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The cardioprotective effect of microRNA-103 inhibitor against isoprenaline-induced myocardial infarction in mice through targeting FADD/RIPK pathway

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Abstract. – OBJECTIVE: The current study investigates the effect of the innovative phosphorothioate modified backbone locked nucleic acid (LNA) of microRNA-103 (miR-103) specifically designed for systemic delivery in the silencing of miR-103 in experimentally induced myocardial infarction (MI). MicroRNA-103 is a small non-coding RNA which regulates Fas-associated protein with death domain (FADD) gene expression, which is a negative regulator for necroptosis occurs during the progression of MI.

MATERIALS AND METHODS: Experimental male mice were allocated into three groups; the first group received normal saline, the second group was injected with isoprenaline and served as the infarcted control, while the third group was treated with LNA miR-103 power inhibitor before isoprenaline injection. Blood and heart samples were used for biochemical analysis of miR-103, FADD, receptor interacting protein kinase (RIPK), nuclear factor-κβ, tumor necrosis factor-α, interleukin-6, troponin-I and creatine kinase-MB (CK-MB) as well as the histological examination of heart tissue.

RESULTS: The treated mice showed marked improvement in the troponin-I and CK-MB levels with almost normal histological structure of heart tissue. Significant inhibition of miR-103 accompanied by increased FADD expression and markedly decreased expression of the other biomarkers were observed in the hearts of the treated mice.

CONCLUSIONS: LNA miR-103 inhibitor is a potent cardioprotective agent and can be a promising treatment against MI through targeting FADD/RIPK pathway.

Key Words:
Myocardial infarction, MicroRNA-103 inhibitor, Fas-associated death domain protein (FADD), Receptor interacting protein kinase (RIPK), Nuclear factor-κβ, Tumor necrosis factor-α (TNF-α).

Introduction

Cardiovascular diseases are the leading cause of death globally taking an estimated 17.9 million lives each year that represent around 31% of all deaths worldwide (WHO, 2018)*. Myocardial infarction (MI) is the myocardial cell death due to prolonged and severe ischemia. Many cell death processes are involved in myocardial cell death, including necroptosis, apoptosis, and autophagy[1]. Necroptosis, the major player in the pathogenesis of MI, is a regulated programmed necrosis that is dependent on a signaling pathway through receptor interacting protein kinase (RIPK)[2]. RIPK is the basic member of a serine-threonine kinase family that transduces inflammatory and cell death signals, including nuclear factor-κβ (NF- κβ) and tumor necrosis factor-α (TNF-α) activation[3]. Fas-Associated Death Domain protein (FADD) is a universal adapter for all TNF receptor-mediated death that functions to inhibit RIPK3 activation, thus blocking necroptosis. FADD is reported to be a negative regulator of the necroptotic pathway and FADD-deficient cells are more susceptible to necroptosis[4].

MicroRNAs are small non-coding RNAs that regulate the expression of genes by preventing the translation process through the destruction of specific target mRNA[5,6]. MicroRNAs have a critical role in the pathogenesis of many cardiovascular diseases, including myocardial infarction, heart failure, and fibrosis[7]. MicroRNA-103

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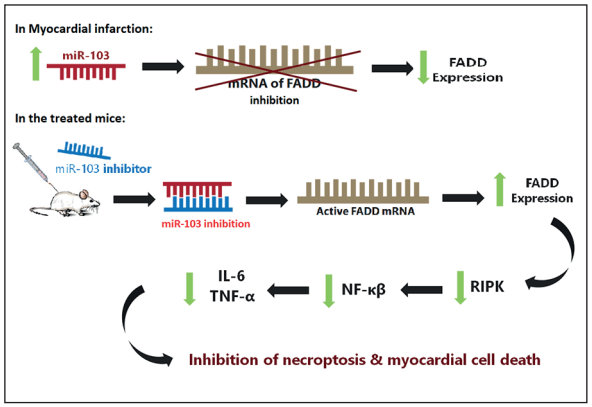

Graphical Abstract: The cardioprotective effect of miRNA 103 inhibitors through targeting the FADD/RIPK pathway. Significant inhibition of miR-103 accompanied by increased FADD expression which is a negative regulator for necroptosis during the progression of MI.

