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Original article

Protective Effects of 2-Methoxyestradiol on Acute Isoproterenol-Induced Cardiac Injury in Rats

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ABSTRACT

Myocardial injury (MI) is an important pathological driver of mortality worldwide., and arises as a result of imbalances between myocardial oxygen demand and supply. In MI, oxidative stress often leads to inflammatory changes and apoptosis. Current therapies for MI are known to cause various adverse effects. Consequently, the development of new therapeutic agents with a reduced adverse event profile is necessary. In this regard, 2-methoxyestradiol (2ME), the metabolic end-product of oestradiol, possesses anti-inflammatory and antioxidant properties. The aim of this research is to assess the impact of 2ME on cardiac injury caused by isoproterenol (ISO) in rats. Animals were separated into six groups; controls, and those receiving 2ME (1 mg/kg), ISO (85 mg/kg), ISO + 2ME (0.25 mg/kg), ISO + 2ME (0.5 mg/kg), and ISO + 2ME (1 mg/kg). 2ME significantly attenuated ISO-induced changes in electrocardiographic changes and the cardiac histological pattern. This compound also decreased lactate dehydrogenase activity, creatine kinase myocardial band and troponin levels. The ability of 2ME to act as an antioxidant was shown by a decrease in malondialdehyde concentration, and the restoration of glutathione levels and superoxide dismutase activity. Additionally, 2ME antagonized inflammation and cardiac cell apoptosis, a process determined to be mediated, at least partially, by suppression of Gal-3/TLR4/MyD88/NF-κB signaling pathway. 2ME offers protection against acute ISO-induced MI in rats and offers a novel therapeutic management option.

1. Introduction

Cardiac disease is a principal contributing factor to death worldwide, ranking first above other diseases, including cancer (Afrasiabi et al., 2020; Reeve et al., 2005). In 2021, the World Health Organization announced that 32% of deaths, globally, were attributable to cardiovascular diseases (CVD), and myocardial injury (MI) was associated with 85% of these cases; its prevalence is increasing on a yearly basis (Afrasiabi et al., 2020; World Health Organization, 2021). Despite the achieved progress in the therapy and management of CVD, efficient treatment for MI and related CVD remains challenging, and so there is an urgent need for novel treatments to improve the therapeutic outcomes of these diseases (Kaur et al., 2006; Zhou et al., 2018). Myocardial injury arises as a consequence of the sudden cessation of blood flow to a region of cardiac tissue, resulting in ischemia and eventually, necrosis of the affected myocardial tissues (Suchal et al., 2016). The loss of cardiac muscles significantly impairs the contractile force of the heart, and ultimately leads to heart failure (Severino et al., 2020).

Oxidative damage is considered to be a major pathophysiological mechanism underlying MI, which subsequently promotes inflammatory cell infiltration (Bayeva et al., 2013; Kaur et al., 2006). Oxidative stress has been demonstrated to induce the production of oxidized low-density lipoproteins and eventually, the onset of atherosclerosis (Ding et al., 2012). During this phase, inflammatory cells produce interleukin-1, reactive oxygen species (ROS), and chemokines via signalling pathways, such as nuclear

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factor-κB (NF-κB), changes which further enhance the inflammatory process (Ong et al., 2018). Concurrently, fibroblasts multiply and differentiate into myofibroblasts, secreting significant quantities of extracellular matrix proteins in order to keep the infarction site structurally intact (Viola et al., 2021). Following MI, there is structural remodeling of the cardiac tissues at the scar site due to granulation tissue cell death and the generation of a cross-linked collagen network (Wilk et al., 2020). Excessive ROS production and inflammatory reactions cause metabolic stress and decreased tissue defense mechanisms, which ultimately result in further cardiomyocyte damage and necrosis (Hearse, 1991; Wilk et al., 2020).

Isoproterenol (ISO) is a β-adrenoceptor non-selective agonist that induces significant myocardial damage (Boarescu et al., 2019). It generates cytotoxic free radicals, which augment lipid peroxidation and reduce the antioxidant capacity, causing severe myocardial damage associated with elevated cardiac enzyme levels (Song et al., 2020). Activation of β-adrenoceptors increases cardiac galectin-3 (Gal-3) expression (Du et al., 2019; Nguyen et al., 2018; Vergaro et al., 2016). A rise in Gal-3 protein levels has been reported in ISO-induced rat models of heart failure; these act as ligands for Toll-like receptor 4 (Xu et al., 2020).

Toll-like receptors (TLRs) are critical for the triggering of inflammatory responses in CVD (Yang et al., 2016). These receptors, TLR2, TLR3, and TLR4, are transmembrane proteins that are expressed in cardiac cells (Feng and Chao, 2011). TLR4 activates NF-κB, a transcription factor involved in modulating pro-inflammatory cytokine expression (Jiang et al., 2015; Shih et al., 2015). TLR4/NF-κB pathway is involved in cardiac ischemia–reperfusion injury, and suppressing this pathway has been reported to reduce MI and inflammation (Mice et al., 2004; Zhang et al., 2017; Zhao et al., 2014). Thus, inhibition of this inflammatory response is critical in protecting against cardiac injury.

2-Methoxyestradiol (2ME) is a metabolic by-product of oestrogen, which is generated through sequential oxidation and *O*-methylation of 17β-oestradiol, reactions facilitated by cytochrome P450 and catechol-*O*-methyltransferase activities, respectively (Perez-sepulveda and Espan, 2013). Previous studies have reported that 2ME possesses antioxidant (Wang et al., 2017), anti-inflammatory (Shand et al., 2011), anti-cancer (Pal et al., 2019), and anti-mitogenic (Dubey et al., 2004) properties. 2ME has also been demonstrated to possess protective activities against various disorders related to inflammation, including rheumatoid arthritis (Stubelius et al., 2011), autoimmune encephalomyelitis (Duncan et al., 2012), acute lung inflammation (Shand et al., 2011) and lung and renal ischemia/reperfusion injuries (Chen et al., 2014; Liao et al., 2021). This anti-inflammatory effect is achieved through the suppression of NF-κB activity (Chen et al., 2014; Liao et al., 2021) which, in turn, inhibits the induction of inflammatory cytokine genes (Plum et al., 2009; Yeh et al., 2011) as well as decreasing ROS production (Mariappan et al., 2009).

Several studies highlight the worth of 2ME use in CVD. It was demonstrated that 2ME exhibited vasoprotective, anti-inflammatory, and anti-hypertrophic effects, suggesting its potential use in the treatment of cardiac hypertrophy and heart failure (Maayah et al., 2018). Additionally, 2ME was reported to protect from bleomycin-induced pulmonary fibrosis and pulmonary hypertension (Tofovic et al., 2009), as well as to reduce carotid artery intimal hyperplasia induced by balloon injury in rats (Azhar et al., 2022). Moreover, a prophylactic role of 2ME against angiotensin II-induced hypertension and cardiovascular and renal injuries has been reported (Salah et al., 2019). However, the possible protective activity of 2ME on acute ISO-induced MI has not been studied. 2ME is well-tolerated and has an outstanding safety record with no documented major side effects (Bruce et al., 2012; Tevaarwerk et al., 2009).

