Diazepam ameliorated myocardial Ischemia-Reperfusion Injury via inhibition of C-C chemokine receptor type 2/Tumor necrosis factor-alpha/Interleukins and Bcl-2-associated X protein/Caspase-3 pathways in experimental rats

Tingting Jiang, 1) Xinghua Ma, 2)* Huimin Chen, 1) Hongfeng Jia, 1) Ying Xiong 1)

1) Department of Anesthesiology, 3201 hospital, Hanzhong, 723000, China
2) Department of Equipment, 3201 hospital, Hanzhong, 723000, China

Corresponding author: Dr. Xinghua Ma

Department of Equipment,
3201 hospital, Hanzhong, 723000,
China

Email ID: mxh3201@sina.com

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ABSTRACT

Myocardial ischemia-reperfusion injury (IRI) is one of the most leading concerns for public health globally. Diazepam, a local anesthetic, has been reported for its cardioprotective potential. The present investigation aimed to evaluate the possible mechanism of action of diazepam against left anterior descending ligation-induced myocardial IRI in experimental rats. IRI was induced in healthy male rats by ligating coronary artery for 30 min and then reperfused for 60 min. The animals were pre-treated with either vehicle or diltiazem (10 mg/kg) or diazepam (1, 2.5, and 5 mg/kg) for 14 days. Compared to the IRI group, diazepam (2.5 and 5 mg/kg) markedly ($p < 0.05$) attenuated IRI-induced alterations in cardiac function and oxido-nitrosative stress. In addition, diazepam prominently ($p < 0.05$) improved cardiac Na$^+$K$^+$ATPase, Ca$^{2+}$ATPase levels and HIF-1$\alpha$ (Hypoxia-Inducible Factor-1 alpha) mRNA expression. It also significantly ($p < 0.05$) down-regulated cardiac mRNA expressions of cTn-I (cardiac troponin I), CCR2 (C-C chemokine receptor type 2), TNF-$\alpha$ (Tumor necrosis factor-alpha), IL (Interleukins)-1$\beta$, and IL-6. In western blot analysis, IRI-induced myocardial apoptosis was reduced by diazepam treatment reflected by a marked ($p < 0.05$) decreased in Bax (Bcl-2-associated X protein) and Caspase-3 protein expression. Diazepam also efficiently ($p < 0.05$) improved IRI-induced histological aberration in cardiac tissue. In conclusion, diazepam exerts cardioprotective effect by inhibiting inflammatory release (CCR2, TNF-$\alpha$, and ILs), oxido-nitrosative stress, and apoptosis (Bax and Caspase-3) pathway during myocardial IRI in experimental rats.

Keywords: apoptosis, C-C chemokine receptor type 2, cardiac troponin I, diazepam, ischemia-reperfusion injury
INTRODUCTION

Acute myocardial infarction (AMI) is one of the leading causes of morbidity and mortality, affecting approximately 17.6 million people worldwide [5]. According to the Chinese disease report (2020), the rate of cardiovascular diseases associated mortality was approximately 45%, and it has been expected that almost 23 million people will suffer from AMI by 2030 [62]. AMI accounts for a heavy economic burden, and the report suggested that the annual healthcare costs for its treatment are $7790, which is expected to grow continuously [23]. Cumulative evidence documented that myocardium ischemic-reperfusion through a timely restoration of coronary blood flow is recommended to prevent the risk of AMI and improve clinical outcomes [20]. Paradoxically, sudden myocardial reperfusion may result in additional damage to myocardial cells thus, this myocardial ischemia-reperfusion injury (IRI) is an unavoidable phenomenon during the management of AMI.

Experimental and clinical studies have suggested that to compensate the demand of oxygen supply to cardiomyocytes during AMI, a sudden blood flow to myocardium caused massive production of reactive oxygen species (ROS), including hydrogen peroxide (H$_2$O$_2$), superoxide (O$_2^-$), and hydroxyl radical (OH) in response to hyperoxia situation [10, 19, 47, 48, 52]. These species initiate a vicious cycle of lipoperoxidation via interaction with lipids present in cardiomyocytes which further leak the cellular content to the cytoplasm via the formation of pores on the cell membrane that further contribute to cell death [19, 35, 47]. Myocardial IRI is characterized by systolic and diastolic dysfunction, alterations in myocardial energy metabolism, myocardial arrhythmia, and decreased flow in blood vessels [49]. Thus, numerous researchers have made efforts to restore these cardiac functions via the attenuation of one or more pathways responsible for IRI.

Current treatment strategies mainly focus on IRI prevention which includes intermittent reperfusion, remote ischemic conditioning (RIC), ischaemic preconditioning (IPC), ischaemic post-conditioning (IPo), and volatile anesthetic conditioning (APC) [16, 19]. Furthermore, other pharmacologic agents who protected mitochondrial function during IRI include sodium nitrite and cyclosporin A, whereas atorvastatin, erythropoietin, atrial natriuretic peptide, delcaserib, and
exenatide modulates IRI-induced salvage kinase prosurvival pathway [19, 48]. However, patient outcomes during their clinical investigation have been mixed. Therefore, despite various advances in pharmaceutical industries, the development of a satisfactory therapeutic strategy for the management of myocardial IRI is still challenging. However, several anesthetics, including isoflurane, desflurane, sevoflurane, and propofol, have reduced myocardial infarctions during pre- or post-conditioning in various clinical settings [34, 36, 39, 43, 63]. This facilitates significant attention for various anesthetics by an array of researchers to increase their interest in developing safe and effective pharmacological strategies to protect myocardial IRI. Thus, to enhance the development of a potential therapeutic intervention for myocardial IRI, an experimental animal model of left anterior descending (LAD) transient ligation has been extensively used [10, 15, 27, 40]. LAD ligation-induced myocardial IRI is a reliable and reproducible experimental model which mimics LV diastolic and systolic dysfunctions [10, 40].