(miR-103) is suggested to play a critical role in the pathogenesis of myocardial cell death and infarction via modulation of FADD expression. The molecular mechanism of miR-103 induced MI is suggested to be a result of the downregulation of FADD expression with subsequent stimulation of the RIPK and TNF-α necroptotic pathways[8]. Thus, the current study suggested that silencing miR-103 through the systemic use of miR-103 inhibitors can provide a promising new approach for the treatment of MI.

Locked Nucleic Acid (LNA) miR-103 inhibitor was used in the current study to be suitable for the systemic delivery in the experimental animals. MiRCURY LNA miRNA power inhibitors are antisense oligonucleotides that have a sequence complementary to their target and have a modified backbone with phosphorothioate (PS). This modification makes them very resistant to enzymatic degradation[9]. Therefore, they are demonstrated to have higher potency and greater stability and to be perfectly suitable for the systemic delivery and *in vivo* applications[10]. Growing studies support that LNA/PS technology provides oligonucleotides with powerful resistance to the enzymatic degradation and markedly improved pharmacokinetics and tissue distribution[11].

The current study aimed to investigate the modulatory effect and the involved molecular mechanism of miRCURY LNA miR-103 power inhibitor against the experimentally induced MI in mice using isoprenaline HCl.

Materials and Methods

Animals

Male mice (15-20 g) were used in the current study. They were purchased from the Egyptian Company for Production of Vaccines, Sera and Drugs (EGYVAC; Cairo, Egypt). Mice were kept in plastic cages under constant conditions of temperature 25 ± 3°C and humidity 50% and allowed free access to water and standard pellet chow.

Animal Ethics Statement

This investigation was approved by the Ethics Committee of October University for Modern Sciences and Arts (MSA University, Giza, Egypt).

Drugs and Chemicals

miRCURY LNA miRNA-103 power inhibitor was purchased from Qiagen Sciences (Germantown, MD, USA). Isoprenaline HCl was obtained from Sigma-Aldrich (St. Louis, MO, USA). All other used chemicals were of analytical grade.

Experimental Design

Mice were randomly allocated into 3 groups (n=6). The first group of mice received sterile saline subcutaneously and served as normal control group. Isoprenaline control group received isoprenaline HCl (100 mg/kg; s.c.) once daily for two successive days for induction of myocardial infarction[12,13]. Finally, the last group was treated with miRCURY LNA miR-103 power inhibitor two hours before the isoprenaline injection in a dose of 4 nmol/mouse; subcutaneously (equivalent to 2.66 mg/kg)[5].

After 24 hours from the last isoprenaline injection, urethane (1.5 g/kg; i.p) was used to anesthetize the animals. Blood samples were collected from the retroorbital plexus and used for the measurement of serum cardiac troponin-I (cTn-I) and creatine kinase-MB (CK-MB). Then, mice were sacrificed by cervical dislocation and the hearts were rapidly isolated.

The hearts were used for total RNA isolation using TRIzol (Invitrogen, Carlsbad, CA, USA) along with the instructions of the manufacturer. Using the Reverse Transcriptase M-MLV (Promega, Madison, WI, USA), RNA was reverse-transcribed into cDNA. The sequences of the primers that were used are shown in Table I.

Small RNA species-enriched RNA was isolated for miRNA quantitative reverse transcriptase PCR as stated in the instructions of the

Table I. Primers sequences.

