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Sinapic acid protects heart against ischemia/reperfusion injury and H9c2 cardiomyoblast cells against oxidative stress

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ABSTRACT

The present study was designed to evaluate antioxidant and cardioprotective potential of sinapic acid (SA) against ischemia/reperfusion (I/R) injury. Cardiac functional recovery after I/R was evaluated by percentage rate pressure product (%RPP) and percentage coronary flow (%CF). Myocardial injury was evaluated by 2,3,5-triphenyltetrazolium chloride (TTC) staining and LDH enzyme leakage. Oxidative stress was estimated by lipid peroxidation level. eNOS protein expression in reperfused heart was assessed using Western blot method. Finally, in order to support the antioxidant effect of SA, *in vitro* protective potential of SA was assessed on H_2O_2-induced oxidative stress in H9c2 cardiomyoblast cells. The overall results demonstrated that I/R induced cardiac dysfunction, injury and oxidative stress was attenuated by SA treatment. Moreover, *in vitro* results also shown that, SA protects H9c2 cells from oxidative stress and modulates mitochondrial membrane permeability transition (MPT). In conclusion, coupled results from both *in vivo* and *in vitro* experiments have confirmed that SA with antioxidant role protects cardiac cells and its functions from I/R induced oxidative stress.

1. Introduction

Coronary heart disease (CHD) is the leading cause of death and disability worldwide. The effects of CHD are usually attributable to detrimental effects of acute myocardial ischemia/reperfusion (I/R) injury, which is also a major challenge during organ transplantation, cardiothoracic, vascular and general surgery [1]. Ischemia or oxygen deprivation, followed by reintroduction of blood flow to the ischemic tissue can cause myocardial damage and cardiac contractile dysfunction, termed as reperfusion injury [2]. I/R aggravated vascular endothelial dysfunction characterized by abrogated endothelium-dependent dilation [3,4].

Antioxidant treatment is considered as a possible approach to prevent I/R injury, because oxidative stress is a key factor that triggers it [5,6]. In the natural antioxidants point of view, recent report has shown that, curcumin pre-treatment attenuates mitochondrial oxidative damage induced by I/R [7]. In a similar way, we aimed to evaluate the potential of sinapic acid (SA) on I/R injury. Sinapic acid, a phenolic acid is a cinnamic acid derivative which possesses 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid. It is obtained from various sources such as rye, fruits and vegetables [8]. It has already been pharmacologically evaluated for its antioxidant [9,10], antihyperglycemic [11] and anti-inflammatory properties [12].

In this study, *ex vivo* rat heart preparations subjected to I/R were used to assess antioxidant and cardioprotective activity of SA. Therefore, the aim of the present study was to: (i) Evaluate protective efficacy of SA on oxidative stress and cell death in rat heart subjected to I/R. (ii) Explore efficacy of SA on recovery of cardiac function and coronary endothelial function after reperfusion. (iii) Confirm the cardioprotective efficacy of SA with H9c2 cardiomyoblast cells through *in vitro* H_2O_2-induced oxidative stress model.

2. Materials and methods

2.1. Animals and ethical statement

Male albino Wistar rats (180–220 g), were obtained from the Central Animal House, Annamalai University. The animals were kept under 12-h light/dark cycles, at 22 °C with food and water

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ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar (Reg No. 160/1999/CPCSEA, Proposal number: 926).

2.2. Experimental protocol

SA was dissolved in corn oil (vehicle – 5 mL/kg) and administered orally to rats using an intragastric tube daily for 7 days. The rats were randomly divided into four groups of six rats per group: (i) Control group pre-treated with vehicle alone for 7 days (isolated rat hearts subjected to continuous perfusion). Isolated rat hearts obtained from the following three groups were subjected to I/R protocol comprise of perfusion with a Krebs buffer solution for 10 min to stabilize the cardiac functions and then subjected to 30 min of global ischemia, followed by 60 min of reperfusion; (ii) I/R hearts pre-treated with vehicle alone for 7 days (Control (I/R)); (iii) I/R hearts pre-treated with sinapic acid (I/R + SA 20 mg/kg body weight); (iv) I/R hearts pre-treated with sinapic acid (I/R + SA 40 mg/kg body weight).