Studies have demonstrated that a number of anesthetics such as propofol, halothane, isoflurane, sevoflurane, lignocaine, procainamide, and bupivacaine protect against myocardial injury improved the outcome via various mechanisms [63, 64]. Diazepam is another benzodiazepine-derived local anesthetic that has been widely used as a sedative, muscle relaxant, anticonvulsant, amnesic, and tranquilizer in clinical settings. Clinically diazepam showed rapid tissue distribution in the adrenal gland, liver, heart, kidney, lungs, and brain [22]. Furthermore, diazepam showed greater partition (Kp: 1.5) coefficients between pericardial fluid and blood [56]. Diazepam binds to gamma-aminobutyric acid (GABA(A)) receptors present in various regions of the spinal cord and brain, which are involved in the induction of sleep, anxiety, control of hypnosis, and memory [11]. Diazepam binding to GABA(A) receptors increases its inhibitory potential, enhancing the frequency of chloride channel opening leading to membrane hyperpolarization and a decrease in neuronal excitability [18]. A researcher reported that diazepam exerts its anxiolytic action via α2-GABAA receptors whereas sedative action via α1 GABAA receptors [11].
It has been suggested that diazepam improves the delivery of oxygen to myocardial tissue with an oxygen-conserving action that might be helpful during coronary heart disease [13]. Furthermore, the administration of diazepam in patients with coronary artery disease was reported to achieve a balance between blood pressure and heart rate [46]. Recently, Al-Abbasi et al., (2020) reported the cardioprotective potential of diazepam via attenuation of troponin I (TnI) and High sensitivity C-reactive protein (hs-CRP) in an experimental model of stress-induced cardiac dysfunctions [3]. Moreover, diazepam treatment reduced the incidence of malignant arrhythmias and inhibited the further spreading of myocardial injury in patients with AMI [44]. In addition, numerous researchers also documented the cardioprotective potential of diazepam during IRI in isolated rat hearts [47, 53]. However, despite the availability of significant evidence for the cardioprotective potential of diazepam, its putative mechanism of myocardial protection during IRI is not yet completely elucidated. Thus, we have undertaken this study to investigate the possible mechanism of action of diazepam against LAD ligation-induced myocardial IRI in experimental rats.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (200-220 g) were obtained from the 3201 hospital, Hanzhong, China. They were maintained at 24 ± 1°C, with a 45-55% relative humidity and a 12:12 hr dark/light cycle. The animals had free access to standard pellet chow and water throughout the experimental protocol. All experiments were carried out between 09:00 and 17:00 hr. The 3201 Hospital animal ethical committee approved all the experimental protocols (approval number: HZ3201-0722). All experiments were carried out between 09:00 and 17:00 hr. All the experimental protocols involved in this experiment were carried out in accordance with 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 (http://www.nc3rs.org/ARRIVE).
Drugs and chemicals

Total ribonucleic acid (RNA) Extraction kit and quantitative Real Time-polymerase chain reaction (qRT-PCR) kit were purchased from MP Biomedicals India Private Limited, India. In addition, the primary antibodies of B-cell lymphoma 2 (Bcl-2, EPR17509, ab182858), Bcl-2-associated X protein (Bax, [EPR18283, ab182733]), caspase-3 (ab2302), and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, [EPR6256, ab128915]) were purchased from Abcam, Cambridge, MA, USA.

Experimental design

The ischemia-reperfusion model was established as previously described [10]. Briefly, SD rats were anesthetized with urethane (1.25 g/kg, i.p.) and restrained in the supine position. Since urethane anesthesia has minimal effects on the cardiovascular and respiratory systems and long-lasting anesthesia with rapid onset following i.p. administration. The animals had an intratracheal cannula inserted and mechanically ventilated using a rodent ventilator (respiration rate 70 min⁻¹, respiration-to-expiration ratio 1:2, and tidal volume 50 ml/kg) procedures. A left parasternal incision was performed through the third and fourth intercostal space, and the pericardium was then opened to expose the heart. Myocardial ischemia was induced by placing a 5-0 silk suture with a slipknot around the left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released, and rats received 60 min of reperfusion. Fifty rats were randomly assigned to five experiment groups (n=15) as follows:

**Group I:** Sham: Rats received normal saline (5 ml/kg) for 14 days. They were subjected to thoracotomy and encircling of the LAD artery with a suture but no ligation.

**Group II:** IRI Control: Rats received normal saline (5 ml/kg) for 14 days. They were subjected to thoracotomy and encircling the LAD artery for 30 min and reperfusion for 60 min.
**Group III:** IRI + Dil (10): Rats received diltiazem (10 mg/kg) for 14 days. They were subjected to thoracotomy and encircling the LAD artery for 30 min and reperfusion for 60 min.

**Group IV:** IRI + Dia (1): Rats received diazepam (1 mg/kg) for 14 days. They were subjected to thoracotomy and encircling the LAD artery for 30 min and reperfusion for 60 min.

**Group V:** IRI + Dia (2.5): Rats received diazepam (2.5 mg/kg) for 14 days. They were subjected to thoracotomy and encircling the LAD artery for 30 min and reperfusion for 60 min.

**Group VI:** IRI + Dia (5): Rats received diazepam (5 mg/kg) for 14 days. They were subjected to thoracotomy and encircling the LAD artery for 30 min and reperfusion for 60 min.

**Group VII:** Dia (5) or Perse: Rats received diazepam (5 mg/kg) for 14 days. They were subjected to thoracotomy and encircling of the LAD artery for 30 min and reperfusion for 60 min. Then, they were subjected to thoracotomy and encircling of the LAD artery with a suture but no ligation.

