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Research Article
Bosentan, an Endothelin Receptor-1 Antagonist, Exerts Cardioprotective Effects During Myocardial Ischemia-Reperfusion Injury in Experimental Rats

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Abstract

Background and Objective: Myocardial infarction is caused by persistent ischemia with or without reperfusion. Bosentan, an endothelin receptor-1 antagonist, is primarily used to treat pulmonary arterial hypertension. However, its effect on myocardial ischemia-reperfusion injury (MIRI) has yet to be evaluated. Thus, the present investigation aimed to investigate the underlying cardioprotective mechanism of bosentan against MIRI in experimental rats. **Materials and Methods:** Sprague-Dawley male rats (200-220 g) were administered either vehicle, diltiazem (10 mg/kg), or bosentan (25, 50 and 100 mg/kg) for 14 days, followed by induction of MIRI by partial ligation of the left anterior descending artery subsequently with reperfusion injury. One way ANOVA was used to evaluate various biochemical, molecular and histological parameters. **Results:** Bosentan (50 and 100 mg/kg) significantly ($p<0.05$) attenuated ischemia-reperfusion injury (IRI)-induced cardiac damage evidenced by improvements in electrocardiographic, hemodynamic and left ventricular function tests. Elevated serum CK-MB, LDH, AST, ALT and ALP levels were effectively decreased ($p<0.05$) by bosentan treatment. It also effectively reduced ($p<0.05$) cardiac ANP, BNP, cTn-I and significantly increased ($p<0.05$) cardiac ATPase enzymes (Na^+K^+ATPase and Ca^{2+}ATPase) and HO-1 levels. Western blot analysis revealed that bosentan therapy inhibited IRI-induced up-regulated CHOP and GRP78-protein expressions. Bosentan improved the IRI-induced histological aberrations in cardiac tissue. **Conclusion:** The findings of the present investigation suggest that bosentan exerts its cardioprotective effects by inhibiting the CHOP and GRP78 expressions.

Key words: Apoptosis, bosentan, CHOP, cTn-I, GRP78, myocardial ischemia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Acute myocardial infarction (AMI) is a cardiovascular disorder that results from the blockage of blood flow to cardiac tissue, which causes morbidity and mortality worldwide[1]. Studies reported that early reperfusion of the ischemic myocardial tissue attenuates myocardial damage and decreases infarct size[2]. However, reperfusion can also injure cardiomyocytes, known as myocardial ischemia-reperfusion injury (MIRI)[3,4]. Several risk factors contribute to the development of cardiovascular disorders, including unhealthy diet (such as high intake of saturated and trans fats, cholesterol and sodium), lack of regular exercise, smoking and tobacco consumption, obesity, family history of heart disease, etc.[5,6]. According to the World Health Organization (WHO), cardiovascular diseases, including AMI, are the leading cause of death globally[7]. Each year, millions of people experience myocardial infarctions, contributing significantly to the burden of non-communicable diseases[7,8]. A recent study reported the global prevalence of myocardial infarctions as 3.8% in individuals with age more than 60 years[9]. In China, the age-standardized mortality rate for Ischemic heart disease was 109.7 per 100,000 individuals[5]. The average healthcare cost for cardiovascular disease in the Chinese population was 41,282 CNY (in 2023)[10]. These higher healthcare expenses were associated with older age cohorts, a higher percentage of males, longer hospital stays, higher rates of comorbidities, more complex medical procedures and emergency admissions[10].

The pathogenesis of MIRI includes the release of vasoconstrictor substances, elevated inflammatory release including numerous cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukins (ILs) Reperfusion, leading to exaggerated localized inflammatory responses and further cardiac injury. An array of investigations has demonstrated that attenuating excessive inflammation can minimize the extent of infarction and alleviate cardiac dysfunction caused by IRI[3,11,12]. Furthermore, the combination of inflammation, oxidative stress, calcium overload and mitochondrial dysfunction contributes to cell death through apoptosis and necrosis[4,13,14]. Thus, recent studies demonstrated that strategies that suppressed the elevated oxidative stress and inflammatory response might be beneficial to managing myocardium damage and attenuation of MIRI[15,16]. Additionally, regular exercise, healthy diet, stress management and stopping tobacco consumption can support prevention of MIRI events. However, lifestyle modifications are often a lifelong process and adherence to prescribed medications is crucial for long-term cardiovascular health.

Current treatment approaches for MIRI include antiplatelet agents, anticoagulants, beta-blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II receptor blockers and cholesterol-lowering agents. However, the effectiveness of these specific interventions may vary based on the individual patient's conditions[17]. Thus, a better treatment option involving a multifaceted approach to minimize tissue damage and optimize recovery during MIRI is needed. Many animal models are available for evaluating various emerging treatments for MIRI. However, the rat MIRI model by transient ligation of the left anterior descending (LAD) is the most widely used animal model to assess the potential of various emerging therapies on left ventricular function[4,15,16].

Bosentan, an Endothelin Receptor-1 (ET-1) antagonist, is primarily used in treating pulmonary arterial hypertension[18,19]. Bosentan ameliorated myocardial infarction-associated mortality in experimental rats[20]. Furthermore, it showed an antidepressant effect via inhibiting circulating IL-6 levels in mice[21]. Researchers also documented its nephroprotective and retinoprotective effect via inhibiting Transforming Growth Factor Beta (TGF-β) in diabetic rats[22]. However, its cardioprotective potential in IRI-induced myocardial damage is yet to be investigated. Thus, the present study aimed to evaluate the potential of bosentan in IR-induced myocardial toxicity in experimental rats.

