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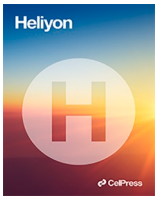

Research article

Cardioprotective effect of antioxidant combination therapy: A highlight on MitoQ plus alpha-lipoic acid beneficial impact on myocardial ischemia-reperfusion injury in aged rats

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ABSTRACT

Objective: (s): Considering the poor prognosis of ischemic heart disease and the diminished effectiveness of cardioprotective interventions in the elderly, it becomes necessary to investigate the interaction of aging with protection during myocardial ischemia/reperfusion injury (IRI). This study was conducted to assess the impact of mitoquinone (MitoQ) and alpha-lipoic acid (ALA) preconditioning on cardioprotection following IRI in aged rats.

Methods: Fifty aged male Wistar rats (22–24 months old) were divided into five groups including Sham, IR, and treatment groups receiving ALA and/or MitoQ. Treatment groups were received 100 mg/kg/day ALA by oral gavage and/or 10 mg/kg/day MitoQ by intraperitoneal injection for 14 consecutive days. An *in vivo* model of myocardial IRI was established through ligation of coronary artery for 30 min and it's reopening for 24 h. The left ventricles were removed at the end of reperfusion to assess oxidative stress indicators, mitochondrial function, and expression of mitochondrial dynamic genes. Myocardial infarct size (IS), hemodynamic parameters, and serum lactate dehydrogenase (LDH) level were also measured.

Results: Combination of MitoQ and ALA reduced oxidative stress, LDH level, and IS in aged hearts subjected to IRI. It also enhanced mitochondrial function and upregulated Mfn1, Mfn2, and Foxo1 and downregulated Drp1 and Fis1 gene expression. Co-administration of MitoQ and ALA partially restored IRI-induced hemodynamic changes to normal state. In all measured parameters, the effect of combined treatment was greater than monotherapies.

Conclusion: The combination therapy of MitoQ and ALA demonstrated considerable therapeutic potential in protecting the aging heart against IRI by improving oxidative stress, mitochondrial function, and dynamics in aged rats.

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1. Introduction

Coronary artery disease is currently the main causes of disability and mortality worldwide [1]. Ischemia/reperfusion injury (IRI) occurs as a result of the returning coronary blood flow into the myocardial tissue after a period of ischemia [2–4]. Besides its therapeutic benefits, reperfusion causes cell death through several mechanisms including increased intracellular calcium, mitochondrial dysfunction, reduced aerobic production of ATP synthesis, increased production of free radicals, and the opening of mitochondrial permeability transition pores [5,6]. Ischemic insult eventually results in decreased mitochondrial oxidative-phosphorylation and increased glycolytic activities and production of lactic acid within cardiomyocytes, leading to elevated release of lactate dehydrogenase (LDH) into circulation in individuals experiencing myocardial infarction (MI) [7]. Moreover, cardiovascular comorbidities, particularly aging, lead to heightened production of reactive oxygen species (ROS) and dysregulation of mitochondrial biogenesis in cardiomyocytes [8]. Therefore, it can be concluded that mitochondrial dysfunction is correspondingly involved in the pathophysiology of both IRI and aging.

During aging, mitochondria undergo structural and functional changes such as reduced activity of its proteins and enzymes [9]. These changes disrupt oxidative-phosphorylation and decrease mitochondrial dynamics by inhibiting mitophagy, releasing proapoptotic cytochrome C, increasing ROS accumulation, and inducing the oxidation of proteins and lipids [10]. It has been reported that mitochondrial dynamics undergo pathological alterations after MI, characterized by a decrease in the expression of fusion proteins, including Mfn2 and Opa1, and an increase in the expression of fission proteins such as Drp1 [10]. Therefore, it appears that targeting mitochondria is crucial in the treatment of myocardial IRI, particularly in the context of aging.

Aging significantly diminishes the efficacy of individual cardioprotective interventions, attributed to numerous negative intracellular alterations. Hence, enhancing the potency of therapeutic interventions becomes imperative in aging conditions [11]. Combination therapy emerges as a practical option to address this challenge, as underscored by the European union-international guideline for cardioprotection [12]. Moreover, the administration of a combination of antioxidants that specifically target mitochondria is highly recommended [13,14].

Mitochondria-targeted antioxidant, mitoquinone (MitoQ), is designed to transport the antioxidant moiety of ubiquinol into the mitochondria [15]. Due to having triphenylphosphonium (TPP), MitoQ has a positive charge that enables it to enter the mitochondrial matrix [15]. Studies have shown that MitoQ is several times more potent than non-targeted antioxidants in preventing mitochondrial oxidative damage [16,17]. MitoQ prevents oxidative damage through inhibiting apoptosis, blocking H2O2, inhibiting the release of cytochrome C, and reducing ROS production and lipid peroxidation [18,19]. It has been shown that MitoQ can be used as a therapeutic target in the treatment of myocardial IRI [20]. Besides, alpha-lipoic acid (ALA) is a cofactor in the mitochondrial dehydrogenase enzyme complex and plays an important role in energy metabolism in mitochondria [21]. Apart from mitochondrial synthesis, ALA is also obtained from dietary sources and accumulates in many tissues. ALA has been shown to possess antioxidative properties and enhance glutathione peroxidase activity, thereby mitigating oxidative stress [22]. This compound demonstrated the potential to reduce infarct size and enhance myocardial function in young rats, providing protection against acute AMI [23,24]. Furthermore, its application exhibited a capacity to maintain cardiac function and preserve structural integrity throughout the progression of left ventricular remodeling [24].

