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Original Article

Ischemic-Postconditioning Improves Myocardial Injury and Fibrosis Following Ischemia/Reperfusion Injury in Diabetic Rats Pretreated with Alpha-Lipoic Acid

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Abstract

Background: Myocardial ischemia/reperfusion (I/R) injury is one of the pre-eminent causes of death. Prevention of I/R injury in diabetic patients is full of ups and downs. This study has investigated the effect of co-administration of alpha-lipoic acid (ALA) preconditioning and ischemic-postconditioning (Post) on myocardial injury and fibrosis in type-II diabetic rats suffering from I/R injury.

Methods: Chronic diabetes was induced by a high-fat supplemented diet and low-dose streptozotocin within 3 months. Diabetic rats received ALA (100 mg/kg/d, orally) for 5 weeks before I/R induction. The myocardium underwent 35 minutes of ischemia through left anterior descending (LAD) coronary artery ligation and 60 minutes of reperfusion. Post was consisted of six cycles of 10/10 seconds algorithm and applied in the early stage of reperfusion. The release of creatine kinase (CK) as the injury enzyme was measured with ELISA. The protein expression of fibrosis markers was evaluated with western blotting and tissue fibrosis was scored through Masson's trichrome staining.

Results: Post alone had no considerable effect on cardiac injury marker CK, while ALA had somehow a positive impact. However, the application of Post in ALA-pretreated rats significantly reduced the injury marker in comparison with the untreated I/R group. In addition, this combination therapy decreased Smad3 and transforming growth factor-β (TGF-β) expression and improved tissue fibrosis as compared with the untreated group.

Conclusion: Combination of ALA pretreatment and Post evoked a cardio-protective effect by improving myocardial injury and attenuating fibrosis induced by diabetes. This combined treatment can be recommended as a useful strategy to restore cardio-protection in hearts with diabetic comorbidity.

Introduction

Ischemic heart diseases (IHDs) cause mortality universally, which is on the rise annually.[1,2] Reflowing blood to the ischemic tissue (reperfusion) is an initial treatment of IHDs. Most studies indicate that reperfusion induces extra damage to the myocardium, causing ischemia/reperfusion (I/R) injury.[3] The chance of myocardial infarction and I/R injury, which leads to death, is particularly higher in diabetic patients.[4] Furthermore, diabetes mellitus is a metabolic malfunction that is increasing throughout the world. Diabetic cardiomyopathy is grown and exacerbated by the contribution of various complications such as high blood sugar (hyperglycemia), accumulated fatty acid, and insulin resistance.[5] Importantly, chronic diabetes significantly impairs the effectiveness of cardio-protective therapeutic interventions.[6] Thus, examining the effectiveness of new cardio-protective strategies in diabetics for reducing I/R damage seems to be one of the priorities of clinical and preclinical investigations.

Cardiac fibrosis is a pathological response that leads to

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malformation in the conductive and mechanical function of the heart and contributes to the development of some I/R-related cardiac disorders including hypertrophy, ventricular stiffness, arrhythmias, and heart failure.[7] Diabetes also makes the heart susceptible to fibrosis.[8] As a result, the progression of fibrosis in diabetes can compromise the diabetic heart's protection against I/R injury and cardiomyopathy.[9] Thus, it is compelling to choose a rational approach in order to increase the efficiency of protective modalities in diabetic hearts. Considering the complexity of the pathophysiology of diabetes and its association with I/R injury, combined therapy may increase the power of cardioprotection in comparison with monotherapies in diabetic cases.