Gene	Forward primer	Reverse primer
FADD	5′-CTGCGCCGACACGATCTAC-3′	5′-CGGGCCAGTCTTTTCCAGT-3′
RIPK	5′-TTTGGCCTGTCCACATTTCAG-3′	5′-GGTTGGCAACTCAACTTCTCTT-3′
NF-κβ	5′-ATGGCAGACGATGATCCCTAC-3′	5′-CGGAATCGAAATCCCCTCTGTT-3′
TNF-α	5′-CCCACTCTGACCCCTTTACT-3′	5′-TTTGAGTCCTTGATGGTGGT-3′
IL-6	5′-CTGCAAGAGACTTCCATCCAG-3′	5′-AGTGGTATAGACAGGTCTGTTGG-3′
GAPDH	5′-CTGGAGAAACCTGCCAAGTA-3′	5′-TGTTGCTGTAGCCGTATTCA-3′

manufacturer (mirVana miRNA isolation kit; Ambion, Austin, TX, USA). Then, miRNA was reverse-transcribed using the Ncode miRNA first-strand complementary DNA synthesis kits (Invitrogen, Carlsbad, CA, USA).

Forward primer sequence was designed to be the corresponding mature sequences of miRNA and S18 RNA (forward: 5'-TGGTGGAGGGATTTGTCTGG-3' and reverse: 5'-TCAATCTCGGGTGGCTGAAC-3') was used after that as the normalizing control. The miR-103a-3p-specific primer as follows: miR-103a-3p forward: GGGAGCAGCATTGTACAGGG, miR-103a-3p reverse: CAGTGCGTGTCGTGGAGT. Quantitative reverse transcriptase PCR was completed by using a Power SYBR Green PCR Master Mix on the CFX96 Instrument (Bio-Rad, Hercules, CA, USA). The relative standard curve method was used for the data analysis.

In addition, sections of the isolated hearts were fixed in formalin solution (10%) and used for the histopathologic assessment of myocardial damage. Heart tissue sections of 3 μm thickness embedded in paraffin were used for detection of NF-κβ, TNF-α and IL-6 expression through the immunostaining with primary antibody polyclonal immunoglobulin-G of mice NF-κβ, TNF-α as well as IL-6 according to the method previously described by[14]. Finally, the grading of the degree of positive immunohistochemical reactions from 1-5 was performed.

Statistical Analysis

Data in the current study were presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test[15] were used for comparing the means of different groups at level of significance $p< 0.05$. All statistical tests were carried out using GraphPad Prism software package, version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

The results of the current study revealed a significant increase in miR-103 expression in heart tissue of mice with isoprenaline-induced myocardial infarction as compared to the normal mice. The isolated hearts from mice pretreated with LNA miR-103 power inhibitor showed significant suppression of miR-103 as compared to the isoprenaline control mice (Figure 1).

In the isoprenaline control mice, the marked increase in miR-103 resulted in significant inhibition of FADD that is considered the target molecule of miR-103 with a further extensive increase in the expression of RIPK, NF-κβ, TNF-α, and IL-6 as determined by RT-PCR (Figures 2, 3, 4). In addition, results of the immunohistochemical analysis demonstrated the significant increase of NF-κβ, TNF-α, and IL-6 in the heart tissue of the infarcted mice (Figure 5). The myocardial cell damage in the isopren-

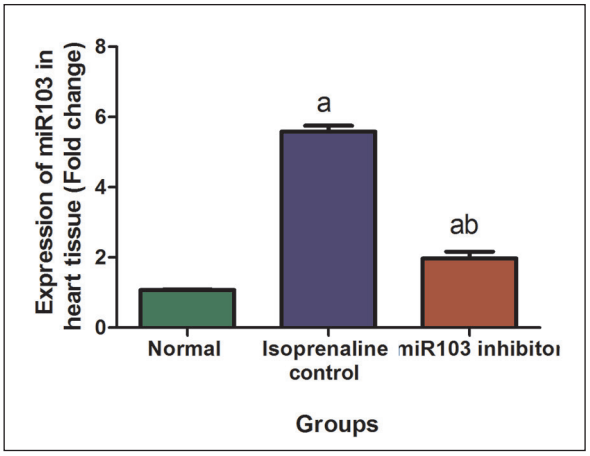

Figure 1. Expression of miR-103 in the heart tissue of mice. Values are presented as mean ± SEM (n = 6). **A,** for the significant difference from the normal group. **B,** for the significant difference from isoprenaline control group (at $p < 0.05$).