Considering the theory of free radicals and the reduction of mitochondrial function in aging, the need to use combined treatments for cardiaoprotection in aging is persuasive. In this way, it seems that the combination of ALA and MitoQ, as antioxidants targeting mitochondria, could be suitable candidates to be used to reduce myocardial damage caused by IRI during aging. Therefore, the aim of this study was to investigate the effect of ALA and MitoQ combination therapy on cardiaoprotection, oxidative stress, and mitochondrial function following IRI in the heart of aged rats.

2. Material and methods

2.1. Animals

Fifty aged male Wistar rats (22–24 months old, weighing 450–500 g) were attained from the Pasteur Institute of Iran and transported to Drug Applied Research Center. Rats (4 rat per cage) were kept under controlled conditions (22 °C temperature, 12-h dark-and 12-h light cycle). Food and water were available *ad libitum* to the animals. All guidelines and experiments were conducted in line with the "Principles of Laboratory Animal Care", specified by the Ethical Committee of Tabriz University of Medical Sciences (No. IR.TBZMED.VCR.REC.1399.479).

2.2. Experimental design

One week after transfer to Research Center and adaptation to the environment, the rats were randomly allocated into 5 groups (n = 10):

1) Sham group (underwent chest surgery without IRI induction)
2) IR group (underwent chest surgery with IRI induction)
3) IR group with ALA (IR + ALA)
4) IR group with MitoQ (IR + MitoQ)
5) Combined group (IR + ALA + MitoQ)

Treatment groups were received 100 mg/kg/day ALA (62320, purity 98%, Sigma-Aldrich, USA) by oral gavage or 10 mg/kg/day MitoQ (GC30416, purity 98%, GLPBIO Technology, USA) by intraperitoneal injection for 14 consecutive days [25–27] before induction of ischemia. The injection volumes ranged from 0.2 to 0.4 mL. ALA and MitoQ were dissolved in 10% corn oil or 1% DMSO, respectively. The injection volumes were 0.2–0.45 mL. Both the Sham and IR groups received the same amounts of corn oil or DMSO as the vehicle.

2.3. Myocardial ischemia-reperfusion injury protocol

According to the previous experimental studies, induction of ischemia was performed as an *in vivo* model in the myocardium of aged male rats [28,29]. In all groups, the animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg) [30–33], and after intubation, their chests were opened to access the heart under sterile conditions. Then, the animals were connected to a mechanical ventilator (with a respiratory rate of 50–60 breaths per minute and a flow volume of 15 mL/kg). To maintain the body temperature of the animal in physiological conditions, a heating pad was placed under the rats. A left thoracotomy was established between the fourth intercostal space, and then the intercostal muscles and the pericardium were separated to fully expose the heart. In the IR groups, to cause regional ischemia, the left anterior descending (LAD) coronary artery was ligated by 6–0 silk suture for 30 min. To create reperfusion, the tied thread on the LAD was opened and reperfusion was established for 24 h. After initiation of reperfusion, the chest and muscle layers were closed by suturing. Tetracycline topical ointment as an antibiotic and buprenorphine (0.05 mg/kg, subcutaneously) as analgesic drugs was given to the animal after surgery [34]. The electrocardiography was recorded using Powerlab data-acquisition system and LabChart-7.3 software (ADInstruments, Australia) and the ST segment elevation during the ischemia phase confirmed the successful induction of ischemia.

2.4. Hemodynamic measurement

Twenty-four hours after reperfusion, rats were anesthetized and the right carotid artery was cannulated with a PE-50 polyethylene cannula for 20 min to measurement of arterial systolic and diastolic pressures (in mmHg) and heart rates (HR; in beats per minutes). After 20 min, to evaluate left ventricular function, the catheter was inserted into the left ventricle (LV) for the measurement of LV pressures. The catheter was connected to a pressure transducer, bridge amplifier, and the PowerLab data acquisition system (ADInstruments, Australia) [11]. HR, LV developed pressure (LVDP), and rate-pressure product (RPP) as an index for cardiac contractility were calculated and analyzed on a computer using LabChart 7.3 software (AD Instruments, Australia).

2.5. Measurement of lactate dehydrogenase (LDH)

In order to measure myocardial cellular damage, the serum LDH level was measured via autoanalyzer device (Alcyon 300, Abbott Labs, Santa Clara, CA, USA) and a LDH assay kit (Pars Azmoon Co., Karaj, Iran). The calorimetric method was conducted following the manufacturer's instructions, and the results were reported in units per liter (U/L) [28].

2.6. Measurement of infarct size (IS)

After 24 h, the LAD was clamped again under anesthesia and 2–3 mL of 2.5% Evans blue dye (Sigma, Germany) was slowly infused through the tail vein. In this way, areas at risk (AAR) were unstained separated from blued, live myocardial tissue. After freezing the hearts at −20°, 2 mm thick cross-sections were prepared from the apex to the base of the frozen hearts. Then the slices were incubated in 2,3,5-triphenyltetrazolium-chloride 1%(TTC, Sigma, Germany) for 15 min dissolved in PBS (pH 7.4), at 37 °C. Later, the slices were fixed in 10% formalin for 24 h. Finally, cardiac slices were photographed and ImageJ software (NIH, Bethesda, Maryland) was used to quantify IS and AAR. AAR and IS were expressed as the percentages of the LV and AAR volumes, respectively [11,35].

2.7. Isolation of cardiac mitochondria

The fresh LV of the myocardium was rapidly removed at the end of reperfusion for mitochondria isolation. Mitochondria isolation was performed at cold temperature at 4 °C. After harvesting the tissue, it was minced in mitochondrial isolation buffer A (containing: 200-mM mannitol, 70-mM sucrose, 10-mM HEPES, and 2-mM EDTA, pH 7.5.) and homogenized by a tissue grinder. The homogenized tissue was centrifuged at 1300 g for 10 min. Then, the supernatant was centrifuged for 10 min at 12,000 g to precipitate the mitochondria. Finally, 100 mL storage buffer (containing: 10-mM HEPES, pH 7.4, 250-mM sucrose, 1-mM ATP, 0.08-mM ADP, 5-mM sodium succinate, 2-mM K2HPO4 and 1-mM DTT pH 7.4, was used to suspend the mitochondrial pellet. The mitochondrial protein concentrations was evaluated using Nanodrop [11,36].