Ischemic post-conditioning (Post) is short episodes of alternating reperfusion and ischemia, which is performed in the initial minutes of reperfusion, and considerably saves non-comorbid heart from I/R injury. It restricts infarct size, recovers cardiac functional efficiency, and improves coronary microcirculation by inducing endogenous cardio-protective signal cascades. However, Post cannot significantly introduce beneficial impacts after I/R injury in diabetic hearts.[10]

Alpha-lipoic acid (ALA) — a leading antioxidant — can remove reactive oxygen species (ROS) and reproduce several types of antioxidants such as vitamin E, vitamin C, and glutathione [11]. Moreover, this compound is effective in I/R hearts in different ways including vasorelaxation, positive metabolic profile, and anti-apoptotic and anti-inflammatory features.[12] It has been shown that ALA has also positive effects on the prevention and treatment of diabetes,[13] and preserves the heart from diabetic cardiomyopathy.[14] It seems that ALA can be more effective when it is used with another intervention.

In the previous study, we found that combined treatment of ALA and Post in diabetic rats had significant antiarrhythmic effects while post alone was not able to protect the heart against I/R injury.[10] Post is a powerful cardioprotection modality but chronic diabetes lowers its ability significantly. Still, there is a need to find out the mechanisms underlying this interfering effect of diabetes with cardio-protection. Understanding these mechanisms in diabetic conditions and the use of combined treatments with careful selection of appropriate combination modalities can be useful to remove barriers to cardio-protection while providing a new therapeutic strategy. Increased myocardial fibrosis in diabetic hearts may be one of the underlying mechanisms. ALA is an antidiabetic agent with antifibrotic features. Accordingly, we hypothesized that pretreatment of diabetic rats with ALA may reduce cardiac fibrosis and thereby prepare an intracellular appropriate situation for Post to show its protective potential. Therefore, this research was focused on the fibrosis changes in diabetic heart as one of the underlying mechanisms and aimed to evaluate the impact of Post on cardiac injury and tissue fibrosis following I/R injury in rats with type-II diabetes pretreated with ALA.

Materials and Methods

Animals and chemicals

Eight-week-old male Wistar rats (50 rats, 200-250 g) were provided from the Laboratory animal breeding center. The animals were freely fed with food and water in a standard animal room with controlled temperature (20-24 °C) and humidity (55%) and were held under a 12-hour dark-light cycle. All of the strategies of this study were conducted in agreement with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (8th Edition, NRC 2011), verified by the ethical committee of the University of Medical Sciences (ethical code: IR.TBZMED.VCR.REC.1400.154). Streptozotocin (STZ) and ALA were bought from Sigma (St. Louis, MO, USA). All materials of Krebs-Henseleit (K-H) solution were obtained from Merck Company (Munchen, Germany).

Induction of type-II diabetes

According to the previous reports,[15] type-II diabetes was induced via a high-fat diet and low-dose STZ strategy. The diabetic period lasted for 12 weeks in order to simulate chronic diabetes. After one week of adaptation, diabetic rats were catered with a high-fat diet (35% normal pellet, 30% lard, 24% casein, 4% sucrose, 1% cholesterol, and 0.3% DL-Methionine) for six weeks. At the beginning of the 7th week and after eight hours of fasting, an intraperitoneal injection of 35 mg/kg of STZ (in citrate buffer, pH 4) was administered to the rats. Blood samples were taken 72 hours after STZ injection through rats' tails and blood glucose level was checked by a glucometer (Elegance CT-X12, Convergent Technologies, Germany). Rats with blood glucose levels higher than 250 mg/dL were considered as diabetics and enrolled for further experiments. Also, the homeostatic model assessment (HOMA) was applied to validate insulin resistance in rats. The plasma levels of insulin were assessed using an ELISA kit according to the instructions provided (Sigma, USA). A maximum of 3 rats were died during the diabetes period before entering to I/R study. These animals were replaced with the other rats.

