



ORIGINAL ARTICLE

Sodium Thiosulfate Preconditioning Ameliorates Ischemia/Reperfusion Injury in Rat Hearts Via Reduction of Oxidative Stress and Apoptosis

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Abstract

Purpose Sodium thiosulfate (STS) has of late been proven efficacious in models of urolithiasis and vascular calcification. However, its cardiovascular effects on ischemia reperfusion injury (IR) have not been revealed. Being an antioxidant and calcium chelator, it is assumed to play a vital role in IR as ROS production and calcium overload are major perpetrators of IR injury.

Methods The cardioprotective effect of STS was evaluated *in vitro* using H9C2 cardiomyocytes and *in vivo* using both isolated rat heart and intact left anterior descending artery (LAD) occlusion models of ischemia reperfusion injury. Finally, *in silico* tools were utilized to establish its possible mode of action. Myocardial injury markers and expression of apoptotic proteins were studied along with myocardial histopathology.

Results STS of 1 mM recovered H9C2 cells from glucose oxidase/catalase-induced apoptosis. The isolated rat heart treated with STS prior to IR injury improved its hemodynamics and reduced the infarct size to 9%. This was supported by the absence of derangement of cardiac fibers from H&E stained section of LAD-occluded rats. Plasma troponin levels decreased by 15% compared to IR and the myocardium showed diminished apoptotic proteins. An *in silico* docking

analysis revealed higher binding affinity of STS for caspase-3 with a binding energy of -60.523 kcal/mol for the complex. **Conclusion** The effectiveness of STS as a cardioprotective agent is attributed to the reduction of apoptosis by binding to the active site of caspase-3 *in silico*, which was substantiated by the reduced expression of caspase-3 and poly ADP ribose polymerase levels.

Keywords Ischemia reperfusion injury · Sodium thiosulfate · Caspase-3 · Langendorff heart · Antioxidant · *In silico*

Introduction

Myocardial ischemia/reperfusion injury (IR) is an undesirable event that occurs during the restoration of blood supply to the ischemic myocardium and is considered one of the primary causes of mortality in the treatment of cardiovascular diseases. Despite several successful therapies reported to reduce reperfusion injury, lack of an effective clinical therapy to ameliorate IR is of great concern. Myocardial IR injury is an intricate process that manifests as irreversible cellular injury which leads to myocardial stunning, arrhythmias, lethal reperfusion injury, and microvascular dysfunction [1].

Reactive oxygen species (ROS)-linked calcium overload has been reported in the myocardium subjected to IR injury, and the source of this oxidative stress is primarily associated with the mitochondria [2]. Sudden increase in ROS can potentially activate cellular stress mechanisms that not only imbalance cellular redox regulation but also change membrane potential. Reperfusion of the ischemic heart alters the mitochondrial membrane potential which opens the mitochondrial permeability transition pore to release cytochrome c, triggering apoptosis [2, 3].

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Therapeutic agents that can reduce oxidative stress or accumulation of calcium in the mitochondria have shown promising results in treating IR injury [4]. Hydrogen sulfide is one such agent that has shown significant benefit as a cardio protective drug against IR injury [5]. H₂S is endogenously produced by cysteine metabolism but is reported to be toxic upon direct exposure at higher concentrations > 20 ppm as per OSHA guidelines. However, its metabolite thiosulfate, a traditional antidote for cyanide toxicity, is safe and non-toxic (LD₅₀ = 2.5 g/kg; intravenous rat). In addition, it is a US FDA-approved drug to treat calciphylaxis [6]. An in vitro study on the effect of sodium thiosulfate in isolated mitochondria subjected to oxidative stress has demonstrated not only its mito-protective effect but also underlines its efficiency in reducing oxidative stress [7].

In this study, cultured cardiomyocytes, ex vivo and in vivo rat models of myocardial ischemia reperfusion injury were employed to explore the cardioprotective effects of sodium thiosulfate and the underlying mechanisms.

Methods

Evaluation of Efficacy of STS in an In Vitro Hypoxia Re-oxygenation Model

HR Model Using Glucose Oxidase/Catalase and Reconfirmation with Cobalt Chloride-Induced Apoptosis [8]

Cardiomyocytes H9C2 were cultured under standard conditions as mentioned in supplementary methods section 1.2.1. Sodium thiosulfate (100, 500, and 1000 μM) was added to cells and pre-treated for 12 h followed by exposure to hypoxia medium employing enzymatic glucose oxidase/catalase (GOX/CAT) hypoxia re-oxygenation system with added 2-deoxyglucose [8]. The concentrations were set based on the data from cytotoxicity assessment for STS (section 1.2.2.). The cells were cultured under hypoxia for 6 h, followed by normal DMEM medium for 16 h to mimic reperfusion. The cell viability of the H9C2 cardiomyocytes was determined using crystal violet assay. The data from three individual experiments were taken into consideration for assessing the efficacy of STS at the above concentrations.

The data obtained from GOX/CAT was reconfirmed with a standard cobalt chloride-induced apoptosis model. Briefly, cells were incubated with medium containing 800 μM CoCl₂ for 6 h followed by replacement with fresh medium to induce apoptosis. STS at above mentioned concentrations were added and incubated for 24 h prior to CoCl₂ incubation. At the end of 12 h after replacement with fresh medium, crystal violet assay was performed to assess the % viability compared to no treatment control. The data from HR models was subjected to non-

linear regression analysis to obtain the EC₅₀ value for STS to estimate the dose to be employed for animal experiments.

Acridine Orange and Ethidium Bromide Staining for Apoptosis Quantification

Apoptosis induced due to HR injury in GOX/CAT model was assessed using AO/EtBr staining as described previously [9]. AO is a vital dye while EtBr is only incorporated by cells that lost membrane integrity. After 16 h of reperfusion, the culture supernatants were removed, trypsinized, and a solution of PBS containing EtBr and AO (25 ng/mL; v/v) was added to cells. Images were acquired using an inverted fluorescence microscope with original magnification of 10×. The percentage of EtBr positive cells, representing the percentage of cell death, was analyzed using progress capture Olympus Axio vert II.

Effect of Sodium Thiosulfate on Isolated Rat Heart Model of Global Ischemia Reperfusion Injury

Induction of Ischemia Reperfusion Injury

All the procedures involving animal experimentation were performed in accordance with the guidelines of the Committee for the purpose of conduct and supervision of experiments on animals (CPCSEA, India), with a prior approval of the Institutional Animal Ethical Committee (IAEC, SASTRA University, No.: 229/SASTRA/IAEC/RPP) as mentioned in the supplementary methods section 1.3.1. The isolated heart preparation was performed as per the method provided in the supplementary section 1.3.2. A typical IR protocol consisted of 30-min ischemia induced by stopping the buffer flow, followed by 60-min reperfusion by resuming the buffer flow. The rats were randomly divided into 5 groups ($n = 6/\text{group}$) viz., normal, ischemia-reperfusion (IR), ischemic preconditioning (IPC), drug control (STS_ctrl), and sodium thiosulfate preconditioning (STS_IPC) at low dose (0.1 mM) and high dose (1 mM). Ischemic preconditioning (IPC) was given after 10-min stabilization window for 15 min in 3 cycles of 2-min ischemia followed by 3-min reperfusion. STS was infused via KH buffer for 15-min period prior to the global ischemia, mimicking the IPC protocol as standard. Hemodynamic parameters such as end diastolic pressure (EDP), developed pressure (DP), rate pressure product (RPP), maximum and minimum dp/dt were calculated from the left ventricular pressure changes using Lab Chart pro package (AD instruments, Australia).

Myocardial Injury Estimation Using Lactate Dehydrogenase (LDH) and Creatine Kinase Activity

The cardiac injury marker levels, mainly LDH and CK, were estimated in both the coronary perfuse and tissue

homogenate. The heart homogenate was prepared in Tris-HCl buffer (pH = 7.4), and the LDH and CK levels were estimated as per the standard instructions of the kit from Sigma-Aldrich (MAK006 and MAK116).

Infarct Size Assessment by Triphenyl Tetrazolium Chloride Staining

TTC (1.5%) in PBS was used to estimate the area of infarct produced due to global IR injury [5]. Briefly, 1-mm sections of heart post-IR were incubated in the above solution for 10 min at 37 °C, and the images were acquired using a zoom stereomicroscope (NIKON-SMZ1270) equipped with a high definition CCD camera (NIKON-DS-Fi2), using NIS-elements documentation tool. The percentage of infarcted tissue (triphenyl tetrazolium chloride negative regions) developed was evaluated using the ImageJ analysis tool (NIH-USA).