2.8. Assessment of cardiac mitochondrial ROS generation

Isolated heart mitochondria were incubated in 40 μl Dichlorodihydrofluorescein diacetate (DCFDA) dye at 25 °C for 20 min in the dark. In the presence of ROS, DCFDA was oxidized to 2′,7′-dichlorofluorescein. The dye was excited at 485 nm wavelength and the amount of light passing through 530 nm was determined using a fluorescent microplate reader (LabTech, USA) (Ex/Em = 485/530 nm). An increase in fluorescent intensity was indicative of an increase in mitochondrial ROS production [37].

2.9. Measurement of cardiac mitochondrial membrane potential changes

Mitochondrial membrane potential changes were evaluated using JC-1 dye (Sigma-Aldrich, USA) according to the manufacturer's instructions. Isolated myocardial mitochondria were incubated for 30 min at 37 °C in JC-1 dye (2.5 mg/mL) in the dark. In normal mitochondria (high membrane potential), JC-1 was accumulated in polymer form and emitted red fluorescent when stimulated. But, in the mitochondria where the membrane potential was reduced and depolarized, JC-1 was in monomeric form and emitted green fluorescent when stimulated. The fluorescent values were recorded by means of the fluorescent reader (LabTech, USA), and the reduction in the red/green fluorescent ratio was indicated as cardiac mitochondrial membrane depolarization [11].

2.10. Assessment of oxidative stress

In order to determine the level of oxidative stress in cardiac cells, oxidative stress markers superoxide dismutase (SOD), glutathione peroxidase (GPX) and malondialdehyde (MDA) were measured. SOD and GPX enzyme activity was calculated in the supernatant solution prepared from cardiac AAR tissue homogenate using the RANDOX kit (RANDOX, UK). SOD and GPX activity was measured at 505 nm and 340 nm, respectively via a spectrophotometer (UV–vis Array, Photonix Ar 2015; Electron Co., Iran). The obtained results were divided by the amount of total protein and expressed per milligrams of protein. Quantification of MDA, as a marker of Lipid peroxidation, was performed by measuring substances reactive to thiobarbituric acid in homogenous heart tissue. MDA level was expressed as nmol/mg protein [38].

2.11. Gene expression analysis

Expression of genes Mfn1, Mfn2, Foxo1, Drp1, Fis1, and GAPDH as the internal control was measured by real-time PCR method. Total RNA was extracted from the myocardial tissue AAR samples using the RNX-Plus solution kit (Yekta Tajhiz Azma, Cat No. FABRK001, Iran) according to the manufacturer's Protocol. Quantity and purity of the RNA were measured using a NanoDrop 1000 (NanoDrop OneC, Thermo Scientific, USA). Reverse transcription of the RNA samples into cDNA was performed using the PrimerScript RT Master Mix Perfect Real-Time Kit (Yekta Tajhiz Azma; Cat No. YT4500, Iran) according to the manufacturer's Protocol. Real-time PCR was carried out using SYBR Green kit (Yekta Tajhiz, Iran) according to the manufacturer's instructions. Real time-PCR measurement was performed by Corbett PCR machine (Rotor-GeneRG-6000, Real Time PCR Analyzer, Corbett, Australia). The primers of genes were designed by Oligo7 software and synthesized by Pishgam Biotech Co. (Iran). The $2^{-\Delta\Delta CT}$ method was used to analyze the results. Table 1 shows the sequences of forward, reverse primers [30].

2.12. Statistical analysis

Data are presented as the mean ± standard error of mean (SEM). Data were statistically analyzed by Graph Pad Prism version 9. The measured parametric variables were analyzed using *one-way ANOVA*. The post hoc *Tukey* test was used to determine the statistical significance between groups. The mean differences were considered significant if $P < 0.05$.

3. Result

3.1. Hemodynamic indices following IRI

Induction of IRI significantly declined arterial systolic blood pressure (Fig. 1A). In the IR group in comparison with the sham group ($p < 0.01$). Monotherapy with ALA or MitoQ ($p < 0.01$) and combination therapy ($p < 0.05$) significantly restored arterial systolic blood pressure compared to the IR group (Fig. 1A). As shown in Fig. 1B, treatment groups including monotherapy and combination therapy significantly increased arterial diastolic blood pressure compared to the IR group ($p < 0.001$). There was no significant difference in mean arterial pressure (MAP) among the groups (Fig. 1C). The HR (Fig. 1D) showed no significant changes among groups except that it was increased in ALA group compared to the IR group ($p < 0.05$). As shown in Fig. 1E, LVDP was significantly decreased in the IR group compared to the sham group ($p < 0.01$), while this parameter considerably increased in the combined group compared to the IR group ($p < 0.05$). Additionally, RPP as a contractility index was decreased significantly in the group exposed to IRI compared to the sham ($p < 0.05$), and the combination therapy of MitoQ and ALA significantly reversed this effect ($p < 0.05$) (Fig. 1F).

Table 1
The sequences of forward and reverse primers of mitochondrial dynamics genes used for real-time PCR analysis.