The diazepam was freshly prepared in three different dosages (1, 2.5, and 5 mg/kg) and administered orally to all groups at a pre-fixed time once daily for 14 days. Diltiazem was used as a positive control (standard) to compare the possible mechanism of action of diazepam. At the end of the experiment, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate at 3 mL/kg and intubated before being artificially ventilated with room air at a frequency of 80 inflations/min on a tidal volume of 1 mL/100 g. Lead II of electrocardiogram (ECG) was recorded via cutaneous needle electrodes. Then, a polyethylene catheter filled with heparinized saline was passed through the right carotid arteries into the left ventricle (LV). The LV pressure was processed via a transducer. The LV function, including the left ventricular systolic pressure (LVP), left ventricular end-diastolic pressure (LVEDP), maximal rates of the rise and decline of LV pressure (±dP/dt<sub>max</sub>) was determined using PowerLab Data Acquisition and Analysis System (ADIInstruments, Australia).

Then, blood samples from each rat were collected into separate vials by a retro-orbital puncture method to determine serum parameters. Then, animals were sacrificed by cervical
dislocation, the heart was rapidly removed and stored at 80°C for biochemical (n = 4) and qRT-PCR analysis (n = 4). Finally, the heart of three rats from each group was isolated and fixed for histopathological evaluation.

**Serum biochemistry**

Serum was separated by centrifugation using Eppendorf Cryocentrifuge (model No. 5810, Germany), maintained at 4°C, and run at a speed of 7000 rpm for 15 min. Serum lactate dehydrogenase (LDH), Creatine Kinase -MB (CK-MB), and alanine aminotransferase (AST) were measured by (UV/VIS spectrophotometer, Jasco V-530, Japan) using reagent kits according to the procedure provided by the manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India).

**Measurement of electrocardiographic, hemodynamic, and left ventricular function**

Blood pressure was measured using a polyethylene cannula (PE 50) filled with heparinized saline (100 IU/ml) and connected to a pressure transducer. The cannula was connected to a transducer, and the signal was amplified by a bio-amplifier. Further, left ventricular systolic pressure was measured using a Millar mikro-tip transducer catheter (Model SRP-320, Millar instrument, INC 320-7051, Houston, TX) inserted into the left ventricle via the right carotid artery and connected to a bio-amplifier. Electrocardiographic, hemodynamic changes and left ventricular (LV) contractile function were recorded by an eight-channel recorder Power lab with LABCHART-6 pro software using a data acquisition system (AD Instruments Pvt. Ltd. with software LABCHART 7.3 pro software, Australia).

**Biochemical estimation**

Tissue homogenate preparation
All animals were sacrificed at the end of the study, and the heart was immediately isolated. Tissue homogenates were prepared with 0.1 M tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA content), nitric oxide (NO content), Na⁺K⁺ATPase and Ca²⁺ATPase as described previously [25, 59].

Determination of cardiac cTnI, HIF-1α, TNF-α, IL-1β, IL-6 and CCR2 mRNA expression by qRT-PCR

The levels of cardiac troponin I (cTnI), Hypoxia-Inducible Factor-1 alpha (HIF-1α), tumor necrosis factor-alpha (TNF-α), interleukins (ILs), and C-C chemokine receptor type 2 (CCR2) messenger ribonucleic acid (mRNA) were analyzed using quantitative RT-PCR as described previously [57]. The primer sequence for a respective gene is cTnI (Forward: 5’-ACTTCCGAGGGCAGCAATCA-3’, Reverse: 5’-GGTTGCTTTGTTCTTCCCTTCAG-3’, base pair (bp): 267), HIF-1α (Forward: 5’-TGCTTGGTGCTGATTGTA-3’, Reverse: 5’-GGTCAGATGACAGATCCA-3’, bp: 209), TNF-α (Forward: 5’-AAGCCTGTAGCCCACGTTCTACC-3’, Reverse: 5’-CAGATAGATGGGCTCATACC-3’, bp: 295), IL-1β (Forward: 5’-TGATGTTCCCATAGACACG-3’, Reverse: 5’-GAGGTGCTGATGTACGTT-3’, bp: 290), IL-6 (Forward: 5’-TAGCCGCCACACAGACAG-3’, Reverse: 5’-GGGTGGCATTTGGTTGGG-3’, bp: 479), CCR2 (Forward: 5’-CAGGGCTTATACCATGGG-3’, Reverse: 5’-AGATGACCATGACAAGTAGCG-3’, bp: 388) and (Forward: 5’-GTCACCACACTGTGCCCATCT-3’, Reverse: 5’-ACAGAGTACTTGCGCTCAGAG-3’, bp: 764).

Determination of cardiac Bax, Bcl-2, and Caspase-3 by western blot assay
Cardiac tissue was sonicated in Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Mumbai, Maharashtra, India). The lysates were centrifuged at 10,000 xg for 10 min at 4°C. Protein concentration was determined using a Bicinchoninic Acid (BCA) assay kit (Beyotime Shanghai, China) on ice for 30 min. Equal amounts of extracted protein samples (50 μg) were separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk at 37°C for 1 hr and incubated overnight at 4°C with the primary antibodies recognized Bcl-2, Bax, and caspase-3. In addition, an anti-rabbit horseradish-linked secondary antibody was used, which was incubated at 37°C for 2 hr. Protein bands were visualized using the Chemiluminescent kit (Bio-Rad Laboratories, Inc., Mumbai, Maharashtra, India), GAPDH served as the loading control.

DNA fragmentation

DNA isolation from cardiac tissue was performed according to the standard phenol chloroform cetyl trimethyl ammonium bromide (CTAB) method mentioned elsewhere [50]. Ten μl of the DNA, isolated from the nerve homogenate, was added to 3 μl of loading buffer (20 ml of glycerol 50%, 25 mg of bromophenol blue, and three drops of 1 N NaOH) and subjected to 2D gel electrophoresis in 2% agarose gel. The gel was examined in a gel documentation instrument (Alpha innotech, Kasendorf, Germany), and a gel image was captured.

