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# Melatonin postconditioning combined with sitagliptin exerts full cardioprotection in diabetic hearts of aged rats through an AMPK-dependent mechanism

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**Abstract:** The presence of multiple comorbidities in patients facing myocardial ischemia-reperfusion (IR) injury is the main obstacle for cardioprotection. This study investigated the effect of melatonin postconditioning combined with sitagliptin pretreatment on cardioprotection in diabetic aged rats by evaluating oxidative stress, apoptosis and involvement of the AMPK/SIRT1 pathway. The type-2 high-fat/streptozotocin experimental model in aged Sprague-Dawley rats (n=78) was used. The animals underwent left coronary occlusion for 30 min, followed by 3 h reperfusion. Diabetic rats were pretreated with sitagliptin (20 mg/kg, i.p.) and received melatonin (10 mg/kg, i.p.) early in reperfusion. Myocardial infarct size, histological changes, oxidative markers, mitochondrial reactive oxygen species (mitoROS) and expression of proteins regulating apoptosis and AMPK/SIRT1 activity were measured. The infarct size-sparing effect of the combination of melatonin plus sitagliptin was greater than that observed in individual treatments ($P<0.01$). Combination therapy significantly reduced IR-induced elevation of 8-isoprostane, mitoROS and proapoptotic proteins Bax and cleaved caspase-3, and increased IR-induced downregulation of mitochondrial superoxide-dismutase, glutathione, anti-apoptotic protein Bcl2, phosphorylated AMPK and SIRT1 ($P<0.01$, $P<0.001$). Inhibition of AMPK via compound-C completely reversed combination-induced cardioprotection. Thus, improving cardiac antioxidative and antiapoptotic responses via upregulation of AMPK/SIRT1 activity may represent a central mechanism through which melatonin plus sitagliptin attenuate myocardial IR injury in diabetic-aged rats.

**Keywords:** diabetes; cardioprotection; aging; AMPK; postconditioning; melatonin; DPP-4

## INTRODUCTION

Ischemic heart disease is the leading cause of death in patients with type 2 diabetes; the incidence of type 2 diabetes increases with age [1,2]. Aging and diabetes increase myocardial infarction (MI) and ischemia-reperfusion (IR) damage-induced cardiac dysfunction, and both interfere with cardioprotection [2]. Appropriate modeling of this condition and performing cardioprotection studies to explore the underlying mechanisms involved in this phenomenon are of great clinical importance.

Important cellular abnormalities, including reperfusion-induced exacerbation of oxidative stress, activation of inflammatory pathways, dysregulated autophagy and apoptosis, play significant roles in the pathophysiology of cardiac IR damage as well as in diabetes and aging [3,4]. Increased reactive oxygen species (ROS) production alters the activity of transcription factors and signaling kinases leading to mitochondrial damage and induction of tissue apoptosis [5]. Thus, augmentation of apoptotic injuries and oxidative stress in the aged diabetic heart requires treatments that, while having potent antidiabetic effects, can overcome these sequential negative events and provide full protection of the heart against IR damage. Metabolic changes due to diabetes and aging reduce the activity of AMP-activated kinase (AMPK) and its downstream target sirtuin-1 (SIRT1) [6]. AMPK and SIRT1 both act as energy (fuel) sensors and share common intracellular targets [7]. Activation of the AMPK/SIRT1 pathway promotes or regulates important biological functions such as cellular longevity, mitochondrial function, autophagy and reduction of oxidative stress and apoptosis [6,7]. The association of the AMPK/SIRT1 pathway

with cardioprotection in ischemic heart without risk factors has been well documented [8,9].

Previous studies have demonstrated that the combination of melatonin with dipeptidyl peptidase-4 (DPP-4) inhibitors or gliptins increased their hypoglycemic and antidiabetic effects [10,11], but it is not yet clear whether this combination therapy can counteract the negative impact of aging and diabetes comorbidities on cardioprotection. Melatonin is an endogenous hormone related to biological circadian rhythms, and its plasma concentration declines during aging [12]. Due to its strong antioxidant and antiinflammatory effects, melatonin has recently attracted considerable attention in cardiovascular studies [13,14]. Numerous studies have reported the protective effects of melatonin preconditioning in various organs and conditions, including rat IR hearts [14,15]. Alternatively, by upregulating AMPK and SIRT1 protein activity, melatonin preconditioning can exert antiapoptotic and antioxidant effects on the heart [16]. However, the effects of melatonin postconditioning, especially in diabetic conditions during aging, are still unknown and the contribution of possible mechanisms also need to be elucidated. Sitagliptin is an important DPP-4 inhibitor that in addition to having strong antidiabetic effects has also exhibited good cardioprotective effects [17,18]. For example, by reducing the activity of apoptotic proteins, this drug suppressed doxorubicin-induced cardiotoxicity in rat [17]. Moreover, when combined with other agents such as trigonelline, sitagliptin had the capability to reduce the biomarkers of diabetic cardiomyopathy and have protective effects on the heart of diabetic rats [19]. However, sitagliptin alone has been unable to reduce the risk of death from heart failure and MI in diabetic patients [20]. This finding justifies the need to use sitagliptin in combination with other drugs in MI patients with cardiovascular comorbidities.