GENE NAME	SEQUENCE OF FORWARD PRIMERS	SEQUENCE OF REVERSE PRIMER
Mfn2-rn	CCTGGGCTTTAGACTCAACCAG	CATCACAATGCCAGACACCAAC
Mfn1-rn	AAGCAACATACAGGAACCCGGAA	GCCAAAAAATGCCACTTTCATATGC
Foxo1-rn	ATAACTGTGCCCCAGGACTCT	TTGAGCCACTCCAGGATCGAC
Drp1	TTCTTCCCAGAGGGACTGGT	GAAATTTACCCCATTCTTCTGCT
Fis1	GGGTTACATGGATGCCCAGA	AGGCACCAGGCGTATTCAAA
GAPDH	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT

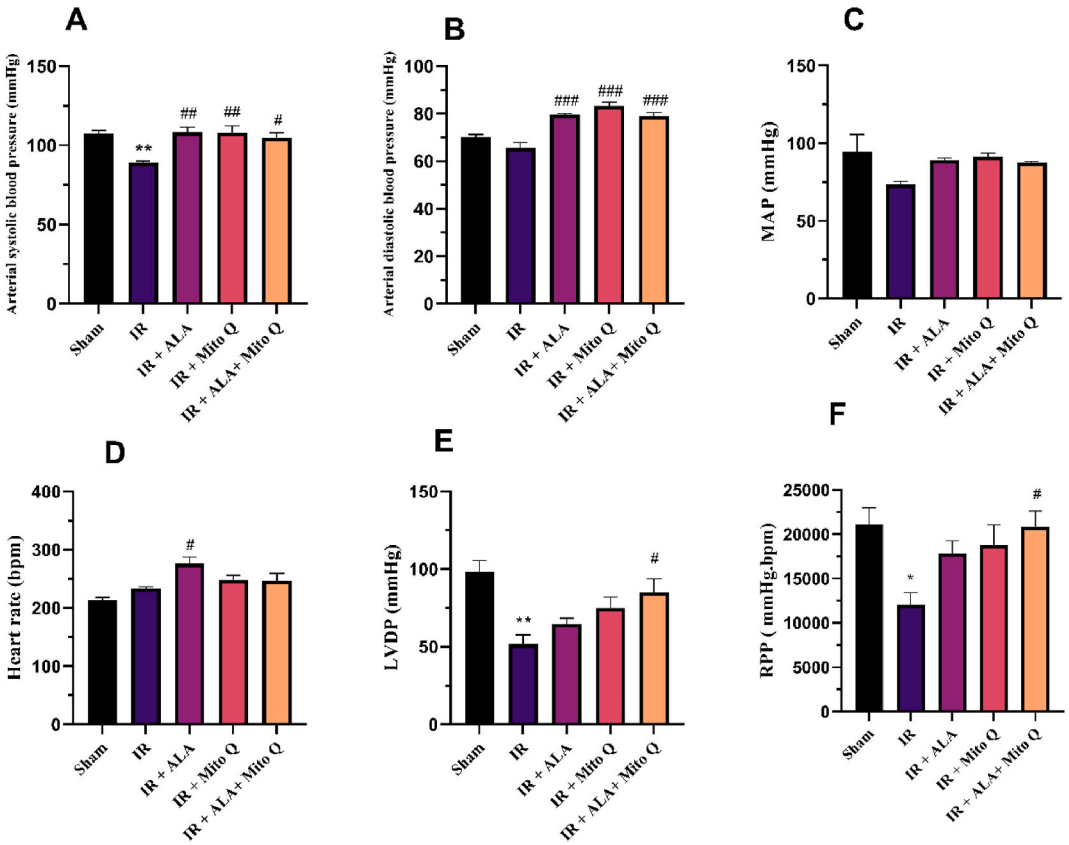

Fig. 1. Hemodynamic indices in aged hearts following IR injury. Arterial systolic blood pressure (A), Arterial diastolic blood pressure (B), Mean arterial pressure, MAP (C), Heart rate (D), Left ventricular developed pressure, LVDP (E) and rate-pressure product, RPP (F). Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM.*p < 0.05 vs. sham group, **p < 0.01 vs. sham group, #p < 0.05 vs. IR group, ##p < 0.01 vs. IR group, ###p < 0.001 vs. IR group. IR: ischemia-reperfusion; ALA: alpha lipoic acid.

3.2. The level of LDH release

The level of LDH in the IR group has raised significantly in comparison with the sham group ($p < 0.05$). Combined treatment with ALA and MitoQ significantly ($p < 0.05$) reduced the activity of LDH compared to IR group. Administration of ALA and MitoQ alone, decreased the level of LDH, but this improvement did not reach a statistically significant level (Fig. 2).

3.3. AAR and infarct size

The volumes of AAR increased in IR group in comparison with sham group ($p < 0.001$). But, there was no significant difference between IR and treatment groups (Fig. 3A). Infarct size (Fig. 3B) in IR group increased significantly compared to the sham group ($p < 0.001$), while in the combined group the infarct size was declined considerably compared to the IR group ($p < 0.05$). In the monotherapy groups, the reduction in infarct size was not statistically significant.

3.4. Mitochondrial function

3.4.1. Cardiac mitochondrial ROS

Our results shown that production of mitochondrial ROS (Fig. 4A) in IR group were high in comparison to sham group ($p < 0.001$). Fig. 4A displays that the level of mitochondrial ROS in monotherapies as well as combination therapy was declined significantly ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively). Moreover, there was a significant difference between the combined group and the ALA group ($p < 0.01$) and the combination therapy was more efficient in decreasing the level of ROS generation.