Histopathological evaluation

The isolated tissue was trimmed into small pieces and preserved in 10% formalin for 24 hr. Specimens were cut in sections of 3-5 μm in thickness by microtome and stained by hematoxylin-eosin. The samples were mounted by disterene phthalate xylene. The photomicrographs of each tissue section were observed using Cell Imaging software for Life Science microscopy (Olympus Soft Imaging Solution GmbH, Munster, Germany).
Statistical analysis

Data were expressed as mean ± standard error means (SEM). Data analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA, USA). Data were analyzed by one-way analysis of variance (ANOVA), and Tukey’s multiple range tests were applied for post hoc analysis. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of diazepam on relative and absolute heart weight, serum CK-MB, LDH, and AST levels of rats

The relative and absolute heart weight, serum CK-MB, LDH, and AST of IRI control group increased significantly ($p < 0.05$) compared to the sham control group. However, administration of diltiazem effectively ($p < 0.05$) attenuated IRI-induced elevated relative and absolute heart weight, serum CK-MB, LDH, and AST as compared to IRI control group. Administration of diazepam (2.5 and 5 mg/kg) noticeably ($p < 0.05$) reduced relative and absolute heart weight, serum CK-MB, LDH, and AST as compared to IRI control group. Notably, diltiazem more effectively ($p < 0.05$) attenuated IRI-induced elevated relative and absolute heart weight, serum CK-MB, LDH, and AST as compared to diazepam. (Table 1 and Supplementary File 1)

Effect of diazepam on electrocardiographic, hemodynamic, and left ventricular function tests in rats

When compared with sham control group (Fig. 1a) and per se treated (Fig. 1f), ischemia-reperfusion resulted in marked ($p < 0.05$) alterations in electrocardiographic, hemodynamic, and left ventricular function tests of IRI control group (Fig. 1b). Diltiazem administration noticeably ($p < 0.05$) inhibited IRI-induced alterations in electrocardiographic, hemodynamic, and left ventricular function tests (Fig. 1c) as compared to IRI control group. Diazepam (2.5 and 5 mg/kg) treatment
effectively \((p < 0.05)\) attenuated IRI-induced alterations in electrocardiographic, hemodynamic, and left ventricular function tests (Fig. 1d and 1e) as compared to IRI control group. (Table 2)

**Effect of diazepam on cardiac oxido-nitrosative stress in rats**

The IRI control group exhibited markedly \((p < 0.05)\) elevated cardiac oxido-nitrosative stress levels compared to the sham control group. Treatment with diltiazem significantly \((p < 0.05)\) inhibited IRI-induced elevated malondialdehyde and nitric oxide levels and replenished superoxide dismutase and glutathione levels compared to IRI control group. Administration of diazepam (2.5 and 5 mg/kg) also prominently \((p < 0.05)\) lessened elevated cardiac oxido-nitrosative stress when compared with IRI control group. Diltiazem more prominently \((p < 0.05)\) attenuated IRI-induced elevated cardiac oxido-nitrosative stress as compared to diazepam. The cardiac superoxide dismutase, glutathione, malondialdehyde, and nitric oxide levels did not differ significantly in the per se treated group, i.e., diazepam (5 mg/kg) and sham control group. (Table 3)

**Effect of diazepam on cardiac ATPase enzymes level in rats**

The activity of cardiac ATPase enzymes \((\text{Na}^+\text{K}^+\text{ATPase} \text{ and Ca}^{2+}\text{ATPase})\) markedly \((p < 0.05)\) decreased in IRI control group as compared to sham control group. However, administration of diltiazem noticeably \((p < 0.05)\) improved the levels of cardiac ATPase enzymes as compared to IRI control group. Diazepam (2.5 and 5 mg/kg) administration also noticeably \((p < 0.05)\) increased \(\text{Na}^+\text{K}^+\text{ATPase}\) and \(\text{Ca}^{2+}\text{ATPase}\) activity when compared with IRI control group. (Table 3)

**Effect of diazepam on the cardiac cTn-I, HIF-1α, CCR2, TNF-α, IL-1β, and IL-6 mRNA expressions in rats**

The cardiac mRNA expressions of cTn-I, CCR2, TNF-α, IL-1β, and IL-6 were up-regulated significantly \((p < 0.05)\), whereas cardiac HIF-1α mRNA expression was down-regulated effectively \((p < 0.05)\) in IRI control group as compared to sham control group. Diltiazem noticeably \((p < 0.05)\)
attenuated IRI-induced alterations in cardiac cTn-I, HIF-1α, CCR2, TNF-α, IL-1β, and IL-6 mRNA expressions compared with IRI control group. Additionally, diazepam (2.5 and 5 mg/kg) also markedly ($p < 0.05$) down-regulated cardiac mRNA expressions of cTn-I, CCR2, TNF-α, IL-1β, and IL-6 as well as up-regulated cardiac HIF-1α mRNA expression as compared to IRI control group. There was no significant difference in cardiac cTn-I, HIF-1α, CCR2, TNF-α, IL-1β, and IL-6 mRNA expressions in per se treated group, i.e., diazepam (5 mg/kg) and sham control group. (Fig. 2)

Effect of diazepam on the cardiac Bax, Bcl-2, and Caspase-3 protein levels in rats