2.3. Langendorff isolated heart experiment

The animals were anaesthetized with an intramuscular injection of ketamine (75 mg/kg body weight). After thoracotomy, the hearts were rapidly excised and placed in cooled (4 °C) Krebs Henseleit bicarbonate solution [composition (in mM): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.3 $CaCl_2$, 25.0 $NaHCO_3$, 11.0 glucose]. The heart was then attached to the cannula through aorta and retrogradely perfused with the Krebs solution maintained at 37 °C and continuously gassed with a mixture of 95% O_2–5% CO_2. Perfusion pressure was kept constant at 80 mmHg. The I/R protocol was followed as described previously [13,14]. An elastic water-filled balloon was introduced into the left ventricle through a left atrial incision and connected to a Pressure Transducer (ADInstruments) linked with a PowerLab data acquisition unit (ADInstruments). The balloon volume was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5–10 mmHg. The percentage rate pressure product [RPP = (LVSP − LVEDP) × HR] and percentage coronary flow were assessed as described previously [15–17]. Coronary effluent was collected for the estimation of lactate dehydrogenase (LDH) activity.

2.4. Measurement of infarct size

Infarct size was measured by staining hearts with 2,3,5-triphenyltetrazolium chloride (TTC) [18]. After Langendorff perfusion, the hearts were sliced into 2–3 mm thick sections perpendicularly to the long axis and incubated in 1% of TTC in phosphate-buffered saline (PBS) for 20 min in a 37 °C incubator followed by fixation for 10 min in 4% paraformaldehyde. From the acquired images infarcted area was analyzed using ImageJ software [19], and normalized infarct size (percentage) was derived by dividing the calculated total infarct size with the total heart volume.

2.5. Lipid peroxidation and LDH activity

Reperfused hearts were sliced into pieces and homogenized in 0.1 M Tris–HCl buffer in cold condition (pH 7.4) to give 20% homogenate (w/v). The homogenate was centrifuged at 560×*g* for 10 min at 4 °C. The supernatant was separated and used for the estimation of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) by the methods of Niehaus and Samuelson [20] and Jiang et al. [21], respectively. Activity of LDH in the coronary effluent was assayed by commercially available kit (Agappe diagnostics, Kerala, India).

2.6. Activity of mitochondrial enzymes

Mitochondrial isolation and enzyme assays were performed as explained previously [22]. The activities of isocitrate dehydrogenase (ICDH), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and α-ketoglutarate dehydrogenase (α-KGDH) were assayed in the reperfused hearts by the methods of King [23], Slater and Borner [24], Mehler et al. [25], Reed and Mukherjee [26], respectively.

2.7. eNOS protein expression – Western blot analysis

Tissue proteins were extracted by homogenization of reperfused hearts in Radio-Immunoprecipitation Assay (RIPA) buffer. Protein concentrations were estimated using the BCA protein assay kit (Merck, India). Protein samples were separated with 10% SDS–polyacrylamide gel electrophoresis gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, USA). Nonspecific binding sites were blocked by 1% bovine serum albumin (BSA) in PBS with 0.05% TWEEN 20 at room temperature for 1 h, then incubated overnight at 4 °C with primary antibody against eNOS (1:1000 dilution) and then incubated with anti-mouse IgG (Sigma–Aldrich, USA) conjugated to horseradish peroxidase. The reaction was developed with a DAB detection system (Merck, India). The densitometric analysis was performed using ImageJ software [19]. Western blot densitometry data of eNOS was normalized to β-actin.