MATERIALS AND METHODS

Drugs and chemicals: Bosentan was procured from Medicines Private Limited, Mumbai. In addition, the primary antibodies of GRP78 (glucose-regulated protein 78), CHOP (C/EBP homologous protein) and GAPDH (Glyceraldehyde 3-Phosphate Dehydrogenase) were purchased from Abcam. The Rat-specific ANP (Atrial natriuretic peptide), BNP (B-type natriuretic peptide), cTnI (cardiac troponin I), HO-1 (Heme oxygenase-1) and ELISA kits (Thermo Scientific, USA), were purchased from respective vendors. All chemicals used were of analytical grade.

Animals: Adult male Sprague-Dawley (SD) rats (n = 105, 200-220 g) were obtained from the Animal House Facility of Xi'an Daxing Hospital, China. The study was conducted in Department of Pharmacology of Xi'an Daxing Hospital from November, 2023 till January, 2024 between 09:00 and 17:00 hrs under animal house condition viz., temperature (24±1°C), relative humidity (45-55%), dark/light cycle (12:12 hrs) with free access to food (standard pellet chow) and *ad libitum* water.

Ethical consideration: The experimental protocol was approved by Animal Ethical Committee of Xi'an Daxing Hospital (ethical approval no. 559974002). All the

experimental protocols involved in the current investigation followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines.

Experimental design and treatment: The SD rats was used to induce ischemia-reperfusion injury around left anterior descending coronary artery using 5-0 silk suture for 30 min and reperfusion for 60 min[3]. Post recovery animals were randomly divided into various groups (n = 15, each) such as IRI control, diltiazem and bosentan and treated orally with dimethyl sulfoxide at a dose of 10 mg/kg, diltiazem at a dose of 10 mg/kg, bosentan at a various dosage of 25, 50 and 100 mg/kg for 14 days. Sham group underwent surgery however did not receive ligation of coronary artery and they were treated with dimethyl sulfoxide at a dose of 10 mg/kg for 14 days. Similarly another group of rats did not underwent surgery however treated with bosentan at a dose of 100 mg/kg for 14 days referred as "*per se*".

At the end of the experiment, Electrocardiogram (ECG) and Left Ventricular End-Diastolic Pressure (LVEDP) was determined in anesthetized rats using PowerLab Data Acquisition and Analysis System (AD Instruments).

Then, blood samples from each rat were collected into separate vials by a retro-orbital puncture method to determine serum parameters including Lactate Dehydrogenase (LDH), Creatine Kinase-MB (CK-MB), Alanine Aminotransferase (AST), Alanine Transaminase (ALT) and Alkaline Phosphatase (ALP) were measured by (UV/VIS spectrophotometer, Jasco V-530) using reagent kits according to the procedure provided by the manufacturer (Accurex Biomedical Pvt. Ltd.)[23,24]. Then, animals were sacrificed by cervical dislocation, the heart was rapidly removed and stored at 80°C for biochemical (n = 6) and qualitative RT-PCR analysis (n = 6). Tissue homogenates were prepared with 0.1 M Tris-HCl buffer (pH 7.4) and a supernatant of homogenates was employed to estimate Na^+K^+ATPase and Ca^{2+}ATPase as described by Kandhare *et al.*[25].

Myocardial tissue homogenate (n = 6) levels of ANP, BNP, cTn-I and HO-1 were determined by using an Enzyme-Linked Immunosorbent Assay (ELISA) (Thermo Scientific, Pierce Biotech Int., USA) according to the manufacturer's instructions. Cardiac GRP78 (ab21685, 1/1000 dilution, 78 kDa) and CHOP (ab256889, 1/500 dilution, 19 kDa) were established using western blot assay kits (Thermo Fisher Scientific, Inc.) and GAPDH (ab128915, 1/10000 dilution, 36 kDa) served as the loading control[26]. Finally, the left ventricle of heart from three rats of each group was isolated and used for histopathological analysis using Hematoxylin and Eosin (H&E) stain according to a method described by Kandhare *et al.*[25].

Statistical analysis: The sample size was calculated based on the power analysis method considering 30% expected attrition using a formula:

Corrected sample size = Sample size/(1- [% attrition/100])

Data were expressed as Mean±Standard Error Means (SEM). Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, California, USA). Data were analyzed by One-way Analysis of Variance (ANOVA) and Tukey's multiple range t-test were applied for *post hoc* analysis. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of bosentan on body weight, relative and absolute heart weights in rats: There was a significant decrease ($p<0.05$) in the body weight of IRI control rats compared to sham. However, diltiazem treatment effectively inhibited ($p<0.05$) IRI-induced decreased body weight compared to the IRI control group. Treatment with bosentan (50 and 100 mg/kg) also showed a significant increase ($p<0.05$) in body weight as compared to the IRI control group. However, diltiazem treatment showed more effective inhibition ($p<0.05$) in IRI-induced decrease in body weight compared to bosentan treatment. There was no significant difference in body weight of the sham and *per se* treated group (Table 1).

