeling and recent reports have shown protective effects of Irbesartan, by suppressing oxidative stress by attenuating endoplasmic reticulum (ER) stress.^[21]

Isoproterenol treated group of animals showed an increase in level of hydroxyproline which correlated to the level of collagen while decreased in level of soluble collagen in Irbesartan treated group of animals indicating inhibition of myocardial cell death. Some studies have reported an association between high ER stress and cardiotoxicity which leads to the cell damage. During chronic inflammatory damage in the hearts of transgenic mice ER stress is activated which over express MCP-1 protein levels leading to heart failure.^[22] In contrast, other research study reported that ER stress may protect the heart during myocardial damage, and even promote myocardial hypertrophic conditions.^[23,24] However, our results agree with the first condition showing that the myocardial damage caused by the administration of ISO as MF inducer, and thus the development of cardiac fibrosis is strongly associated with ER stress.

The dose of 10 to 30 mg/kg/day of irbesartan were used during study period which lies within the normal range of use of this drug. Additionally, irbesartan has been described to have little adverse effects, and to be effective against several diseases conditions, which is reliable with the cardioprotective role that we observed in the study after ISO induced cardiac fibrosis.^[4] Finally, Irbesartan could be

a protective factor against cell death by necrosis/apoptosis which induced due to ISO administration, by decreasing oxidative stress, the decrease in level of collagen deposition. Overall, results showed that cardiac fibrosis induced by ISO is mediated by ER stress activation. Therefore, Irbesartan prevents the cardiac fibrosis due to ER stress conditions, collagen secretion and cardiomyocyte loss.

Thus, irbesartan possesses anti-fibrotic activity and showed the protection against myocardial fibrosis. This protection is due to regulation of oxidative stress and soluble collagen level. Irbesartan might attenuate the endoplasmic reticulum stress and thus inhibit myocardial fibrosis *via* inhibition of oxidative stress. The regulation of antioxidant defensive enzymes may be involved in anti-fibrotic effect of irbesartan.

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