2.8. H9c2 cardiomyoblast cell culture

Rat embryonic cardiomyoblast derived H9c2 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and a combination of penicillin–streptomycin (1%) in a humidified 5% CO_2 atmosphere at 37 °C.

2.9. In vitro oxidative stress and mitochondrial transmembrane potential study

In order to evaluate the cytotoxic effect of SA, viability was checked with MTT assay on SA treated (25–100 μM) H9c2 cells. The assay was performed by seeding H9c2 cells in the concentration of 1×10^4 cells/well in 96-well plate. For assessment of protective potential of SA against oxidative stress, different concentrations of SA (0.1, 1 and 10 μM) were incubated with H9c2 cells for 2 h, and then co-incubated with 500 μM of H_2O_2 for further 18 h [27,28]. For viability analysis, MTT solution (5 mg/mL) was added to each well, and incubated for 4 h at 37 °C. After incubation, optical density was measured on a microplate reader at 570 nm. With the 10 μM dose of SA, the level of intracellular reactive oxygen species (ROS) formation was quantified with fluorimetry using redox-sensitive fluorescent probe 2, 7-dichlorodihydrofluorescin diacetate (DCF-DA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Further, to examine mitochondrial membrane permeability transition (MPT), H9c2 cells were incubated with 5 μg/mL Rhodamine 123 at 37 °C for 30 min [29]. The images were acquired using the Olympus IX71 inverted fluorescence microscope.

2.10. Statistical analysis

Values are represented as mean ± SD for six rats in each group. Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using SPSS version 20. The limit of statistical significance was set at $P < 0.05$.

3. Results

3.1. SA improves cardiac functional recovery in the reperfused heart

Oral pre-treatment with SA significantly ($P < 0.05$) improved the percentage rate pressure product (%RPP) recovery and percentage coronary flow (%CF) recovery in hearts subjected to I/R procedure when compared with untreated I/R hearts (Fig. 1A and B).

3.2. SA modulates LDH activity and infarct size in the reperfused heart

Untreated I/R hearts showed significantly elevated LDH activity and increased percentage infarct area (Fig. 2A and B). Pre-treatment of SA in I/R subjected hearts significantly restored the above when compared with untreated I/R heart ($P < 0.05$).

3.3. SA inhibits lipid peroxidative damage

Table 1 depicts increased lipid peroxidative markers such as TBARS and LOOH in the I/R heart due to the oxidative burst driven by reperfusion. Meanwhile, SA pre-treatment significantly ($P < 0.05$) reduced the levels of TBARS and LOOH in the reperfused hearts.

3.4. SA restores heart mitochondrial function

Activities of ICDH, α-KGDH, SDH and MDH were significantly decreased in the mitochondria of reperfused hearts (Table 2). Pre-treatment with SA significantly ($P < 0.05$) increased the activities of these enzymes in I/R hearts when compared to untreated I/R group.

3.5. Effect of SA on eNOS protein expression

eNOS protein expression was downregulated in the reperfused heart compared with continuously-perfused heart. SA pre-treatment significantly ($P < 0.05$) enhanced the protein expression of eNOS in I/R heart compared with untreated I/R hearts (Fig. 3A and B).

3.6. Protective effect of SA on H_2O_2-induced oxidative stress in H9c2 cardiomyoblast cells – in vitro study

The results of MTT assay explored that SA does not interfere with the viability of H9c2 cells up to 100 μM (Fig. 4A). Moreover, SA treatment increased the viability in H_2O_2-treated cells in a concentration dependent manner (Fig. 4B). 10 μM dose of SA was chosen for the evaluation of total ROS level and mitochondrial transmembrane potential. The elevation of oxidative stress upon H_2O_2 treatment and the amelioration effect of SA were shown by DCF-DA fluorimetry analysis, which showed SA significantly ($P < 0.05$) protects cells from ROS generation (Fig. 4C).