Compared to sham rats, relative and absolute heart weights were markedly increased ($p<0.05$) in IRI control rats. Diltiazem significantly decreased ($p<0.05$) relative and absolute heart weights compared to IRI control rats. Bosentan (50 and 100 mg/kg) treatment also showed a significant decrease ($p<0.05$) in relative and absolute heart weights compared to the IRI control group. However, diltiazem treatment showed more marked inhibition ($p<0.05$) in IRI-induced increased relative and absolute heart weights as compared to bosentan treatment (Table 1).

Effect of bosentan on serum CK-MB, LDH, AST, ALT and ALP levels: A significant increase ($p<0.05$) in serum CK-MB, LDH, AST, ALT and ALP levels was observed in IRI control rats than sham rats. Administration of diltiazem markedly inhibited ($p<0.05$) elevated levels of these serum biomarkers than the IRI control group. Bosentan (50 and 100 mg/kg) treatment also showed significant attenuation of ($p<0.05$) IRI-induced elevated serum CK-MB, LDH, AST, ALT and ALP levels as compared to the IRI control group. However, diltiazem had a more significant reduction ($p<0.05$) in serum CK-MB, LDH, AST,

Table 1: Effect of bosentan on Ischemia-reperfusion injury (IRI)-induced alterations in relative and absolute heart weight, serum CK-MB, LDH, ALT, AST, ALP and cardiac ATPase enzymes in rats

Parameter	Sham	IRI control	IRI+Dil (10)	IRI+Bos (25)	IRI+Bos (50)	IRI+Bos (100)	Bos (100)
Body weight (gm)	227.50±2.14	219.30±2.60#	220.70±4.19*,$	222.50±2.55	224.00±4.26*,$	227.30±4.51*,$	222.30±3.04
Heart weight (gm)	0.47±0.05	0.99±0.11#	0.57±0.05*,$	0.94±0.10	0.71±0.08*,$	0.67±0.05*,$	0.51±0.05
Heart weight/body weight ($\times 10^{-3}$)	2.06±0.21	4.50±0.48#	2.58±0.23*,$	4.21±0.42	3.14±0.33*,$	2.96±0.26*,$	2.27±0.20
Serum CK-MB (IU/L)	987.10±103.10	1810.00±79.15#	1145.00±51.79*,$	1627.00±50.65	1371.00±78.85*,$	1147.00±117.60*,$	1129.00±80.22
Serum LDH (IU/L)	1111.00±35.28	2445.00±77.62#	1211.00±35.28*,$	2334.00±74.09	1811.00±35.28*,$	1411.00±35.28*,$	1161.00±35.28
AST (mg%)	121.40±8.58	311.10±8.73#	159.00±10.34*,$	268.00±9.79	210.90±12.22*,$	170.10±7.92*,$	146.90±7.70
ALT (mg%)	33.31±6.40	171.90±5.84#	50.72±4.95*,$	154.60±4.68	96.73±6.06*,$	60.54±6.66*,$	42.87±7.10
ALP (mg%)	91.58±7.43	274.10±9.48#	131.00±6.43*,$	247.90±7.74	217.50±9.35*,$	151.40±9.19*,$	118.90±11.54
Na^+K^+ATPase (µmoles/mg of protein)	5.85±0.15	3.32±0.18#	5.18±0.15*,$	3.79±0.15	4.38±0.16*,$	4.87±0.19*,$	5.52±0.13
Ca^{2+}ATPase (µmoles/mg of protein)	3.86±0.13	1.44±0.16#	3.46±0.15*,$	1.71±0.16	2.54±0.15	3.12±0.12	3.76±0.12

Data are expressed as Mean±SEM (n = 6) and analyzed by one-way ANOVA followed by Tukey's multiple range tests. *p<0.05 as compared to the IRI-control group, #p<0.05 as compared to the sham, $p<0.05 as compared to one another. IRI: Ischemia-reperfusion Injury control rats, Dil (10): Diltiazem (10 mg/kg, p.o.) treated rats, Bos (25): Bosentan (25 mg/kg, p.o.), Bos (50): Bosentan (50 mg/kg, p.o.) and Bos (100): Bosentan (100 mg/kg, p.o.) treated rats. AST: Alanine Aminotransferase, ALT: Alanine Transaminase, ALP: Alkaline Phosphatase, CK-MB: Creatine Kinase-MB and LDH: Lactate Dehydrogenase

Table 2: Effect of bosentan on ischemia-reperfusion injury (IRI)-induced alterations electrocardiographic, hemodynamic and left ventricular function tests changes in rats