The present work has been designed in order to delineate the potential protective activity of 2ME on acute ISO-induced MI in rats, and to elucidate possible underlying mechanisms.

2. Materials and methods

2.1. Chemicals

2ME and ISO were acquired from Merck (Rahway, NJ, USA). The rest of the chemicals used were of optimal commercial quality.

2.2. Preparation and administration of isoproterenol

ISO was freshly prepared using normal saline, and subcutaneously (SC) administered (85 mg/kg) for two consecutive days (days 13 and 14) (Halim et al., 2018).

2.3. Animals

The animal facility at King Abdulaziz University's Faculty of Pharmacy provided the forty-eight male Wistar rats utilized in the study, which ranged in weight from 200 to 230 g. The animals were housed in conditions that included a 12-hours light/dark cycle, a constant temperature of 22 °C, a humidity of 60–70%, and complete access to food and water. Ethical approval for the animal experiments was obtained from the Faculty of Pharmacy's Research Ethics Committee (Reference # PH-1443–39).

2.4. Experimental protocol

Animals were arbitrarily divided into six groups. Each group contained 8 rats. Group 1 was a control group that received no medications, while rats in group 2 were administered 2ME 1 mg/kg intraperitoneally (IP) for 14 consecutive days and injected SC with normal saline. Group 3 rats received only ISO, as mentioned above. Rats in groups 4, 5, and 6 received 0.25, 0.5, and 1.0 mg/kg IP 2ME, respectively, for 14 days, in addition to ISO.

Twenty-four hours after the second ISO treatment, anesthesia was induced in the rats by administering IP ketamine (50 mg/kg) and xylazine (5 mg/kg) injections (Ritschl et al., 2015). Electrocardiographic (ECG) tracing was performed. Then, blood was withdrawn from the retroorbital plexus, left to stand for 15 min, and spun for 10 min (3000 rpm at 4 °C) in order to separate the serum. Animals were decapitated and their hearts were carefully dissected and washed with saline solution. Parts of cardiac tissue were kept in formalin solution (10%) and subjected to immunohistochemical and histopathological examinations. The remaining cardiac tissues were flash frozen using liquid nitrogen, and then stored with the serum at −80° C for further analyses.

2.5. Cardiac tissue homogenization

Cardiac tissues were homogenized in phosphate-buffered saline (PBS) solution (ice-cold, pH 7.4). Homogenate aliquots were spun (10,000 × g) for 20 min at 4 °C prior to supernatant collection for cardiac oxidative and inflammatory markers analysis.

2.6. Electrocardiography

Anesthetized sedated animals (as previously described) were prepared for ECG tracing. A thermostatically regulated heating blanket kept rectal temperatures at 37.5 °C whilst the ECG was performed. Electrodes were sited on the skin of the right, left, and front hind limbs. Recording of the ECG was carried out utilizing Power Lab model 8/35 (AD Instruments, Sydney, Australia). Heart

rate, RR interval, QRS complex, QT interval, QT_c interval, and amplitude of the ST segment were determined.

2.7. Histopathological examination

The cardiac tissues, preserved in formalin, were embedded in paraffin and sectioned into 5 μm thick specimens. The tissues were subsequently stained with Sirius red, hematoxylin and eosin (H&E), and Masson's trichrome stains. Slides were examined by a pathologist, who was blinded to the study protocol, using a Nikon light microscope (Tokyo, Japan).

2.8. Determination of cardiac injury parameters

Serum lactate dehydrogenase (LDH) and creatine kinase myocardial band (CK-MB) activities, together with troponin levels, were examined using colorimetric kits (Cat. No. SEB370Ra, SEA479Ra, and SEA478Ra, respectively, Cloud-Clone Corp, Houston, TX, USA).

2.9. Determination of oxidative stress parameters

Malondialdehyde (MDA) content, reduced glutathione (GSH) levels and the superoxide dismutase (SOD) activity in cardiac homogenates were assessed using ELISA kits (Cat. No. MD 2529, GR 2511 and SD 2521, respectively, Biodiagnostics, Giza, Egypt). The sample protein concentrations were measured with the use of a bicinchoninic acid (BCA) protein assay kit (Biovision® Inc., CA, USA).

2.10. Immunohistochemistry staining

Deparaffinization of the cardiac specimens was performed, followed by rehydration of the tissues using serial dilutions of ethanol. Deparaffinized specimens were boiled for 10 min in 0.1 M citrate buffer (pH 6). The slides were blocked using 5% bovine serum albumin in a tris-buffered saline solution (TBS) for 2 h at room temperature. The slides were kept for 12 h at 4 °C with the following primary antibodies: cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6). These were obtained from Abcam®, Cambridge, UK (Cat. No. ab15191, ab178945, ab19870, ab220210 and ab9324, respectively). After washing with TBS, the slides were incubated with a specific secondary antibody, relative to the reactivity of the primary antibody (Cell & Tissue Staining Kit, Cat. No. CTS002 and CTS006, R&D systems, Minneapolis, MN, USA). A Nikon light microscope (Tokyo, Japan) was used to visualize tissue. Image analysis (Image J, 1.8.0, NIH, Bethesda, MD, USA) was carried out for image quantification.

2.11. Expression of Bcl-2, Bax and Caspase-3 mRNA

In order to evaluate the expression of Bcl-2, Bax, and Caspase-3 (CASP-3) mRNA expression, a real-time reverse transcription polymerase chain reaction (RT-PCR) assay was performed. TRIzol was used to extract total RNA, which was subsequently used as a template for the synthesis of cDNAs using an Omniscript RT kit (Cat. No. 205113, Qiagen, MD, USA). The RT-PCR step was conducted using a SYBR Green Master Mix (Cat. No. 180830, Qiagen, MD, USA). The used primers' sequences are shown in Table 1. A delta-delta Ct (ΔΔCt) method was employed for data analysis; normalisation was performed using β-actin (Livak and Schmittgen, 2001).

Table 1
Primers' sequences of Bcl-2, Bax, CASP-3 and β-actin.