3.4.2. Mitochondrial membrane potential (MMP)

Induction of myocardial IRI in aged rats significantly increased the mitochondrial membrane depolarization compared to the sham group (Fig. 4B). The combined treatment prevented the excessive depolarization of the mitochondrial membrane. As shown in Fig. 4B the red/green intensity (indicating the membrane polarization near to resting level) was considerably declined in the IR group

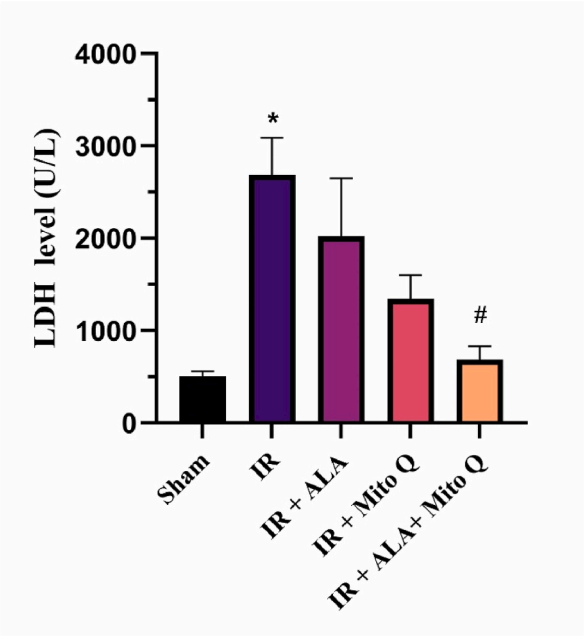

Fig. 2. Levels of lactate dehydrogenase (LDH) released from aged hearts following IR injury. Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM. *p < 0.05 vs. sham group, #p < 0.05 vs. IR group. IR: ischemia-reperfusion; ALA: alpha lipoic acid.

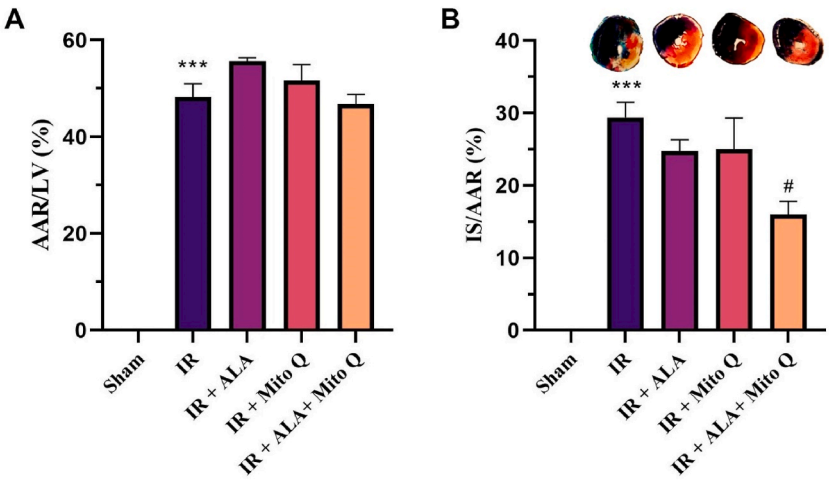

Fig. 3. Percentages AAR (A) and infarct sizes (B) in aged hearts following IR injury. Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM. ***p < 0.001 vs. sham group, #p < 0.05 vs. IR group. IR: ischemia-reperfusion; ALA: alpha lipoic acid.

compared to the sham group ($p < 0.001$). Combination therapy induced a significant increase in the red/green intensity compared to IR group and ALA group ($p < 0.001$, $p < 0.01$, respectively).

3.5. Oxidative stress indicators

As shown in Fig. 5A to B, the level of SOD and GPX enzyme activity as enzymatic antioxidants, were decreased significantly in the group exposed to IRI compared to the sham group ($p < 0.01$, $p < 0.001$, respectively). Combined treatment increased both indices compared to the IR group ($p < 0.05$, $p < 0.001$, respectively). A significant difference in the GPX marker was also observed between the combination therapy and monotherapies ($p < 0.05$ compared to ALA group, $p < 0.01$ compared to MitoQ group). Additionally, MDA (Fig. 5C), as a marker of lipid peroxidation, was significantly increased in the IR group compared to the sham group ($p < 0.05$), and its level was reduced after combination therapy in comparison to untreated IR group ($p < 0.01$).

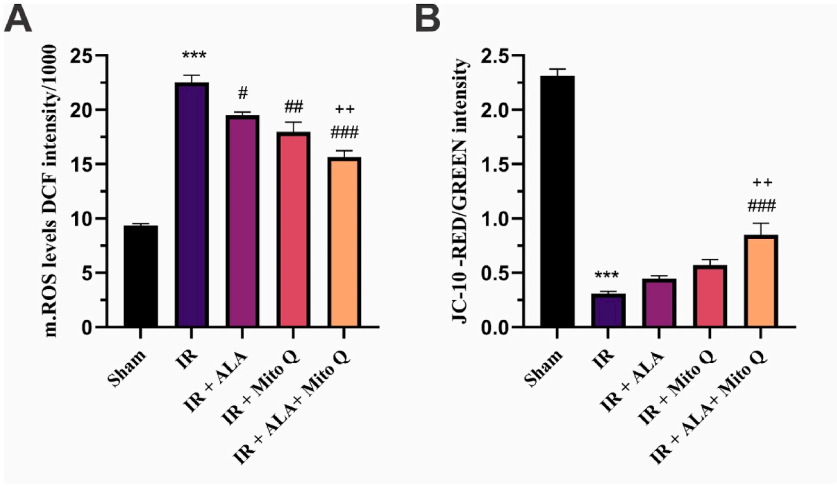

Fig. 4. Cardiac mitochondrial ROS production levels (DCF intensity) (A) and mitochondrial membrane potential changes (red/green JC-1 intensity) (B) in aged hearts following IRI. Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM. ***p < 0.001 vs. sham group, #p < 0.05 vs. IR group, ##p < 0.01 vs. IR group, ###p < 0.001 vs. IR group, ++ p < 0.01 vs. ALA group. m.ROS: mitochondrial reactive oxygen species; IR: ischemia-reperfusion; ALA: alpha lipoic acid.