According to the findings of the above studies, both melatonin and sitagliptin could share common intracellular pro-survival targets and therefore their combined application could minimize the complications of cardiovascular risk factors in patients with MI. However, it is not known whether cardioprotection by exogenous melatonin and its combination with the DPP-4 inhibitor sitagliptin is achievable in aged hearts with chronic type 2 diabetes as a commonly occurring comorbidity, and whether this cardioprotection involves the activity of AMPK and SIRT1 proteins as important regulators of energy metabolism during diabetes and aging. This study was designed to confirm the hypothesis that postconditioning with melatonin in combination with sitagliptin pretreatment can confer substantial cardioprotection by reducing apoptosis and oxidative stress in the diabetic heart of aged rats, and whether the AMPK/SIRT1 pathway is involved in this protection.

## MATERIALS AND METHODS

### Animals

Seventy-two male Sprague Dawley rats aged 22±2 months with a body weight of 300-325 g were obtained from the Experimental Animal Center of the Hospital and housed under standard animal room conditions (12 h day/night cycling at 22±2°C and 55% humidity). The animals had free access to food and water throughout the experiment. The procedures in the animal experiments followed the Laboratory Animals Guideline of the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised in 1996) and were approved by the local Animal Care Committee under the ethical code of CXDNF-2019KL.

### Reagents

Evans blue, triphenyl-tetrazolium chloride (TTC), hematoxylin and eosin (H&E) dyes, melatonin, streptozotocin (STZ), compound-C (CC), mitochondrial isolation kit and DCFD dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sitagliptin was obtained from Novartis, Switzerland. The assay kits for 8-isoprostane, manganese-superoxide dismutase (MnSOD) and glutathione were purchased from Jiancheng Bioengineering Institute (Nanjing, China). The primary antibodies against phosphorylated or total forms of AMPK, and SIRT1, B-cell lymphoma 2 (Bcl2), Bcl-2-associated X protein (Bax), cleaved caspase 3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as well as secondary antibody were obtained from Cell Signaling Technology (Boston, MA, USA).

### Induction of diabetes

The type 2 diabetic model was established according to the scheme of high-fat diet and low-dose STZ [21]. After two weeks of acclimation, the rats were fed a

high-fat diet (62% calories obtained from fat) for 4 weeks and then received 35 mg/kg of STZ (dissolved in 0.1 mol/l citrate buffer, pH 4.5) by intraperitoneal (i.p.) injection; 72 h later, fasting plasma glucose was measured and values above 250 mg/dL were considered diabetic. The total high fat regimen period was extended for ten weeks.

**Myocardial ischemia and reperfusion injury**

Diabetic aged rats were anesthetized by an i.p. injection of sodium pentobarbital (50 mg/kg) and were then ventilated by endotracheal intubation using a small animal ventilator (Taimeng Technology, China). After lateral thoracotomy and exposure of the heart, the left anterior descending (LAD) coronary artery was ligated for 35 min by a 4-0 silk ligature to induce regional myocardial ischemia. Successful ischemia was confirmed by observation of blanching of the area distal to the ligation place. Myocardial reperfusion was initiated by release of the ligature and lasted 3 h. Rats in the control group received thoracotomy and threading without any ligation.

**Experimental design**

For measurement of myocardial infarct size, 42 diabetic aged rats were divided into seven groups (6 rats each) as follows: the control group received only thoracotomy without LAD ligation; the IR group received IR injury via 35 min LAD ischemia followed by 3 h reperfusion; the IR+Mela group received IR injury and melatonin (10 mg/kg, i.p.) at the onset of reperfusion; the IR+Sita group received sitagliptin pretreatment (20 mg/kg, i.p.) for 4 weeks before surgery and IR injury; the IR+Mela+Sita group received sitagliptin pretreatment (20 mg/kg, i.p.) for 4 weeks before the IR injury, as well as melatonin (10 mg/kg, i.p.) at the onset of reperfusion, the IR+CC group received IR injury and two injections of AMPK inhibitor CC (250 μg/kg, i.v. (intravenous)) before ischemia and at the onset of reperfusion, and finally, the IR+CC+Mela+Sita group received sitagliptin pretreatment (20 mg/kg, i.p.) for 4 weeks before the IR injury, as well as AMPK inhibitor CC (2.5 mg/kg, i.p.) and melatonin (10 mg/kg, i.p.) at the onset of reperfusion. The selected dosages of melatonin [22], sitagliptin [17] and CC [23] were the most protective doses commonly used in previous studies. In addition, for measurement of biochemical, molecular and histological parameters, another 36 diabetic aged rats were allocated into six groups: control, IR, IR+Mela, IR+Sita, IR+Mela+Sita and IR+CC+Mela+Sita. Six rats were considered for each group.

**Infarct size measurement**

For determination of the extent of areas at risk and infarct sizes, Evans blue and TTC staining were performed on transverse sections of left ventricles (5-6 cuts of ≈2 mm thickness), as described [16]. Digital visualizing software (Image-Pro, Media Cybernetics, USA) was used to measure the percentages of areas at risk relative to the total volumes of left ventricles and infarct sizes relative to areas at risk.