Surgical preparation and isolated heart perfusion

Heparin-sodium (500 IU/kg) and a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg) were injected into rats intraperitoneally. The hearts were immediately removed as soon as the chests were opened. Next, the isolated hearts were cannulated and retrogradely perfused through the aorta with a K-H buffer gassed with 5% CO_2 and 95% O_2 at 37 °C ± 0.5 °C, and pH of 7.4, in Langendorff perfusion apparatus (ML176-V; AD Instruments, New South Wales, Australia) at a constant pressure of 80 mm Hg. The composition of K-H buffer in mmol/L included: KCl 4.7; NaCl 118; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; and glucose 11.1. Following a 15-minute stabilization period with a 12–14 mL/min coronary flow rate, the hearts went through left anterior descending (LAD) coronary

artery ligation for 35 min to make regional ischemia. Then, LAD re-opened for 60 minutes to develop reperfusion. Controlled rats experienced the same operation without induction of LAD ischemia-reperfusion. An approximate 70% reduction of coronary flow in the ligation period, and the full recovery of coronary flow within early reperfusion showed successful I/R cycles.

Experimental design

The diabetic rats were categorized into 5 groups as follows (n = 10/each group):

- Control (without I/R): The hearts received 110 minutes full perfusion.
- IR: The hearts received 35 min ischemia and 60 minutes reperfusion.
- IR + Post: The hearts received 35 min ischemia and 60 minutes reperfusion, and the Post algorithm was done in the initial minutes of reperfusion by 6 alternating cycles of 10 s ischemia followed by 10 s reperfusion.
- IR + ALA: Rats were pretreated with ALA (100 mg/kg/d) via gastrogavage for 5 weeks, and then the same procedure was conducted on their hearts as in the IR group.
- IR + ALA + Post: Rats were pretreated with ALA (100 mg/kg/d) via gastrogavage for 5 weeks, and then the same procedure was conducted on their hearts as in the IR + Post group.

The body weight of rats was recorded weekly, and the heart weight was measured at the end of reperfusion. The ratio of heart weight to body weight was used as an auxiliary index for characterizing diabetic cardiomyopathy. In each group, 5 rats were utilized for histopathological evaluation, while the remaining 5 rats were allocated for molecular experiments.

Cardiac creatine kinase assay

The amount of coronary effluent was collected in the first 15 minutes of reperfusion, and the amount of creatine kinase (CK) was measured using the specific kit and calorimetrically immunoassay method according to the instructions of the manufacturer (Pars Azmoon, Iran). The values of this indicator were reported as U/L.

Western blotting

Western blot technique was used to measure the expression of myocardial Smad3 and transforming growth factor-β (TGF-β) proteins. Myocardial samples were homogenized in a RIPA lysis buffer (Sigma, MO, USA). The obtained mixture underwent centrifugation (10 000 RCF, 10 minutes, 4 °C) in order to gather the supernatants. Bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA) was employed to compute the concentration of protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the same quantity (≈50 μg) of protein extracts. Next, electrophoresis was employed to transfer proteins to polyvinylidene difluoride (PVDF) membranes (Sigma, MO, USA). A 5% skimmed milk was used to block membranes in Tris-buffered Saline-Tween 20 (TBST, pH 7.4) at room temperature. The blocked membranes were then incubated for a night with primary antibodies against Smad3, and TGFβ, as well as glyceraldehyde 3-phosphate dehydrogenase or GAPDH (1:1000, Cell Signaling Technology, USA). After washing, membranes were incubated with anti-rabbit secondary antibody (1:7000, Cell Signaling) for a whole hour on a shaker at room temperature. An enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA) was used to visualize the antibody-substrate reactions. Finally, the densitometry evaluation was done to measure the intensity of protein bands. All protein quantities were normalized with the corresponding GAPDH band intensity.