Gene	Forward sequence	Backward sequence
Bcl-2	TGATAACCGGGAGATCGTGA	AAAGCACATCCAATAAAAAGC
Bax	CCTGAGCTGACCTTGGAGCA	GGTGGTTGCCCTTTTCTACT
CASP-3	CTCGGTCTGGTACAGATGTCGATG	GGTTAACCCGGGTAAGAATGTGCA
β-actin	TCCGTCGCCGGTCCACACCC	TCACCAACTGGGACGATATG

2.12. Western blots

A radio-immunoprecipitation assay buffer with protease and phosphatase inhibitors was used for cardiac tissue lysis prior to protein concentrations assessment using the BCA protein assay kit (Biovision® Inc., CA, USA). Samples were loaded onto a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% non-fat milk for 1.5 h. The primary antibodies against TLR4 (1:2000), MyD88 (1:2000), Gal-3 (1:1000), and NF-κB (1:1000) (Biovision® Inc., CA, USA (Cat. No. 3253, K965, 3244R, A2232 and 3038, respectively) were added to the membranes and allowed to incubate at 4 °C in the blocking solution overnight. After washing for three times with 0.005% TBS-Tween20, the membranes were incubated with the secondary antibody at 1:20,000 for 1 h at room temperature (Cat. No. ab205720). The ChemiDoc MP Imaging System (Bio-Rad Laboratories, Dubai, UAE) was used to visualize the signals. Image J analysis software (1.8.0–112, NIH, MD, USA) was utilized in order to quantify the proteins relative to the reference protein, β-actin.

2.13. Statistics

Data were expressed as mean ± standard deviation (SD). Statistical differences were calculated using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. GraphPad Prism (Prism 9.1, GraphPad Software, Inc., La Jolla, CA, USA) was employed for analysis. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of 2ME on ISO-induced electrocardiographic changes

Fig. 1 and Table 2 show the ECG patterns (heart rate, PR interval, QRS complex duration, RR interval duration, QT interval, QT corrected (QTc), and ST segment amplitude) observed in the rats. Administration of ISO significantly elevated heart rate, QT and QTc intervals, and ST segment amplitude, and reduced PR, QRS complex, and RR interval durations. 2ME, at the three dose levels, antagonized the ISO-induced ECG changes. 2ME at the highest dose (1 mg/kg), significantly restored QT, PR, and RR intervals, as well as ST segment duration.

3.2. Effect of 2ME on ISO-induced histological changes

H&E staining of cardiac specimens from the control and 2ME (1 mg/kg) groups revealed histologically normal cardiac architecture. Sections from the ISO group and rats treated with 2ME (0.25 mg/kg) showed evidence of cardiotoxicity. These manifestations included severe cardiac fibrosis with myxomatous degeneration, and mononuclear inflammatory cell infiltration. Rats pretreated with 2ME (0.5 mg/kg) showed improved cardiac injury with moderate cardiac fibrosis, myxomatous degeneration, and mononuclear inflammatory cell infiltration. Rats administered ISO + 2ME (1 mg/kg) exhibited the highest degree of protection,

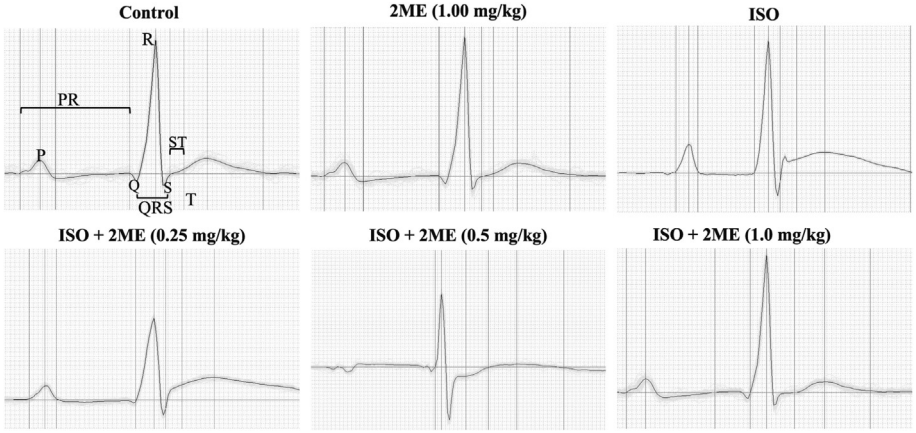

Fig. 1. Effect of 2ME on ISO-induced electrocardiographic changes in rats.

Table 2
Effect of 2ME on ISO-induced changes in rats' ECG tracing.

	HR (beat/min)	PR interval (ms)	QRS complex (ms)	RR interval (ms)	QT interval (ms)	QTc (ms)	ST segment amplitude (μV)
Control	286 ± 11.8	50.7 ± 4.86	35.1 ± 6.71	204 ± 12.8	54.4 ± 4.47	119 ± 2.01	47.9 ± 6.01
2ME 1 mg/kg	284 ± 13.0	51.1 ± 3.10	34.4 ± 5.93	214 ± 10.5	58.0 ± 5.28	126^{a} ± 2.02	51.0 ± 3.46
ISO	$312^{a,b}$ ± 7.32	$41.69^{a,b}$ ± 3.94	28.9 ± 2.17	$183^{a,b}$ ± 5.03	$75.9^{a,b}$ ± 5.60	$173^{a,b}$ ± 1.71	$151^{a,b}$ ± 22.4
2ME 0.25 mg/kg + ISO	$307^{a,b}$ ± 9.49	$40.6^{a,b}$ ± 3.55	29.8 ± 5.99	$188^{a,b}$ ± 6.81	$74.5^{a,b}$ ± 5.16	$169^{a,b,c}$ ± 1.70	$96.0^{a,b,c}$ ± 26.6
2ME 0.5 mg/kg + ISO	296^{c} ± 8.88	45.3^{b} ± 2.59	34.1 ± 5.71	197^{b} ± 8.49	$72.63^{a,b}$ ± 6.76	$161^{b,c,d}$ ± 2.50	$81.9^{a,b,c}$ ± 12.1
2ME 1 mg/kg + ISO	$283^{c,d}$ ± 9.02	$49.3^{c,d}$ ± 4.46	34.75 ± 2.71	$218^{c,d,e}$ ± 10.8	$65.37^{a,c,d}$ ± 3.20	$142^{a,b,c,d,e}$ ± 1.41	$69.3^{c,d}$ ± 12.0

Results are displayed as mean ± SD (n = 8). ISO; isoproterenol, 2ME; 2-methoxyestradiol, ms; millisecond, μV; microvolt. a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$. e: significantly different from ISO + 2ME (0.5 mg/kg) group at $p < 0.05$.

demonstrating almost normal cardiac muscle with a reduced level of inflammatory cell infiltration (Fig. 2).

Masson's trichrome and Sirius red stains are widely applied to characterize collagen and fibrous tissue deposition. In this regard, ISO induced abundant fibrosis, whereas 2ME demonstrated the ability to attenuate these effects (Fig. 3).