Molecular Docking Studies of Sodium Thiosulfate with Caspase-3

Molecular Docking Studies

The docking studies were performed using Auto Dock 4.2 software. The preparation of protein and ligand was done as mentioned in supplementary methods section 1.4. Caspase-3 was treated to be rigid during the docking simulation. Grid box was constructed and positioned on the centroid of the co-crystallized inhibitor (AC-DEVD-CHO). The dimension of the grid box is set to $60 \times 60 \times 60$ with the default spacing of 0.375 Å between grid points. The grid box included all the important binding site amino acids including Arg 64(A), Gly 122(A), Gln 161(A), Cys 163(A), Ser 205(B), ARG 207(B), Asn 208(B), and Phe 250(B). All the required map files for docking simulations were generated using Auto Grid 4. The Lamarckian genetic algorithm was used for the docking simulation. The docking simulation was performed for 50 runs, and the simulation results were written in the docking log file (dlg). The estimated binding free energy, inhibition constant, and binding modes were analyzed using Auto Dock Tools (ADT), chimera, and discovery studio visualizer.

MM/GBSA Binding Free Energy Calculation

The molecular mechanics energies combined with generalized Born and surface area continuum solvation (MM/GBSA) methods are popular approaches to estimate the free energy of the binding of small ligands to biological macromolecules. The most widely used computational methods in drug design are docking and scoring [10], thereby the binding mode of the drug is predicted, followed by an estimate of the binding affinity. The binding free energy was calculated by $\Delta G_{Bind} = \Delta E_{MM} + \Delta G_{Solv} + \Delta G_{SA}$ where ΔE_{MM} is the difference in the

minimized energies between the protein ligand complexes. ΔG_{Solv} is the difference in the GBSA solvation energy of the protein ligand complex and sum of the solvation energies for the protein and ligand. ΔG_{SA} is the difference in the surface area energies for the complex and some of the surface area energies in the protein and ligand. Using MM/GBSA, the binding free energies of caspase-3-thiosulfate complex and caspase-3 AC-DEVD-Cho inhibitors were calculated and compared for accuracy using Schrodinger.

In Vivo Assessment of Cardioprotection Using Left Anterior Descending Artery Ligation Model

LAD Ligation Model

The rats were anesthetized using halothane (1.5% with O₂), supported on a rodent ventilator (70 strokes/min at tidal volume of 10 mL/kg), and placed on a thermal heating pad to maintain 37 °C. After 10 min of stabilization time, an incision was made between the 3rd and 4th intercostal ribs to locate the heart. The pericardium was detached, to locate the left anterior descending artery. A 7–0 suture was passed below the LAD, and a slipknot was made to occlude the blood flow. Ischemia was created for 30 min, which was confirmed by the appearance of regional epi-cardial cyanosis and the knot was released for reperfusion of the myocardium for 2 h. Successful reperfusion visualized from the arterial blood flow and appearance of hyperemia over the surface of the previous cyanotic segment [11].

Experimental Groups

The rats were divided into 4 groups ($n = 6$ per group) consisting of normal/ sham control, IR group, IPC consisting of 3 cycles of 2-min ischemia followed by 3 min of reperfusion before the main ischemic event and STS 1 mM infused via jugular vein for 15 min prior to ischemia. At the end of reperfusion time, blood was collected in K₂EDTA tubes and the heart was isolated and immediately flash frozen in liquid nitrogen for storage at –80 °C, until further analysis. Plasma was analyzed to confirm the elevated levels of cardiac injury markers such as cardiac troponin I and lactate dehydrogenase, using the automated AIA-2000 platform (Tosoh Corporation, Tokyo, Japan).

Western Blot Analysis for Apoptosis Markers

The apoptosis-related proteins mainly caspase-3 (CST #9665), cleaved caspase-3 (CST #9664), PARP, and cleaved PARP (CST #9542) were determined with Western blotting analysis [12]. Myocardial tissue samples from the ischemic zone were homogenized with an ice-cold RIPA buffer. Protein concentration was determined using Bradford reagent

(Bio-Rad, USA). Equal concentrations of proteins were mixed with SDS sample buffer and denatured at 75 °C for 5 min. The samples were resolved with 8% SDS-PAGE gel and transferred on to 0.45-μm PVDF membranes. The membranes were blocked with 5% defatted milk in 0.1% Tris-buffered saline with Tween (TBST) for 1 h and then probed with primary antibodies: caspase-3, cleaved caspase-3, PARP, and cleaved PARP antibodies (Cell Signaling Technologies, USA), at 4 °C overnight. After being washed for three times with TBST, the membranes were incubated with anti-rabbit secondary antibodies (CST #7074) in TBST at room temperature for 1 h. Membranes were washed thrice with TBST, visualized on Chemi-Doc XRS (BioRad, USA), using chemiluminescent detection system (ECL, BioRad, USA). The housekeeping protein β-actin (CST #8457) was used as protein loading control. The relative band intensities were measured by image analysis software Quantity-One (BioRad, USA).

Histopathological Examination of Ventricle Sections

For histopathological evaluation, heart tissues fixed in 10% neutral buffered formalin were embedded in paraffin to make tissue blocks. The blocks were then cut into 5-μm sections and stained with hematoxylin and eosin (H&E). At least three hearts from each group were examined under light microscope (NIKON, JAPAN) for any pathological changes.

Assessment of Lipid Peroxidation and Antioxidant Status of Cardiac Myocardium

The heart tissue was homogenized in Tris-HCl buffer pH = 7 and used for estimation of lipid peroxidation based on thiobarbituric acid reactive species assay [13]. The antioxidant status of myocardium was assessed by estimating reduced glutathione and the antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase as per standard protocols referred in the supplementary methods section 1.2.3.

Mitochondrial Enzyme Activity Assessment

The mitochondria from the heart were isolated using sucrose method by density-based separation [7]. Briefly, heart tissue was homogenized in isolation medium (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EDTA, 0.2% BSA, pH = 7.4) and centrifuged at 600×g for 10 min at 4 °C. The supernatant was subjected to centrifugation at 14,000×g for 10 min at 4 °C to obtain a crude mitochondrial pellet. The pellet was washed with two such cycles, and their protein content was assessed using Bradford reagent (BioRad, USA). The mitochondrial enzymes such as

NADH dehydrogenase, malate dehydrogenase, and succinate dehydrogenase were assessed in these samples according to standard protocols referred in supplementary methods section 1.2.4, using Synergy H1 spectrophotometer (BioTek, USA).

Statistical Analysis

Data were presented as the mean ± SD. Graph Pad Prism 7.0 was used for all of the statistical analyses. Significant differences between the groups were analyzed using one-way ANOVA followed by post hoc analysis by Dunnett's test. For an unbiased analysis, data investigators were blinded to treatment and control groups.

Results

Sodium Thiosulfate Protects H9C2 from Hypoxia Re-oxygenation-Induced Cell Death, Intracellular ROS Accumulation and Preserves Mitochondria

H9C2 cardiomyocyte cell lines were used to study the effect of sodium thiosulfate on oxidative stress. Based on a cytotoxicity assessment by crystal violet assay at graded STS concentrations (mM) of 400, 200, 150, 100, 80, 60, 40, 30, 20, 10, 1, and 0.5 (Supplementary Fig. S1), three doses (10, 1, and 0.5 mM) were selected below the IC₅₀ value of 27.54 mM to assess the impact on GOX/CAT and CoCl₂-induced oxidative stress on cell viability. The HR and CoCl₂ models induced 46 and 44% cell death, respectively, and upon treatment with STS, cell viability improved in both the models at 10 mM (HR 82%, CoCl₂ 74%) and 1 mM (HR 77%, CoCl₂ 63%) significantly ($P < 0.05$) compared to stress control (Fig. 1a). The results from both the models were analyzed using non-linear regression, and the EC₅₀ for HR model was found to be 0.131 mM, while that of the CoCl₂ model was 0.115 mM.

In addition, we evaluated the apoptotic index using AO/EtBr assay in the HR model (Fig. 1b) and found several EtBr positive cells in the HR control group (Fig. 1d). STS pre-treatment at 1 mM (Fig. 1g) had lower EtBr positive cells when compared to the other two concentrations (10 and 0.5 mM) as observed from the fluorescence intensity (Fig. 1b).