Histological evaluation of myocardial fibrosis

Hearts were dipped in paraffin and cut into pieces with a thickness of about 5-μm and transferred on corresponding slides for Masson's trichrome staining according to its protocol.[16] Then, their fibrotic changes were evaluated with a light microscope (Olympus, Japan). Image pro-insight software (Media Cybernetics, USA, version: 9.00) was used to examine the photomicrographs of each cross-section. Twenty random microscopic visions (2530 μm × 2530 μm) of photomicrographs of randomly selected left ventricular slices were utilized to measure the severity of positive reactions (blue-dyed pixels/total pixels) of fibrosis. Next, the average rate of the pixel-based intensities from three sections for each heart was examined in each group to calculate the severity of fibrosis.[17]

Statistical analysis

SPSS software v25 was used to analyze data statistically (SPSS Inc., Chicago, IL, USA). All values were reported as means ± standard error of the mean (SEM), and analyzed with Repeated ANOVA (hemodynamic parameters) or one-way ANOVA (other parameters) followed by Tukey post-hoc test. $P < 0.05$ was calculated as statistically significant.

Results

Characteristics of diabetic rats

At the end of the diabetic periods, the blood samples were taken to measure the biochemical parameters in diabetic rats. Fasting blood glucose was 540 ± 15 (mg/dL), insulin level was 7.2 ± 0.6 (μu/mL), HOMA1 was 9.3 ± 0.2, body and heart weights were 311 ± 14 (g) and 1.61 ± 0.04 (g), respectively, and heart/body weight was 0.53 ± 0.01 (%). However, pretreatment of diabetic rats with ALA a caused significant decrease in hyperglycemia (346 ± 12 mg/dL; $P < 0.01$), an increase in plasma levels of insulin (9.3 ± 0.4 μu/mL; $P < 0.05$), and reduction of HOMA-IR index (8.2 ± 0.5; $P < 0.05$), body weight (305 ± 9 g; $P > 0.05$), heart weight (1.48 ± 0.06 g; $P < 0.05$), and heart/body

weight ratio (0.49 ± 0.01 %; $P < 0.05$), as compared to the untreated diabetic rats.

Myocardial CK release

After induction of I/R injury, the level of CK ($P < 0.05$) release into the coronary effluent increased in comparison to the control group (Figure 1). Preconditioning with ALA, alone or in combination with Post, decreased CK release considerably as compared with those of IR group ($P < 0.05$, or $P < 0.001$). Nevertheless, there was no significant difference in CK levels between IR + Post and IR groups. Besides, combined treatment significantly reduced the amount of CK release in comparison to the IR + Post group ($P < 0.05$) (Figure 1).

Cardiac expression of Smad3 and TGF-β proteins

The expression levels of Smad3 ($P < 0.01$) and TGF-β ($P < 0.001$) in the hearts of the IR group were significantly raised compared to the control group (Figure 2A, and Figure 2B). The application of Post at early reperfusion had no substantial effects on reducing Smad3 and TGF-β proteins. On the contrary, ALA pretreatment remarkably downregulated the expression of these proteins ($P < 0.05$) as compared to the IR group. In addition, administration of both therapeutic interventions simultaneously reduced the expression of Smad3 and TGF-β in comparison to not only the IR group ($P < 0.001$) but also IR + Post and IR + ALA groups ($P < 0.05$ and $P < 0.01$). These findings demonstrated the strong efficacy of combination therapy rather than monotherapies on the regulation of pro-fibrotic signaling proteins in diabetic hearts.

Histopathological observation of fibrosis

Masson's staining indicated thickness of left ventricles and interstitial fibrosis due to the accumulation of extracellular matrix (ECM), movement, and multiplication of macrophages and neutrophils in addition to fibroblasts. Changes illustrating tissue fibrosis in the IR group were almost similar to the control group. Post had no effects on myocardial fibrosis; in contrast, ALA pretreatment, alone or combined with Post, reduced fibrosis considerably in comparison with the IR group (Figure 3A).

Fibrosis severity was also scored according to the pixels of fibrotic areas in ventricular-stained sections. The fibrosis degree in the IR group was almost similar to the Control group. Post had no considerable effect on the reduction of fibrosis in diabetic hearts. However, the severity of fibrosis reduced noticeably by using ALA ($P < 0.05$). Additionally, the grade of cardiac fibrosis in combination therapy was similar to the IR + ALA group and significantly lower than the IR group ($P < 0.01$) (Figure 3B).