3.3. Effect of 2ME on ISO-induced alterations in cardiac injury markers

Serum LDH, CK-MB activities, and troponin levels were found to be raised in rats challenged with ISO relative to the control group. Coadministration of 2ME, in a dose-dependent manner, ameliorated ISO-induced increases in the studied markers. Fig. 4A and B

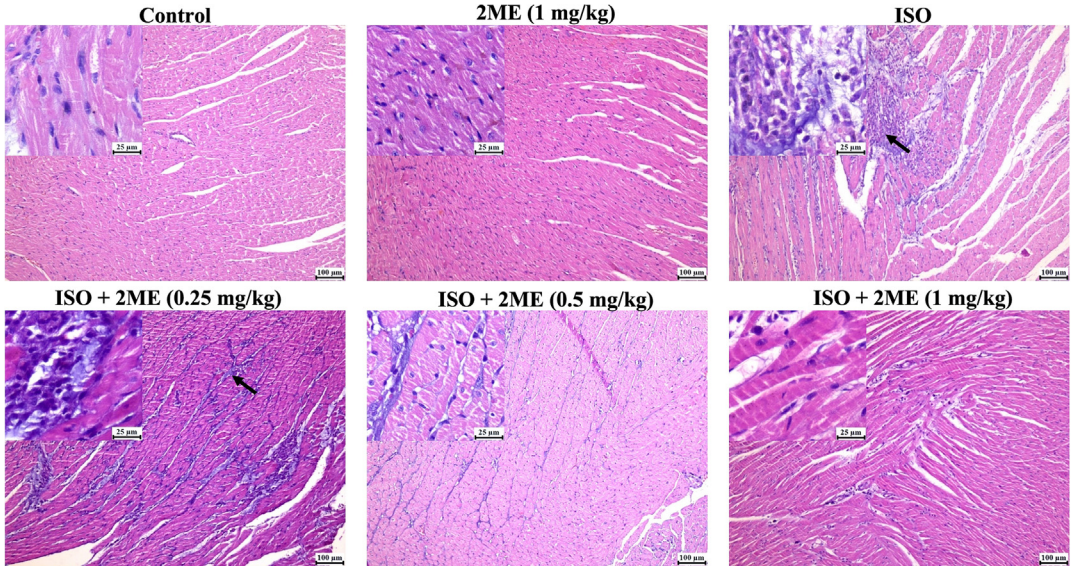

Fig. 2. Effect of 2ME on ISO-induced histological changes in cardiac tissues stained with hematoxylin and eosin stain. Scale bars are 100 μm for the large micrograph and 25 μm for the insert micrograph. ISO; isoproterenol, 2ME; 2-methoxyesterdiol. ISO group showed severe cardiac fibrosis and mononuclear inflammatory cell infiltration (black arrow).

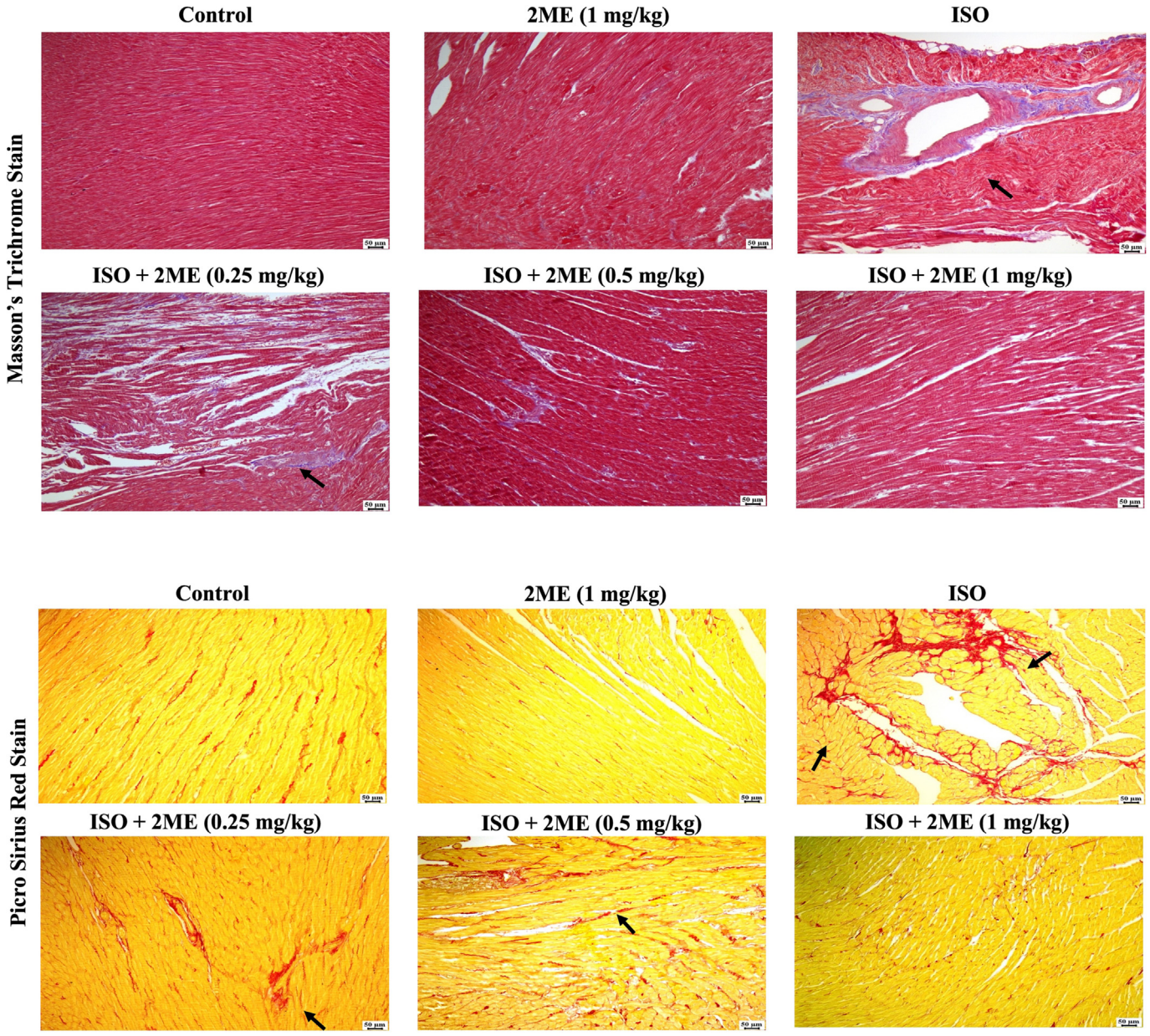

Fig. 3. Effect of 2ME on ISO-induced histopathological changes in cardiac tissues stained by Masson's trichrome (MT) and Sirius red (SR) stains. ISO group shows severe fibrosis (black arrow). Scale bar is 50 µm. ISO; isoproterenol, 2ME; 2-methoxyestradiol. Groups 1 (control) and 2 (2ME 1 mg/kg) show a normal cardiac pattern. Group 3 (ISO) shows abundant fibrosis. Groups 4 (ISO + 2ME 0.25 mg/kg) and 5 (ISO + 2ME 0.5 mg//kg) show moderate to low level of fibrosis. Group 6 (ISO + 2ME 1 mg//kg) evidences the least amount of fibrosis.

show that prior treatment with the highest 2ME dose (1 mg/kg) significantly prevented the enhanced LDH and CK-MB activities in response to the ISO challenge and restored them to normal levels. Fig. 4C demonstrates that 2ME at 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg significantly attenuated the ISO-induced levels of troponin by approximately 14%, 33% and 39%, respectively.