Therefore, we fixed a 1 mM dose to check effectiveness of STS in reducing oxidative stress by measuring the activities of antioxidant enzymes like SOD, catalase, GR, and GPx in the GOX/CAT model. Supplementary Fig. S2 represents the activities of these enzymes which were significantly ($P < 0.05$) lowered due to HR and showed an improvement with STS pre-treatment when compared with the control cells. STS

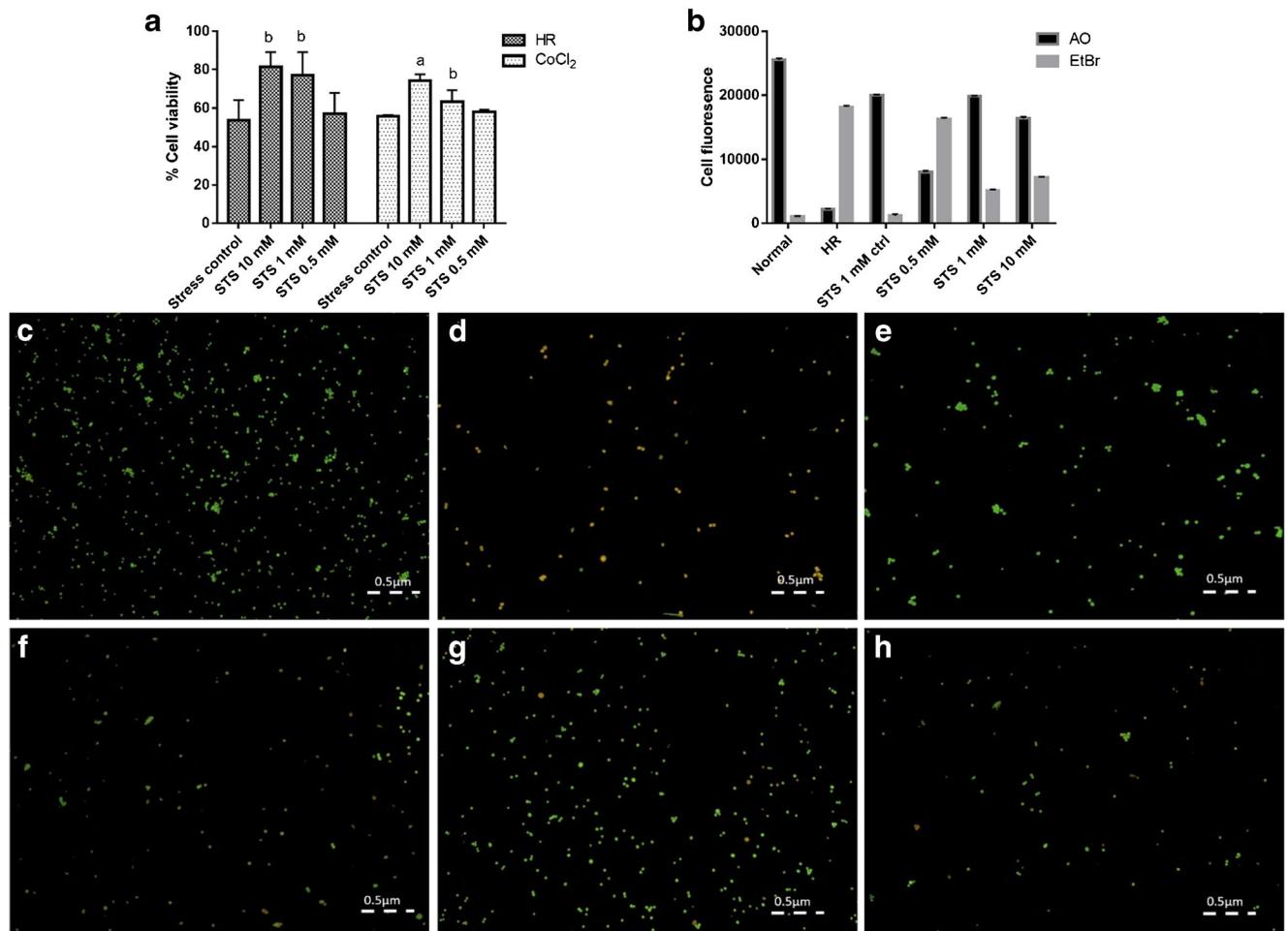


Fig. 1 H9C2 cardiomyocytes were treated with sodium thiosulfate (STS) at various concentrations to assess for its effectiveness against **a** hypoxia re-oxygenation (HR) injury by enzymatic GOX/CAT model and chemical induced CoCl_2 model, using crystal violet assay. The anti-apoptotic effect of STS was **b** quantified by AO/EtBr assay and the representative images

of regions are presented for **d** normal, **e** HR, **f** STS 1 mM control, **g** STS 0.5 mM, **h** STS 1 mM, and **i** STS 10 mM. The graphs represent mean \pm SD of three independent experiments. $^aP < 0.001$, $^bP < 0.05$ vs. the respective stress control

pre-treatment also improved the endogenous antioxidant, GSH, levels close to normal.

Mitochondrial dysfunction, being one of the cardinal features of reperfusion injury, was assessed in H9C2 cells subjected to HR in GOX/CAT model by measuring mitochondrial swelling behavior and the activities of marker enzymes such as NADH dehydrogenase, MDH and SDH. Supplementary Fig. S3 shows mitochondrial swelling behavior in different mitochondrial energy states, namely, non-energized and energized (either with succinate or glutamate malate as substrate) conditions (refer to y-axis). Treatment of the cells with 1 mM STS prior to HR improved the physiology of both energized and non-energized mitochondria, indicating the possibility that STS can act as an electron donor to the mitochondrial electron transport chain. The above observations were well supported by the improved activity of ferricyanide sensitive NADH dehydrogenase, and TCA cycle enzymes succinate dehydrogenase and malate dehydrogenase.

In Silico Studies in Thiosulfate Reveal Its Affinity Towards Caspase-3, Which Is Predicted to Be Responsible for Its Anti-apoptotic Efficiency

Molecular docking simulation results show that thiosulfate has a strong binding affinity for caspase-3 (Fig. 2). The estimated lowest binding energy and inhibition constant are -6.48 kcal/mol and $17.89 \mu\text{M}$, respectively. From the docking results, it was identified that the thiosulfate molecule forms strong hydrogen bonds with Arg 64(A), Gln 161(A), and Arg 207(B). Table 1 lists the hydrogen bond distances, all of which are within the acceptable range of 2.6 to 3.1 \AA [14]. Docking analysis also revealed that the amino acid Cys 163(A) is present in close proximity of 3.79 \AA to one of the free sulfur atoms in the thiosulfate molecule (Fig. 2c). Also, the His 121(A) is present at a distance of 5 \AA from one of the charged sulfur atoms (Fig. 2c). On calculating the MM/GBSA, binding free energy for caspase-3-thiosulfate and