Discussion

This study showed that combined treatment with ALA preconditioning and Post additively affected the hearts of rats with type II diabetes exposed to I/R injury. The main findings of this work were a significant reduction of CK release and attenuation of fibrosis following combined treatment. Solitary use of ALA decreased cardiac fibrosis, which leads to cardiac injury improvement, while single therapy with Post did not have significant results. Remarkably, data proved that the reduction of myocardial

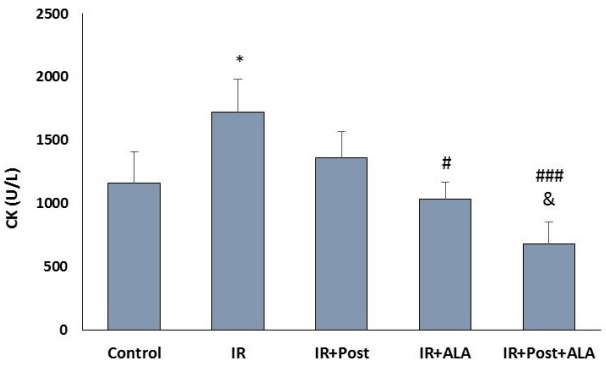

Figure 1. The levels of CK release into the coronary effluent in diabetic hearts subjected to 35 min ischemia and 60 min reperfusion. The data were expressed as mean ± SEM (n = 5). $^{*}P < 0.05$, and $^{**}P < 0.01$ vs. Control group, $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.001$ vs. IR group, $^{\&}P < 0.05$ vs. IR + Post group. IR: ischemia reperfusion; Post: ischemic postconditioning; ALA: alpha lipoic acid; CK: creatine kinase

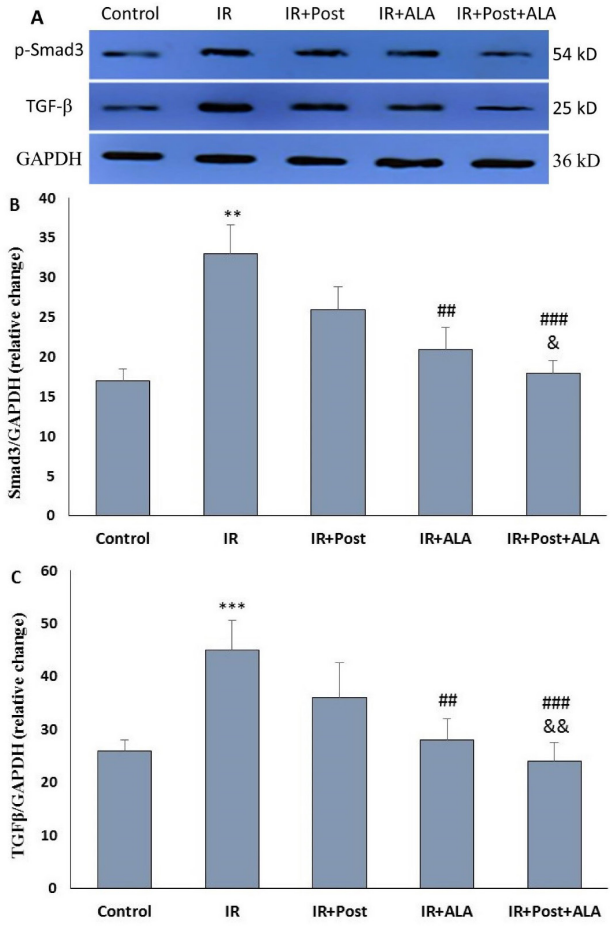

Figure 2. The representative immunoblots (A) and the expression of Smad3 (B) and TGFβ (C) proteins in diabetic hearts subjected to 35 min ischemia and 60 min reperfusion. The data were expressed as mean ± SEM (n = 5). $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. Control group, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. IR group, $^{\&}P < 0.05$, and $^{\&\&}P < 0.01$ vs. IR + Post group. IR: ischemia reperfusion; Post: ischemic postconditioning; ALA: alpha lipoic acid