3.4. Effect of 2ME on ISO-induced alterations in cardiac oxidative stress markers

Fig. 5A demonstrates the generation of ISO-augmented oxidative stress, as evidenced by increased cardiac MDA. Pre-treatment with 2ME at doses of 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg significantly antagonized the increased MDA by 17%, 40%, and 47%, respectively. Notably, 2ME at the higher two doses restored MDA to normal levels. Conversely, the ISO challenge significantly depleted GSH and exhausted SOD activities (Fig. 5B and 5C). 2ME administration at the respective used doses increased GSH by 133%, 167%, and 189% relative to the ISO group. Similarly, 2ME ameliorated the exhaustion of SOD activity associated with ISO at all tested doses. Treatment with 2ME (1 mg/kg) significantly normalized the GSH levels and SOD activity.

3.5. Immunohistochemistry staining

As can be seen in Fig. 6, the ISO challenge significantly increased the levels of the pro-inflammatory mediators, COX-2 and iNOS, the transcriptional factor, NF-κB, and the cytokines, TNF-α and IL-6. However, pre-treatment with 0.25 mg/kg 2ME induced a significant decrease in the ISO-enhanced expression of the later markers.

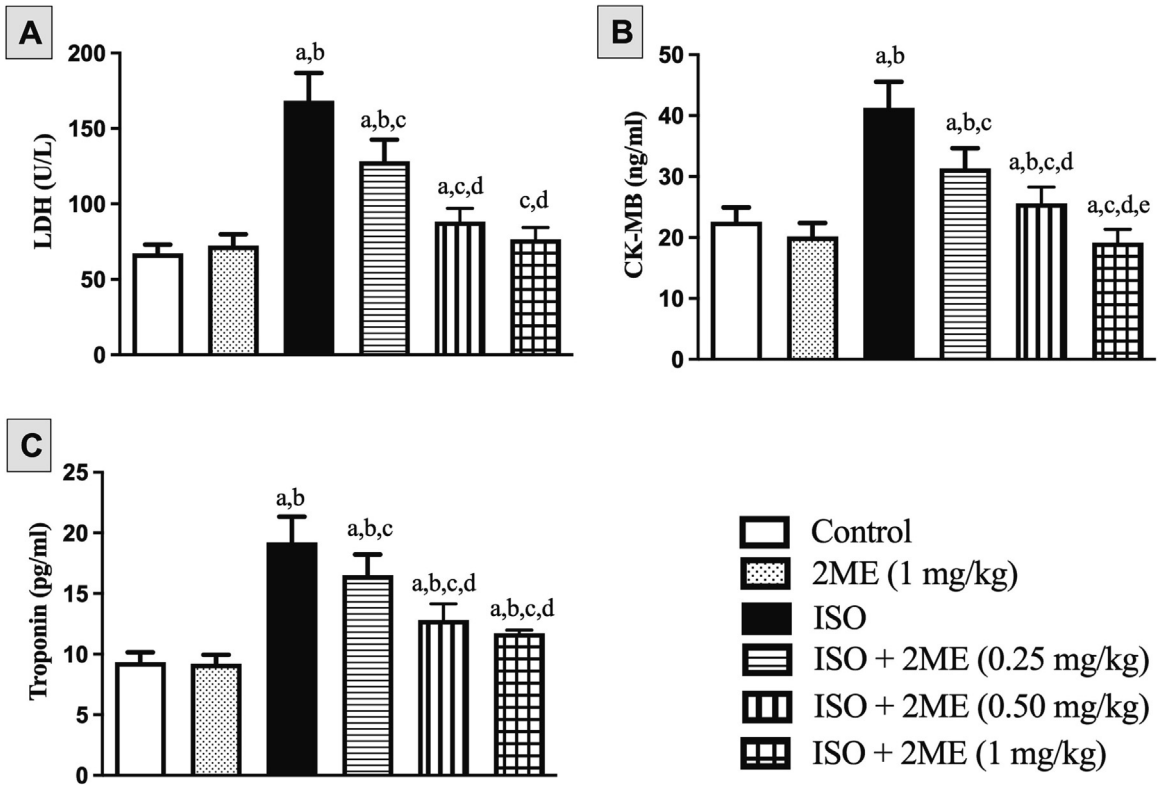

Fig. 4. Effect of 2ME on serum cardiac injury markers in ISO-treated rats. (A) LDH activity, **(B)** CK-MB activity, **(C)** troponin levels. Data are displayed as mean ± SD (n = 8). ISO; isoproterenol, 2ME; 2-methoxyestradiol. a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$. e: significantly different from ISO + 2ME (0.5 mg/kg) group at $p < 0.05$.

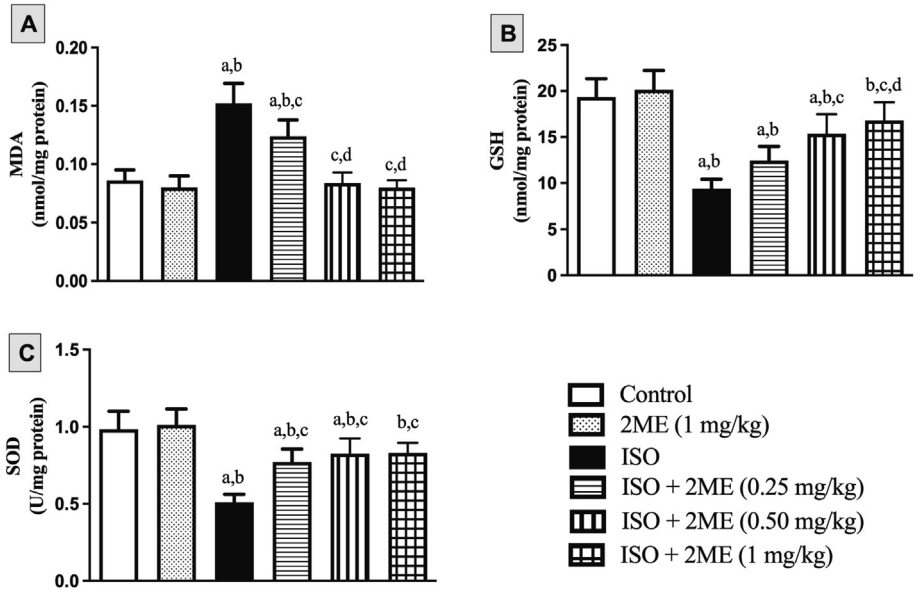

Fig. 5. Effect of 2ME on oxidative status in ISO-induced cardiotoxicity in rats. (A) MDA, **(B)** GSH, **(C)** SOD activity. Data are displayed as mean ± SD (n = 8). ISO; isoproterenol, 2ME; 2-methoxyesterdiol. a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$. e: significantly different from ISO + 2ME (0.5 mg/kg) group at $p < 0.05$.