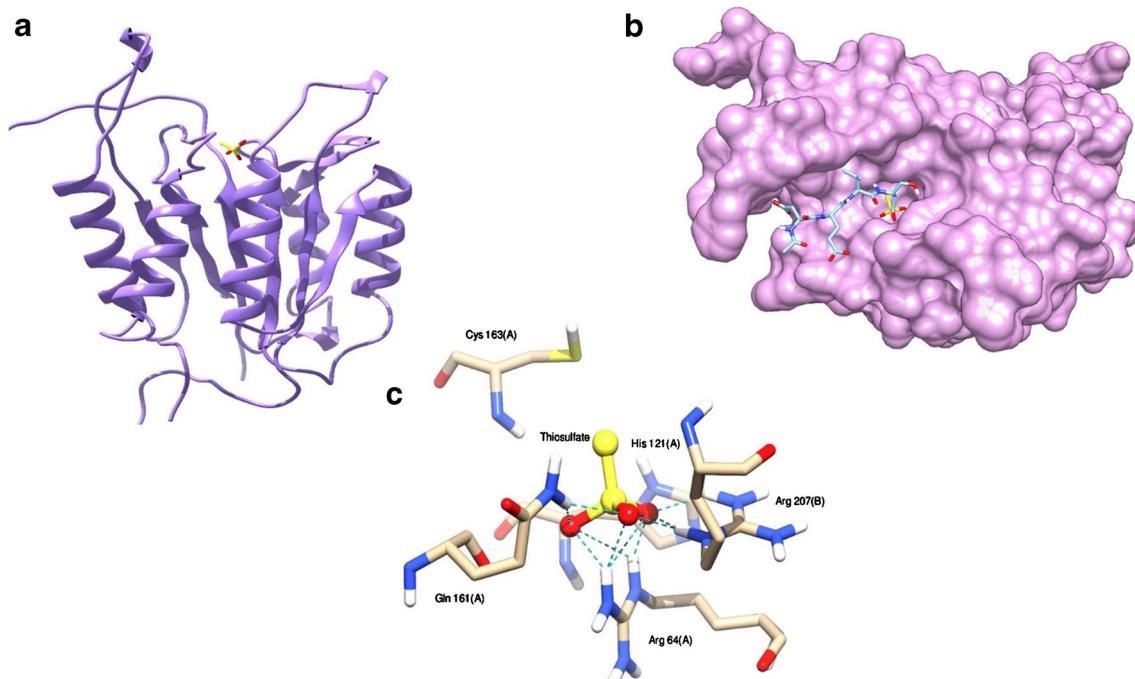


Fig. 2 The bonding interactions of thiosulfate with caspase-3 is represented along with the amino acid residue, type of bond, and bond distances as calculated from the molecular docking simulation analysis. The binding modes of **a** thiosulfate (yellow), **b** AC-DEVD-CHO (blue) and

thiosulfate (yellow) superimposed in the active site of caspase-3 is represented along with the **c** hydrogen bonding interactions (blue dashes) between thiosulfate and caspase-3 complex at Arg 64 (A), Gln 161 (A), and Arg 207 (B) represented by sticks

caspase-3-AC-DEVD-ChO complexes were found to be – 60.523 and – 43.146 kcal/mol.

Sodium Thiosulfate Mediated Functional Recovery of Rat Heart Subjected to Ischemia Reperfusion Injury in a Langendorff Perfusion Model

To determine the therapeutic implications of STS on IR injury, we employed an ex vivo Langendorff model and measured the various parameters like hemodynamics, infarct size by (TTC) staining, cardiac injury markers such as lactate dehydrogenase (LDH) and creatine kinase (CK),

caspase-3 activity, and DNA fragmentation. As illustrated in Table 2, end diastolic pressure (EDP), developed pressure (DP), rate pressure product (RPP), and force of contraction ($\pm dp/dt$) were measured to assess the left ventricular function, at the end of reperfusion phase. The hemodynamic data presented in Table 2 revealed that IR injury significantly ($P < 0.05$) elevated the hemodynamic parameters like EDP (9.8-fold) and reduced the DP (52%) and RPP (60%), affecting the contraction/relaxation force ($\pm dp/dt$) compared to the levels obtained for normal perfusion. IPC recovered (EDP 2.7-fold, DP 95%, RPP 2.15-fold) the heart from IR. However, in the STS_IPC treatment groups, the levels of these parameters only improved

Table 1 The bonding interactions of STS with caspase-3 is represented along with the amino acid residue, type of bond, and bond distances as calculated from the molecular docking simulation analysis

S. No.	Amino acid	Number of hydrogen bonds	Amino acid residue atom	Ligand atom	Bonding type	Bond distance (Å)
1	Arg 64(A)	3	NE	O3	Hydrogen bonding	2.68
			NH2	O3		3.41
			NH2	O4		2.82
2	Gln 161(A)	1	NE2	O5	Hydrogen bonding	2.95
3	Arg 207(B)	1	NH1	O3	Hydrogen bonding	3.04
			NE	O3		2.81
4	His 121(A)	1	Imidazole ring	O3	Electrostatic (pi anion)	4.99

Table 2 The hemodynamic changes observed in isolated rat hearts subjected to ischemia reperfusion (IR) injury and the effect of STS treatment is represented as mean \pm SD of 6 independent experiments

Groups (<i>n</i> = 6/group)	EDP (mm Hg)	DP (mm Hg)	RPP (mm Hg beats/min $\times 10^3$)	Max dp/dt (mm Hg/s)	Min dp/dt (mm Hg/s)
N	5 \pm 2	98 \pm 3	96 \pm 3	1737	-2141
IR	49 \pm 3 ^a	47 \pm 3 ^a	40 \pm 2 ^a	835 ^a	-3212 ^a
STS_ctrl	7 \pm 2	104 \pm 2	113 \pm 4	1903	-1981
IPC	18 \pm 4 ^b	92 \pm 4 ^b	86 \pm 3 ^b	1612 ^b	-2644
STS IPC 0.1 mM	40 \pm 2 ^a	42 \pm 3 ^a	55 \pm 1 ^a	1031 ^b	-3001
STS_IPC_1mM	22 \pm 3 ^b	86 \pm 2 ^b	88 \pm 4 ^b	1694	-2617

EDP end diastolic pressure, DP developed pressure, RPP rate pressure product = heart rate \times EDP, $\pm dp/dt$ maximum/minimum force of contraction

^a *P* < 0.001, ^b *P* < 0.05 vs. the normal

with the higher dose of 1 mM (EDP 4.5, DP 8.3, RPP 12-fold) compared to the IR group (Table 2). The observed hemodynamic changes were evident from the reduced infarct size (9%) for 1 mM dose of STS compared to the 0.1 mM (28%) (Fig. 3a, b). These findings were supported by the activities of cardiac injury markers like LDH and CK in the coronary perfusate (Fig. 3c, d) which were found to be lowered in STS 1 mM compared to the IR group while their corresponding tissue levels showed significant improvement (Fig. 3e, f). Hence, the 1 mM dose of STS was used for further *in vivo* experiments. The reduction in release of injury markers (LDH and CK) in the perfusate and improvement of hemodynamic response upon administration of 1 mM STS was comparable with that of IPC.

To assess the efficiency of STS as an anti-apoptotic agent, DNA fragmentation and caspase-3 activity were evaluated. DNA fragmentation implying apoptotic cell death was high in IR rat heart and improved significantly with STS pre-treatment (Supplementary Fig. S4a). The anti-apoptotic effect demonstrated by STS at 1 mM was comparable to that of the IPC procedure. A DNA control from normal heart not subjected to any perfusion was included to compare the effects of perfusion. The lower caspase-3 activity in the STS-treated heart compared to the IR group supported the observed anti-apoptotic effect of sodium thiosulfate (Supplementary Fig. S4b). IPC showed a 25% reduction in caspase-3 activity in the heart while STS 1 mM showed only a 15% reduction compared to IR form isolated rat heart model.

Sodium Thiosulfate Protects the Rat Heart from Ischemia Reperfusion-Induced Apoptosis in LAD Model

The immune cell-mediated apoptotic mechanism is negated in the isolated model, and therefore, the cardioprotective effects of STS against IR-induced

apoptosis were reconfirmed in LAD model. Caspase-3, cleaved-caspase-3, PARP, and cleaved-PARP immunoblots revealed a lower expression of active caspase-3 and thereby lower cleaved-PARP levels, after STS treatment compared to the IR control (Fig. 4a, b). The observed anti-apoptotic effect of STS was as effective as existing preconditioning technique where in STS_IPC reduced apoptosis by 92% for both markers compared to the 88% reduction by IPC, compared to IR group. The reduction in cardiac injury marker levels in plasma such as cardiac troponin I and LDH substantiated our findings that STS preconditioning provides significant cardioprotection in an *in vivo* model of myocardial ischemia reperfusion injury (Fig. 4c, d). On comparison with IPC group, STS was effective in reducing the plasma LDH close to normal while the troponin level remained unchanged.

In addition, the histopathological examination confirmed that STS treatment prominently suppressed the myocardial injury, inflammatory cell infiltration, and interstitial oedema induced by IR (Fig. 5).