**Determination of myocardial histopathological changes**

Left ventricular samples from ischemic zones were prepared and immersed in 10% formaldehyde and then subjected to paraffin-embedded sectioning (5 μm thickness) followed by staining with H&E. Intercellular swelling, inflammatory cell infiltration and necrotic lesions were assessed by a blind histologist using an optical microscope and the changes were scored as mild, moderate and severe changes or normal tissue.

**Measurement of myocardial content of 8-isoprostane, manganese superoxide-dismutase (MnSOD) and glutathione**

After 3-h reperfusion, the hearts of animals were isolated and the left ventricles were separated and transferred to a deep freezer in liquid nitrogen. Then the samples were homogenized in lysis buffer containing protease inhibitors leupeptin, aprotinin and pepstatin A, and centrifuged at 12000 ×g for 15 min to obtain supernatants. A bicinchoninic acid (BCA) protein quantification kit was used to assess the protein concentrations of samples. The levels of free 8-isoprostane, as the key marker of oxidative stress, and the levels of the antioxidant enzyme MnSOD and glutathione in heart supernatants were determined using related enzyme immunoassay kits (Cayman Chemical, Ann Arbor, USA) based on the manufacturers' protocol. The values of parameters were expressed according to the protein concentration of each sample.

### Isolation of myocardial mitochondria and determination of mitochondrial ROS

Fresh left ventricles were washed and homogenized in buffer A containing 0.3 mmol phenyl-methyl-sulfonyl fluoride (PMSF), 1 $Na_3VO_4$, 1 mmol NaF, 1 mmol EDTA, 10 mmol Tris-HCl, 250 mmol sucrose and protease inhibitor. The solutions were serially centrifuged at 1000 and 10000 ×g. Thereafter, the pellets were resuspended in buffer B containing 0.3 mmol phenylmethyl sulfonyl fluoride (PMSF), 1 mmol NaF, 1 mmol $Na_3VO_4$, 10 mmol EDTA, 20 mmol Tris-HCl, 150 mmol NaCl, 1% NP-40 and protease inhibitor, and centrifuged at 21000 ×g for 10 min. The resultant supernatant was used as the mitochondrial fraction. Dichlorohydro-fluorescein diacetate (DCFDA)-based fluorometry was employed to measure the mitochondrial ROS level in myocardial samples. The mitochondrial supernatants were incubated with 2 μM DCFDA dye for 30 min at 37°C and then the excitations and emissions were measured at 480 nm and 530 nm, respectively, using a fluorescent reader. The mitoROS value was quantified as the fluorescence intensity per mg protein of supernatant.

### Western blotting

Fifty micrograms of total protein from left ventricular supernatants were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (PVDF, Millipore, USA). The membrane was blocked with 5% skim milk and incubated with primary antibodies as follows: anti-Bax, anti-Bcl2, anti-cleaved caspase 3, anti-tAMPA, anti-pAMPK, anti-SIRT1 and anti-GAPDH (1:1000, Cell Signaling Technology, USA) overnight. After removing nonspecific antibody binding by washing blocked membranes three times with washing buffer, they were re-incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 2 h at 24°C and then with enhanced chemiluminescence (Millipore, USA) for 10 min. The blots were exposed to X-ray film and visualized. The blot intensities were analyzed with Image-lab software (Bio-Rad, USA).

### Statistical analysis

The sample size was six rats in each group. Data are shown as the mean±standard error. Statistical analyses of variables were performed using Kruskal-Wallis and one-way analysis of variance (ANOVA); $P<0.05$ was considered statistically significant.

## RESULTS

### Combination of Mela+Sita reduced myocardial infarct size and CC reversed this effect

TTC staining was performed to determine the effect of melatonin and sitagliptin and their combination therapy on myocardial infarct size in diabetic aged rats (Fig. 1). No significant difference in the areas at risk was identified in all experimental groups, indicating similar realization of the IR injury in rats (Fig. 1A). MI size was significantly increased in the IR group as compared to the control rats ($P<0.001$) (Fig. 1B). Administration of melatonin or sitagliptin alone tended to reduce the IR-induced increase in infarct size, but these effects were not statistically significant. In contrast, combined treatment with melatonin and sitagliptin significantly reduced MI size when compared with the IR group ($P<0.001$). More importantly, the infarct-sparing effect of the combined treatment was better than that of individual treatments ($P<0.05$) (Fig. 1B). Finally, administration of CC to inhibit AMPK activity significantly abolished the effect of the combination therapy on infarct size ($P<0.05$).

### Combination of Mela+Sita improved myocardial histological changes and CC abrogated this effect

Cardiac histopathological investigation among the experimental groups revealed that IR induction in diabetic aged rats caused considerable histopathological changes in terms of increased intercellular expansion and edema, inflammatory cell infiltration, sarcoplasmic vacuolation and eccentric nuclei, when compared with the control group (Fig. 2). Melatonin or sitagliptin, when administered individually, reduced moderately the IR-induced histopathological changes. More importantly, the combination therapy with melatonin and sitagliptin had a greater influence in correcting these inflammatory and necrotic changes compared to individual therapies. Conversely, AMPK inhibition by CC completely abrogated the protective effect of the combination therapy on myocardial histology in aged diabetic rats (Fig. 2).