The cardiac Bax and Caspase-3 protein levels were increased significantly ($p < 0.05$), whereas cardiac Bcl-2 protein level was decreased markedly ($p < 0.05$) in the IRI control group as compared to sham control group. IRI-induced variations in cardiac Bax, Bcl-2, and Caspase-3 protein levels were effectively ($p < 0.05$) inhibited by diltiazem treatment as compared to IRI control group. Diazepam (2.5 and 5 mg/kg) administration also noticeably ($p < 0.05$) decreased cardiac Bax and Caspase-3 protein levels as well as significantly ($p < 0.05$) increased cardiac Bcl-2 protein level as compared to IRI control group. Diltiazem treatment more effectively ($p < 0.05$) attenuated IRI-induced variations in cardiac Bax, Bcl-2, and Caspase-3 protein levels as compared to diazepam. However, cardiac Bax, Bcl-2, and Caspase-3 protein levels did not differ significantly in per se treated group, i.e., diazepam (5 mg/kg) and sham control group. (Fig. 3a-d)

Effect of diazepam on the cardiac DNA fragmentation

Ischemia-reperfusion injury caused a higher degree of apoptosis reflected by maximum fragmentation of DNA compared to the sham control group. Administration of diltiazem and diazepam (5 mg/kg) showed a lower degree of DNA fragmentation, suggesting amelioration of IRI-induced apoptosis as compared IRI control group. There was minimal DNA fragmentation in the normal and per se group (Fig. 3e).
Effect of diazepam on IRI-induced cardiac histopathological alteration in rats

Cardiac tissue from sham control group and per se treated group, i.e., diazepam (5 mg/kg), showed the normal architecture of myocardiocytes and myocardial muscles with mild interstitial inflammation (Fig. 4a and 4f). However, ischemia-reperfusion caused significant ($p < 0.05$) damage to cardiac tissue reflected by myocardial degeneration, interstitial inflammation, necrosis and hemorrhage in IRI control group (Fig. 4b) as compared to sham control group. Diltiazem treatment effectively ($p < 0.05$) attenuated IRI-induced alteration in the cardiac architecture reflected by decreased myocardial degeneration, interstitial inflammation, necrosis, and hemorrhage (Fig. 4c) compared to IRI control group. Diazepam (2.5 and 5 mg/kg) administration also noticeably ($p < 0.05$) reduced IRI-induced myocardial degeneration, interstitial inflammation, necrosis and hemorrhage as compared to IRI control group (Fig. 4d and 4e). (Fig. 4g)

DISCUSSION

Myocardial ischemia-reperfusion injury (IRI) is an unavoidable vicious consequence of several cardiac surgeries leading to cardiomyocyte death. Due to the scarcity of effective therapeutic intervention for the management of myocardial IRI, it has become an important subject of investigation in cardiovascular diseases [19, 20]. Numerous anesthetic agents such as desflurane, isoflurane, propofol, and sevoflurane have been shown to exert their cardioprotective efficacy against myocardial infarction clinically [34, 36, 39, 43, 63]. In the present investigation, we have also evaluated the potential of diazepam against LAD ligation-induced myocardial IRI in experimental rats. The current study found that diazepam attenuated myocardial injury by inhibiting inflammatory release (CCR2, TNF-$\alpha$, and ILs), oxido-nitrosative stress, and apoptosis (Bax and Caspase-3), thus improves myocardial function (Supplementary File 2).

Cumulative evidence suggested that clinically cardiac ischemia is characterized by various findings ranging from diffuse chest pain to alteration in ECG outcomes such as heart rate, ST segment, QRS interval, QTc Interval, Q wave, and T wave [20, 33, 37, 58]. The narrow QRS complex
depicted ventricular depolarization or quicker cardiac ejection. However, prolongation in QRS interval represents delayed ventricular depolarization, suggesting the inability of cardiac tissue towards the ejection, which may be due to tissue ischemia or infarction [64]. Thus, ECG findings have been suggested as an important prognostic and non-invasive tool for quicker diagnosing ischemic heart disease [52]. Injury to myocardial tissue results in loss of reliability of the left ventricle after its contraction, which causes a decrease in the volume of the left ventricle chamber, which increases LVEDP [17, 41]. Thus, LVEDP is documented as a reliable indicator of cardiac damage post-IRI. Additionally, the rate of rising and fall in LVEDP as well as a performance of ventricular determined by $dP/dt_{\text{max}}$ and $dP/dt_{\text{min}}$. IRI caused alteration in the electrocardiographic, hemodynamic, and left ventricular functions in the present study, suggesting the overall cardiac dysfunction. Conversely, administration of diazepam inhibited IRI-induced alterations in heart functions revealing its anti-arrhythmic potential. The putative mechanism behind the anti-arrhythmic potential of diazepam may be due to its profound effects on cardiac regulation via positive allosteric modulators of GABA\textsubscript{A} receptors [6]. The previous researcher documented elevated heart rate post diazepam (6 mg/kg) administration [42].

Inflammation is an important mediator of cell necrosis during myocardial ischemia [33, 36]. The myocardial infarction can cause an influx of inflammatory cytokines, including TNF-\(\alpha\) and ILs (IL-1\(\beta\) and IL-6), into the infarcted cardiac tissue [47, 61]. CCR2 chemokine has been suggested to recruit TNF\(\alpha\)-producing monocytes at myocardial infarcted area via the formation of the CCL2 concentration gradient [65, 66]. This excessive recruitment of monocytes resulted in left ventricular remodeling, thus contribute to myocardial dysfunction. Furthermore, monocytes secrete TNF-\(\alpha\), which aggravates the inflammatory reaction and boosts neutrophil and other pro-inflammatory cytokines [7, 14, 21]. IL-1\(\beta\) has been suggested to initiate neutrophil cell adhesion to endothelial cells [29]. IL-6 is another critical pro-inflammatory cytokine closely associated with myocardial injury [55]. In the present study, myocardial ischemia-reperfusion induces the expression of CCR2 chemokine and aggravates the myocardial injury via the release of pro-inflammatory cytokines (TNF-\(\alpha\) and ILs). Nevertheless, administrations of diazepam attenuated elevated levels of chemokine and
cytokines, which is consistent with the observation of previous researchers [24, 67]. Furthermore, the present investigation evident the presence of cardiac hypertrophy reflected by a significant increase in heart weight which is in line with findings of previous researchers [60]. Thus, the hypertrophy of cardiomyocytes may attribute to the release of inflammatory and apoptotic mediators post IRI. However, diazepam pre-treatment inhibited inflammatory influx, which plays a vital role in halting cardiac hypertrophy. This notion was further supported by the histological findings of cardiac tissue from diazepam-treated rats, showing inhibition of inflammatory infiltration.