Sodium Thiosulfate Ameliorated Reperfusion Injury Associated Oxidative Stress in Rat Heart and Preserved the Mitochondrial Enzymes

To provide further evidence for the oxidative impairment that leads to cardiac injury, we evaluated the lipid peroxidation level in the cardiac tissue from the levels of malondialdehyde. A significant elevation (*P* < 0.001) of MDA was observed in reperfusion control rat as compared with control animal (Fig. 6a). Preconditioning the myocardium with STS at 1 mM produced a significant decline in the level of MDA and improved the endogenous antioxidant GSH level (Fig. 6b) compared to IR group. The improved antioxidant enzymes (SOD, catalase, GPx, and GR) in STS pre-conditioned rat heart (Fig. 6c–f) supports this finding. The lipid peroxidation and antioxidant enzyme levels in the STS-pre-treated rat hearts were

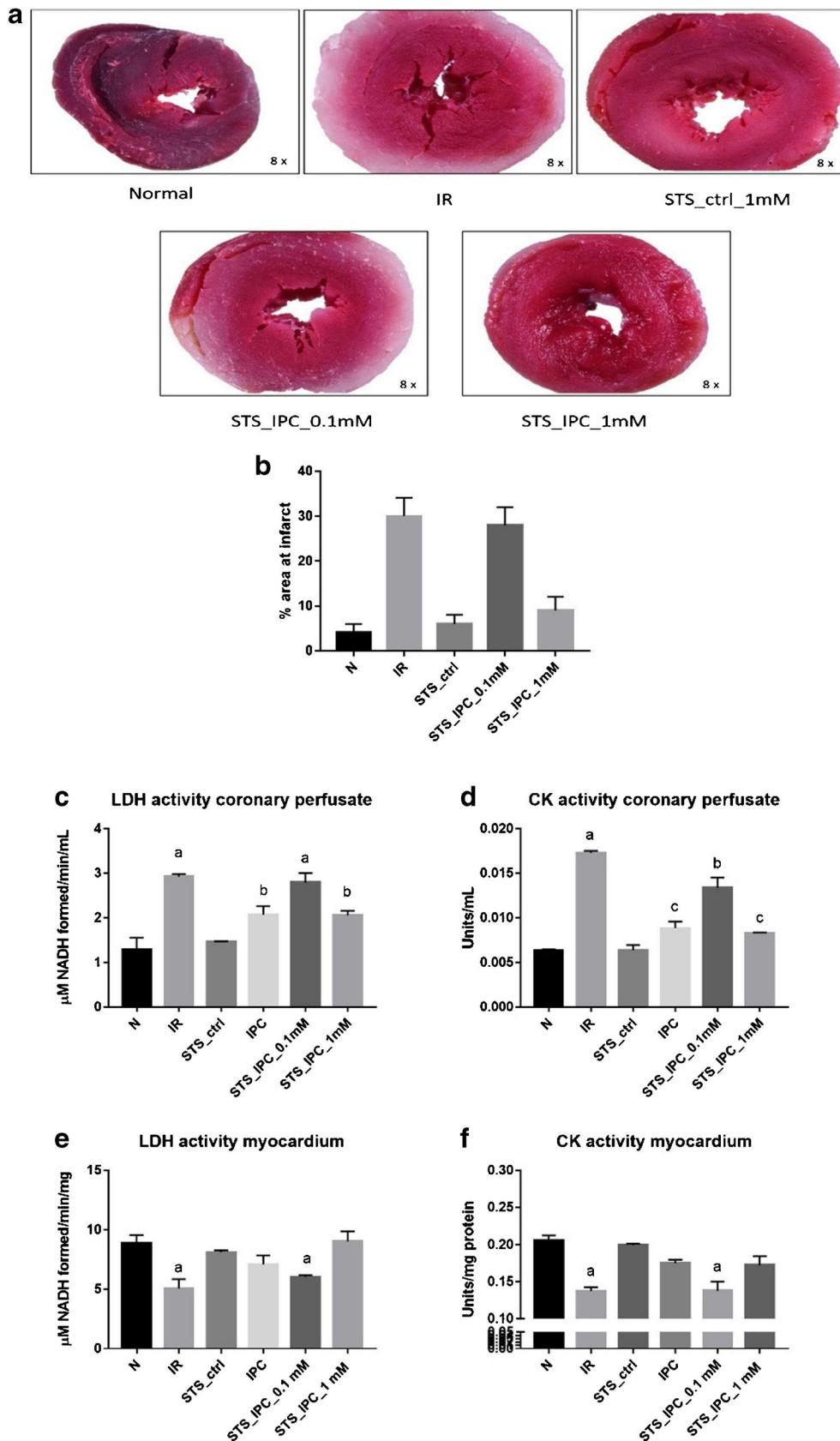


Fig. 3 The TTC stained images of **a** rat hearts subjected to IR injury and subsequent treatment with STS from representative of each group is presented along with the **b** percentage area at infarct calculated for six independent observation, represented by mean \pm SD. **a** $P < 0.001$, **b** $P < 0.05$ vs. the normal. The cardiac injury markers namely lactate dehydrogenase and creatine kinase were evaluated from the **c**, **d** coronary perfuse and **e**, **f** myocardium to assess the extent of injury and effect of STS treatment in an isolated rat heart model of IR injury. The graphs represent mean \pm SD of six independent experiments. **a** $P < 0.001$, **b** $P < 0.05$, **c** $P < 0.02$ vs. the respective normal control

comparable to that of classical cardio-protective mechanism of IPC, which was used as a positive control.

STS Preserves Mitochondrial Enzyme Activity Required for Myocardial Functional Recovery After IR

The mitochondrial super complex activity for complex I was assessed in-gel using CN-PAGE. The STS treatment showed improved activity for complex I compared to the IR group (Fig. 7a, b) from rats exposed to ex vivo IR injury. Additionally, the mitochondrial ferricyanide sensitive NADH dehydrogenase activity improved in the in vivo LAD hearts, which supported the findings in vitro (Fig. 7c). The TCA cycle enzymes malate dehydrogenase and succinate dehydrogenase also showed improved recovery comparable to that of positive control IPC (Fig. 7d, e).

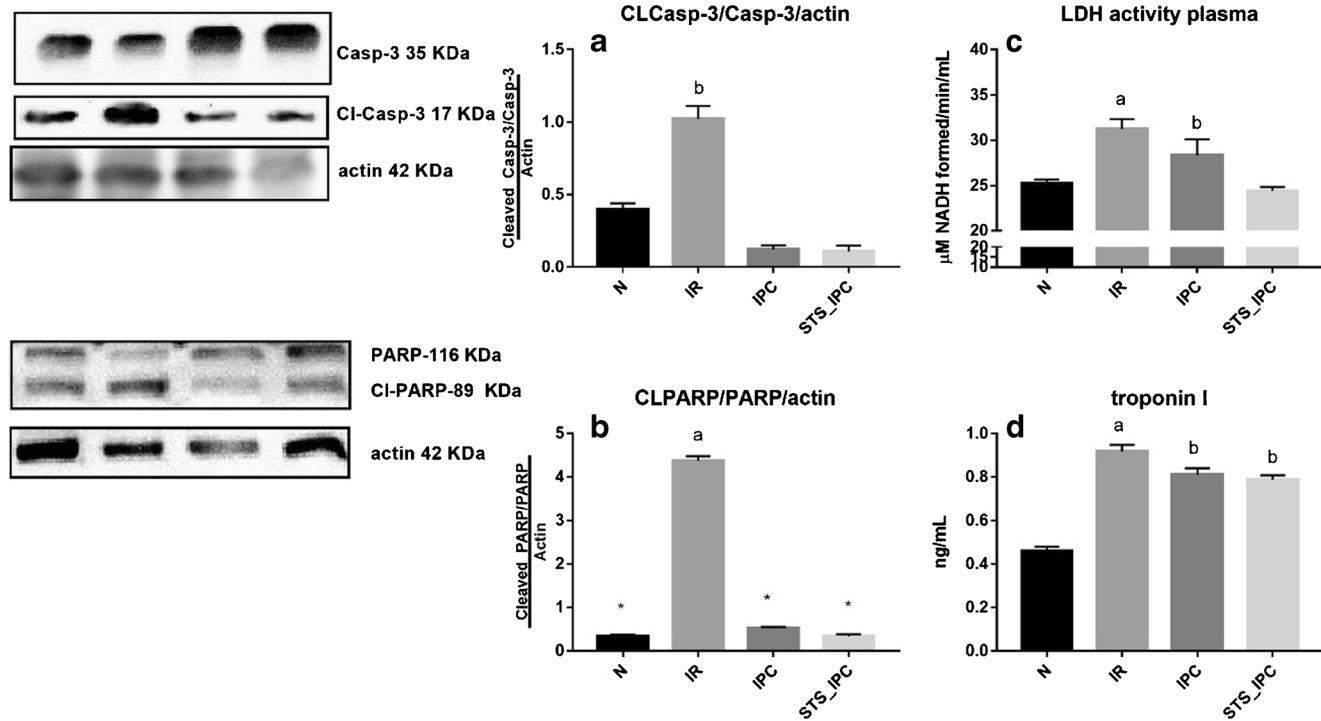


Fig. 4 Western blot and injury marker analysis of rat heart subjected to LAD occlusion and subsequent treatment with STS is represented for following proteins **a** cleaved caspase-3/caspase-3 and **b** cleaved PARP to PARP **c** LDH activity and **d** cardiac troponin I (cTnI) in plasma. All the

In Vitro Radical Scavenging Potential of STS

In order to check if thiosulfate's antioxidant ability is due to enhancement of antioxidant system or an innate potential to scavenge the radicals generated during reperfusion injury, varying concentrations of STS were used to scavenge nitric oxide, superoxide, and hydroxyl radicals. Supplementary Fig. S5 represents the respective radical scavenging potential in terms of % scavenging and compared it with a standard. STS was effective in scavenging the radicals generated in the order of nitric oxide > superoxide > hydroxyl.