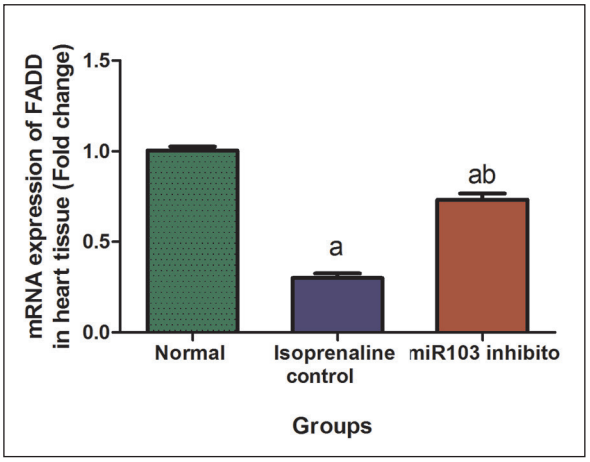

Figure 2. Expression of FAS-associated death domain protein (FADD) in the heart tissue of mice. Values are presented as mean ± SEM (n = 6). **A,** for the significant difference from the normal group. **B,** for the significant difference from isoprenaline control group (at $p < 0.05$).

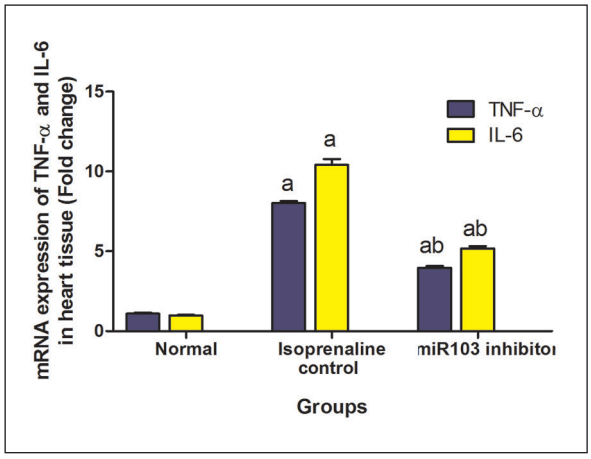

Figure 4. Expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the heart tissue of mice. Values are presented as mean ± SEM (n = 6). **A,** for the significant difference from the normal group. **B,** for the significant difference from isoprenaline control group (at $p < 0.05$).

aline control mice was revealed via the significant increase in the serum levels of CK-MB and troponin-I (Figure 6) and also histopathological examination, which showed multiple focal degenerations of the myocardium with inflammatory cells infiltration (Figure 7).

The powerful effect of miR-103 inhibition was detected through the significant increase in FADD expression in hearts of the pretreated mice as compared to the isoprenaline control mice with further inhibition of RIPK, NF-κβ, TNF-α, and IL-6 expression in hearts of the treated mice (Figures 2, 3, 4). The significant decrease in myocardial levels of NF-κβ, TNF-α, and IL-6 shown by immunohistochemistry further supported the beneficial effect of miR-103 inhibition.

The significant decrease in myocardial contents of the powerful effect of miR103 inhibitor in myocardial infarction is shown in Figure 5. The cardio-protective effect of miR103 inhibitor was reflected through the significant decrease in the serum levels of CK-MB and troponin-I of the treated mice (Figure 6) as well as the histological examination that showed almost normal histological structure of the heart tissue (Figure 7).