3.6. Expression of mitochondrial dynamics genes

Our results indicated that induction of IRI caused a significant decrease in the expression of mitochondrial fusion genes such as Mfn1 (Fig. 6A) ($p < 0.05$), Mfn2 (Fig. 6B) ($p < 0.001$) and Foxo1 (Fig. 6C) ($p < 0.01$) in comparison with the sham group, while treatment with ALA and MitoQ in combine manner increased the expression level of these fusion genes compared to IR group ($p < 0.05$, $p < 0.001$, $p < 0.05$, respectively). Although IRI increased the expression of genes involved in mitochondrial fission like Drp1 (Fig. 6D) ($p < 0.001$) and Fis1(Fig. 6E) ($p < 0.01$) compared to sham group, the combined treatment significantly reversed this effect ($p < 0.05$) (Fig. 6).

4. Discussion

Aging not only predisposes the heart to ischemic disorders but also diminishes the efficacy of therapeutic interventions in cardioprotection and hampers the process of myocardial function improvement following ischemia [11]. This study evaluated the hypothesis that whether combination therapy with MitoQ and ALA could exert enhanced cardioprotective effects in aged rats subjected to IRI. The study's findings demonstrated that preconditioning with MitoQ and ALA reduced myocardial infarct size, the level of the injury marker LDH, mitochondrial ROS production, as well as the expression of mitochondrial fission genes and lipid peroxidation following IRI. Additionally, combination therapy not only modified blood pressure variations induced by IRI and prevented excessive depolarization of the mitochondrial membrane, but also improved myocardial function, increased mitochondrial fusion genes, and strengthened the potency of the endogenous antioxidant system.

Studies have indicated reduced effectiveness of individual therapeutic interventions in the aged heart, highlighting the clinical importance of identifying protective strategies [3,4,11,12]. Previous research has investigated the effects of ALA and MitoQ in young animals, demonstrating their efficacy in reducing infarct size and providing cardioprotection when administered individually [20,23,24]. To avoid redundancy with similar studies and to extend our prior investigations, the present study was specifically conducted on aged rats. Our primary objective was to assess the impact of ALA and MitoQ, both individually and in combination, on cardioprotection in aged rats. Thus, it did not aim to compare the effects these compounds in old rats with young rats. Our findings indicate that in aged rats, monotherapy with ALA and MitoQ is not effective, comparing with the IR group receiving no treatment. Consequently, we opted for combination treatment to enhance the effectiveness of these compounds and address this issue. So, the hypothesis centered on whether ALA and MitoQ, as monotherapy or combination therapy, could reduce cardiac injury in aged rats, rather than exploring age-related reductions in their effectiveness. Accordingly, the combined treatment exhibited superior effects across all measured parameters.

Previous studies have indicated that aging alters the expression of genes involved in the production and elimination of ROS in heart tissue [8,39]. Consequently, the aged heart becomes more susceptible to cardiovascular diseases due to increased oxidative stress during this period. Increased ROS damage mitochondria, DNA, and other organelles and molecules, ultimately leading to cellular aging and cell death [40]. Studies have reported that because of the reduced antioxidant ability in aging, the healing process after IRI is diminished in elderly patients [11,41]. The present study confirmed the increase in oxidative stress in aged rat heart following IRI, as evidenced by a decline in the levels of endogenous antioxidants such as SOD and GPX, along with an increase in lipid peroxidation. However, the combined treatment of MitoQ and ALA corrected these alterations. Previous research has reported that ALA exerts its

