Non-coding RNA Research 10 (2025) 192–197

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

Molecular insight of miRNA-217 role in the pathogenesis of myocardial infarction: Promising diagnostic biomarker and therapeutic target

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ARTICLE INFO

Keywords:
miRNA-217
MAPK
PI3K
Cardioprotective
Dihydromyricetin

ABSTRACT

Background: Globally, myocardial infarction (MI) is one of the main causes of death. This study aims to investigate the role of miR-217 in the pathogenesis through targeting MAPK and PI3K/AKT signaling pathways in experimental model of myocardial infarction and studying the possible cardioprotective role of dihydromyricetin (DHM) through modulation of this pathway.

Methods: Dihydromyricetin was injected (100 mg/kg; p.o.) in isoprenaline induced myocardial infarction rat model for 14 days. Rats were anaesthetized and blood samples were taken for serum separation, estimation of creatine kinase-MB (CK-MB), and troponin-I levels after 24 h had passed since the last isoprenaline injection. In addition, the hearts were also used for the other biochemical studies and the histological evaluation.

Results: DHM resulted in a significant suppression of the elevated levels miR-217 and MAPK compared to the MI control group and restored the normal level of serum CK-MB. Furthermore, DHM successfully restored the oxidative balance and halted the pro-inflammatory mediators in the cardiac tissue.

Conclusion: Accordingly, our experiment emphasizes the anti-ischemic property that has been demonstrated through modulation of expression level of miR-217 and consequent deactivation of MAPK and PI3K/AKT signaling pathways, and this was assured by halting downstream pro-inflammatory markers.

1. Introduction

During the last two decades, and despite the variance of income levels, ischemic heart diseases have remained the leading cause of death globally. Ischemic myocardium is a pathologic consequence of either severe imbalance between oxygen supply and demand for the myocardium or obstruction of reperfusion source from the coronary arteries, which results in cardiomyocyte apoptosis which leads to Myocardial Infarction (MI) [1].

The post-ischemic inflammatory response is multi-influenced by critical pathophysiological processes such as complement activation, and macrophage and neutrophils infiltration which induce further release of reactive oxygen species (ROS) and pro-inflammatory cytokines [2].

Mitogen-activated protein kinase (MAPK) signaling pathway is among the molecular pathophysiologic processes involved in MI. MAPK signaling is known to orchestrate several intracellular processes contributing to macrophage and monocytes activation. It is activated through phosphorylation of the kinases at their tyrosine/threonine residues. Consequently, this leads to activation of three main downstream cascades which are ERK (extracellular signal-related kinase), JNK (c-Jun N-Terminal kinase) and p38. Oppositely, MAPK Phosphatases (MKP) is responsible for MAPK deactivation by dephosphorylation of their tyrosine residue, threonine residues, or both [3]. The downstream cascades are involved in modulation of pro-inflammatory and pro-fibrotic processes which influence cardiac remodeling thereafter. By controlling cardiomyocyte size and survival, angiogenic processes, and inflammatory responses, the PI3K/AKT pathway plays crucial roles in the pathophysiology of several human disorders, including heart diseases [4]. It has been reported that the PI3K/Akt pathway plays a role in the occurrence, progression, and treatment of MI. It is involved in survival, proliferation, apoptosis, and other physiological or pathological processes [5].

Nuclear-factor-kappa-B (NF-κB) exhibits a pivotal role in the development of ischemic myocardium. NF-κB is exaggerated as a response to the external stimuli including ROS stress, cytokines, and microbial

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https://doi.org/10.1016/j.ncrna.2024.09.007
Received 6 July 2024; Received in revised form 21 August 2024; Accepted 9 September 2024
Available online 10 September 2024

antigens. Additionally, it acts as an inducer for several proinflammatory molecules such as TNF-α and Il-6 which exhibit detrimental damage to the myocardium during an ischemic attack [6].

MicroRNAs (miRs) are short non-coding RNA molecules. They function as post-transcriptional gene expression modulators, thus used for diagnostic and therapeutic experiments [7,8]. MicroRNAs have been proven to modulate several cardiovascular diseases [9,10]. miR-217 was found to be involved in several myocardial pathologies such as cardiac hypertrophy and ischemia/reperfusion injuries. It has also been proven to induce cardiac hypertrophy through inhibition of the protective Phosphatase and Tensin Homolog (PTEN). Additionally, its down-regulation demonstrated protection against ischemia/reperfusion injuries by deactivating MAPK and NF-κB pathways and restoring mitochondrial function [11]. However, upregulation of miR-217 was involved in further development of atherosclerotic process in coronary arteries through downregulation of endothelial Nitric Oxide Synthase (eNOS) [12], which leads to inevitable increased risk of cardiac disorders.