Parameter	Sham	IRI control	IRI+Dil (10)	IRI+Bos (25)	IRI+Bos (50)	IRI+Bos (100)	Bos (100)
Heart Rate (BPM)	346.60±11.04	255.40±6.79#	319.30±12.52*,$	263.80±4.03	294.00±5.81*,$	332.60±4.78*,$	336.80±6.99
QRS interval (ms)	13.09±0.52	31.09±0.52#	17.09±0.52*,$	28.09±0.52	23.09±0.52*,$	20.09±0.52*,$	13.09±0.52
QT Interval (ms)	42.00±0.52	78.00±0.52#	59.50±0.52*,$	75.33±0.52	69.33±0.52*,$	52.83±0.52*,$	52.83±0.52
QTc Interval (ms)	132.70±7.10	173.00±2.71#	141.70±5.13*,$	165.00±6.14	145.00±6.11*,$	146.00±1.57*,$	134.70±1.84
RR interval (ms)	139.80±3.62	201.30±3.98#	164.00±4.35*,$	191.70±4.00	176.50±3.33*,$	155.30±4.24*,$	146.00±4.38
SBP (mmHg)	107.00±4.00	159.70±3.17#	117.50±4.52*,$	154.30±2.64	132.70±3.40*,$	125.70±4.10*,$	119.30±3.84
DBP (mmHg)	83.50±3.34	121.80±4.06#	97.67±4.69*,$	112.70±2.60	103.70±5.28*,$	106.70±3.80*,$	92.00±4.37
MABP (mmHg)	69.27±2.18	108.80±2.52#	74.97±0.98*,$	109.90±2.69	95.47±2.25*,$	83.97±4.47*,$	78.87±2.34
Pulse pressure (mmHg)	30.67±0.76	36.50±0.56#	33.83±0.79*,$	35.83±1.08	34.67±0.99*,$	33.67±0.92*,$	31.83±0.83
LVEDP (mmHg)	5.33±0.95	19.50±3.72#	6.83±0.65*,$	17.33±3.10	14.17±2.04*,$	10.50±1.06*,$	6.67±0.80
LVESP (mmHg)	84.51±0.42	114.7±5.93#	89.31±3.81*,$	110.3±1.51	106.9±2.40*,$	98.33±3.88*,$	87.93±2.56
Systolic duration (ms)	47.02±4.53	73.56±2.06#	46.69±2.54*,$	72.42±0.94	61.67±3.23*,$	56.54±3.86*,$	54.52±2.22
Diastolic duration (ms)	97.13±4.40	145.10±4.40#	102.10±4.40*,$	142.10±4.40	127.10±4.40*,$	107.10±4.40*,$	127.10±4.40
$Max_{dp/dt}$	4071.00±154.10	2304.00±296.50#	3877.00±115.00*,$	2642.00±215.20	3116.00±219.70*,$	3452.00±128.70*,$	3686.00±110.10
$Min_{dp/dt}$	-2555.00±102.50	-1911.00±211.30#	-2527.00±160.70*,$	-1748.00±131.30	-2072.00±130.90*,$	-2279.00±126.30*,$	-2387.00±191.90
Contractility index	54.17±2.44	27.33±1.73#	48.83±3.20*,$	33.00±1.61	36.00±1.46*,$	45.33±2.75*,$	50.83±3.26
Tau (ms)	5.00±0.63	10.83±0.87#	4.83±0.40*,$	9.67±0.76	8.17±0.83*,$	6.33±0.61*,$	5.67±0.84
Pressure time index	18.67±1.17	25.83±0.48#	19.67±1.02*,$	24.33±0.61	22.17±0.54*,$	21.17±0.79*,$	19.33±0.76

Data are expressed as Mean±SEM (n = 6) and analyzed by one-way ANOVA followed by Tukey's multiple range tests. *p<0.05 as compared to the IRI-control group, #p<0.05 as compared to the sham, $p<0.05 as compared to one another. IRI: Ischemia-reperfusion Injury control rats, Dil (10): Diltiazem (10 mg/kg, p.o.) treated rats, Bos (25): Bosentan (25 mg/kg, p.o.), Bos (50): Bosentan (50 mg/kg, p.o.) and Bos (100): Bosentan (100 mg/kg, p.o.) treated rats. SBP: Systolic blood pressure, DBP: Diastolic blood pressure and LVEDP: Left Ventricular End-Diastolic Pressure

ALT and ALP levels compared to bosentan treatment. Compared with sham rats, no significant differences were observed in the *per se* treated group in serum CK-MB, LDH, AST, ALT and ALP levels (Table 1).

Effect of bosentan on cardiac ATPase enzymes: There was a significant increase (p<0.05) in cardiac ATPase enzymes (Na^+K^+ATPase and Ca^{2+}ATPase) in IRI control rats than sham rats. Administration of diltiazem effectively inhibited (p<0.05) IRI-induced elevated cardiac Na^+K^+ATPase and Ca^{2+}ATPase levels compared to the IRI control group. Treatment with bosentan (50 and 100 mg/kg) significantly increased (p<0.05) cardiac ATPase enzymes (Na^+K^+ATPase and Ca^{2+}ATPase) as compared to the IRI control group. Diltiazem showed more effective inhibition (p<0.05) in IRI-induced elevated cardiac Na^+K^+ATPase and Ca^{2+}ATPase levels compared to bosentan treatment. There was no significant difference in cardiac ATPase enzymes in the sham and *per se* treated group (Table 1).

Effect of bosentan on electrocardiographic, hemodynamic and left ventricular functions in rats: Electrocardiographic, hemodynamic and left ventricular functions were effectively altered (p<0.05) in IRI control rats compared to sham rats. Administration of diltiazem markedly attenuated (p<0.05) IRI-induced hemodynamic, left ventricular functions and electrocardiographic changes than the IRI control group. Bosentan (50 and 100 mg/kg) administration also effectively inhibited (p<0.05) hemodynamic, left ventricular functions and electrocardiographic changes as compared to the IRI control group. The electrocardiographic, hemodynamic and left ventricular functions did not differ significantly in the sham and *per se* treated group (Table 2).