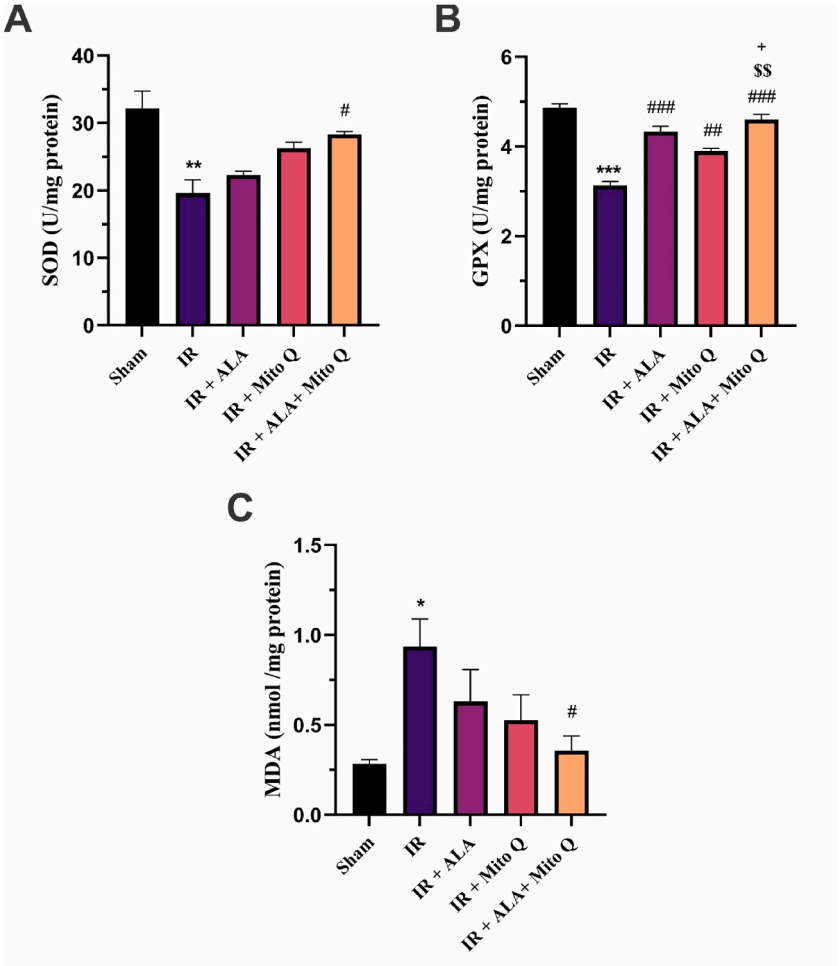

Fig. 5. Antioxidant enzymes activity of SOD (A) and GPX (B) and MDA content (C) in aged hearts following IRI. Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM. *p < 0.05 vs. sham group, **p < 0.01 vs. sham group, ***p < 0.001 vs. sham group, #p < 0.05 vs. IR group, ##p < 0.01 vs. IR group, ###p < 0.001 vs. IR group, ###p < 0.001 vs. IR group, + p < 0.05 vs. ALA group, $ $ p < 0.01 vs. MitoQ group. SOD: superoxide dismutase; GPX: gluthatione peroxidase; MDA: malondialdehyde; IR: ischemia-reperfusion; ALA: alpha lipoic acid.

protective function by directly scavenging ROS and restoring endogenous antioxidants like glutathione, vitamin E, and vitamin C [42]. Studies have also demonstrated ALA's efficacy in reducing myocardial IRI in both *in vivo* and *in vitro* conditions [25,43]. Similarly, MitoQ has shown protective effects in various contexts, such as promoting follicular growth and maturation, inhibiting ROS and oxidative damage, and regenerating mitochondrial membrane potential [44], as well as protecting against radiation-induced oxidative damage in the testis [45]. Notably, these previous studies on ALA and MitoQ have been conducted in young animal models and humans without considering risk factors for cardiovascular diseases [20,23,24]. Thus, in our experiment, the combined treatment of MitoQ and ALA demonstrated a promising additive effect in reducing oxidative stress caused by IRI in aged rats.

Our results indicated higher mitochondrial ROS production and increased mitochondrial membrane depolarization in the IR group. Conversely, MitoQ and ALA combination therapy reduced these mitochondrial function indicators compared to the IR group. During early ischemia, mitochondrial ROS production increases due to the partial availability of oxygen and the inability of cytochrome C oxidase to consume the remaining oxygen [46,47]. Under stress conditions like ischemia, drastically reduced tissue oxygen and nutrient reserves lead to excessive calcium release and subsequent dephosphorylation of cytochrome C and other mitochondrial proteins, preparing the mitochondrial electron transport chain (ETC) for over-activation during reperfusion. The subsequent hyper-activity of ETC components, when coupled with the reintroduction of oxygen during reperfusion, results in increased ROS production, initiating the process of cell death and apoptosis [48,49]. ALA has been observed to increase the activity of various complexes of the respiratory chain, mitochondrial membrane potential, and ATP production in an Alzheimer's cell model, affirming its role in improving mitochondrial function [50]. Additionally, oral administration of MitoQ in elderly patients with endothelial dysfunction reduced mitochondrial ROS, highlighting its potential in mitigating oxidative stress [51].

MI is associated with impaired mitochondrial function and dynamics, emphasizing the role of mitochondrial dynamics regulation

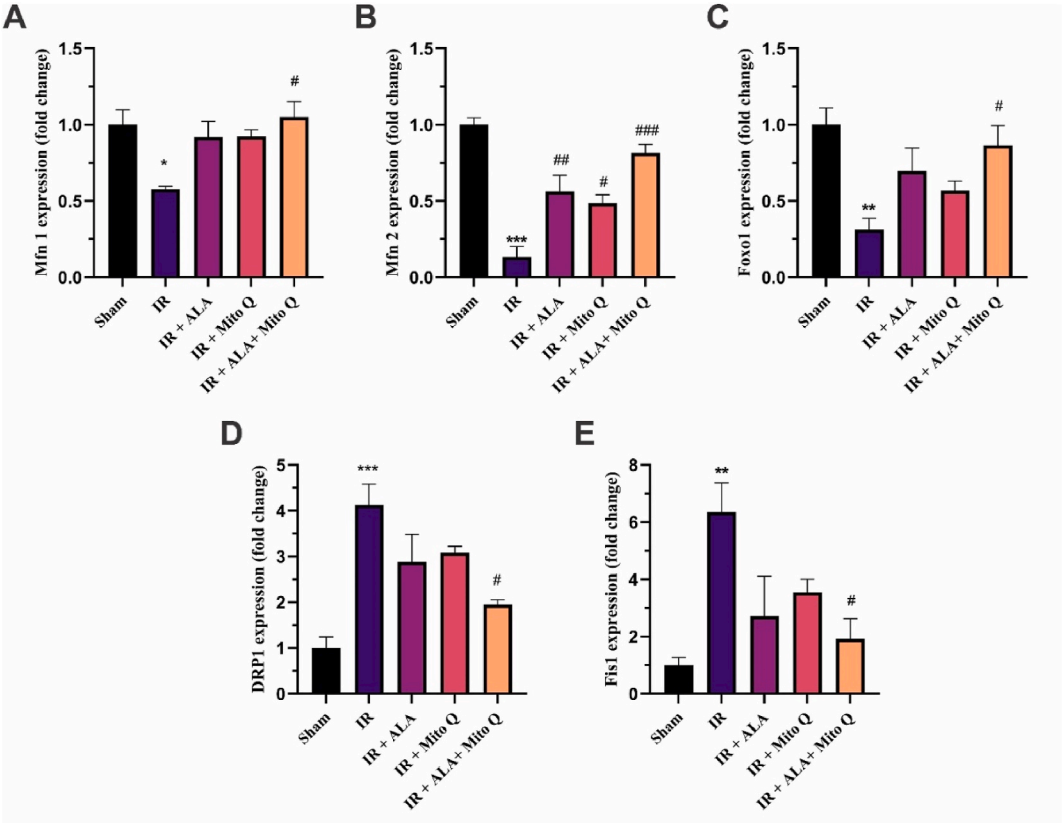

Fig. 6. Expression of mitochondrial dynamics genes in aged hearts following IRI. Mfn1 (A), Mfn2 (B), Foxo1 (C), Drp1 (D) and Fis1 (E). Data were analyzed using *one-way ANOVA* followed by *Tukey's* post hoc test, and presented as Mean ± SEM. *p < 0.05 vs. sham group,**p < 0.01 vs. sham group, ***p < 0.001 vs. sham group, #p < 0.05 vs. IR group, ##p < 0.01 vs. IR group, ###p < 0.001 vs. IR group. IR: ischemia-reperfusion; ALA: alpha lipoic acid.