Dihydromyricetin (DHM), Ampelopsin, is a bioactive flavonoid that is extracted from *Hovenia dulcis, Rhamnaceae, and Cedrus deodara, Pinaceae*. It is mostly abundant in the stems and leaves of *Ampelopsis grossedenata*, *Vitacea* (Vine tea). Vine tea has been used as traditional Chinese medicine for ages for its antipyretic and cough alleviation effect [13]. Pharmacological experiments on extracted DHM have shown promising results for its anti-alcohol intoxication and withdrawal effect, improving insulin resistance, and scavenging reactive oxygen species [14]. It has additionally been shown to exert cardioprotective role through alleviation of myocardial ischemia/reperfusion injuries, Adriamycin-induced cardiotoxicity, and Angiotensin-II Cardiac hypertrophy [15].

The goal of the current study is to determine whether DHM could have cardioprotective effects against isoprenaline-induced MI by evaluating MicroRNA-217 and the MAPK/NF-κB pathways.

2. Materials and methods

- **Animals:**

While using animals in this research, we adhered to National Institutes of Health regulations. The study protocol was approved by the MSA University's ethics committee. In this study, male Sprague-Dawley rats (150–200 g) were utilized. The animals were kept in a facility that was kept at a constant temperature of 25 °C and allowed free access to food and water. All institutional and national guidelines for laboratory animal care and use were followed and the study was approved from the ethics committee of MSA University (Approval no. PH3/REC3/2023PD).

- **Drugs and chemicals:**

Dihydromyricetin was supplied from Double Wood Supplements (USA) and isoprenaline HCl were purchased from Sigma-Aldrich (USA). All other chemicals used were of analytical grade.

- **Experimental design:**

The rats were divided into three groups at random, each with six rats. The groups were normal, MI control groups (subcutaneous 100 mg/kg isoprenaline HCl in the last two days of the experiment) and a third group treated with dihydromyricetin (100 mg/kg; p.o.) for 14 days and isoprenaline in the last 2 days of the experiment. The doses and duration of both isoprenaline and dihydromyricetin were chosen based on previous studies [16,17]. Rats were anaesthetized with urethane (1.5 g/kg; i.p) 24 h after the last isoprenaline injection and blood samples were collected for serum separation and evaluation of creatine kinase-MB (CK-MB) levels. The hearts were also utilized for other biochemical studies and the histological analysis.

2.1. Biochemical assays

A standard kit was used to measure the serum CK-MB level (CAT no.: DEIA-FN285, Creative diagnostics, NY, USA). The following common ELISA kits were used to examine the IL-6 levels in the heart (CAT no. SEA079Ra; Cloud-clone corp, TX., USA). Additionally, commercial kits were used to analyze the heart tissue's SOD activity, GSH content, and MDA content (Bio-diagnostics, Cairo, Egypt).

RNA was extracted from heart tissue using Trizol (Invitrogen; Auckland, New Zealand), and then reverse-transcribed into cDNA with the reverse transcriptase M-MLV (Promega; Madison, WI, USA) in accordance with the manufacturer's instructions for miRNA-217 (miR-217) and mitogen-activated protein kinase (MAPK) quantitative RT-PCR analysis. In the current experiment, the forward and reverse primers for MAPK were 5′- CGAAATGACCGGCTACGTGG -3′ and 5′- CACTT-CATCGTAGGTCAGGC -3′, respectively and for PI3K, forward primer 5′-CTCTCCTGTGCTGGCTACTGT-3′ and reverse primer 5′-GCTCTCGGTTGATTCCAAACT-3′. Additionally, the forward and reverse primers for β-actin were 5′- CTGAGAGGGAAATCGTGCGT -3′ and 5′-TTGTTGGCATAGAGGTCTTTA -3′. Following the manufacturer's instructions, small RNA species-enriched RNA was isolated for miRNA quantitative RT-PCR (mirVana miRNA isolation kit; Ambion, Austin, TX, USA). Using Ncode miRNA first-strand complementary DNA synthesis kits, miRNA was reverse-transcribed (Invitrogen; Auckland, New Zealand). The forward primer sequence was created to correspond to mature miRNA sequences, and U6 snRNA was used as a normalizing control (5′-CTCGCTTCGGCAGCACATATACT-3′ and reverse primer: 5′-ACGCTTCACGAATTTGCGTGTC-3′). The specific primer for miR-217 is as follows: Both the forward and reverse sequences of miR-217 are 5′-TACTGCATCAGGAACTGACTGGA -3'. A Power SYBR Green PCR Master Mix was used to perform quantitative reverse transcriptase PCR (CFX96 Instrument; Bio-Rad, USA). Relative standard curve analysis was used to determine the results of the data analysis.