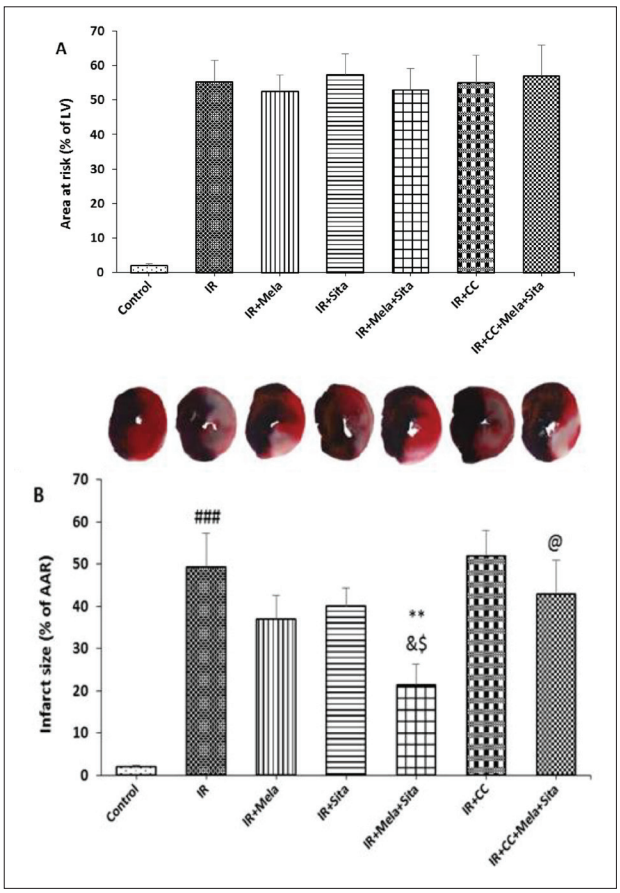


**Fig. 1.** Effect of melatonin (Mela), sitagliptin (Sita) and their combination on myocardial area at risk, AAR (A) and infarct size (B) in diabetic aged IR hearts. LV – left ventricle, IR – ischemia-reperfusion, CC – compound C. N=6 in each group. ###P<0.001 vs the Control group; **P<0.01 vs the IR group; &p<0.05vs. IR+Mela group; $P<0.05vs. IR+Sita group; @P<0.05 vs the IR+Mela+Sita group.

## The combination of Mela+Sita suppressed myocardial oxidative stress and CC inhibited this effect

The levels of 8-isoprostane and MnSOD and glutathione were measured to quantify oxidative stress of the myocardium; mitoROS generation was also measured. As shown in Fig. 3A-D, the myocardial contents of 8-isoprostane (P<0.01) and mitoROS (P<0.001) were significantly increased and MnSOD (P<0.01) and glutathione (P<0.001) were significantly reduced in the IR group in comparison to the control group. Neither melatonin alone nor sitagliptin alone significantly affected the changes in 8-isoprostane and glutathione compared with the IR group. Melatonin alone increased MnSOD levels and reduced mitoROS generation, and sitagliptin alone reduced only mitoROS generation, in comparison to the IR group (P<0.05, Fig. 3B). However, combination therapy significantly reduced the IR-induced increase in 8-isoprostane level and mitoROS generation (P<0.001) and upregulated the levels of MnSOD (P<0.01) and glutathione (P<0.05) when compared with the IR group. Additionally, the effects of combination therapy on the reduction of 8-isoprostane and mitoROS were significantly greater than those of any individual treatment (P<0.05) (Fig. 3A and 3D). Inhibition of AMPK by CC significantly reversed these antioxidative effects of combination therapy in myocardial IR injury in diabetic aged rats (Fig. 3A-D).