in addressing complications caused by MI [52,53]. Our study revealed a decrease in the expression of mitochondrial fusion genes (Mfn1, Mfn2, and Foxo1) and an increase in fission genes (Drp1 and Fis1) following MI in aged rats, while combination therapy reversed these detrimental changes. Consistent with our findings, Jiang et al. reported pathological changes in mitochondrial dynamics post-MI in rats, characterized by decreased Mfn2 and Opa1 expression and increased Drp1 expression [54].

The induction of IRI has been demonstrated to lower blood pressure in rats, leading to a reduction in blood supply to various tissues [2,3]. This study revealed that both individual interventions (ALA or MitoQ) and their combination restored and normalized blood pressure in rats. However, since monotherapy did not completely correct all cardiac parameters, the observed improvement in blood pressure by monotherapies may be attributed to their extra-cardiac mechanisms, such as effects on vascular tone or mechanisms related to the nervous system. Further studies are warranted to elucidate these potential mechanisms. In addition, fluctuations in HR among the groups were not statistically significant, except for the ALA group alone, where a slight increase in HR was observed for reasons that remain unclear and were not elucidated in this study.

The effects of monotherapies were significant in some parameters but not in others, displaying inconsistency and unreliability. In contrast, the effects of combination therapy were significant across all cardiac parameters and endpoints, indicating greater stability and reliability. Furthermore, the impact of combination therapy surpassed that of individual therapies, making it challenging to rely solely on monotherapies for cardioprotection in the aging heart. These findings indicate that in aging, the therapeutic potency and effectiveness of interventions individually may not be sufficient to activate pathways and intracellular protective mediators. However, under conditions of simultaneous intervention, the impact of each treatment is enhanced along the mentioned pathways, leading to cumulative and adequate protection. Combination therapies have been shown to have greater protective potential in the aging heart compared to monotherapies [11,55]. For instance, combined treatment of ALA with acetyl-L-carnitine in patients with coronary artery diseases improved blood pressure regulation and vascular tone [56]. Similarly, MitoQ in combination with endurance exercise had beneficial effects on cardiac function in hypertensive patients [57]. Therefore, combining multiple protective modalities may overcome potential limitations of monotherapies in cardioprotection for aged subjects. The clinical perspective of this manuscript lies in its exploration of a potential therapeutic strategy, involving the combination of MitoQ and ALA, to address the challenges posed by aging and ischemic heart conditions. In clinical scenarios, a significant proportion of patients experiencing IRI are elderly individuals, and the intricate mechanisms involved in the pathogenesis of IRI in aging necessitate combined treatments. Given that ALA and MitoQ are

potent antioxidants with demonstrated positive effects when used individually, their combined use as preventive approach becomes crucial for older individuals with comorbidities predisposing to IRI, such as obesity, hypertension, dyslipidemia, etc. Additionally, in patients already experiencing IRI, there is potential for employing the combination of ALA and MitoQ as an intervention immediately prior to reperfusion therapy. The application of these compounds as the postconditioning modality is another clinical aspect not addressed in the current study. However, administering ALA and MitoQ at the onset of reperfusion therapy and beyond might be effective, highlighting the necessity for additional supplementary studies.

The study has other limitations, including its reliance on experiments conducted in aged rats, limiting the generalizability of the results to diverse human populations with varying clinical conditions or comorbidities. While emphasizing the important role of mitochondrial function and oxidative stress, the study may not fully capture the complexity of the aging heart's response to ischemia, and additional exploration of other contributing factors and pathways is warranted. The relatively short duration of the study (24 h of reperfusion) does not permit an exploration of the long-term effects, sustainability, and potential side effects of the combined antioxidant therapy in aging subjects. The study acknowledges the necessity for supplementary investigations to elucidate different surviving mechanisms affected by the combined treatments, highlighting avenues for future research to enhance the overall understanding of potential interventions in myocardial IRI in aging. Finally, extending the study to clinical trials involving elderly individuals is crucial for confirming the efficacy of the proposed combination therapy.

5. Conclusion

To sum up, the present study demonstrates that combined antioxidant therapy with ALA and MitoQ is more effective than individual monotherapies in mitigating the consequences of myocardial IRI in aging. The findings suggest that this combination therapy has the potential to enhance cardioprotection, improve mitochondrial function, and counteract oxidative stress in the aging heart, providing a basis for future research and potential clinical applications. This suggests that therapeutic strategies based on combination treatments targeting mitochondria could offer a promising approach against myocardial IRI in elderly individuals. Further supplementary studies are necessary to elucidate the role of various survival mechanisms and signaling pathways influenced by these combined treatments in the context of aging.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

CRediT authorship contribution statement

Zohreh Zavvari Oskuye: Writing – original draft, Software, Formal analysis, Data curation. **Keyvan Mehri:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Behnaz Mokhtari:** Writing – review & editing, Software. **Soleyman Bafadam:** Formal analysis, Data curation. **Samira Nemati:** Software, Data curation. **Reza Badalzadeh:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28158.

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