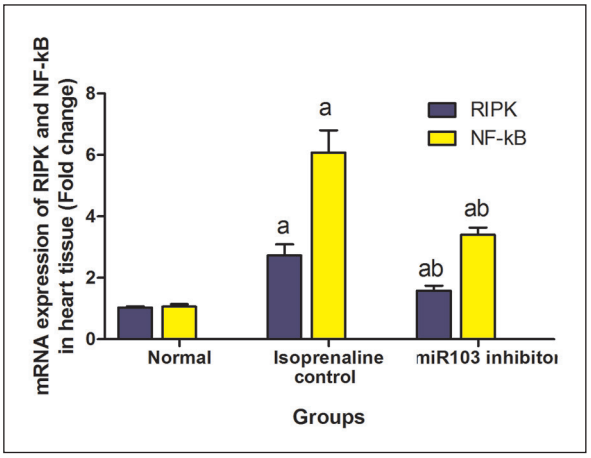

Figure 3. Expression of receptor interacting serine/threonine protein kinase (RIPK) and nuclear factor-κβ (NF- κβ) in the heart tissue of mice. Values are presented as mean ± SEM (n = 6). **A,** for the significant difference from the normal group. **B,** for the significant difference from isoprenaline control group (at $p < 0.05$).

Discussion

Myocardial cell death plays a critical role in the pathogenesis of myocardial infarction (MI). A growing number of studies have emphasized the importance of miRNAs in regulating the incidence of cardiomyocytes death via apoptosis, necroptosis and autophagy[16]. MiR-103 was reported to have an important role in regulating necroptosis and cardiomyocyte death through targeting Fas-associated protein with death domain (FADD). A previous study has demonstrated the marked overexpression of miR-103 in the infarcted areas that is associated with the inhibition of the expression of FADD resulting in sensitization of the cells to necroptosis induction[8].