Cellular apoptosis is a critical pathophysiological pathway during IRI-induced cardiac failure [31, 33]. Bcl-2 (B-cell lymphoma 2) is a regulatory protein that plays a vital role in regulating mitochondrial-dependent cellular apoptosis [2]. On the other hand, Bax (Bcl-2 associated X) is a pro-apoptotic protein responsible for mitochondrial apoptosis via the release of cytochrome c and formation of its apoptosome complex with APAF-1 (apoptotic protease-activating factor-1)[12, 26, 38]. Further, this apoptosome causes DNA fragmentation through activation of caspase-3 and promoting activity of CAD (caspase-3-activated DNase) enzyme [1, 51]. As mitochondria are the important site for apoptosis, a balance between these pro-apoptotic and anti-apoptotic proteins plays a vital role in mitochondrial apoptosis [30]. Additionally, researchers have established the link between IRI-induced elevated oxidative stress and activation of caspase-3 through the release of mitochondrial cytochrome C [28]. Clinically, an autopsy of cardiac tissue from the ischemic patient showed elevated apoptotic protein expressions in cardiomyocytes [33]. The findings of the present study also suggested that ischemia-reperfusion caused induction of apoptosis in mycardiocytes reflected by elevated Bax and Caspase-3 protein expressions along with increased DNA fragmentation. Interestingly, administration of diazepam attenuated IRI-induced apoptosis via down-regulation of Bax, Caspase-3, and DNA fragmentation levels depicting its anti-apoptotic property.

It is well established that hypoxia plays a vital role during myocardial ischemia [16, 52, 53]. HIF-1α, a transcriptional regulator highly sensitive to hypoxia and during normal physiological conditions, remains stable via inhibiting proline hydroxylase (PHD) activity [9]. However, during hypoxia, elevated oxidative stress induces transactivation of PHD, which further degraded the
expressions of HIF-1α [8, 9]. Furthermore, hypoxia also leads to degradation of intracellular ATP levels, which further causes failure of ATP-dependent transport systems, including Na⁺K⁺ATPase and Ca²⁺ATPase [4, 45, 68]. Abnormalities in these transport systems eventually increase the extracellular concentration of K⁺, which further contributed to reducing conduction velocity and myocardial contractility [4, 45]. In the present study, LAD transient ligation causes initiation of hypoxia where the levels of HIF-1α, Na⁺K⁺ATPase, and Ca²⁺ATPase decrease; however, administration of diazepam significantly restored these alterations suggesting its cardioprotective property.

At present, diltiazem has been used as a first-category therapeutic regimen for managing myocardial ischemia [52]. Diltiazem, a calcium channel blocker, has been reported to inhibit the influx of calcium ions in myocardial smooth muscle cells at the time of depolarisation [32]. It also decreases intracellular calcium levels, thus increases smooth muscle relaxation. The FDA has approved diltiazem to manage hypertension, atrial arrhythmia, and chronic stable angina [54]. However, it is associated with several side effects, including bradycardia, edema, headache, fatigue, and dizziness. Sometimes chronic administration of diltiazem may cause myocardial infarction, congestive heart failure, and hepatotoxicity [54]. In a randomized clinical study, administration of diazepam (15 mg, p.o.) showed a reduction in the incidence of arrhythmias and preventing further spreading of myocardial injury [44]. Thus, diazepam may provide a beneficial effect in the management of myocardial injury induced by ischemic-reperfusion. However, validation is needed in the larger group of cardiac surgery patients susceptible to myocardial ischemia-reperfusion.

In conclusion, our results of the present study demonstrate that diazepam exerts cardioprotective effect against LAD ligation-induced myocardial IRI in experimental rats. Furthermore, the cardioprotective potential of diazepam on ischemia injury was mediated by inhibiting inflammatory release (CCR2, TNF-α, and ILs), oxido-nitrosative stress, and apoptosis (Bax and Caspase-3) pathway thus, it can be considered as a potential candidate for the treatment of the myocardial ischemia-reperfusion injury.
CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Fig. 1. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced altered in electrocardiographic parameters. Representative images of electrocardiographic recording from sham (a), IRI control (b), IRI + diltiazem (10 mg/kg) (c), IRI + diazepam (2.5 gm/kg) (d), IRI + diazepam (5 gm/kg) (e) and diazepam (5 gm/kg) (f) treated rats.
Fig. 2. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations in cardiac troponin I (a), Hypoxia-Inducible Factor-1 alpha (b), C-C chemokine receptor type 2 (c), Tumor necrosis factor-alpha (d), Interleukins (IL)-1β (e) and IL-6 (f) mRNA expressions in rats.