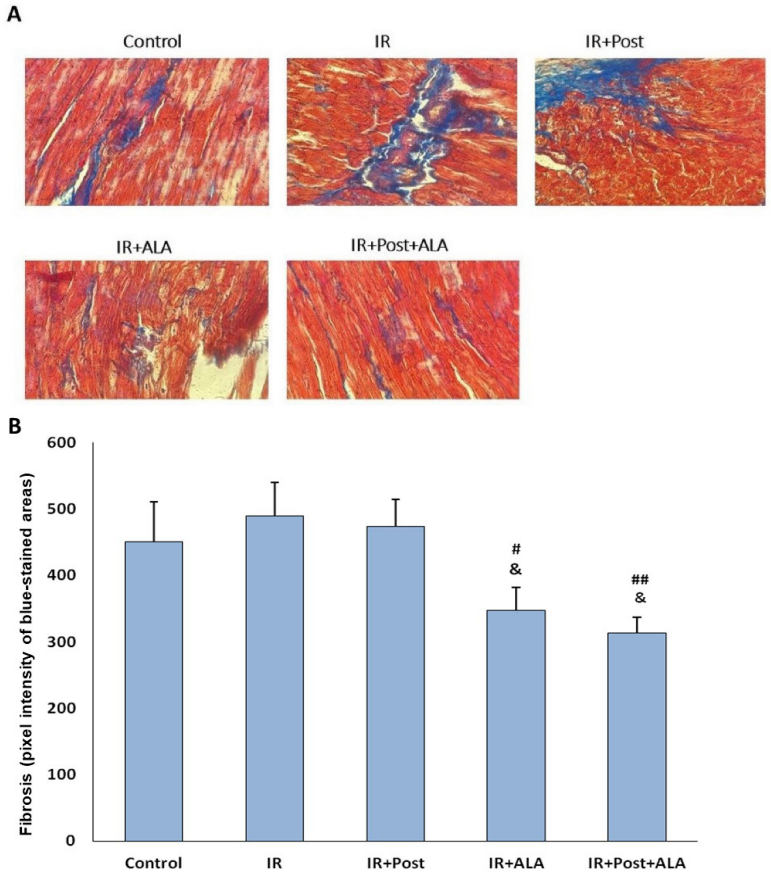

Figure 3. Masson's trichrome staining (A), and fibrosis severity scoring (B) in diabetic hearts subjected to 35 min ischemia and 60 min reperfusion. The data were expressed as mean ± SEM (n = 5). In A, red parts indicate cardiomyocytes and blue parts indicate fibrosis. $^{\#}P < 0.05$ vs. IR group, $^{\&}P < 0.05$ vs. IR + Post group. IR: ischemia reperfusion; Post: ischemic postconditioning; ALA: alpha lipoic acid

fibrosis by ALA pretreatment considerably increased the cardio-protective potential of monotherapy with Post in diabetic hearts.