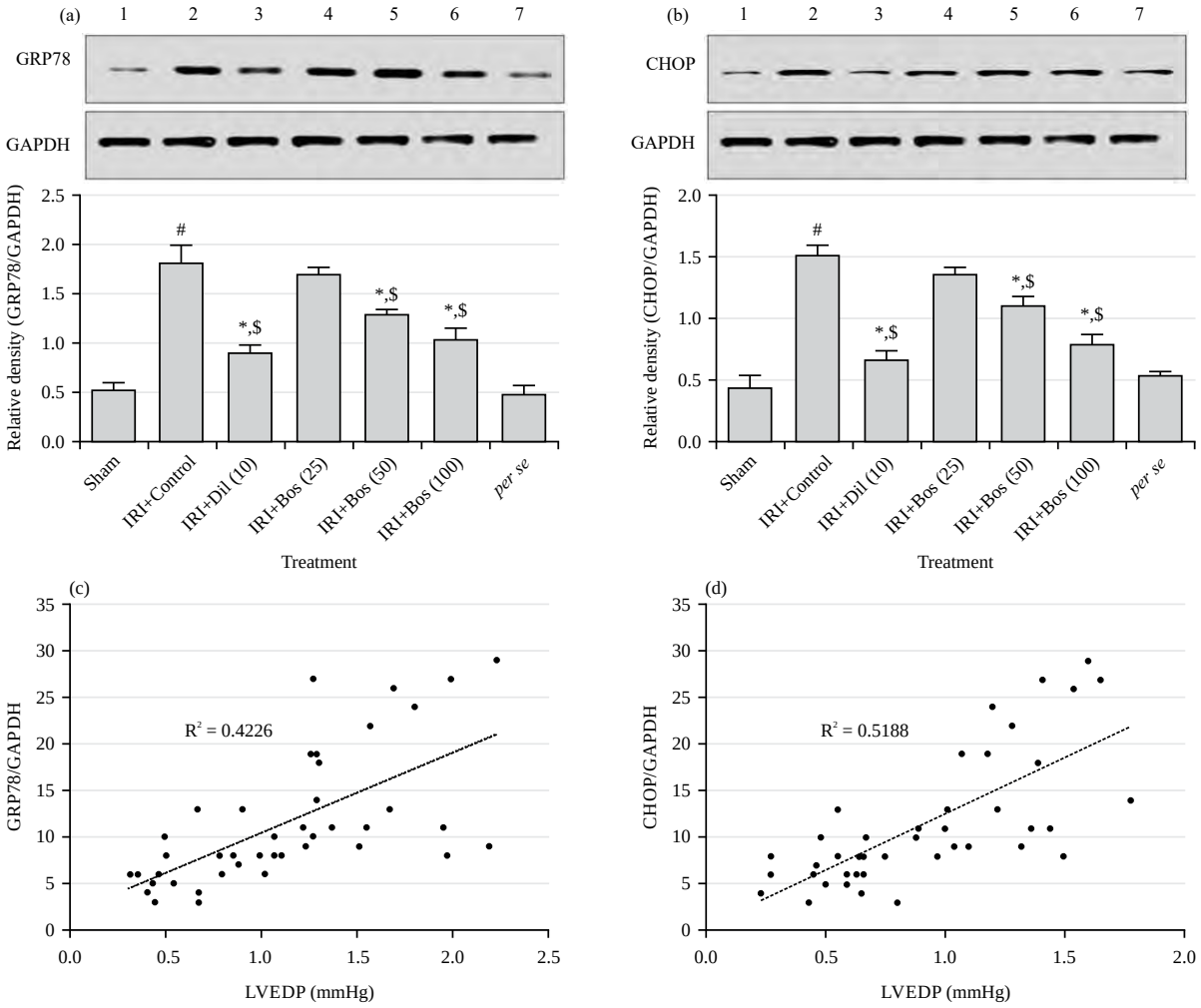

Fig. 1(a-d): Effect of bosentan on Ischemia-Reperfusion Injury (IRI)-induced alterations in cardiac (a) GRP78, (b) CHOP protein expressions in rats. A simple regression of cardiac (c) GRP78 and (d) CHOP with LVEDP. Correlation coefficients were determined using a two-sided Fisher test

Data are expressed as Mean±SEM (n = 6). *p<0.05 as compared to the IRI-control group, #p<0.05 as compared to the sham, $p<0.05 as compared to one another. IRI: Ischemia-reperfusion Injury control rats, Dil (10): Diltiazem (10 mg/kg, p.o.) treated rats, Bos (25): Bosentan (25 mg/kg, p.o.), Bos (50): Bosentan (50 mg/kg, p.o.) and Bos (100): Bosentan (100 mg/kg, p.o.) treated rats. GRP: Glucose-regulated protein and CHOP: C/-EBP homologous protein

Effect of bosentan on the cardiac ANP, BNP, cTn-I and HO-1 levels in rats: A significant increase (p<0.05) in cardiac ANP, BNP, cTn-I and a considerable decrease (p<0.05) in cardiac HO-1 levels were noted in IRI control rats when compared with sham rats. Treatment with diltiazem efficiently decreased (p<0.05) cardiac ANP, BNP and cTn-I and showed a significant increase (p<0.05) in cardiac HO-1 levels than the IRI control group. Bosentan (50 and 100 mg/kg) treatment effectively attenuated (p<0.05) IRI-induced alterations in cardiac ANP, BNP, cTn-I and HO-1 levels as compared to the IRI control group. However, diltiazem showed more effective inhibition (p<0.05) in IRI-induced alterations in cardiac ANP, BNP, cTn-I and HO-1 levels than bosentan treatment. There was no significant difference in cardiac ANP, BNP, cTn-I and HO-1 levels in the sham and *per se* treated group (Table 3).