3.7. Inhibitory effect of SA on mitochondrial membrane permeability transition (MPT) by H_2O_2

Exposure of H9c2 cells to H_2O_2, caused a dramatic change in MPT observed with morphological alterations shown as diffuse

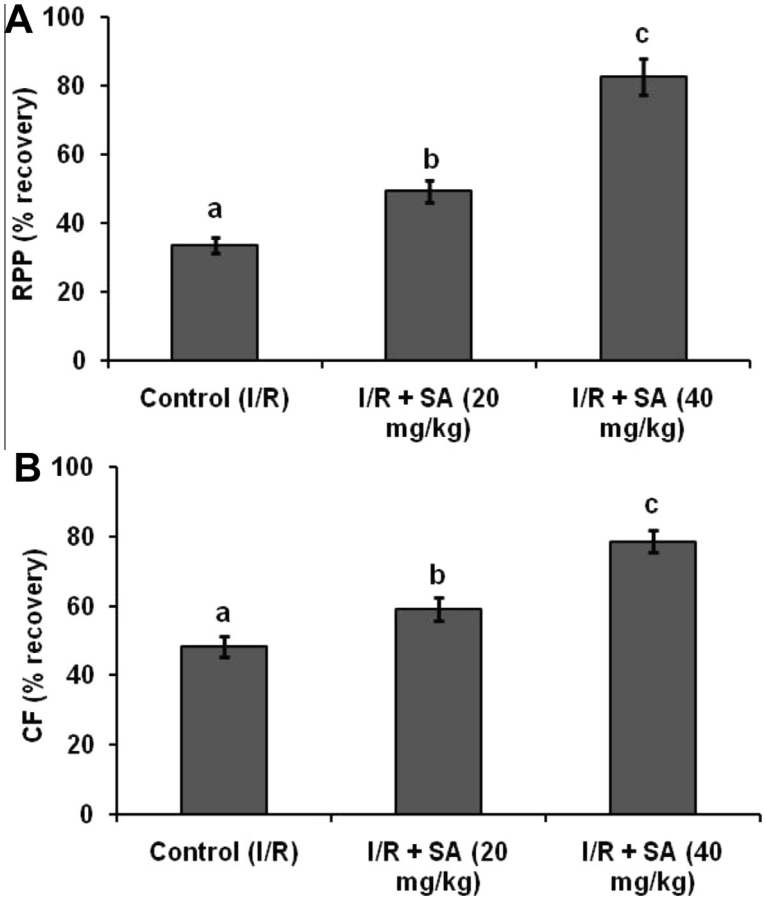

Fig. 1. Effect of SA on cardiac functional recovery. (A) and (B) shows the %rate pressure product (%RPP) recovery and %coronary flow (%CF) recovery, respectively in untreated I/R and SA-treated I/R hearts. Values are represented as mean ± SD ($n = 6$). Values not sharing common letter (a, b and c) are significant with each other at $P < 0.05$.