For Western blot analysis of TNF-α, sections of each lung were homogenized using radioimmunoprecipitation assay (RIPA) buffer. Protein quantification was done according to the manufacturer's instruction using anti-TNF-α (cat #53-7321-82) and anti-β-actin (1:1000; cat#: PA5-16914) antibody (Thermo Fisher Scientific, Waltham, MA, USA). Results were expressed as arbitrary units after normalization for β-actin protein expression [18,19].

- **Histopathological assessment of myocardial damage:**

The heart tissues were washed and fixed in formalin solution (10 %) for 72 h from the various groups. Trimmed samples were processed in successive grades of alcohols, cleared in Xylene, infiltrated into Paraplast tissue embedding medium, and then embedded. Using a rotatory microtome, tissue sections (5 n thick) were cut, H&E-stained, and after which it was examined under a light microscope (Leica Microsystems GmbH, Wetzlar, Germany) [20].

- **Statistical analysis:**

The normality of the data was checked using Shapiro-wilk test. The means of the various groups were compared using the one-way ANOVA test, which was followed by the Tukey-Kramer multiple comparisons test. Dunn's multiple comparisons test was used after the Kruskel-Wallis test to assess the histopathological scores. The cutoff point for significance was set at $p < 0.05$. The GraphPad Prism software, version 5, was used to conduct all statistical analyses (GraphPad Software, Inc., USA).

3. Results

- **Effect on serum CK-MB:**

The current study's findings showed that isoprenaline injection significantly raised the serum levels of the cardiac biomarker CK-MB by 540.5 % compared to normal rats (95 % CI of diff: 751.2 to 448.8). On the other hand, dihydromyricetin therapy returned serum CK-MB levels to normal by decreasing its level by 77.8 % as compared to the MI-control group (Fig. 1A).

- **Effect on cardiac levels of TNF-α and IL-6:**

The current findings demonstrated a considerable increase in TNF-α and IL-6 levels in the cardiac tissue of the MI control group by 435.2 % and 181.8 %, respectively as compared to the rats' normal hearts. The levels of TNF-α and IL-6 in the heart tissue of the dihydromyricetin-treated group, in contrast to the MI control group, were significantly suppressed by 70.53 % and 52.9 %, respectively (Fig. 1B & C).

- **Effect on cardiac levels of miR-217 and MAPK:**

The current study's findings demonstrated a significantly higher level of miR-217 in the heart tissue of the MI control rats by 1348.3 % along with a significantly higher level of cardiac relative MAPK expression by 356.8 %, compared to the heart tissue of the normal group (95 % CI of diff: 16.40 to 10.08 and 4.89 to 2.77; respectively). Dihydromyricetin resulted in a significant suppression of the elevated levels miR-217 by 68.4 % and MAPK by 41.9 % compared to the MI control group (95 % CI of diff: 6.38 to 13.07 and 0.93 to 3.18; respectively) (Fig. 2).

- **Effect on cardiac levels of PI3K and AKT:**

The current study also demonstrated a considerable increase in PI3K and AKT levels in the cardiac tissue of the MI control group by 109.2 % and 277.7 %, respectively as compared to the rats' normal hearts. The levels of PI3K and AKT in the heart tissue of the dihydromyricetin-treated group, in contrast to the MI control group, were significantly suppressed by 48.1 % and 62.3 %, respectively (Fig. 3).