Effect of bosentan on the cardiac GRP78 and CHOP protein levels in rats: Cardiac GRP78 and CHOP protein levels were markedly up-regulated (p<0.05) in IRI control rats compared to sham rats. Administration of diltiazem effectively inhibited (p<0.05) IRI-induced up-regulation in cardiac GRP78 and CHOP protein levels compared to the IRI control group. Treatment with bosentan (50 and 100 mg/kg) also significantly down-regulated (p<0.05) cardiac GRP78 and CHOP protein levels as compared to the IRI control group. Diltiazem treatment showed more effective inhibition (p<0.05) in

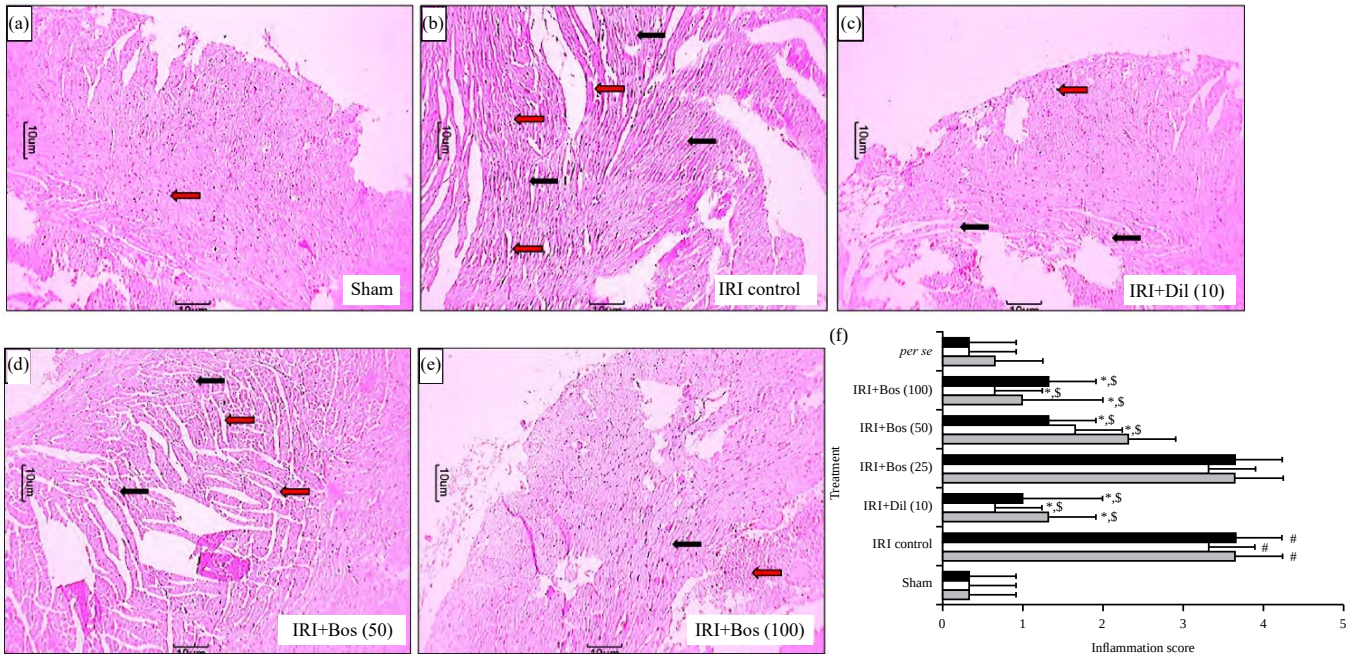

Fig. 2(a-f): Effect of bosentan on ischemia-reperfusion injury (IRI)-induced alterations in cardiac histopathology in rats Photomicrograph of sections of the heart from, (a) Sham, (b) IRI control, (c) IRI+diltiazem (10 mg/kg), (d) IRI+bosentan (50 mg/kg), (e) IRI+bosentan (100 mg /kg) and (f) Treated rats. Images at 40X and the quantitative representation of histological score

Data are expressed as Mean±SEM (n = 3) and One-way ANOVA followed by the Kruskal-Wallis test was applied for *post hoc* analysis, *p<0.05 as compared to the IRI-control group, #p<0.05 as compared to the sham, $p<0.05 as compared to one another. Myocardial degeneration (black arrow) and interstitial inflammation (red arrow)

Table 3: Effect of bosentan on ischemia-reperfusion injury (IRI)-induced alterations in cardiac cardiac troponin I, atrial natriuretic peptide, brain natriuretic peptide and heme oxygenase-1 levels in rats

Parameters	Sham	IRI control	IRI+Dil (10)	IRI+Bos (25)	IRI+Bos (50)	IRI+Bos (100)	Bos (100)
Cardiac troponin I (pg/mg)	1.19±0.09	4.63±0.11#	1.72±0.11*,$	4.51±0.10	3.59±0.08*,$	2.46±0.08*,$	1.40±0.08
Atrial natriuretic peptide (pg/mg)	29.10±2.33	71.30±3.29#	41.97±1.65*,$	64.12±2.95	58.75±1.99*,$	43.81±2.26*,$	29.73±1.05
Brain natriuretic peptide (pg/mg)	84.71±4.45	117.40±2.87#	92.35±4.72*,$	115.90±3.99	103.30±3.44*,$	100.30±3.14*,$	89.89±3.85
Heme oxygenase-1 (pg/mg)	4.88±0.15	1.79±0.33#	4.49±0.10*,$	1.39±0.36	2.43±0.33*,$	2.96±0.24*,$	4.30±0.36