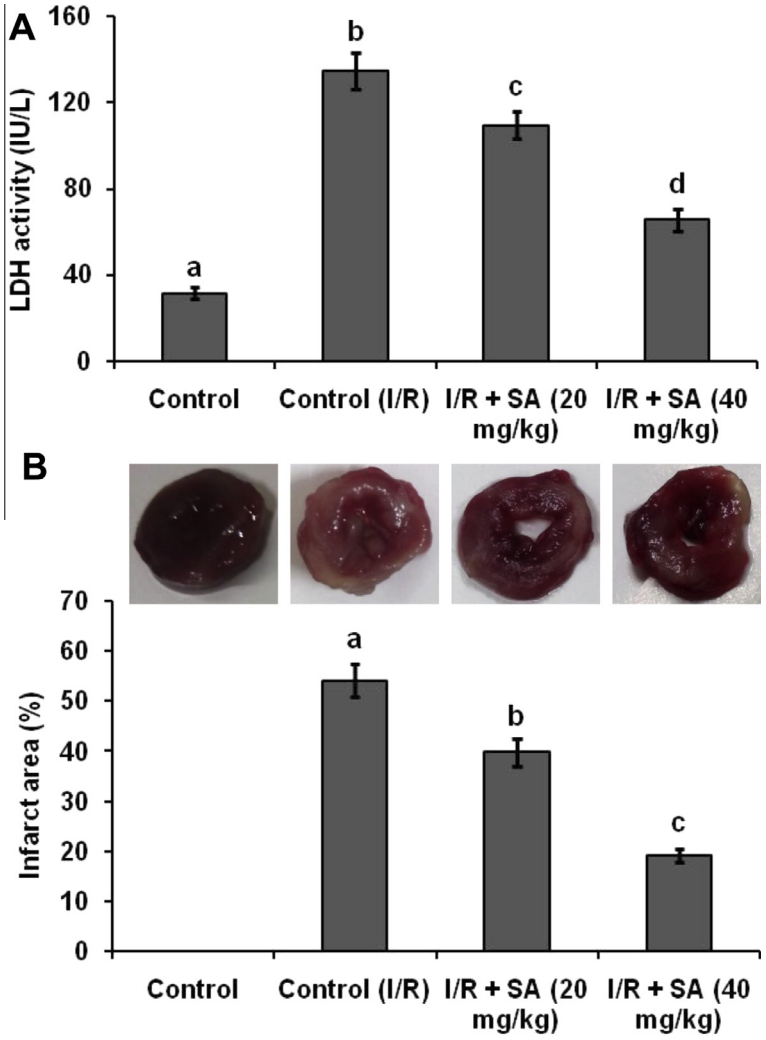

Fig. 2. Effect of SA on cardiac injury. (A) LDH activity in the coronary effluent. Values are represented as mean ± SD ($n = 6$). (B) Representative photomicrographs of the infarct area of untreated I/R and SA-treated hearts, showing infarct (white zones) and non infarct (red zones) after TTC staining and their respective infarct area in percentage determined using ImageJ software. Values are represented as mean ± SD ($n = 3$). Values not sharing common letter (a, b, c and d) are significant with each other at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Effect of SA on lipid peroxidation products in reperfused hearts.

Parameter	Control	Control (I/R)	I/R + SA (20 mg/kg)	I/R + SA (40 mg/kg)
TBARS (mmol/100 g wet tissue)	0.61 ± 0.04[a]	1.43 ± 0.08[b]	1.17 ± 0.06[c]	0.79 ± 0.05[d]
LOOH (mmol/100 g wet tissue)	69.23 ± 3.84[a]	108.31 ± 8.15[b]	95.24 ± 5.43[c]	78.35 ± 4.82[d]

Values are represented as mean ± SD. ($n = 6$). Values not sharing common superscript (a, b, c and d) are significant with each other at $P < 0.05$.

Table 2
Effect of SA on the activities of mitochondrial enzymes in reperfused hearts.

Parameter	Control	Control (I/R)	I/R + SA (20 mg/kg)	I/R + SA (40 mg/kg)
ICDH	694.17 ± 37.23[a]	489.23 ± 26.45[b]	557.37 ± 26.62[c]	644.52 ± 31.18[d]
α-KGDH	62.41 ± 4.12[a]	40.22 ± 2.14[b]	46.42 ± 2.89[c]	54.33 ± 3.12[d]
SDH	243.26 ± 14.31[a]	162.53 ± 10.64[b]	183. ± 10.23[c]	213.52 ± 14.32[d]
MDH	316.4 ± 19.47[a]	258.35 ± 13.22[b]	276.35 ± 12.3[c]	301.4 ± 14.56[d]

Activity is expressed as nM of NADH oxidized/h/mg protein for ICDH; nM of ferrocyanide formed/h/mg protein for α-KGDH; nM of succinate oxidized/min/mg protein for SDH; nM of NADH oxidized/min/mg protein for MDH. Values are represented as mean ± SD ($n = 6$). Values not sharing common superscript (a, b, c and d) are significant with each other at $P < 0.05$.