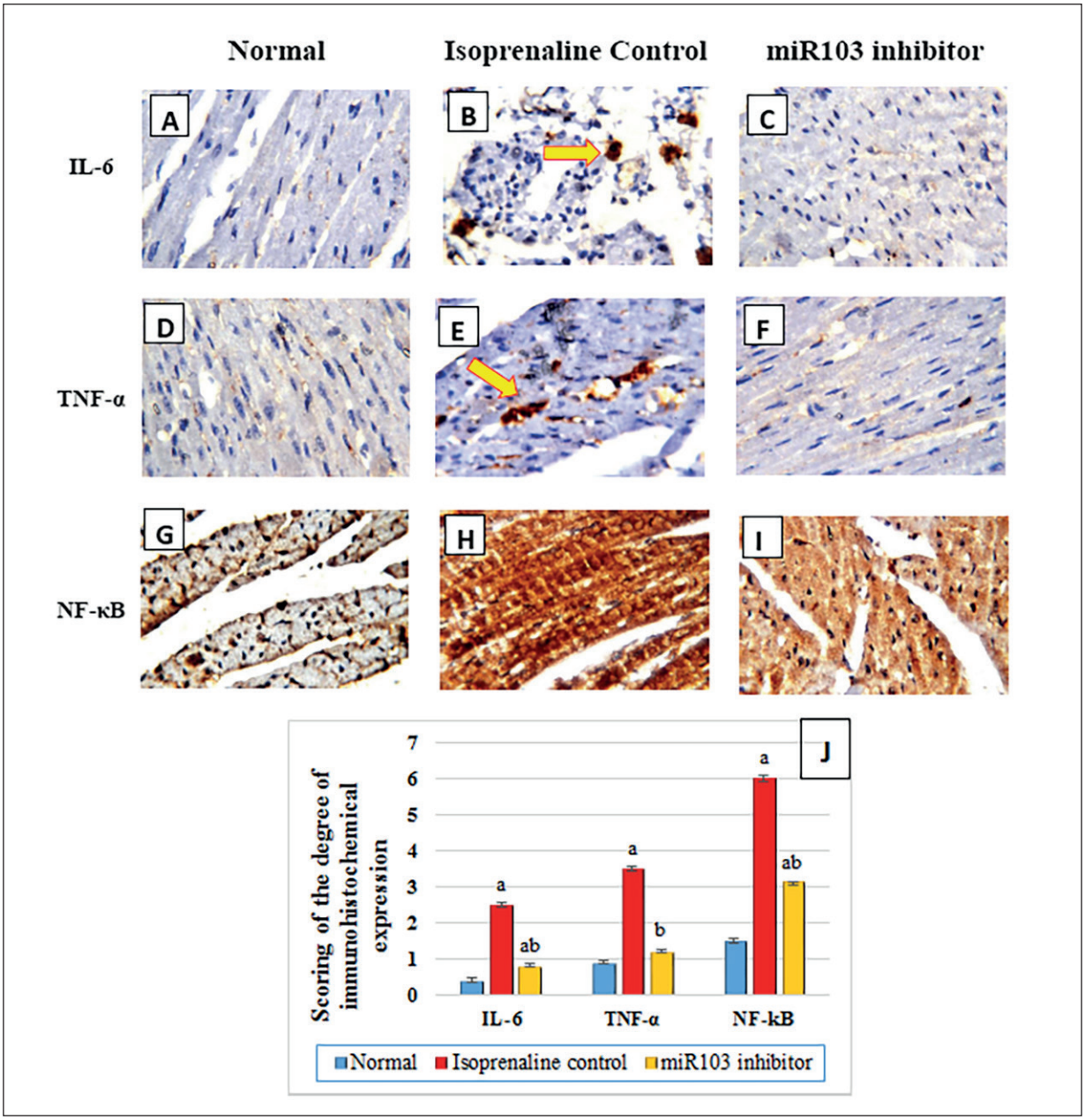

Figure 5. Immunostaining of IL-6, TNF-α and NF-κβ in the heart tissue of mice. Heart sections from normal mice (**A, D** and **G**) showed a small degree of immunostaining for IL-6 and TNF-α with moderate degree of immunostaining for NF-κβ. Conversely, isoprenaline produced marked increase in the immunohistochemical expression of IL-6, TNF-α, and NF-κβ in heart tissue (**B, E,** and **H**). Sections from mice treated with miR103 inhibitor showed significant decrease in the immunohistochemical expression of IL-6, TNF-α, and NF-κβ (**C, F** and **I**) as compared to the isoprenaline control mice. **J,** It represents a comparative quantification of the immunohistochemical expression for IL-6, TNF-α, and NF-κβ in heart tissue of mice from all groups. The severity of the immune reaction depends on the intensity and distribution of the brown color. **A,** for the significant difference from the normal group. **B,** for the significant difference from isoprenaline control group (at $p < 0.05$).

Hence, the current study investigated the effect of miR-103 inhibition as a potential therapeutic target for myocardial infarction treatment. The miRCURY LNA-phosphorothioate (PS) oligonucleotides technology was used to silence miR-103 efficiently with potential *in vivo* systemic application. Recently, LNA antisense oligonucleotides were used to inhibit the non-coding RNAs *in vivo*