Data are expressed as Mean±SEM (n = 6) and analyzed by one-way ANOVA followed by Tukey's multiple range tests. *p<0.05 as compared to the IRI-control group, #p<0.05 as compared to the sham, $p<0.05 as compared to one another. IRI: Ischemia-reperfusion Injury control rats, Dil (10): Diltiazem (10 mg/kg, p.o.) treated rats, Bos (25): Bosentan (25 mg/kg, p.o.), Bos (50): Bosentan (50 mg/kg, p.o.) and Bos (100): Bosentan (100 mg/kg, p.o.) treated rats

IRI-induced up-regulated cardiac GRP78 and CHOP protein levels as compared to bosentan treatment (Fig. 1a and b).

Linear correlation analysis suggested that IRI-induced variations in GRP78 and CHOP were numerically correlated with LVEDP (r^2 = 0.4226 and 0.5188) (Fig. 1c and d).

Effect of bosentan on IRI-induced cardiac histopathological alteration in rats: Figure 2a depicted the normal architecture of myocardial tissue without evidence of bleeding, cell edema, or necrosis in sham control rats. However, IRI caused significant damage to the myocardial tissue, reflected by a significant increase (p<0.05) in cardiac inflammatory infiltration, necrosis, myocardial degeneration and hemorrhage in IRI control when compared to sham control (Fig. 2b). Data from Fig. 2c depicted that diltiazem treatment effectively attenuated (p<0.05) IRI-induced myocardial tissue damage reflected by decreased cardiac inflammatory infiltration, necrosis, myocardial degeneration and hemorrhage than the IRI control group. Compared to the IRI control group, bosentan (50 and 100 mg/kg) treatment also showed a significant decrease (p<0.05) in cardiac inflammatory infiltration, necrosis, myocardial degeneration and hemorrhage as compared to the IRI control group (Fig. 2d-f).

DISCUSSION

Acute myocardial infarction, in particular, causes ischemia damage to the myocardium and thus, reperfusion therapy is considered the best treatment for protecting the myocardium when it is at risk[9,17]. However, in clinical practice, reperfusion can abnormally worsen cardiac damage, a condition known as myocardial ischemia-reperfusion injury (MIRI)[27]. In the present investigation, treatment with bosentan showed inhibition of myocardial damage and cardiac dysfunction following MIRI.

Chronic ischemic caused damage to myocardial tissue resulted in changes in ECG. In the present study, MIRI caused physiologic changes in myocardial tissue reflected by ECG alternations, however, administration of bosentan effectively attenuated these MIRI-induced ECG alterations. Treatment with bosentan in patients with pulmonary arterial hypertension showed amelioration in hemodynamic, left ventricular functions and electrocardiographic changes. Previous investigators well established the link between myocardial ischemia-reperfusion injury and left ventricular dysfunction[28]. The MIRI can cause significant alterations in left ventricular function and the extent of the injury to myocardial tissue may result in the development of left ventricular dysfunction[16]. A sudden reintroduction of oxygen during reperfusion leads to increased production of reactive oxygen species (ROS), causing oxidative stress and triggering an inflammatory response, contributing to tissue damage[29,30]. Additionally, disruption of calcium homeostasis during ischemia and reperfusion can lead to intracellular calcium overload, activating destructive enzymes[31]. The IRI can also impair the function of the microvasculature in the myocardium, further contributing to left ventricular dysfunction and myocardial infarction[4,15,32]. These modifications can be evaluated and confirmed with the testable findings, such as ECG changes and a rise in blood troponin levels. The ECG monitoring includes P wave, QRS complex and T wave representing electrical conduction by myocardiocytes, viz., depolarization of the atria and ventricular depolarization followed by repolarization[33-37].

In the present investigation, bosentan treatment significantly reduced IRI induced myocardial damage reflected by lowered levels of cardiac enzymes (ALT, AST and ALP) in serum followed by reduced histological abnormality induced by IRI. An increased levels of ALT, AST and ALP in blood depicted the cardiac dysfunction[38-40] and during IRI an altered permeability of myocardial tissue results in leakage of ALT, AST and ALP into blood stream denoted damage to myocardial tissue[16,41,42]. The histopathological changes further confirmed this notion, reflecting inflammatory infiltration, myocardial degeneration and myocardial necrosis. Furthermore, elevated cardiac tissue weights (relative and absolute) suggested damage to cardiac tissue during ischemic reperfusion[16,43]. Previous researcher also documented that bosentan attenuated elevated levels of the ALT, AST and ALP in serum and finding of present investigation is in line of results of Abbas *et al.*[44].