It is important to mention that we previously conducted a comparison between control and diabetic rats to assess the extent of I/R damage.[18] The results showed that while the intensity of damage in diabetic rats was not significantly higher than in controls, diabetic rats did not respond to Post or pharmacologic treatment alone. However, the combined therapy was more effective than individual treatments in diabetic rats, whereas the effects of combined therapy and single treatments were similar in non-diabetic rats. Therefore, in the present study, our analysis focused exclusively on diabetic rats to examine the role of fibrosis in this context. Fibrosis is defined by an excessive gathering of collagen and other ECM constituents.[19] Fibroblasts are nonmyocyte cells, which include ≈26% of the total cells in rats' hearts. After ischemic injury, cardiac fibroblasts transform into endothelial cells, contributing to the neovascularization of impaired myocardium.[20] These cells synthesize collagen I and III, developing ECM. Collagens assemble around intramyocardial coronary arteries, and then fibrosis expands within cardio-myocytes in both ventricles. Remarkably, disturbance in synthesis and degeneration of ECM can cause severe cardiac dysfunction and heart failure.[21] These processes are more severe in the diabetic heart.[22] Overall, researchers have suggested that the association between diabetes and metabolic cardiomyopathy is complicated, and, it is a reason for the increase of I/R damages and the compromised cardio-protection during chronic diabetes.[9] Normally, the cardiac fibroblasts remain inactive and do not proliferate. Production of collagen I and III increase following myocardial I/R injury. So, fibrosis is considerably activated by cardiac injury which causes myocardial dysfunction.[23] Thickening of ECM decreases energy in cardio-myocytes, so the muscle workload increases in response to imposed stress. Therefore, cells die because of necrosis or apoptosis. Fibrosis interrupts the coordination of myocardial cells in both systole and diastole which damages signal conduction through cardio-myocytes. Finally, the fibroblasts synthesize a new matrix to replace the damaged cells and scars are formed based on the size of the lesion. This process is called reparative fibrosis.[21] This study proved that fibrosis in diabetic hearts considerably increased after induction of I/R injury and this was associated with cardiac injury and dysfunction at the reperfusion phase.

Transforming growth factor-β1 (TGF-β1) is a profibrotic cytokine that stimulates the secretion of ECM proteins in hearts and causes tissue fibrosis and dysfunction.[24] Additionally, the deletion of Smad3 has

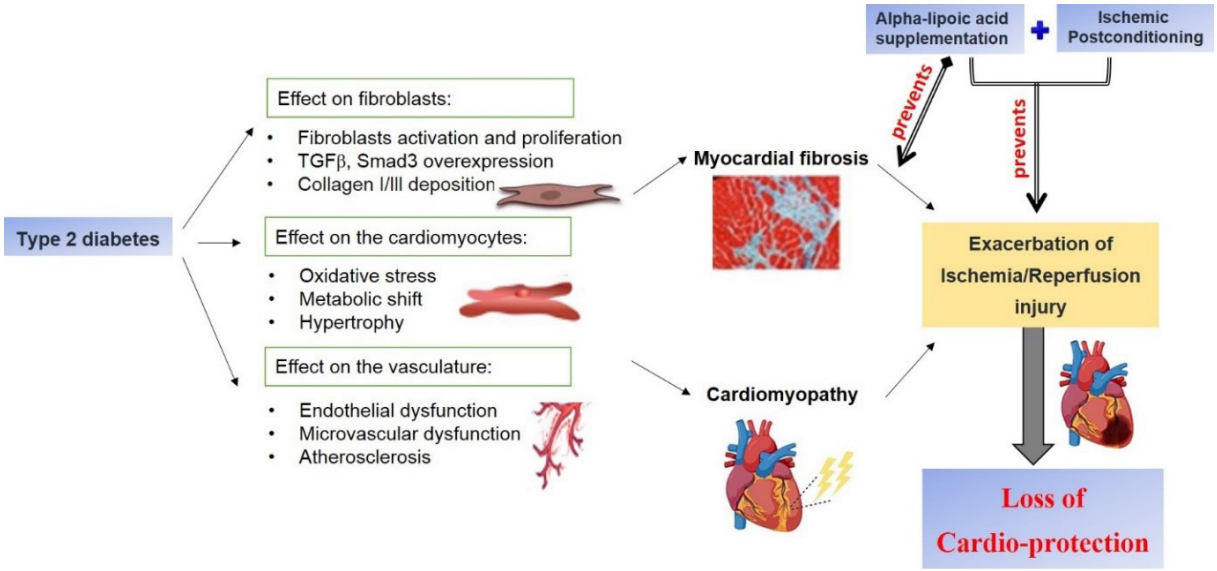

Figure 4. Schematic diagram of diabetes-induced loss of cardioprotection and its prevention by the combination of alpha lipoic acid and ischemic postconditioning