- **Effect on cardiac levels of SOD, GSH, and MDA:**

Additionally, the oxidative stress biomarker MDA was significantly elevated in the hearts of the MI control group by 64.5 % while SOD and GSH were significantly suppressed by 49.1 % and 42.8 % respectively compared to the normal heart samples. Contrarily, dihydromyricetin therapy led to a significant reduction in cardiac MDA content by 38.1 % as well as an increase in cardiac SOD and GSH by 62.0 % and 67.1 %, respectively, when compared to the MI-control group (Fig. 4).

- **Effect on histological examination of heat tissue:**

Normal myocardial bundles with one cardiomyocyte with a central nucleus were visible in normal heart tissue. Multiple areas of myocardium degeneration were produced by isoprenaline injection, along with diffuse infiltration of inflammatory cells throughout the myocardial bundles. On the other hand, the dihydromyricetin-pretreated rats' hearts exhibited few focal areas of inflammatory infiltration and degenerative changes, and most of the myocardium displayed an intact histological structure (Fig. 5).

4. Discussion

Cardiovascular diseases possess a major global health burden, and MI is the most prevalent among them [21]. In the present study, we aimed to investigate the role of the most abundant bioactive compound of vine tea, dihydromyricetin, against the inflammatory response of isoprenaline-induced MI.

Our MI induction model with isoprenaline was preferred to the other models such as coronary artery ligation, due to its relatively safe and valid outcomes. The Isoprenaline model avoids invasive procedures that result in high mortality rates not only from the procedures themselves but also from the high risk of infections and pneumothorax [22].

Oxidative stress play a major role in the pathogenesis of several diseases including cardiovascular, pulmonary and neurodegeneration diseases [23–27] Isoprenaline induces oxidative stress in the myocardium which is expressed by releasing Malondialdehyde (MDA) and reduction of antioxidants such as glutathione (GSH) and Superoxide dismutase (SOD) [28].

Our experiment showed that pre-treatment of mice with DHM showed significant improvement against ROS activity through reduction of MDA and stabilizing of ROS scavengers; SOD and GSH. This has also been observed in high-fat-fed and LDL-deficient mice with induced aortic and hepatic inflammation [29].

The produced oxidative stress in the myocardium aggravated further release of proinflammatory molecules such as TNF-α and IL-6, thus increasing their levels markedly. However, pre-administration of dihydromyricetin led to reducing the released TNF-α and IL-6 in isoprenaline-induced MI, comparatively to the control group. These results were consistent with other experiments that confirmed the anti-inflammatory property of DHM, however, on Lipopolysaccharide (LPS)-

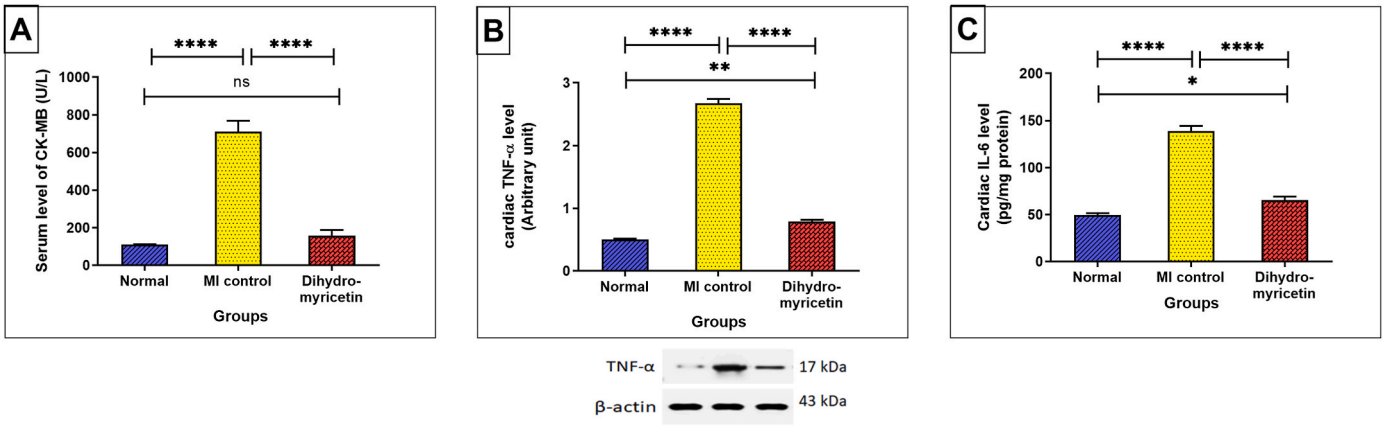

Fig. 1. Biochemical investigations among the different groups: [A] Serum creatine kinase-MB (CK-MB) and [B] cardiac tumor necrosis factor-α (TNF-α) and [C] cardiac interleukin-6 (IL-6) level: Data are offered as mean ± SEM; significant differences from the normal group and the MI control group are indicated by *, **, ***, **** for ($p < 0.05$, 0.01, 0.001, and 0.0001 respectively.