This effect of 2ME was further improved by increasing the dose to 0.5 mg/kg. Interestingly, the upregulated expression of COX-2, iNOS, NF-κB and TNF-α was significantly restored to normal levels following administration of the highest 2ME dose (Fig. 6).

3.6. Effect of 2ME on ISO-induced alterations in the apoptotic markers' mRNA expression

Anti-apoptotic activity of 2ME in the heart tissues was elucidated by determining the mRNA expression of Bax, Bcl-2, and

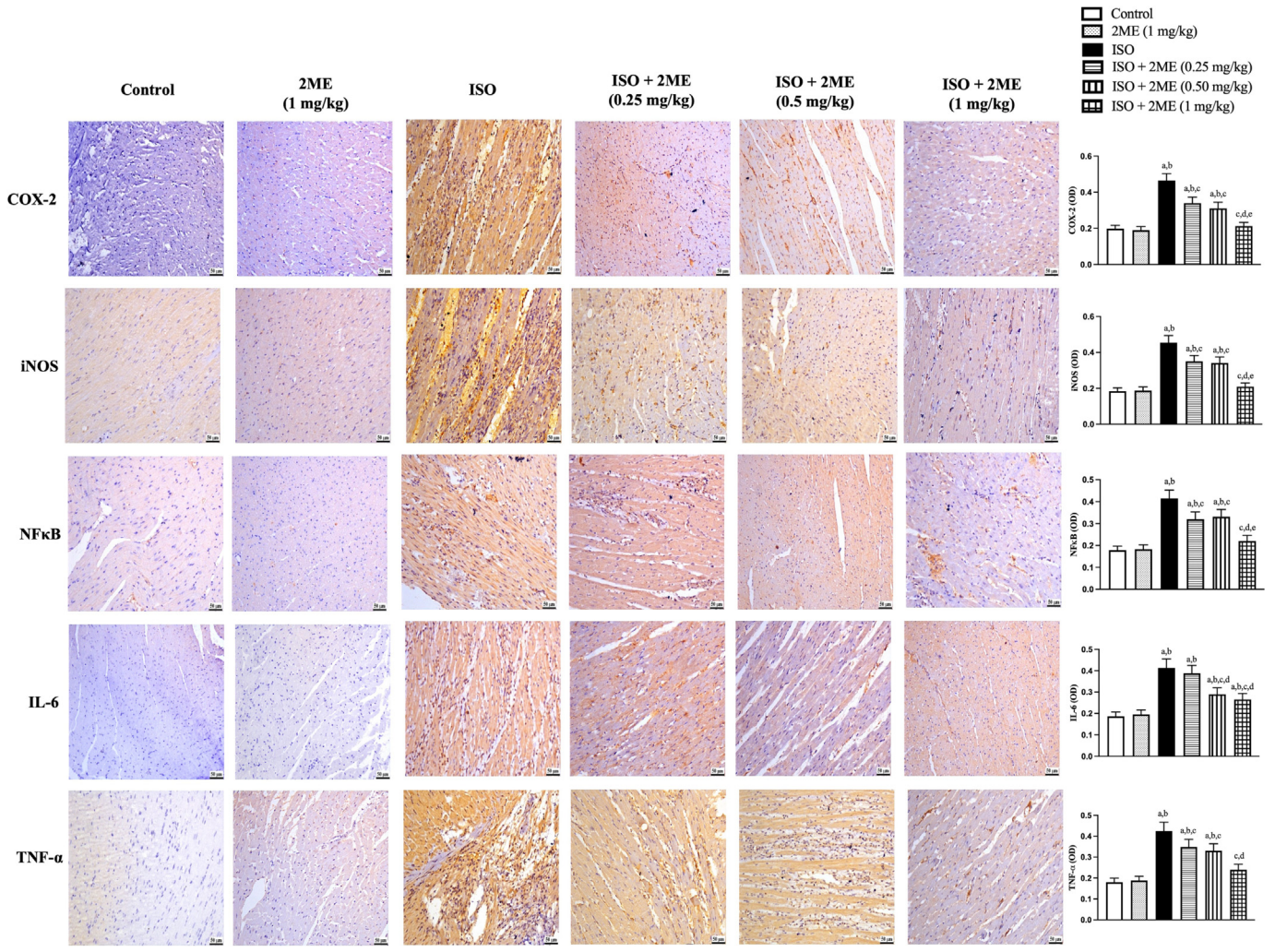

Fig. 6. Effect of 2ME on expression of the pro-inflammatory mediators, COX-2, and iNOS, transcription factor, NF-κB, and cytokines, IL-6 and TNF-α, in ISO-induced cardiotoxicity in rats. ISO; isoproterenol, 2ME; 2-methoxyesterdiol. OD; relative density. a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$. e: significantly different from ISO + 2ME (0.5 mg/kg) group at $p < 0.05$.

CASP-3. Challenging rats with ISO significantly increased Bax mRNA expression (Fig. 7A). However, 2ME, at the used doses, significantly ameliorated this effect by 12%, 32%, and 44%, respectively, in comparison with the effects observed in the ISO group. ISO significantly reduced Bcl-2 mRNA expression. As shown in Fig. 7B, 2ME antagonized this effect and increased Bcl-2 mRNA expression by 25%, 50%, and 88%, respectively, at the used 2ME doses when compared to ISO group. Additionally, ISO illustrated significant pro-apoptotic activity, enhancing CASP-3 mRNA expression. As shown in Fig. 7C, 2ME mitigated the increase in CASP-3 mRNA expression by 6%, 38%, and 44%, respectively, at the used 2ME doses in comparison with ISO group.

3.7. Effect of 2ME on ISO-induced alterations in inflammatory protein expression

Expression levels of the inflammatory proteins, TLR4, MYD88, Gal-3, and NF-κB, were investigated as part of efforts to clarify the mechanisms underlying the protective effects of 2ME against ISO-induced MI. Data presented in Fig. 8 show a significant enhancement of expression of the aforementioned proteins in ISO-challenged group relative to the control cohort. Compared to the group exposed to ISO, pre-treatment of rats with the highest dose of 2ME significantly attenuated the enhanced expression of these proteins (Fig. 8).

4. Discussion

The aim of current study was to evaluate the possible protective effects of 2ME on cardiac injury induced by ISO in rats. The protective effect of 2ME on the altered electrical activity in rats was studied. ISO elevated the ST segment, prolonged the QT and QTc intervals, increased the heart rate, and shortened the RR, PR, and QRS intervals in comparison to the control rats. Studies conducted by Jain et al. (2018), Shaikh et al. (2019), and Xu et al. (2020) reported similar changes in rat ECG parameters (Jain et al., 2018; Shaikh et al., 2019; Xu et al., 2020). The use of 2ME, in this study, effectively counteracted these changes.