prevented cardiovascular inflammation and fibrosis in hypertension, myocardial infarction, and obese diabetes.[25] According to previous reports, TGF-β/Smad3 signaling is a crucial pathway associated with cardiovascular diseases.[26] Following phosphorylation by TGF-β, Smad3 moves to the nucleus and connects to Smad-binding oligonucleotides available in the regulatory areas of particular genes, so collagen type I gene expression levels can change.[27] Therefore, if this pro-fibrotic process in the ischemic heart is inhibited by targeted interventions, the heart can be protected from further damage. Despite this, in the present study, Post administration was not effective enough in activating the cell survival mechanisms and reducing the activity of detrimental pathways, including the TGF-β1/Smad3 pathway in the diabetic heart. Nevertheless, in rats pretreated with ALA for 5 weeks, the protective effects of the Post returned to the diabetic heart. Since the extent of tissue fibrosis in IR hearts receiving ALA alone was at its lowest level. It can be concluded that higher fibrosis in the diabetic heart may prevent the effectiveness of the Post. However, by correcting fibrosis through pre-treatment with ALA, the efficacy of the second intervention on protective mechanisms increases, and hence, the simultaneous use of interventions produces their additive effects and overcomes the interference of diabetes on cardio-protection.

ALA has other beneficial effects such as vasorelaxation, a positive metabolic profile as well as an anti-inflammatory potential.[12] Additionally, according to the previous study, pretreatment of diabetic rats with ALA reestablished the infarct size-limiting effect of Post via regulating autophagy and improving mitochondrial function.[28] Therefore, it is inferred that the reduction of cardiac fibrosis can be attributed to both the recovery of cardiac autophagy and mitochondrial function, which improves the function of the heart. Also, the improvement of mitochondrial function by ALA intervention in diabetic subjects can lead to a better effect of Post in the combined treatment group. Because mitochondria are considered the main end-effector of Post,[28] and mitochondrial dysfunction in diabetic hearts can prevent the effectiveness of the intervention. The other question is whether there are other signaling pathways involved or not. Confirmation of the contribution of these hypotheses requires further study. An important drawback of this study is that we did not take into account the impact of our interventions on female rats. Our decision to use male rats exclusively was motivated by the desire to maintain experimental consistency with prior studies in the field of myocardial I/R injury and minimize potential variations resulting from physiological differences between males and females. Nonetheless, we recognize the significance of conducting research on both sexes and acknowledge the necessity for future studies involving female rats, as they may encounter more pronounced complications in this context.

Conclusion

Based on the results, it can be demonstrated that combination therapy with ALA and Post increases the potency of each other, resulting in better cardio-protection against I/R damage in rats with type-II diabetes. This protection was associated with previously reduced myocardial fibrosis (Figure 4). By focusing on diabetic rats, we aimed to address a specific clinical scenario where individuals with diabetes are more susceptible to cardiac complications following ischemic events. However, further study is needed to answer the limitations of this research and to give a deep insight into how various mechanisms and signaling pathways protect diabetic hearts in combination therapy.

Authors' Contribution

Conceptualization: Sanaz Gholami, Poul Flemming Høilund-Carlsen, Reza Badalzadeh, Alireza Alihemmati.

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Writing–original draft: Sanaz Gholami, Behnaz Mokhtari, Aniseh Javadi.
Writing–review & editing: Poul Flemming Høilund-Carlsen, Reza Badalzadeh, Alireza Alihemmati.

Competing Interests

The authors proclaimed no possible conflicts of interest regarding the research, authorship, and/or publication of this article.

Data Availability Statement

Data will be available on inquiry from the corresponding author.

Ethical Approval

All steps and processes of this experimental examination were confirmed by the Institutional Animal Ethical Committee at the Molecular Medicine Research Center (ethical code: IR.TBZMED.VCR.REC.1400.154).

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