Discussion

Myocardial ischemia reperfusion injury still remains a cause of mortalities involved in cardiovascular disease complications and poses a challenge for clinicians in the treatment of the same to ensure safe outcomes of effective reperfusion of the myocardium. In the present study, we propose that sodium thiosulfate can effectively treat myocardial IR injury by attenuating cardiac contractile dysfunction and reducing apoptosis associated with mitochondrial dysfunction, apart from being an antioxidant.

We have reported previously that hydrogen sulfide, one of the endogenous metabolites of thiosulfate, attenuated myocardial IR via the preservation of interfibrillar mitochondria [5,

blots were normalized to control beta actin. The graphs represent mean \pm SD of three independent experiments for blotting and six independent experiments for LAD and cTnI. ^a $P < 0.05$, ^b $P < 0.02$ vs. the respective normal control

Fig. 5 Histopathological examination of myocardium from rat heart subjected to IR injury from in vivo model and its subsequent treatment with STS. The images were obtained at 10 \times magnification represented by **a** normal, **b** IR, **c** STS control, **d** STS 1 mM. The IR injury caused derangement of fibers, infiltration of inflammatory cells with lot of oedematous spaces. STS at 1 mM concentration preserved the myocardium with only slight derangement of fibers. The figures are representative of three sections per group

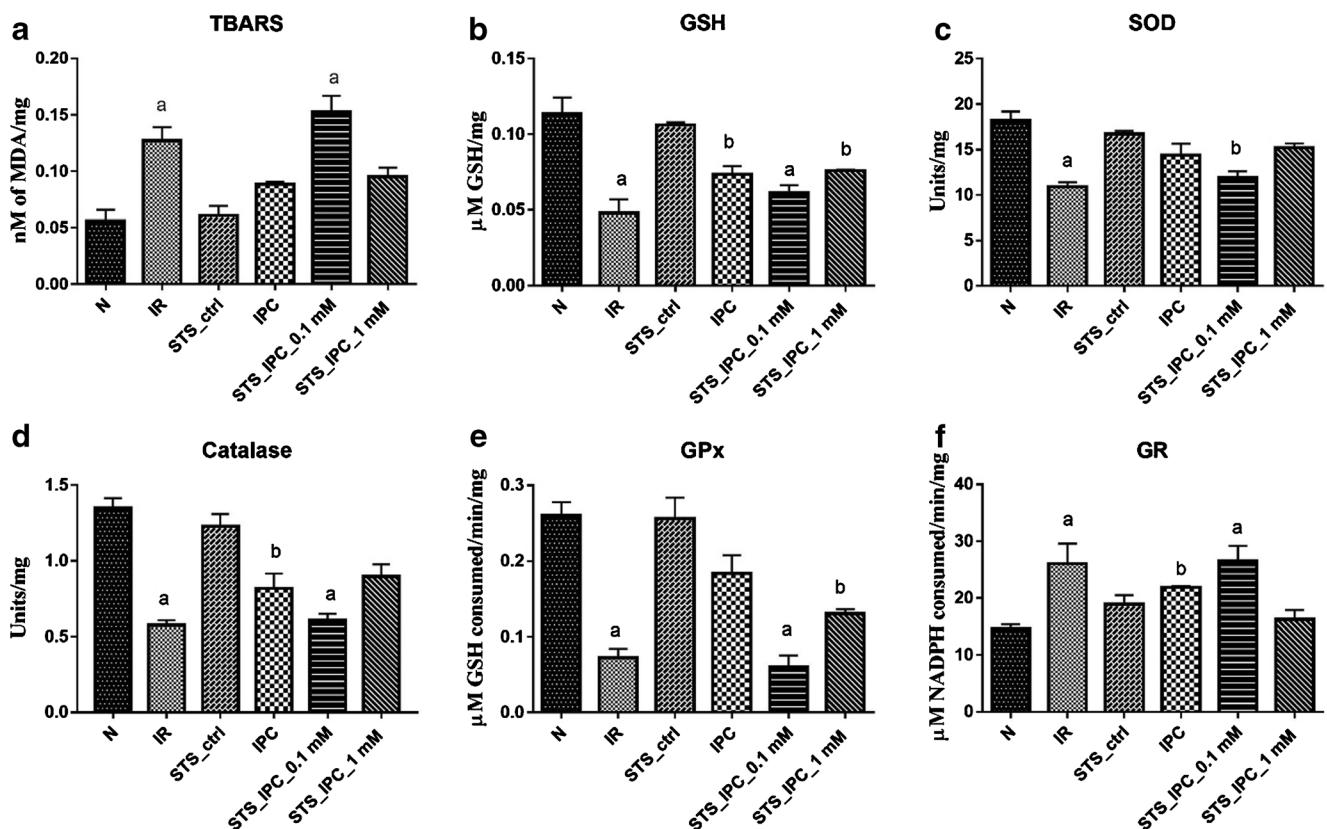
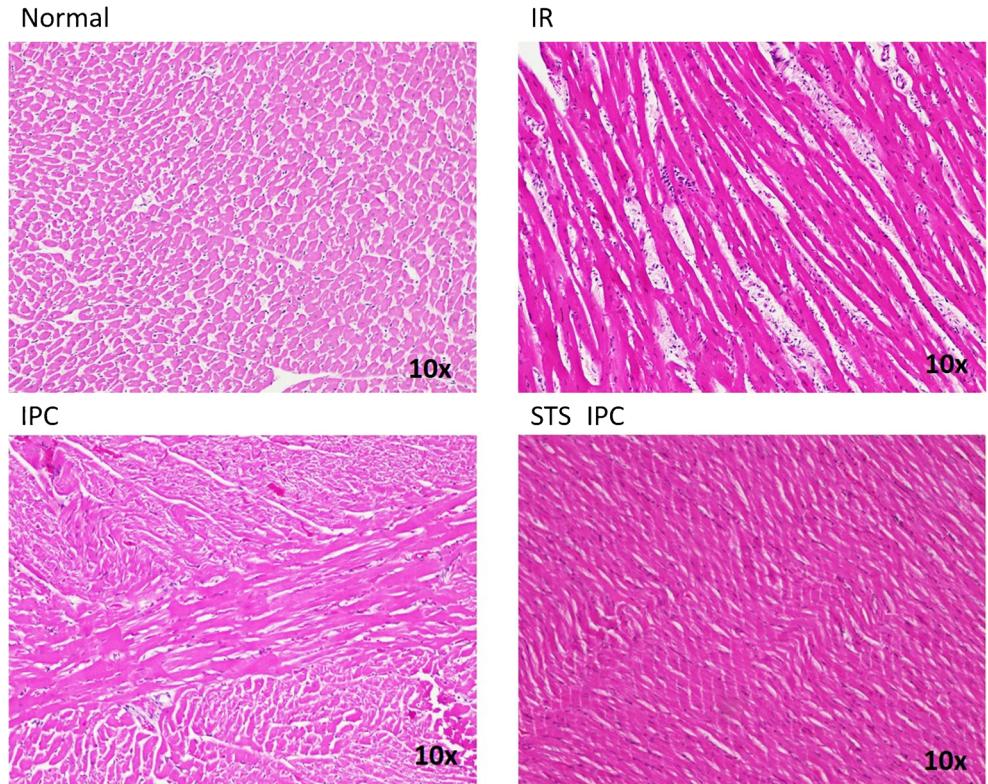
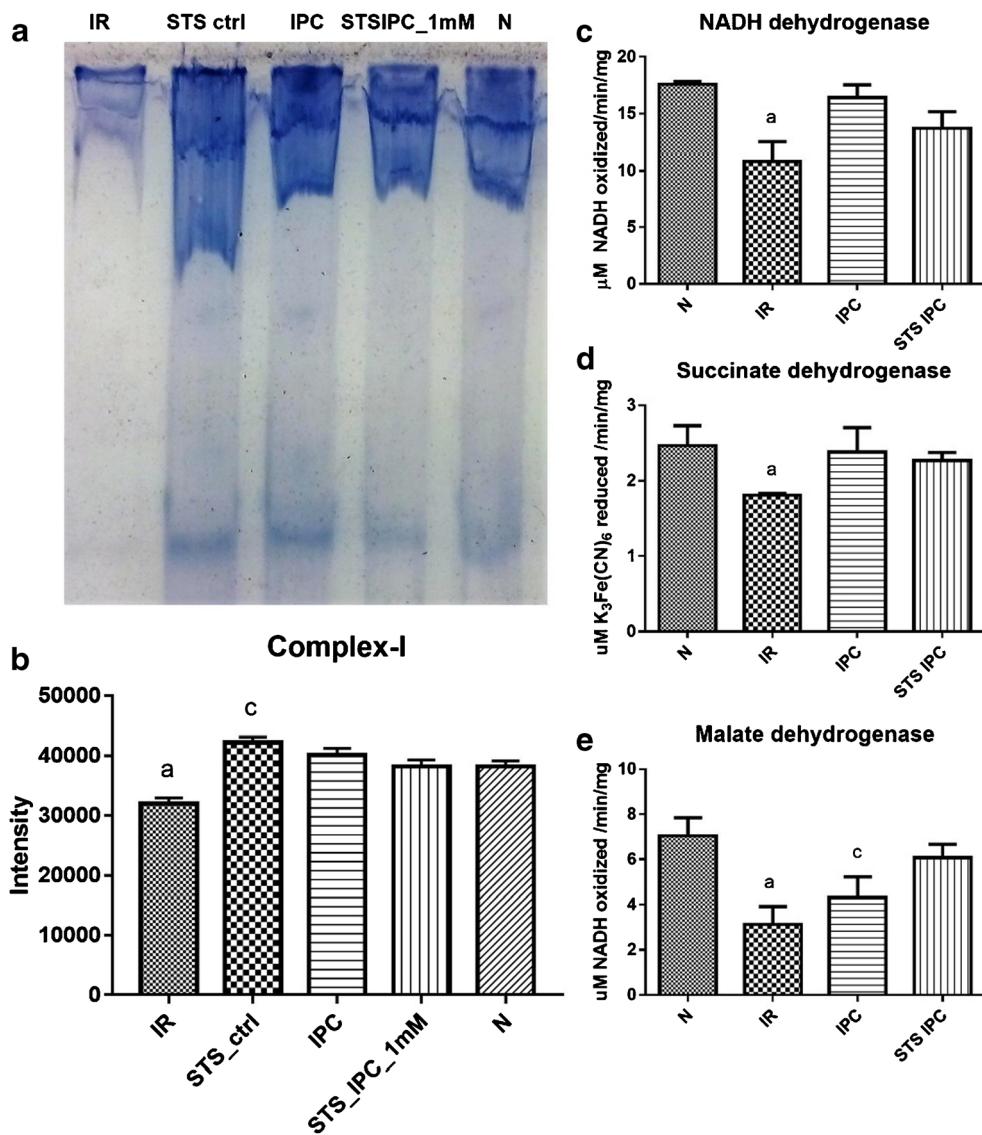