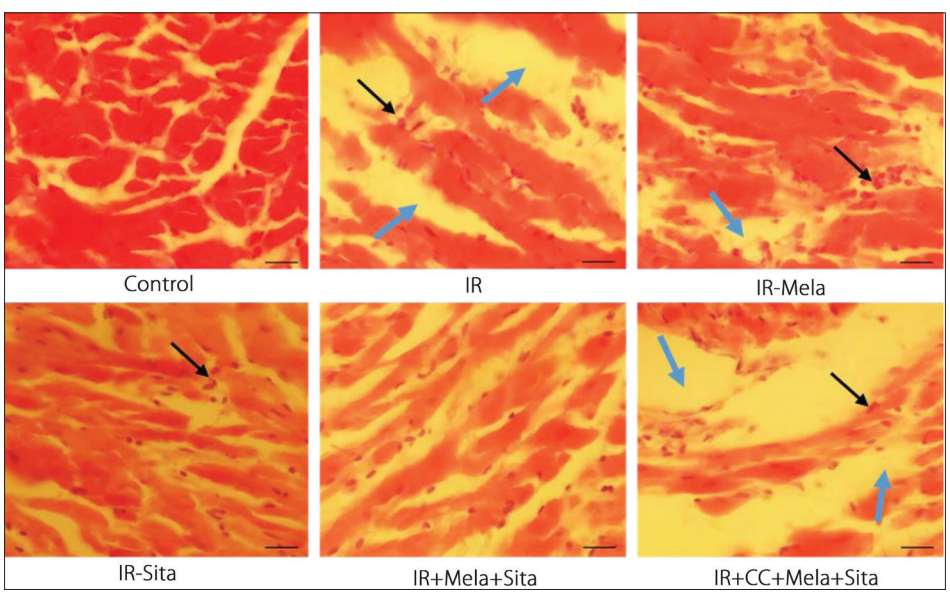


**Fig. 2.** Effect of melatonin (Mela), sitagliptin (Sita) and their combination on myocardial histopathological changes in diabetic aged IR hearts. Intercellular expansion and edema, infiltration of inflammatory cells, sarcoplasmic vacuolation and eccentric nuclei were more evident in the IR group. Combination therapy with melatonin and sitagliptin had greater influence in correcting these inflammatory and necrotic changes in comparison to individual treatments. Blocking AMPK activity by CC completely abolished these protective effects. IR – ischemia-reperfusion, CC – compound C. N=6 in each group. Bar=40 μm. Thick blue arrows indicate intercellular expansions and narrow black arrows indicate necrotic nuclei.

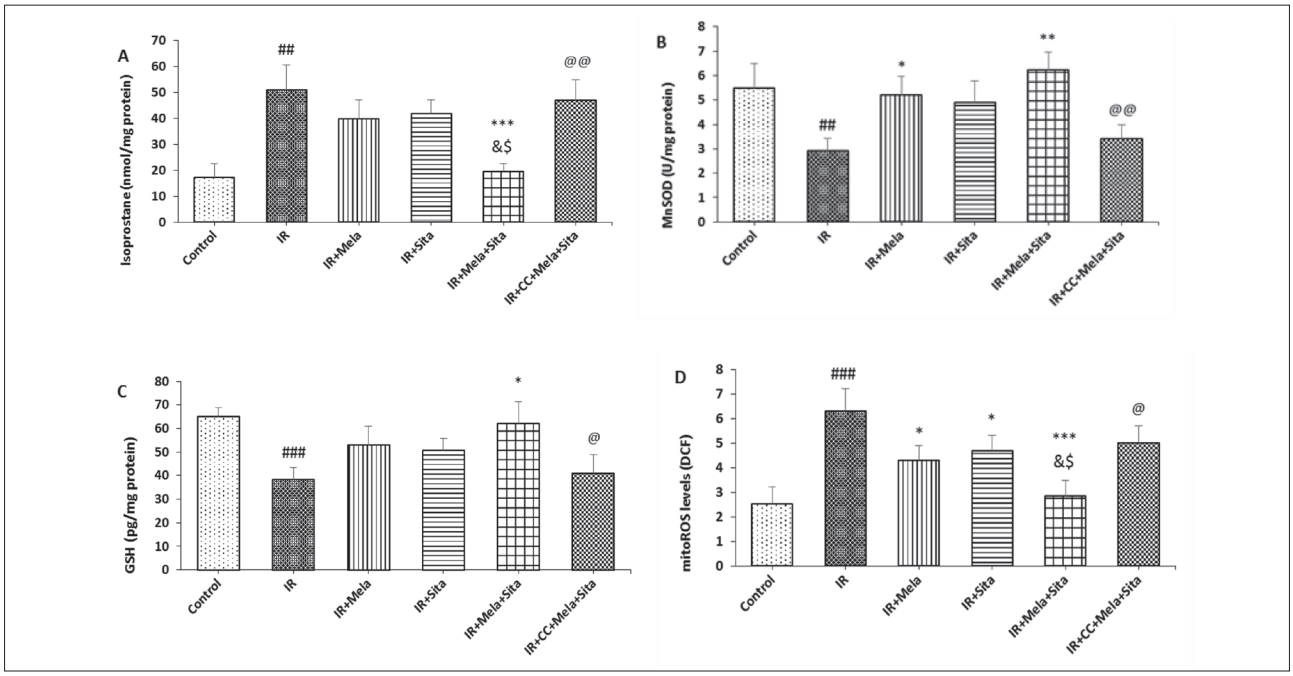


**Fig. 3.** Effect of melatonin (Mela), sitagliptin (Sita), and their combination on intracellular-mitochondrial oxidative stress: 8-isoprostane (**A**), mitochondrial or manganese-dependent superoxide dismutase, MnSOD (**B**), glutathione, GSH (**C**) and mitochondrial ROS, mitoROS (**D**) in diabetic aged IR hearts. IR – ischemia-reperfusion, CC – compound C. N=6 in each group. ##P<0.01, ###P<0.001 vs the control group; *P<0.05, **P<0.01, ***P<0.001 vs the IR group; &P<0.05 vs. IR+Mela group; $P<0.05 vs. IR+Sita group; @P<0.05, @@ P<0.01 vs the IR+Mela+Sita group.