In the present study, H&E staining of cardiac tissues subjected to an ISO challenge showed severe cardiac fibrosis with myxomatous degeneration, mononuclear inflammatory cell infiltration, and myocardial degeneration. The extensive fibrosis was confirmed by the application of Sirius red and Masson's trichrome stains. Such changes have been reported previously (Allawadhi et al., 2018; Liu et al., 2018; Salah et al., 2019). 2ME in the present study mitigated against these changes and the highest dose used almost

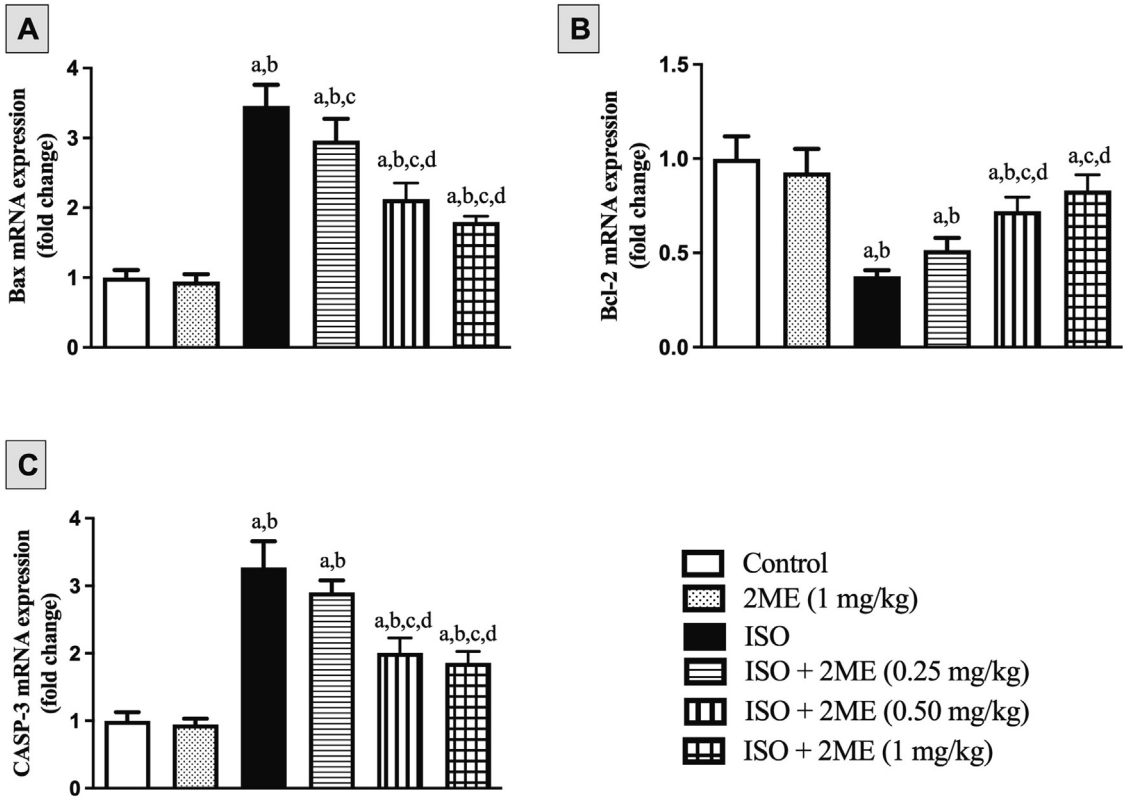

Fig. 7. Effect of 2ME on cardiac mRNA expression of (A) Bax, (B) Bcl-2, and (C) caspase-3 in ISO-treated rats. ISO; isoproterenol, 2ME; 2-methoxyestradiol. a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$.

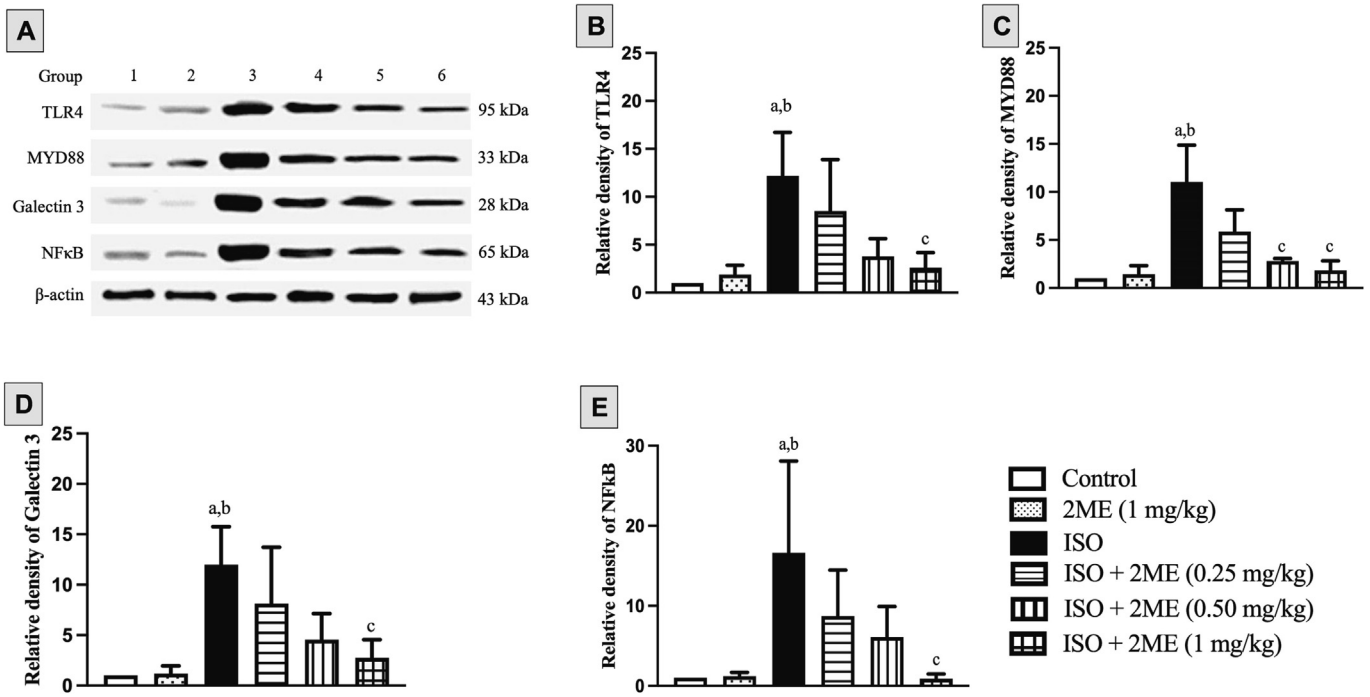

Fig. 8. Effect of 2ME on TLR4, MYD88, Galectin3, and NF-κB protein expression in ISO-induced cardiac injury in rats. (A) Western blot for different experimental groups, group 1 (control), group 2 (2ME 1 mg/kg), group 3 (ISO), group 4 (ISO + 2ME 0.25 mg/kg), group 5 (ISO + 2ME 0.5 mg//kg) and Group 6 (ISO + 2ME 1 mg/kg). The semi-quantification of **(B)** TLR4, **(C)** MYD88, **(D)** Galectin 3, and **(E)** NF-κB expression is shown as the relative density of protein bands normalized to β-actin and to controls. Data are expressed as mean ± SD (n = 3). a: significantly different from the control group at $p < 0.05$. b: significantly different from the 2ME (1 mg/kg) group at $p < 0.05$. c: significantly different from the ISO group at $p < 0.05$. d: significantly different from ISO + 2ME (0.25 mg/kg) group at $p < 0.05$.

restored the histological pattern of cardiac tissue to that seen in the controls.