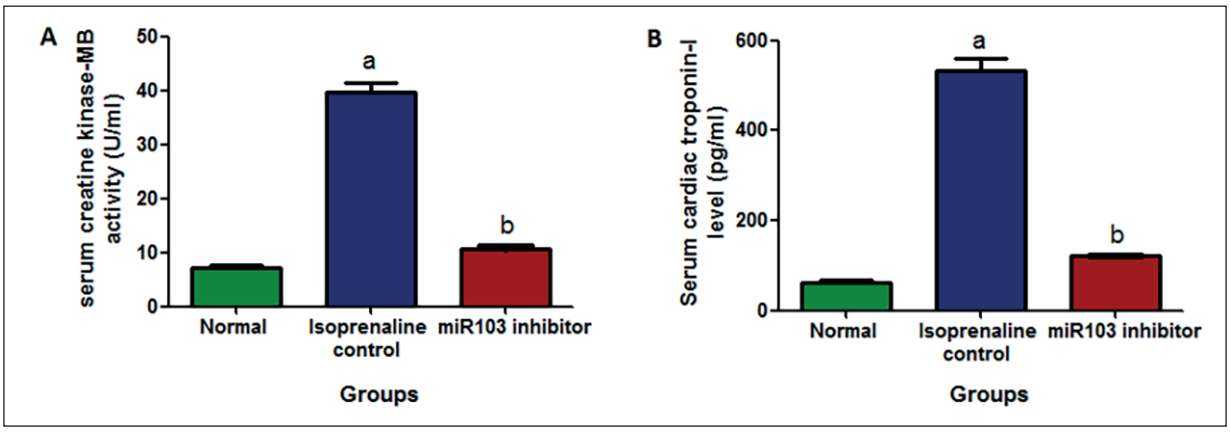

Figure 6. Serum levels of creatine kinase-MB (CK-MB) and cardiac troponin-I. Values are presented as mean ± SEM (n = 6). a: for the significant difference from the normal group, b: for the significant difference from isoprenaline control group (at $p < 0.05$).

as a potential therapy that silence the disease-associated miRNAs[17,18].

Results of the present study demonstrated the marked increase of miR-103 in heart tissue upon induction of myocardial infarction in mice using isoprenaline accompanied by significant inhibition of the relative expression of FADD. These results are in agreement with Wang et al[8] who approved