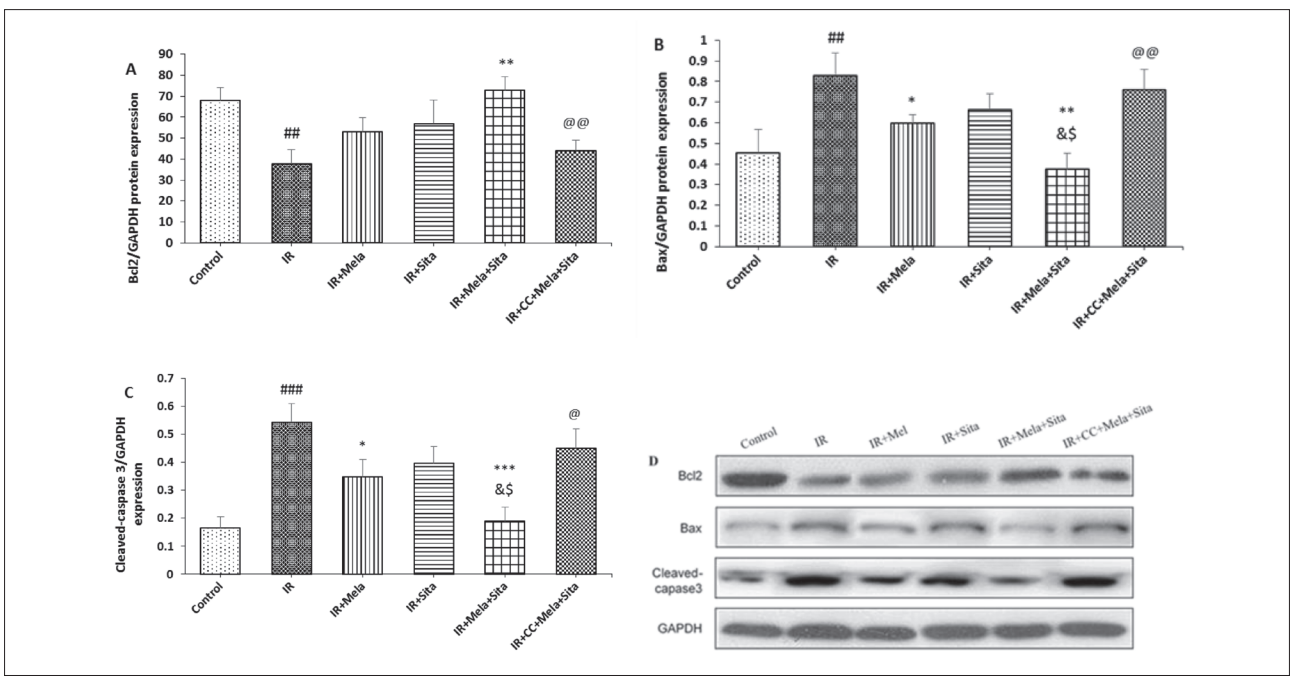


**Fig. 4.** Effect of melatonin (Mela), sitagliptin (Sita), and their combination on anti-apoptotic protein Bcl2 expression (**A**) and pro-apoptotic proteins Bax (**B**), cleaved caspase 3 (**C**) and their representative immunoblots (**D**) in diabetic aged IR hearts. IR – ischemia-reperfusion, CC – compound C. N=6 in each group. ##P<0.01, ###P<0.001 vs. Control group; *P<0.05, **P<0.01, ***p<0.001 vs the IR group; &P<0.05 vs the IR+Mela group; $P<0.05 vs the IR+Sita group; @P<0.05, @@P<0.01 vs the IR+Mela+Sita group.