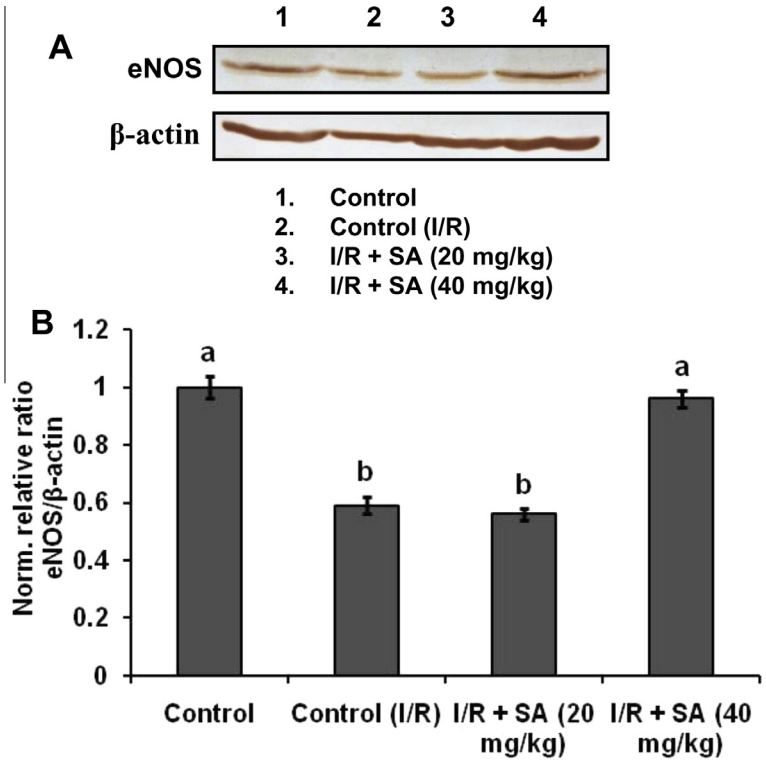

Fig. 3. Effect of SA on eNOS protein expression. Western blot analysis of eNOS (A) and its normalized value with β-actin (B). Values are represented as mean ± SD ($n = 3$). Values not sharing common letter (a and b) are significant with each other at $P < 0.05$.

form (Fig. 4D). Whereas, SA prevented the changes of MPT and morphological features in H_2O_2-treated H9c2 cells shown as punctuated form thereby prevents mitochondrial function (Fig. 4D).

4. Discussion

In this study, the effect of SA was evaluated on myocardial injury with the aid of Langendorff isolated heart system, which subjects isolated rat heart to I/R protocol. Ischemia followed by reperfusion triggers cell death and its suppression is a logical strategy to protect cardiomyocytes [30,31]. Although many drugs highlights on myocardial ischemia intervention, few nutraceutical agents have been recognized to play an effective role and currently, antioxidant therapy has turned into a promising pharmacological approach [32,33].

In order to assess the cardioprotective role of SA on I/R heart, different indices of myocardial function were assessed in this study. Among them percentage RPP recovery, percentage CF recovery and percentage infarct size are being considered as the major parameters, which explains the functional recovery of heart after reperfusion. Our results have shown that, SA pre-treatment prevents I/R induced cardiac contractile dysfunction and improves the functional recovery. Moreover, SA pre-treatment attenuates the level of LDH enzyme leakage in the myocardial effluent [34]. TTC staining, which is a well-accepted method to determine myocardial infarct size provides a reliable index of myocardial necrosis [18]. Result of TTC staining indicates the protective role of SA on myocardial infarction.