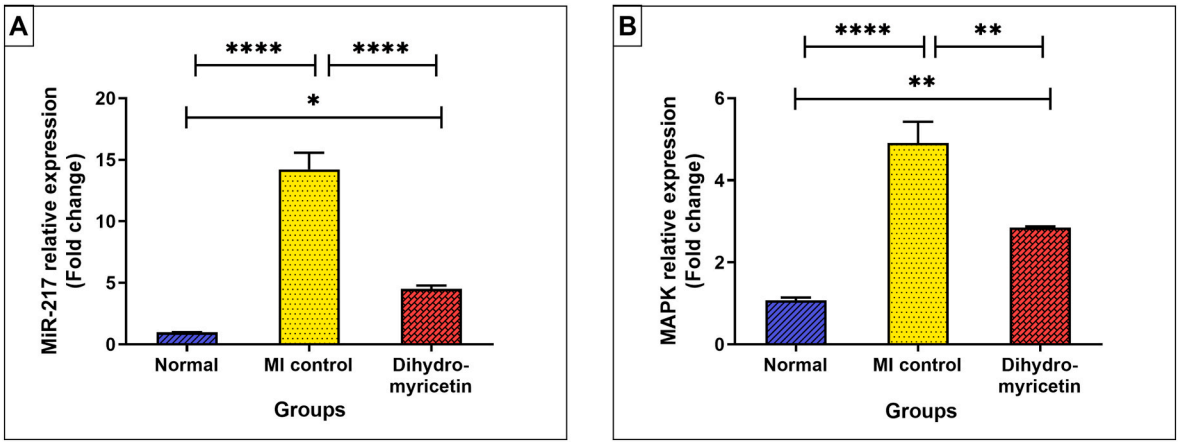

Fig. 2. Cardiac expression levels of miR-217 and mitogen-activated protein kinase (MAPK) among the different groups:
Data are offered as mean ± SEM; significant differences from the normal group and the MI control group are indicated by *, **, ***, **** for ($p < 0.05$, 0.01, 0.001, and 0.0001 respectively.

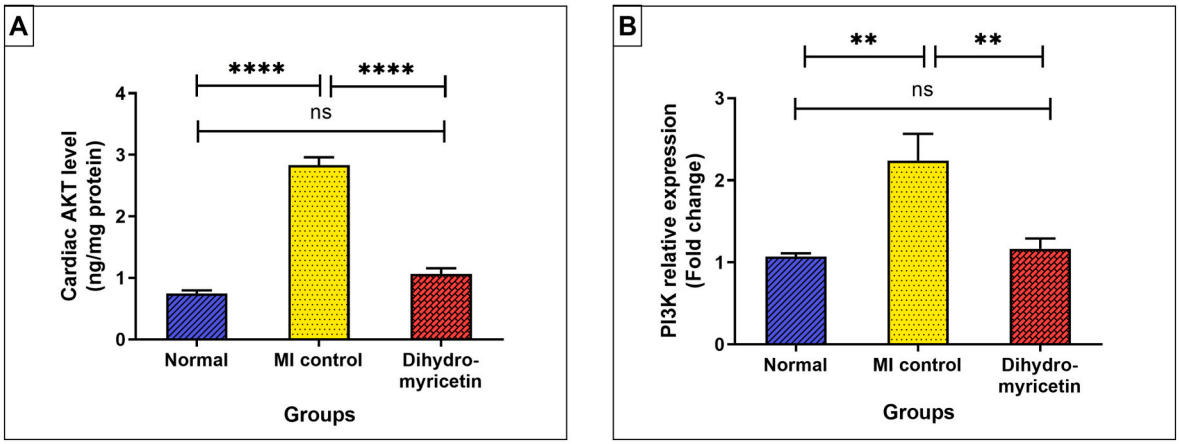

Fig. 3. Cardiac expression levels of PI3K and AKT among the different groups:
Data are offered as mean ± SEM; significant differences from the normal group and the MI control group are indicated by *, **, ***, **** for ($p < 0.05$, 0.01, 0.001, and 0.0001 respectively.