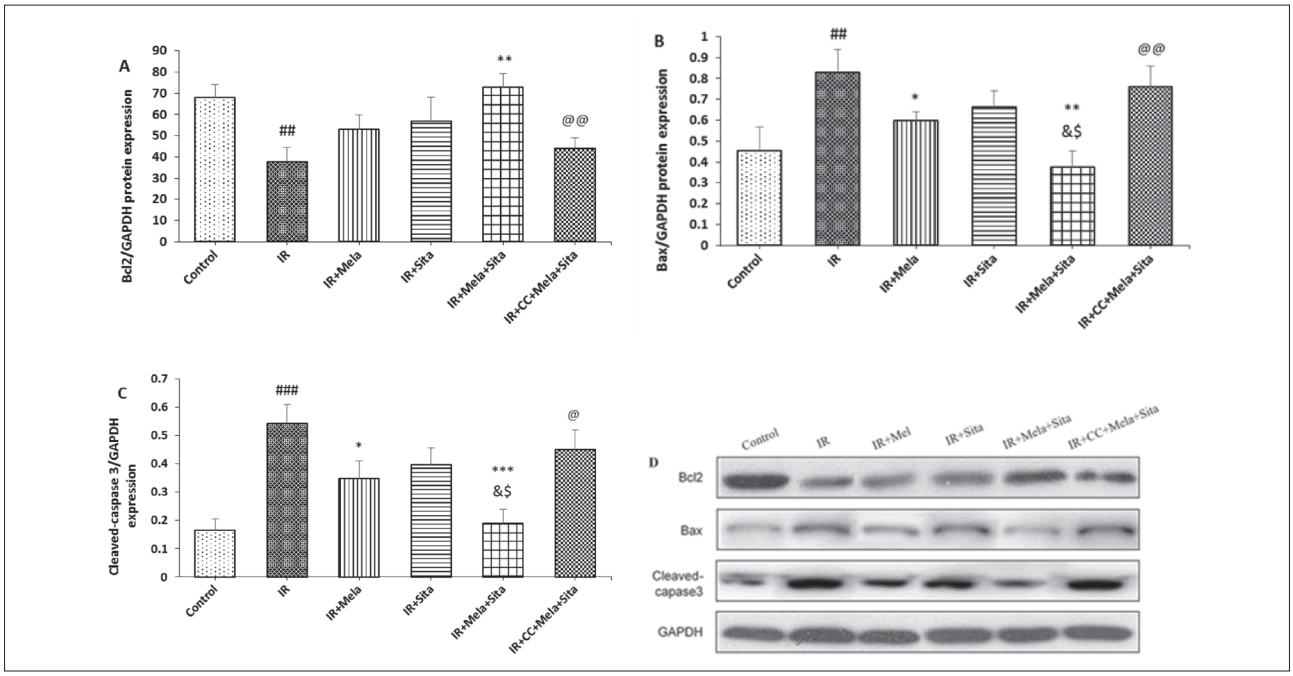


**Fig. 5.** Effect of melatonin (Mela), sitagliptin (Sita) and their combination on protein expression of total AMPK – t-AMPK (**A**), phosphorylated AMPK – p-AMPK (**B**), SIRT1 (**C**) and their representative immunoblots (**D**) in diabetic aged IR hearts. IR – ischemia-reperfusion, CC – compound C. N=6 in each group. ##P<0.01, ###P<0.001 vs the Control group; *P<0.05, **P<0.01, ***p<0.001 vs the IR group; &P<0.05 vs the IR+Mela group; $P<0.05 vs the IR+Sita group; @@P<0.01, @@@P<0.001 vs the IR+Mela+Sita group.

## Combination of Mela+Sita attenuated myocardial apoptosis and CC abolished this effect

The effect of treatments on IR-induced myocardial apoptosis in diabetic aged rats was assessed by quantifying the alterations of proapoptotic and antiapoptotic proteins by immunoblotting (Fig. 4). Myocardial IR injury significantly decreased protein expression of antiapoptotic Bcl-2 (P<0.01, Fig. 4A) and increased protein expression of proapoptotic Bax (P<0.01, Fig. 4B) and cleaved caspase 3 (P<0.001, Fig. 4C) in comparison to the IR group. Sitagliptin alone had no effects on the expression of these apoptosis-related proteins, but melatonin alone significantly downregulated the expression of Bax and cleaved caspase 3 (P<0.05) and not the expression of Bcl2. In contrast, combination therapy markedly reversed the IR-induced changes of expression of all three proteins as compared with the IR group (P<0.01 and P<0.001). Again, the potency of the combined treatment was greater than that of either melatonin or sitagliptin alone on the regulation of Bax and cleaved-caspase 3 expression (P<0.05) (Fig. 4B, C). Administration of CC to the combination therapy-receiving IR-exposed rats increased myocardial apoptosis significantly (with P<0.05 for Bcl2/Bax and P<0.01 for cleaved-caspase 3).

## Combination of Mela+Sita downregulated myocardial AMPK/SIRT1 pathway activity and CC blocked this effect

To further explore the underlying molecular mechanism of combination therapy-mediated cardioprotection, we determined AMPK/SIRT1 pathway expression in diabetic aged rat heart after IR injury. The expression of total AMPK (t-AMPK) protein did not differ between groups (Fig. 5A). IR induction significantly downregulated the expression of the phosphorylated form of AMPK (p-AMPK) (P<0.001) and SIRT1 (P<0.01) in comparison to control rats (Fig. 5A, B). Melatonin alone enhanced the expression of both proteins when compared with the IR group (P<0.05), but the effects of sitagliptin on them were not significant. However, combination therapy significantly upregulated the expression of p-AMPK and SIRT1 both in comparison to the IR group (P<0.001) as well when compared to either individual treatment

(P<0.05). Finally, the simultaneous administration of AMPK inhibitor, CC, significantly reduced the expression of p-AMPK (P<0.001) and SIRT1 (P<0.01) as compared to the group that received combination therapy (Fig. 5). Taken together, the data suggest that the combination of melatonin and sitagliptin could protect the hearts of diabetic aged rats against IR injury-induced oxidative stress and apoptosis through the activation of the AMPK/SIRT1 signaling pathway.

## DISCUSSION

Based on the main findings of this study, first, we showed that the combination of melatonin with sitagliptin was superior to individual treatments in protecting the heart of aged diabetic heart against IR damage. Second, this combined therapy exerted its cardioprotective action by lowering mitochondrial and intracellular oxidative stress (and increasing their antioxidant capacity), as well as by reducing the apoptosis of cardiac cells. Third, the activity of the AMPK/SIRT1 signaling pathway played a major role in the protective effect of the combined therapy in the presence of these two additional comorbidity conditions co-occurring with myocardial IR injury. To the best of our knowledge, this is the first study to demonstrate the protective potential of the combined therapy and its possible mechanisms in the aged diabetic heart against the IR insult.

Under normal physiological conditions, the production of ROS and their removal by antioxidant enzymes is in balance. However, a sharp increase in ROS production during cardiac ischemia in the presence of aging and diabetes comorbidities causes severe oxidative stress damage and consequent cell death [24]. The antioxidant effects of melatonin (widely) [25] and sitagliptin (to a lesser extent) [26] have been reported in different tissues. However, in the present study, their single use had only a mild effect on oxidative stress parameters; the production of ROS was inhibited only by melatonin, and the antioxidant enzyme MnSOD was upregulated by both compounds, but they did not individually affect other parameters of oxidative stress, including lipid peroxidation and glutathione. Conversely, the combination therapy with both was capable of restoring all indicators of oxidative stress more powerfully to the values of the control group and even in some cases (ROS, isoprostane) had a stronger effect compared to the effect of the individual treatments. These effects were associated with strong cardioprotection by the combination therapy. Similar changes were observed in the levels of proapoptotic proteins Bax and caspase-3, and antiapoptotic factor Bcl2.