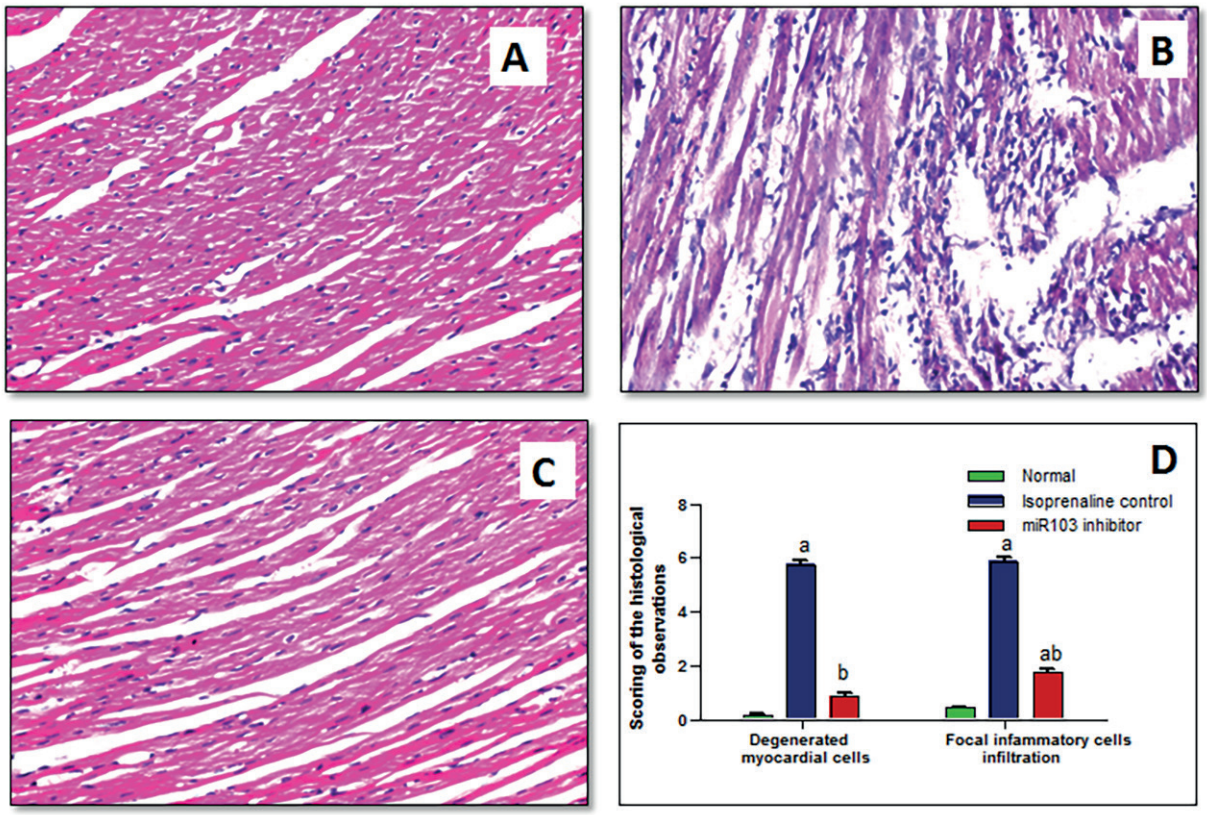

Figure 7. Histological structure of the heart tissue in mice with isoprenaline-induced myocardial infarction. (H&E×16). **A,** Normal histological structure of the myocardial bundles with one centrally nucleated cardiomyocyte in normal mice. Isoprenaline injection produced degenerated myocardium in multiple focal areas with inflammatory cells infiltration in a diffused manner all over the myocardial bundles **(B)**. Alternatively, pretreatment with miR103 inhibitor showed only few inflammatory cells infiltration with almost normal histological structure of heart tissue with **(C)**. Scoring of the histological observations in the myocardium **(D)**.

the role of miR-103 in inducing myocardial injury in a mouse model of ischemia/reperfusion through targeting FADD. FADD was previously proved to be an important negative regulator of necroptosis[19]. Upon trying to elucidate the main molecular mechanism by which the suppressed FADD can induce necroptosis and hence myocardial infarction, an apparent increase in the relative expression of RIPK, NF-κβ, TNF-α and IL-6 in the infarcted hearts was observed in the current study revealing their important role in necroptosis and myocardial cell death. These results support previous research that has suggested necroptosis to be dependent on the activation of receptor interacting serine/threonine-protein kinase (RIPK) 1 and 3, which is negatively regulated by FADD[20]. In addition, results of the present study are consistent with another study that reported a prominent NF-κβ expression concomitantly with the overexpression of RIPK3 in a cell line where necroptosis was associated[21]. The activation of NF-κβ transcription in patients with coronary artery diseases is suggested to further promote the expression of many target genes, including IL-6 and TNF-α that play a major role in the systemic inflammatory response and myocardial injury[22].

Silencing of miR-103 in the current study antagonized the induction of necroptosis and myocardial infarction in the mouse infarcted model with isoprenaline and these results were demonstrated through the significant decrease in the serum levels of CK-MB and troponin-I in the hearts of the treated mice as well as the marked improvement of the histological structure of the hearts.

The present study highlighted the potential role of LNA miR-103 power inhibitor in the treatment of MI through targeting the FADD/RIPK pathway. The results revealed normalization in the miR-103 expression in heart tissue accompanied with a significant increase in FADD expression upon treatment with miR-103 power inhibitor. Moreover, hearts of the treated mice revealed a marked suppression of the expression levels of the proinflammatory mediators and cell death inducers; RIPK, NF-κβ, TNF-α and IL-6 when compared to the infarcted control mice clarifying the molecular mechanism of the modulatory effect of miR-103 inhibitor on myocardial infarction as illustrated in the graphical abstract. These results demonstrate the potential activity of inhibiting miR-103 in the prevention of myocardial cell death and infarction which can be a novel promising treatment for MI and prevention of its major life-threatening cardiovascular complication.

Conclusions

LNA miR-103 power inhibitor is suitable for systemic use and can be considered as a promising treatment for the prevention of myocardial injury and infarction. These data provide the rationale for the clinical development of LNA miR-103 inhibitor for the treatment of MI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

Participated in research design: Zaafan and Abdelhamid. Conducted experiments: Zaafan and Abdelhamid. Performed data analysis: Abdelhamid. Contributed to the writing of the manuscript: Zaafan and Abdelhamid.

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