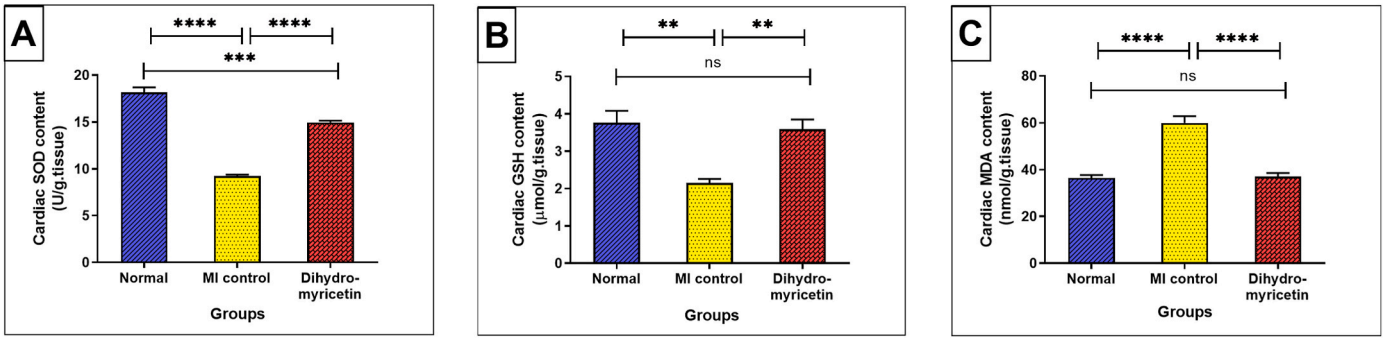

Fig. 4. Oxidative stress biomarkers in heart tissue of the different groups: [A] superoxide dismutase (SOD), [B] reduced glutathione (GSH), and [C] malondialdehyde (MDA):
Data are offered as mean ± SEM; significant differences from the normal group and the MI control group are indicated by *, **, ***, **** for ($p < 0.05$, 0.01, 0.001, and 0.0001 respectively.

treated mice [30], and C57BL/6 mice with pulmonary inflammation [31].

Meanwhile, miR-217 expression level was upregulated in the induction group. This was observed with consequent upregulation of MAPK/NF-κB pathway. It has been previously reported that downregulation of miR-217 is beneficial for myocardial ischemia through modulation of MAPK as well as NF-κB. These results highlight the beneficial role of downregulating of miR-217 which was observed again in other studies against inflammatory atherosclerosis, and cardiac fibrosis [32]. In addition, the miR-217 was reported to increase