Fig. 6 The effect of STS treatment on lipid peroxidation product and antioxidant status of myocardium post-IR injury in an isolated heart model is represented by **a** TBARS, **b** GSH, **c** SOD, **d** catalase, **e** GPx, and **f**

GR. The graphs represent mean \pm SD of six independent experiments.
^a $P < 0.001$, ^b $P < 0.05$ vs. the respective normal control

Fig. 7 The mitochondrial enzyme activity post-IR injury was evaluated from isolated rat hearts to assess the effectiveness of STS perfusion. Complex 1 activity was evaluated by **a**, **b** in-gel using the NBT reduction, followed by quantification and **c** ferricyanide sensitive NADH dehydrogenase. The TCA cycle enzyme activity was evaluated for **d** succinate dehydrogenase and **e** malate dehydrogenase. The activities were compared to a classical cardioprotective ischemic preconditioning (IPC) mechanism. The graphs represent mean \pm SD of six independent experiments. $^aP < 0.001$, $^cP < 0.02$ vs. the respective normal control

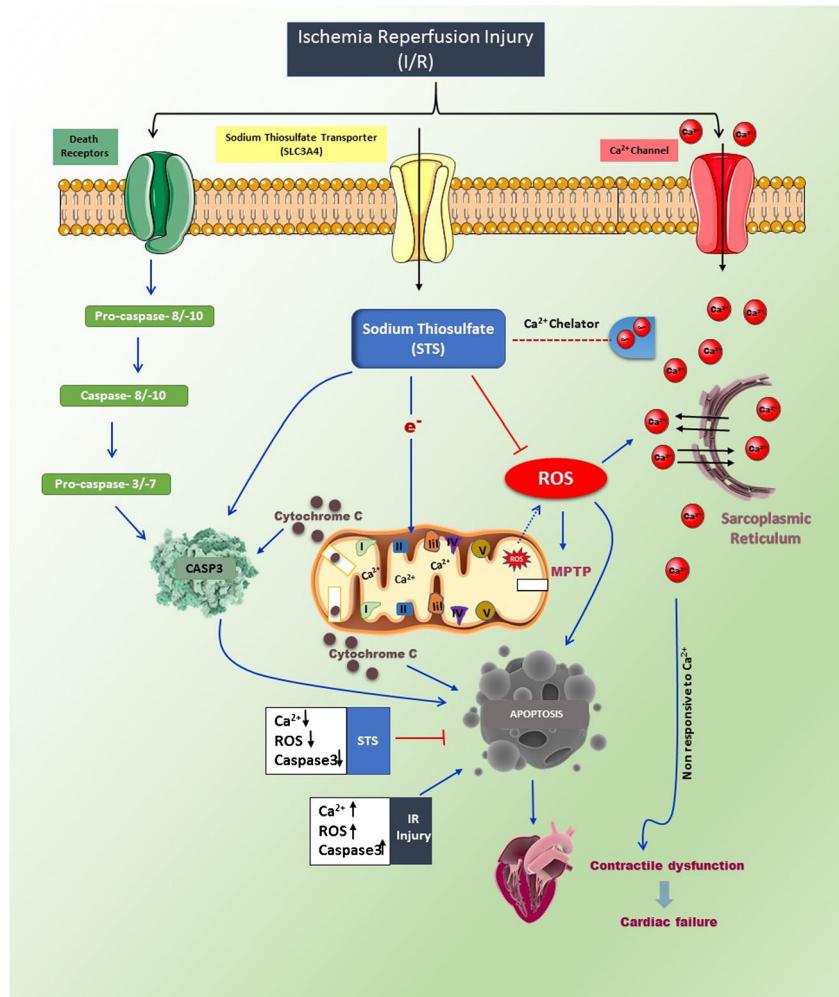


[15]. In this study, we examined the effect of STS on myocardial ischemia reperfusion, because of its effectiveness in the treatment of renal [16], vascular [17], heart [18], and lung [19] related complications. In addition to this, hydrogen sulfide, which is a promising molecule for the treatment of myocardial IR injury, displays a biphasic toxic effect that depends on its tissue level concentration, which is a major obstacle for its clinical use [20]. Evidence from the earlier studies and our own work emphasizes that STS has a wide safety margin for its tissue action and possess calcium chelation effect, minimizes oxidative stress, acts as an anti-inflammatory agent, modulates mitochondrial potassium ATP channel, and acts as a hydrogen sulfide donor [21].