Challenging rats with ISO in this study raised serum LDH, CK-MB, and troponin levels. The elevation of cardiac markers has been reported to result from their diffusion into the blood circulation (Chen et al., 2021; Gyongyosi et al., 2019; Thangaiyan et al., 2020). Pre-treatment with 2ME, dose-dependently, ameliorated such pathological abnormalities.

Cardiac diseases can result from the augmented generation of ROS relative to the exhausted capacity of antioxidant defense mechanisms (Tsutsui et al., 2011). In the current study, ISO elevated MDA levels and reduced GSH levels and SOD activity, findings which are similar to reports published in previous studies (Chen et al., 2022; Kalkan et al., 2018). 2ME has been previously shown to exhibit antioxidant activities in the lungs (Wang et al., 2017), liver (Neamatallah et al., 2019), and prostate (Abdel-naim et al., 2018). In this study, 2ME intervention, dose-dependently, decreased the accumulation of MDA and increased the levels of SOD activity and GSH content compared to ISO group.

Inflammation plays a significant role in the pathogenesis of ISO-induced cardiac toxicity (Hasan et al., 2020; Kumar et al., 2020). The current investigation revealed that, similarly to previously published reports, challenging rats with ISO upregulated COX-2, iNOS, IL-6 and TNF-α receptor expression (Al-Taweel et al., 2017; Amirshahrokhi and Zohouri, 2021; Cao et al., 2022; Mishra et al., 2018; Othman et al., 2017; Xu et al., 2020). In contrast, 2ME, in a dose-dependent manner, demonstrated significant anti-inflammatory activity as it reduced the amplified expression of the aforementioned proinflammatory cytokines and mediators, findings which are compatible with data from earlier studies (Cho et al., 2017; Liao et al., 2021; Neamatallah et al., 2019).

Caspase-3 activation is a chief determinant of cellular apoptosis (Porter and Jänicke, 1999). The ratio of the apoptotic protein, Bax, and the antiapoptotic protein Bcl-2, determines the activity of caspase-3 and, consequently, apoptosis pathway activity (Mackey et al., 1998). In this study, ISO reduced Bcl-2 and increased Bax and CASP-3 gene expression. This finding aligns with previous reports relating to cardiac (Ma et al., 2018; Othman et al., 2017) and renal tissues (Hasan et al., 2020). The current results also indicate that 2ME, in proportion to the dose used, antagonized the apoptotic activity of ISO as it downregulated Bax and CASP-3 gene expression concurrently with an increase in Bcl-2 gene expression. 2ME has been reported to inhibit apoptosis in the kidneys (Chen et al., 2014) and lungs (Liao et al., 2021) by decreasing CASP-3 gene expression and increasing Bcl-2 gene expression. 2ME has also been observed to diminish Bax expression in left ventricular hypertrophy (Maayah et al., 2018).

Galectin-3 is a binding protein that controls cell adhesion processes. This protein plays a central role in cardiac tissue remodeling, especially in hypertrophy and fibrosis (de Boer et al., 2011; Dumic et al., 2006). Increased Gal-3 expression is observed in many CVDs (Sygitowicz et al., 2022). It is also a positive regulator of TLR4, which promotes cytokine secretion and induces a pro-inflammatory response (Wang et al., 2019a). TLRs are associated with CVD. They play a significant role in myocardial inflammation as they activate the expression of several pro-inflammatory cytokine genes (Vaure and Liu, 2014). They are bound to the MyD88 (adapter molecule), on activating NF-κB (Wang et al., 2019b; Yang et al., 2016).

NF-κB is a principal cytoplasmic regulator of immune homeostasis and inflammation (Mitchell and Carmody 2018; Jing and Li, 2019; Mishra et al., 2018). Once activated, NF-κB undergoes nuclear translocation as regulates the expression of pro-inflammatory mediators and cytokines (Liu et al., 2017). Interestingly, NF-κB is a critical transcription factor in the context of CVD pathogenesis (Chen et al., 2016). 2ME is known to mediate anti-inflammatory activity by suppressing the gene expression of NF-κB in the heart (Maayah et al., 2018) and lungs (Liao et al., 2021).

The current work demonstrates the role of the upregulated expression of Gal-3/TLR4/MyD88/NF-κB signal transduction pathway in the pathogenesis of MI. 2ME significantly reduced such augmented expression in the ISO-challenged groups. Similarly, amelioration of myocardial fibrosis and inflammation in ISO-challenged rats through Gal-3/TLR4/MyD88/NF-κB pathway downregulation has been previously reported (Jin et al., 2020; Xu et al., 2020). Targeting these downstream proteins/kinases allowed 2ME to suppress the inflammatory process and exert its cardioprotective effects.

This research provides insights regarding the potential protective effects of 2ME against acute ISO-induced cardiac injury in rats. However, there are some limitations in the current study. Firstly, it did not investigate acute or chronic 2ME toxicities. Such information is crucial for determining its safety profile that permits its clinical use. Additionally, it examined acute model of cardiac injury. Whilst the acute model provides valuable insights, it provides only a limited vision of the disease process. To have a comprehensive understanding, it is important to investigate 2ME in a chronic model, such as coronary artery ligation, which shows long-term cardiac changes. Lastly, additional signaling pathways, such as mitogen-activated protein kinase, may allow more understanding of the mechanisms of cardiac injury and identify potential alternative targets for therapeutic intervention.

5. Conclusion

The current study indicates the cardioprotective effects of 2ME in acute ISO-induced injury as indicated by amelioration of the altered histopathological pattern, reduction in cardiac injury and inflammatory marker levels, and inhibition of apoptotic gene expression. The cardioprotective effect of 2ME is suggested to be mediated, at least in part, by downregulation of Gal-3/TLR4/MyD88/NF-κB signaling pathway. This study suggests that 2ME represents a potential therapy against myocardial injury and identifies Gal-3/TLR4/MyD88/NF-κB as a promising therapeutic target.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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