Our results displayed an elevated lipid peroxidation product upon I/R, which indicates an accelerated oxidative burst upon reperfusion. Under oxidative stress conditions, membrane permeability and membrane bound enzymes/ion channels were altered by products of lipid peroxidation and exhibits direct cardiac depression at ventricular myocyte level [35]. These dysfunctional responses also resulted from redox modification of proteins involved in excitation–contraction coupling and/or mitochondrial energy production [36]. It is well-known that SA have antioxidant and anti-lipid peroxidation potential [9,10,37]. In this connection, we here show that SA reduces the lipid peroxidation level and protects cardiac cells from membrane damage owing to its antioxidant potential.

I/R induced coronary endothelial dysfunction reduces vasodilatation that contributes to adverse cardiac effects. It was previously mentioned that, substances, which protect cardiac function during I/R, might also protect coronary endothelial function [38]. In this study, coronary flow was reduced after reperfusion due to impaired endothelial relaxation whereas SA prevents the above. Recent reports showed that endothelial dysfunction in coronary arteries, probably due to decreased nitric oxide bioavailability caused by increased endothelial superoxide production [39]. Endothelial cells of cardiac vasculature highly express eNOS and its inhibition/downregulation during I/R leads to reduced production of nitric oxide, which consequently ends with coronary dysfunction [40]. Roy et al. [9,10] already reported that SA have superoxide and hydroxyl radical scavenging potential in a dose dependent manner. From these relationships, we sought that SA pre-treatment prevents coronary endothelial dysfunction in reperfused heart through scavenging free radicals along with elevating the level of nitric oxide by upregulation of eNOS expression.

Mitochondria plays a critical role in constant supply of energy to the heart and its dysfunction is associated with cardiac contractile dysfunction and hypertrophy. In order to reveal the mechanisms behind the mitochondrial dysfunction in heart failure, researchers mainly focussed on TCA cycle and electron transport chain components [41]. It was already explored that under oxidative stress conditions, functions of several key TCA cycle enzymes were perturbed [42]. Further, Nulton-Persson et al. [43] assessed the activities of electron transport chain complexes and Krebs cycle enzymes in isolated rat heart mitochondria, which displayed that H_2O_2 disrupted the activities of α-KGDH, SDH and aconitase.