Our results of single therapies with melatonin or sitagliptin on the parameters of oxidative stress or apoptosis are inconsistent with the findings of previous studies [25,26]. In previous studies, their effects were not studied in the presence of cardiovascular risk factors, as they were examined after IR injury on the hearts of young animals, or because just one risk factor was considered, showing that in the presence of comorbidity the effects of the individual treatments on cardiac protection were reduced or abolished [4]. To overcome these problems, we used aged rats with type 2 diabetes and the experimental IR heart model, which thus has a considerable clinical similarity significance. We observed that the presence of these two main comorbidities at the same time negatively affected the protective effects of either melatonin or sitagliptin alone on MI size and protective mediators. Extensive changes in intracellular metabolism and cell survival signaling kinase activities in both type 2 diabetes and aging are the most important factors that undermine the protective effects of different interventions [4,27,28]. Augmented oxidative stress following reperfusion injury causes cardiomyocyte mitochondria to fail, with mitochondrial dysfunction leading to ROS overproduction and exacerbation of these events [29]. In diabetic circumstances, the severity of these events will be higher than in no-comorbidity conditions. Oxidative stress caused by mitochondrial damage releases proapoptotic factors and activates the pathway of cell apoptosis and cardiomyocyte death [29]. Thus, mitochondrial homeostasis is closely related to the extent of cardiac IR damage during diabetes in aging hearts.

The AMPK/SIRT1 pathway plays a key role in cell survival and mitochondrial homeostasis in cardiac cells [6,9]. This pathway also has antidiabetic and antiaging benefits in addition to having cardioprotective effects [30]. To gain insight into the signaling pathways responsible for the antioxidant and antiapoptotic effects of combination therapy in the aged diabetic heart, we examined the role of the AMPK pathway. Results showed that combination therapy reduced the apoptotic and oxidative damage induced by cardiac

IR through activation of AMPK and its downstream target SIRT1 in cardiomyocytes. AMPK activation has been shown to induce cardiac resistance against IR injury by activating mitochondrial ATP-dependent potassium channels as well as by reducing oxidative stress and cardiac apoptosis [31]. In our study, AMPK phosphorylation caused by the combined therapy was sufficient to counteract IR-induced infarct size and apoptosis in the diabetic heart. CC administration, along with exacerbating oxidative stress, was able to reverse the effect of the combination therapy by reducing Bax and Casp3 expression and increasing Bcl2 expression. CC administration could also significantly reduce the combination therapy-induced upregulation of SIRT1 activity. Therefore, phosphorylation and enhancement of AMPK activity by the combination therapy and its suppression by CC is strong confirmation of the contribution of the AMPK pathway in protection of the diabetic heart by the combined therapy in aged rats. Activation of SIRT1 following AMPK phosphorylation by the combined therapy can balance mitoROS production by increasing MnSOD, thereby reducing the production of Bax and other proapoptotic mediators. SIRT1 is also required for mitochondrial biogenesis and stimulates SOD and catalase expression through peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) deacetylation, leading to upregulation of mitofusin-2 (needed for mitochondrial fusion), forkhead box transcription factor one (FOXO-1) and nuclear factor erythroid 2-related factor 2 (NRF2) (needed for mitochondrial redox signaling) [32]. However, animals lacking the SIRT1 gene should be used in future studies to confirm the causative role of SIRT1 in protection.

Sitagliptin works by inhibiting DPP-4 and thereby increasing the hormone glucagon-like peptide-1 (GLP-1) activity in cells, thus having diverse activities to reduce insulin resistance, regulate intracellular energy homeostasis and ion channel function, boost mitochondrial function and improve the lipid profile [33]. These actions may contribute to the cardioprotective advantage of sitagliptin in combination therapy in aged diabetic rats. Melatonin also has membrane receptor-dependent and non-dependent effects [34]. However, the possible role of melatonin membrane receptors in its protective effects in the heart of aged diabetic rats is unknown and further studies are needed to elucidate their participation. To confirm the findings of this study, it is also necessary to explore the effect of the combination of sitagliptin and melatonin on the process of mitochondrial biogenesis and function in the diabetic heart during aging.

In conclusion, we showed that the administration of a combined therapy of melatonin and sitagliptin to aged diabetic rats reduced myocardial infarct size induced by IR injury by suppressing mitochondrial-dependent oxidative stress and apoptosis; the combined effect of melatonin and sitagliptin was superior to individual interventions. The cardioprotective effect of the combined therapy was accomplished through the activation of the AMPK/SIRT1 pathway in the diabetic heart of aged rats. This study revealed that the combination of melatonin and sitagliptin could have promising application in elderly diabetic patients with ischemic heart disease. Further clinical studies are warranted before a definitive conclusion can be reached.

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**Author contributions:** All authors designed the project, performed the experimentations, analyzed and interpreted the data. ML was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

**Conflict of interest disclosure:** The authors do not have any conflicts of interest to disclose.

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