In the present study, the ANP, BNP, cTn-I and HO-1 levels were elevated in cardiac tissue, which aligns with previous Liu *et al.*[11]. However, administration of bosentan showed a marked reduction in the elevated levels of these markers. Recent studies also reported the cardioprotective effect of bosentan via amelioration of ANP, BNP and cTn-I in 5-fluorouracil intoxicated and pulmonary hypertensive rats[31]. The ANP and BNP are circulating hormones used as diagnostic and prognostic indicators in cardiovascular disease[45]. During ischemic conditions, the ratio of B-Cell Lymphoma protein 2 (Bcl-2) to Bcl-2 associated X (Bax) also varies and is the main regulating indicator for determining the survival rate of the cardiomyocytes[46-48]. Heme Oxygenase-1 (HO-1) is another important enzyme that degrades heme and produces carbon monoxide and biliverdin to stimulate iron release, thereby exerting cardioprotective effects by mitigating myocardial tissue injury through regulating cellular signal transduction, inflammatory responses and mitochondrial functions[49]. In addition, cardiac troponin (cTn-I), expressed in skeletal and cardiac myocytes, has long been employed in diagnosing AMI. The cTn-I plays a crucial role in cardiac muscle contraction and its elevated levels indicate myocardial damage due to conditions such as cardiac arrest or MIRI[4]. A recent study found cardiac troponin I autoantibody promotes myocardial dysfunction through PTEN signaling activation[50]. Thus, the relationship between MIRI and cTn-I is crucial for diagnosing and managing acute cardiac events[51].

Present study findings showed that bosentan enhanced the enzymatic activity of Na^+/K^+-ATPase in the reperfusion phase and the elevation of Ca^{2+}ATPase activity, leading to the myocardium's cardioprotective function. The Ca^{2+} and Na^+ flow into the myocardium during myocardial ischemia and Ca^{2+} overload is considered a critical factor in the onset of ischemic myocardial injury[52]. A membrane protein called sodium/potassium-ATPase (Na+/K+-ATPase) actively transports Na^+ and K^+ ions across the plasma membranes of most higher eukaryotes[53]. In clinical and experimental studies, it has been shown that a reduction in cardiac Na^+/K^+-ATPase

activity contributes to impairments in ventricular contractility[54]. On the other hand, the cardiac isoform of sarco/endoplasmic reticulum Ca^{2+}ATPase (SERCA2a) is crucial in regulating the excitation/contraction coupling. The SERCA2a expression is markedly decreased in experimental and clinical heart failure, resulting in aberrant Ca^{2+} handling and contractility[55]. According to a previous study, bosentan significantly prevented acute lung injury by increasing the activity of Na^+/K^+-ATPase[56]. These outcomes were consistent with a recent study, which supports the cardioprotective properties of bosentan in ameliorating heart ischemia damage.

Findings of the present study suggested that bosentan provides cardioprotective benefits by down-regulating expression of GRP78 and CHOP, thus preventing cardiac myocytes from abrupt death induced by an ischemia episode, hence preserving heart cell viability. The effect of bosentan in ameliorating the expression of GRP78 and CHOP in the rat cardiac tissues was reported for the first time in the present study. Myocardial necrosis during MIRI is primarily caused by cell apoptosis brought on by endoplasmic reticulum (ER) stress. The primary mechanism by which apoptosis is initiated under ER stress is through the C/EBP (CCAAT-enhancer-binding proteins) homologous protein (CHOP) pathway[57,58]. The CHOP is a transcription factor particularly associated with cellular stress responses, including those triggered by myocardial ischemia-reperfusion injury[41,59]. Furthermore, CHOP is a downstream target of the unfolded protein response, a cellular pathway activated during ER stress to restore homeostasis[59]. The CHOP has also been associated with oxidative stress and implicated in promoting apoptosis, which is another key feature of IRI[60]. Another significant protein in the CHOP pathway is Glucose-Regulated Protein 78 (GRP78)[61]. The GRP78 is a key molecular chaperone located in the endoplasmic reticulum (ER) of cells[62]. It plays a crucial role in the various cellular processes, including those involved in the response to stress and injury, regulation of protein folding and the unfolded protein response. During MIRI, GRP78 is involved in the cellular response to ER stress[63]. Targeting GRP78 and the UPR pathways is a potential therapeutic strategy for conditions affecting ER stress, including MIRI. Thus, various researchers examined CHOP and GPR78 protein expression pathways to identify the molecular mechanism responsible for the cardioprotective activities of different moieties[62,63].

Bosentan has been widely used in clinical settings for the management of patients with severe chronic heart failure[29], pulmonary arterial hypertension[18,64], pulmonary arterial hypertension in adults with congenital heart disease[65] and mildly symptomatic pulmonary arterial hypertension[66]. Thus, bosentan can be an important therapeutic for managing myocardial ischemia-reperfusion injury.

CONCLUSION

In the present investigation, bosentan treatment attenuated myocardial ischemia-reperfusion injury (MIRI)-induced myocardial damage in experimental rats. This protective efficacy of bosentan against IRI-induced cardiac damage was exerted via inhibiting the GRP78/CHOP pathway in the cardiomyocytes. To the best of our knowledge, this is the first study reporting a correlation between bosentan and attenuation of MIRI-induced myocardial damage. Based on these findings, bosentan might represent a new cardioprotective drug during IRI. However, further randomised clinical studies are needed to confirm the potential of bosentan against IRI.

SIGNIFICANCE STATEMENT

The current work has evaluated the effect of bosentan against left anterior descending ligation-induced myocardial ischemia-reperfusion injury in experimental rats. This study evaluated an array of biochemical and molecular evidence that showed that bosentan exerted a cardioprotective effect against myocardial IRI probably via a mechanism involving (a) Inhibition of CK-MB, LDH, AST and cTn-I, (b) Downregulating CHOP and GRP78 protein levels in the cardiomyocytes. This study will deliver valuable information to researchers and physicians to find an alternative healthcare product for management of ischemia-reperfusion injury during various cardiac surgeries.

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