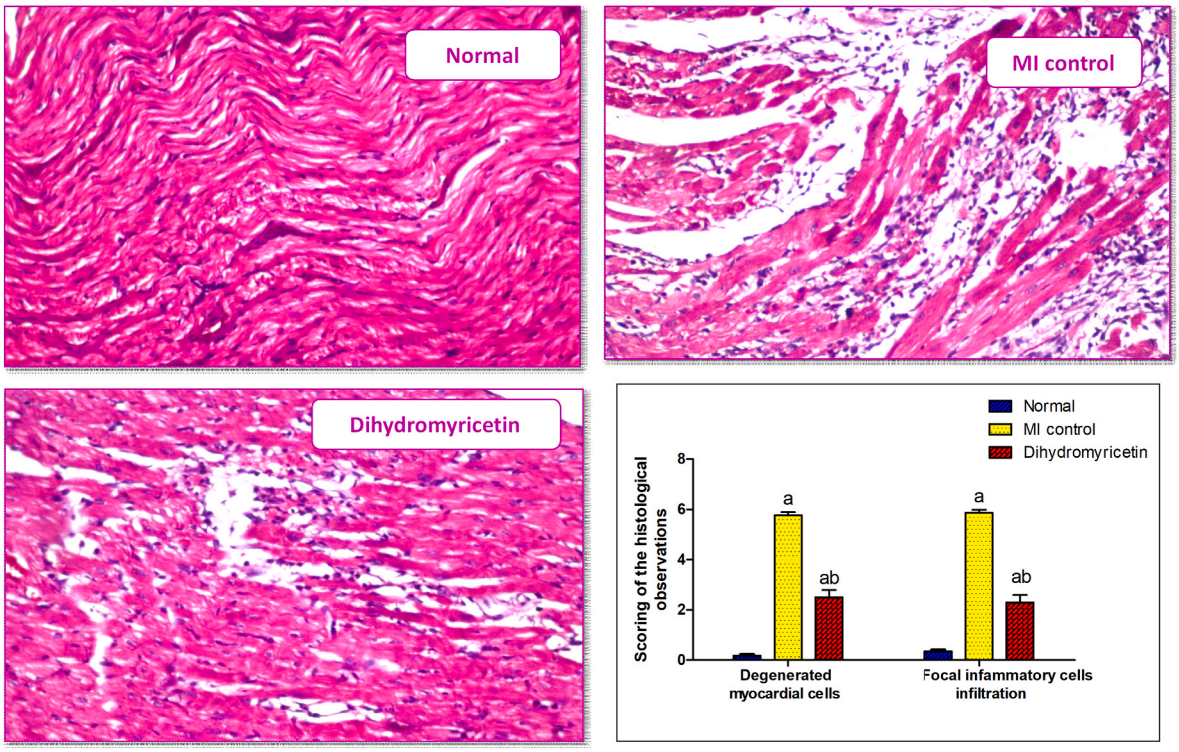

Fig. 5. Histological examination of heat tissue of the different groups:
Data are offered as mean ± SEM**;** significant differences from the normal group and the MI control group are indicated by a and b, respectively ($p < 0.05$).

oxidative damage of cardiomyocytes [33]. Another study has illuminated the underlying mechanism that how miR-217 inhibition exerted a protective effect on myocardial ischemia reperfusion injury via modulation of MAPK signaling pathway and the pro-inflammatory TNF-α and IL-6 [34].

Furthermore, several studies have reported the same anti-inflammatory property of DHM in LPS-treated mice with Acute Lung Injury through downregulation of MAPK pathway separately [30]. It was also reported against Receptor activator of nuclear factor kappa-B ligand (RANKL)-treated RAW264.7 macrophage cells and LPS-treated RAW264.7 cells through NF-κB pathway. Moreover, DHM was found to diminish MAPK/NF-κB signaling in an experiment on mice with LPS-induced inflammation [35]. These matches our results where DHM treatment showed less relative expression of miR-217 and resulted as well in downregulation of MAPK expression levels, and, consequently, their downstream cytokines.

Moreover, the current study demonstrated a significant suppression in the cardiac levels of PI3K and AKT in the dihydromyricetin treated rats as compared to the MI-control rats. The modulation of this signaling pathway is suggested to play a crucial role in the cardioprotective effect of dihydromyricetin and modulation of myocardial level of miR-217. PI3K/AKT signaling pathway has been reported to play fundamental role in the pathological processes leading to atherosclerosis and initiating plaques formation and rupture. Atherogenic stimuli such as IFN-γ, TGF-β, and TNF-α can also activate PI3K/AKT signaling. PI3K/AKT pathway can also modulate adhesion of platelets, and expression of inflammatory molecules [4]. A previous study demonstrated that miR-217 mainly exerts its function by regulation of the WNT, MAPK, and PI3K/AKT signaling pathways that are considered important molecular targets of miR-217 [36].

Accordingly, our findings highlight DHM's overall cardioprotective properties. Furthermore, it suggests the anti-ischemic property that has been demonstrated through modulation of expression level of miR-217 and consequent deactivation of MAPK signaling pathway. This was assured by halting downstream pro-inflammatory markers; TNF-α and IL-6, as well as resolving oxidative stress resembled by modulation of MDA and upregulation of ROS scavengers; GSH and SOD. As a result, we can conclude that dihydromyricetin plays a cardioprotective role in isoprenaline-induced MI by modulating miR-217 and subsequently regulating the MAPK and PI3K/AKT signaling pathways.

Conflict of interest

The authors declared no potential conflict of interest with respect to the research, authorship, and publication of this article.

CRediT authorship contribution statement

Mai A. Zaafan: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Amr M. Abdelhamid:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization.

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.

The author Mai A. Zaafan was not involved in the editorial review or the decision to publish this article.

The authors declare there is no financial interests/personal relationships which may be considered as potential competing interests:

The authors declared no potential conflict of interest with respect to the research, authorship, and publication of this article.

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