Data are expressed as mean ± S.E.M (n=4) 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; Dia (1): diazepam (1 mg/kg, p.o.); Dia (2.5): diazepam (2.5 mg/kg, p.o.) and Dia (5): diazepam (5 mg/kg, p.o.) treated rats. cTnI: cardiac troponin I; CCR2: C-C chemokine receptor type 2; HIF-1α: Hypoxia-Inducible Factor-1 alpha; ILs: Interleukins; TNF-α: Tumor necrosis factor-alpha.
Fig. 3. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations in cardiac BCL2 Associated X (Bax), B-cell lymphoma 2 (Bcl-2), and Caspase-3 protein expressions in rats (a). Quantitative representation of protein expression of Bax (b), Bcl-2 (c), and Caspase-3 (d) in rats. Effect of diazepam on cardiac DNA fragmentation (e).

Data are expressed as mean ± S.E.M (n=4). *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; Dia (1): diazepam (1 mg/kg, p.o.); Dia (2.5): diazepam (2.5 mg/kg, p.o.) and Dia (5): diazepam (5 mg/kg, p.o.) treated rats. Bax: BCL2 Associated X; Bcl-2: B-cell lymphoma 2.
Fig. 4. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations in cardiac histopathology in rats.

Photomicrograph of sections of the heart of from sham (a), IRI control (b), IRI + diltiazem (10 mg/kg) (c), IRI + diazepam (2.5 gm/kg) (d), IRI + diazepam (5 gm/kg) (e) and diazepam (5 gm/kg) (f) treated rats. Images at 40X. The quantitative representation of histological score (g). 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.
Table 1. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations in relative and absolute heart weight, serum Creatine Kinase-MB, Lactate dehydrogenase, Alanine Aminotransferase in rats.

| Parameters                  | Sham     | IRI Control | IRI + Dil (10) | IRI + Dia (1) | IRI + Dia (2.5) | IRI + Dia (5) | Dia (5)    |
|-----------------------------|----------|-------------|----------------|---------------|----------------|---------------|------------|
| Heart weight (gm)           | 0.40 ± 0.01 | 0.81 ± 0.03<sup>a</sup> | 0.48 ± 0.03<sup>a</sup> | 0.75 ± 0.07 | 0.63 ± 0.07<sup>a</sup> | 0.54 ± 0.02<sup>a</sup> | 0.50 ± 0.03 |
| Heart weight / Body weight  | 1.71 ± 0.04 | 3.52 ± 0.15<sup>a</sup> | 2.14 ± 0.11<sup>a</sup> | 3.25 ± 0.28 | 2.76 ± 0.30<sup>a</sup> | 2.30 ± 0.09<sup>a</sup> | 2.17 ± 0.13 |
| Serum CK-MB (IU/I)          | 1,073.00 ± 44.11 | 2,062.00 ± 56.63<sup>a</sup> | 1,185.00 ± 39.01<sup>a</sup> | 2,048.00 ± 64.43 | 1,632.00 ± 51.22<sup>a</sup> | 1,435.00 ± 55.70<sup>a</sup> | 1,033.00 ± 56.47 |
| Serum LDH (IU/I)            | 1,212.00 ± 73.23 | 2,634.00 ± 107.50<sup>a</sup> | 1,627.00 ± 92.86<sup>a</sup> | 2,685.00 ± 111.10 | 2,014.00 ± 95.56<sup>a</sup> | 1,638.00 ± 87.84<sup>a</sup> | 1,222.00 ± 68.46 |
| AST (mg %)                  | 110.90 ± 11.89 | 358.70 ± 11.35<sup>a</sup> | 139.00 ± 9.26<sup>a</sup> | 334.80 ± 10.70 | 249.50 ± 8.68<sup>a</sup> | 139.60 ± 11.77<sup>a</sup> | 121.00 ± 8.98 |

Data are expressed as mean ± S.E.M (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; Dia (1): diazepam (1 mg/kg, p.o.); Dia (2.5): diazepam (2.5 mg/kg, p.o.) and Dia (5): diazepam (5 mg/kg, p.o.) treated rats. AST: Alanine Aminotransferase; CK-MB: Creatine Kinase-MB; LDH: Lactate dehydrogenase.
### Table 2. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations electrocardiographic, hemodynamic, and left ventricular function tests changes in rats