The tissue injury/cell death associated with myocardial IR injury manifests as necrotic or apoptotic injury that ultimately impairs the ischemic heart's ability to recover during reperfusion. The ability of STS to reduce apoptotic injury was assessed

initially with H9C2 cardiomyocytes subjected to HR injury (enzymatic model with GOX/CAT system) and was re-confirmed with a chemically induced apoptotic model (cobalt chloride induction). Cobalt chloride induces apoptosis by activation of caspase3/8 and perturbs the mitochondrial membrane potential ($\Delta\Psi$) thereby inhibiting electron transport [22]. On the other hand, hypoxia induced in H9C2 by the GOX/CAT method and subsequent incubation of cells under normoxia leads to apoptosis, based on glucose depletion along with accumulated free radicals playing a central role in cell death via hypoxia inducible factor [8]. Pre-treatment of H9C2 cells with STS at 10 and 1 mM concentrations maintained a higher cell viability in both these models (Fig. 1a), which was supported by the recovery of anti-oxidant enzyme activities (Supplementary Fig. S2). In addition, mitochondrial dysfunction caused by HR was reduced by 1 mM STS, proving its ability to preserve mitochondria (Supplementary Fig. S3).

Fig. 8 Ischemia reperfusion injury is characterized by calcium overload, increased ROS production, leading to release of cytochrome-c and activation of apoptosis due to mitochondrial dysfunction. The culmination of these effects is contractile dysfunction progressing in to cardiac failure. Sodium thiosulfate, by chelating calcium, scavenging ROS, donating electrons to the electron transport chain (ETC), and modulating the mitochondrial permeability transition pore (MPTP) is known to prevent mitochondrial damage. Additionally, it is found to interact with caspase-3 thereby inactivating the apoptosis cascade. Thus, STS is able to prevent contractile dysfunction of the myocardium, aiding in its recovery from reperfusion injury



Once we identified that STS has the potential to reduce apoptosis, our next task was aimed at determining the mode of action of this molecule. As a way forward, we initiated an in silico analysis in search of thiosulfate's binding affinity to any of the apoptotic mediator molecules, in the cascade of cell death event. Caspase-3 protein, a member of the cysteine-aspartic acid protease family, is well known to play a key role in the sequential activation of caspase cascade that takes part in the execution phase of cell apoptosis and is identified as an attractive therapeutic target for heart failure [23]. The active site of caspase-3 contains cysteine (Cys-163) and histidine (His-121) residues that stabilize the intermediates formed during the peptide bond cleavage mediated by cysteine proteases. A previous study demonstrated that nitric oxide can inhibit caspase-3 by S-Nitrosation of Cys-163 [24] and its importance was verified by the rapid turnover of the enzyme that is dependent on the activity of the mature enzyme [25]. We performed a docking stimulation analysis, found that thiosulfate fits perfectly in the active site and is stabilized within the binding pocket of caspase-3 via strong hydrogen bonding with Arg-64, Gln-161, Arg-207, and pi anion interaction with His 121. The

strength of affinity of STS and known caspase-3 inhibitor AC-DEVD-CHO were compared using MM/GBSA free energy calculations, which showed more negative ΔG_{bind} (-60.523 kcal/mol) for STS-caspase-3 complex as opposed to STS-AC-DEVD-CHO complex (-43.146 kcal/mol). These interactions could prevent the access of the natural substrate to its binding site and hence prevent cardiomyocyte apoptosis. Binding of thiosulfate to caspase-3 reduced the turnover rate of active enzyme that in turn regulated the apoptotic events.

Further, we used the *in vivo* animal experiment (LAD ligation) model to validate the *in silico* findings, where the apoptosis was induced via myocardial ischemia reperfusion. The protective effect of STS identified earlier in cardiomyocytes (H9C2) towards reperfusion injury was reconfirmed in intact animal from the cardiac marker enzyme levels (Fig. 4c, d) and histopathology (Fig. 5). Additional analysis through immunoblot in STS preconditioned rat heart validated the anti-apoptotic effect of STS where we found a significant decline in myocardial caspase-3 activity, indicating the possible interactions of STS and caspase-3, in agreement with the *in silico* findings (Fig. 2 and Fig. 4).

The cell mediated by apoptosis is a tightly regulated event that initiates from either mitochondria (intrinsic) or death receptors (extrinsic). In order to understand the ability of STS to prevent energy-dependent apoptotic cascade thereby rendering cardioprotection, we used the isolated rat heart model, where the system is devoid of any hormonal or CNS influences and the apoptosis will be mitochondria dependent. We utilized two doses of STS (0.1 and 1 mM) to evaluate the efficacy of the molecule to recover the isolated rat heart subjected to ischemia reperfusion, and a dose-dependent cardiac recovery was observed from the hemodynamics, TTC stain, and cardiac injury markers in this study (Table 2 and Fig. 3). Further analysis of the cardiac apoptosis (reduced active caspase-3 activity along with a limited DNA fragmentation) reconfirmed the effectiveness of STS (Supplementary Fig. S4). Evidence from the previous study showed that thiosulfate causes increased persulfidation of cleaved caspase-3 at Cys-163 and this oxidation can result in caspase inactivation [21].

One of the central mediators of myocardial ischemia reperfusion injury is the increased level of ROS and related oxidants formed during the reperfusion phase. Many studies have shown that inhibiting the oxidative stress experienced during reperfusion phase of the ischemic myocardium is considered to be a viable approach for the treatment of IR-associated abnormalities [26]. Many studies, including our own, have shown that STS is a powerful antioxidant and can act as calcium chelator [16]. In concordance with previous findings, we observed that STS significantly reduces the oxidative damage, measured by the MDA levels and increased activity of antioxidant enzymes (SOD, catalase, GPx) in the myocardium compared to the reperfusion control groups. In general, the protection mediated by the antioxidant enzyme system under such conditions of oxidative injury is very complex and requires the evaluation of STS either with respect to its ability to scavenge free radicals or suppress its release. To understand this further as to STS has the potential to scavenge free radicals or to prevent its release, we initiated an *in vitro* free radical (nitric oxide, hydroxyl, superoxide) scavenging activity experiment with STS and the results suggest that it possesses the capacity to scavenge free radicals, in particular nitric oxide. Several lines of studies have demonstrated that the radical scavenging activity of many biological antioxidants such as glutathione, thioredoxin, and glutaredoxin are vested with sulfur groups [27–29]. Furthermore, oxidizing conditions in the extracellular environment of the myocardium prevail during reperfusion phase, and thus, a need for molecules to maintain the reducing environment is a prerequisite for the cellular homeostasis. This is normally done by glutathione, thioredoxin, glutaredoxin, and has been found to be insufficient during reperfusion [30]. Thus, STS not only being rich in sulfur but also by providing a strong reducing environment confers cardioprotection.

Mitochondrial dysfunction contributes to cardiac dysfunction and myocyte injury during reperfusion via the release of free radicals and altered calcium homeostasis. Deteriorated

mitochondria can act as source of further ROS release and thus maintenance of functional mitochondria has long been recognized to be a critical function, which is a prime requirement of therapeutic agents developed against ischemia reperfusion. The prominent role of STS in mediating cardioprotection in the present study is presumably by preserving mitochondrial function as observed from the improved activities of the mitochondrial enzymes, especially the ones that determine the flow of electrons through complex I. Recent data from both LAD and Langendorff animal model for IR indicate that mitochondrial complex I is the most sensitive indicator to assess the structural and functional integrity of the mitochondrial respiratory system. Given the role of complex I in generating ROS thereby damaging cardiolipin during IR, preservation of complex I activity with STS in the present study emphasizes the mitochondrial and cardioprotective effects of STS.

Sodium thiosulfate can reduce the infarct size in hearts undergoing ischemia reperfusion injury *in vitro* and preserve the myocardial architecture in an *in vivo* model. The underlying mechanism may be attributed to reduction of apoptosis by preventing caspase-3 activation and scavenging of ROS (Fig. 8). This protective mechanism of STS is not dependent on its conversion to H₂S as our recent publication using the isolated rat heart model proved that STS did not improve the H₂S levels in rat heart subjected to IR [31], as blocking endogenous H₂S production by using PAG declined its ability to provide cardio protection. STS has a larger therapeutic window as its IC₅₀ = 27.54 mM and LD₅₀ = 2000 g/kg IV are at higher levels to achieve a toxic dose. Further studies of its use in cardiac abnormalities associated with calcium deposition such as vascular calcification or complications such as diabetes/hypertension can be of help in taking this drug from bench to bedside.

Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All the procedures involving animal experimentation were performed in accordance with the guidelines of the Committee for the purpose of conduct and supervision of experiments on animals (CPCSEA, India), with a prior approval of the Institutional Animal Ethical Committee (IAEC, SASTRA University, No.: 229/SASTRA/IAEC/RPP).

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