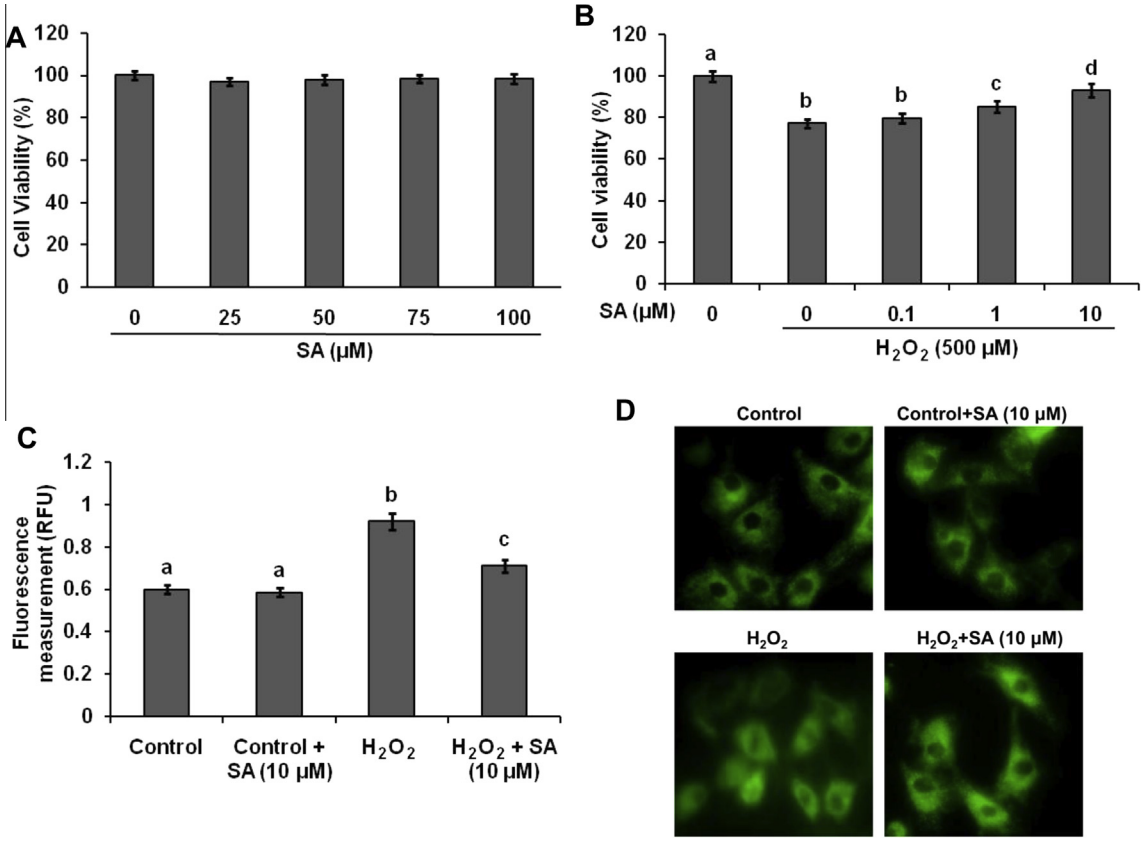

Fig. 4. Effect of SA on H_2O_2-induced oxidative stress in H9c2 cardiomyoblast cells. (A) The cytotoxic effect of SA (25–100 μM) was evaluated in H9c2 cells. (B) Effect of different concentrations of SA (0.1, 1 and 10 μM) on H_2O_2-induced oxidative stress. (C) Intracellular ROS formation was quantified using DCF-DA. The results are represented as mean ± SEM of three independent experiments. Values not sharing common letter (a, b, c and d) are significant with each other at $P < 0.05$. (D) Dramatic change in MPT was observed in H_2O_2 alone treated group with morphological alterations shown as diffuse form. SA prevented the changes of MPT and morphological features in H_2O_2-treated H9c2 cells (H_2O_2 + SA 10 μM) shown as punctuated form. The results were the representative of three independent experiments.

In the current study, oxidative stress upon I/R inactivates mitochondrial enzymes whereas SA pre-treatment shown to be protective which may due to its antioxidant property, thereby improves the energy metabolism and cardiac functional recovery.

The observations of the *in vitro* study revealed a concentration dependent protective effect of SA on H_2O_2-induced injury in H9c2 cells. Oxidative stress, an imbalance between ROS production and elimination, results in over accumulation of intracellular ROS initiating the apoptosis of the cell [44]. Among these ROS, H_2O_2 is the dominant form in cells since it is much more stable in comparison with other ROS [45]. We could argue that some proportion of the ability of SA to reduce H9c2 cardiomyoblast cell injury caused by H_2O_2 could be due to scavenging of ROS. Consistent with oxidative stress results, SA prevented changes in mitochondrial membrane permeability transition and morphological features in oxidative stress induced cells. Considering the results of mitochondrial enzymes activity, these data also insist the protective effect of SA on cardiac cell mitochondria against oxidative stress through prevention of membrane permeability. Our result is comparable with the previous report shows that antioxidant quercetin protects H9c2 cardiomyoblast cells against hydrogen peroxide-mediated disruption of mitochondrial membrane permeability transition (MPT) [29]. In this connection, we conclude that cardioprotective efficacy of SA is due to its antioxidant and mitochondrial protective property.

In conclusion, this study demonstrated that treatment with SA protects cardiac function, coronary flow and mitochondrial function in heart subjected to I/R. *In vitro* studies on H9c2 cardiomyoblast cells confirm the cardioprotective effect of SA attributed to its antioxidant property. The overall study suggests that further pharmacological evaluation of SA could be helpful as an adjuvant therapy for myocardial I/R injury.

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