| Parameters               | Sham        | IRI Control | IRI + Dil (10) | IRI + Dia (1) | IRI + Dia (2.5) | IRI + Dia (5) | Dia (5)  |
|--------------------------|-------------|-------------|----------------|---------------|----------------|---------------|----------|
| Heart Rate (BPM)         | 369.20 ± 10.94* | 271.00 ± 10.89* | 349.80 ± 11.42* | 287.20 ± 7.51 | 319.00 ± 9.01* | 343.00 ± 11.04* | 351.00 ± 8.80 |
| QRS interval (ms)        | 12.80 ± 0.58  | 32.40 ± 0.68* | 17.40 ± 0.68* | 28.80 ± 0.37  | 22.60 ± 0.75* | 20.60 ± 0.93* | 13.60 ± 0.51 |
| QT interval (ms)         | 48.17 ± 2.59  | 88.50 ± 1.57* | 57.50 ± 2.01* | 86.00 ± 3.14  | 72.83 ± 2.55* | 64.17 ± 2.06* | 57.50 ± 2.34 |
| QTc Interval (ms)        | 125.30 ± 5.30 | 175.70 ± 5.89* | 146.80 ± 3.34* | 169.80 ± 4.55 | 154.20 ± 6.69* | 144.20 ± 6.81* | 135.50 ± 5.92 |
| RR interval (ms)         | 144.30 ± 6.09 | 204.50 ± 4.64* | 161.80 ± 3.44* | 198.80 ± 3.37 | 183.70 ± 5.59* | 170.80 ± 5.16* | 152.50 ± 3.73 |
| SBP (mmHg)               | 106.70 ± 1.87 | 161.30 ± 4.72* | 124.00 ± 4.48* | 151.80 ± 4.09 | 137.30 ± 5.06* | 119.30 ± 1.82* | 108.70 ± 2.89 |
| DBP (mmHg)               | 83.50 ± 3.72  | 117.50 ± 2.51* | 89.33 ± 3.70* | 110.50 ± 2.79 | 98.50 ± 3.22* | 96.83 ± 4.11* | 91.00 ± 4.05 |
| LVEDP (mmHg)             | 5.33 ± 0.21   | 11.67 ± 0.56* | 7.50 ± 0.43* | 11.17 ± 0.75  | 8.67 ± 0.67* | 7.50 ± 0.56* | 5.83 ± 0.70  |
| Max\(_{dp/dt}\)          | 4.011.00 ± 151.70 | 1.995.00 ± 134.50* | 3.656.00 ± 117.60* | 2.518.00 ± 124.10 | 2.858.00 ± 126.40* | 3.499.00 ± 116.90* | 3.880.00 ± 167.80 |
| Min\(_{dp/dt}\)          | -2.708.00 ± 88.37 | -1.970.00 ± 74.60* | -2.382.00 ± 69.35* | -1.886.00 ± 36.34 | -2.245.00 ± 58.68* | -2.547.00 ± 35.78* | -2.530.00 ± 85.90 |
| Pressure time index      | 17.33 ± 0.33  | 24.33 ± 0.33* | 20.17 ± 0.75* | 23.67 ± 0.95  | 21.33 ± 0.67* | 21.83 ± 0.40* | 18.83 ± 0.87 |
| Contractility index      | 56.33 ± 1.50  | 33.83 ± 1.17  | 47.17 ± 1.70* | 35.00 ± 1.93  | 42.17 ± 1.40* | 44.17 ± 1.78* | 56.50 ± 1.09 |
| Tau (ms)                 | 3.50 ± 0.56   | 10.83 ± 0.65  | 5.83 ± 0.54  | 10.17 ± 0.48  | 9.17 ± 0.31* | 6.50 ± 0.67* | 5.00 ± 0.73  |

Data are expressed as mean ± S.E.M (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; Dia (1): diazepam (1 mg/kg, p.o.); Dia (2.5): diazepam (2.5 mg/kg, p.o.) and Dia (5): diazepam (5 mg/kg, p.o.) treated rats. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LVEDP: Left ventricular end-diastolic pressure.
Table 3. Effect of diazepam on Ischemia-Reperfusion Injury (IRI)-induced alterations cardiac oxido-nitrosative stress and ATPase enzymes in rats

| Parameters                          | Sham    | IRI Control | IRI + Dil (10) | IRI + Dia (1) | IRI + Dia (2.5) | IRI + Dia (5) | Dia (5)  |
|-------------------------------------|---------|-------------|----------------|---------------|-----------------|---------------|----------|
| SOD (U/mg of protein)               | 10.53 ± 0.27 | 3.73 ± 0.44* | 9.23 ± 0.48*<sup>+</sup> | 4.13 ± 0.17 | 6.61 ± 0.35*<sup>+</sup> | 8.71 ± 0.46*<sup>+</sup> | 10.48 ± 0.65 |
| GSH (µg/mg protein)                 | 32.94 ± 1.01 | 17.78 ± 1.27* | 29.13 ± 0.98*<sup>+</sup> | 18.85 ± 1.26 | 22.26 ± 0.85*<sup>+</sup> | 25.80 ± 1.03*<sup>+</sup> | 30.61 ± 1.10 |
| MDA (nmol/l/mg of protein)          | 2.52 ± 0.21  | 5.69 ± 0.29* | 3.33 ± 0.30*<sup>+</sup> | 5.04 ± 0.23 | 4.81 ± 0.27*<sup>+</sup> | 3.97 ± 0.23*<sup>+</sup> | 3.11 ± 0.19 |
| NO (µg/mg of protein)               | 216.40 ± 30.70 | 704.00 ± 28.39<sup>*</sup> | 317.40 ± 37.31*<sup>+</sup> | 635.20 ± 27.03 | 516.00 ± 29.32*<sup>+</sup> | 377.40 ± 37.64*<sup>+</sup> | 247.40 ± 24.21 |
| Na⁺K⁺-ATPase (µmoles/mg of Protein) | 5.78 ± 0.31  | 2.86 ± 0.37<sup>*</sup> | 4.99 ± 0.38*<sup>+</sup> | 3.09 ± 0.18 | 4.54 ± 0.18*<sup>+</sup> | 5.01 ± 0.39*<sup>+</sup> | 5.17 ± 0.33 |
| Ca²⁺-ATPase (µmoles/mg of Protein)  | 3.44 ± 0.33  | 1.61 ± 0.31<sup>*</sup> | 3.14 ± 0.21*<sup>+</sup> | 1.61 ± 0.25 | 2.33 ± 0.27*<sup>+</sup> | 2.86 ± 0.36*<sup>+</sup> | 3.01 ± 0.25 |

Data are expressed as mean ± S.E.M (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, <sup>*</sup>p < 0.05 as compared to the sham, <sup>+</sup>p < 0.05 as compared to one another. IRI: Ischemia-reperfusion Injury control rats; Dil (10): diltiazem (10 mg/kg, p.o.) treated rats; Dia (1): diazepam (1 mg/kg, p.o.); Dia (2.5): diazepam (2.5 mg/kg, p.o.) and Dia (5): diazepam (5 mg/kg, p.o.) treated rats. SOD: Superoxide dismutase; GSH: Glutathione peroxidase; MDA: Malondialdehyde; NO: Nitric Oxide.
Supplementary File 1: Morphological representation of section of heart from sham (a), Ischemia-Reperfusion Injury (IRI)-control (b), IRI + diltiazem (10 mg/kg) (c), and diazepam (5 gm/kg) (d) treated rats.
Supplementary File 2: Diazepam ameliorated myocardial Ischemia-Reperfusion Injury