



Fisetin Preserves Interfibrillar Mitochondria to Protect Against Myocardial Ischemia-Reperfusion Injury

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Abstract

According to our previous study, fisetin (3,3',4',7-tetrahydroxyflavone), a bioactive phytochemical (flavonol), reportedly showed cardioprotection against ischemia-reperfusion injury (IRI) by reducing oxidative stress and inhibiting glycogen synthase kinase 3 β (GSK3 β) [1]. GSK3 β is said to exert a non-mitochondrial mediated cardioprotection; therefore, distinct mechanisms of GSK3 β on the regulatory effect of mitochondria need to be addressed. The two distinct mitochondrial subpopulations in the heart, namely interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM), respond differently to disease states. The current study aimed to understand the effect of fisetin on the subpopulation-specific preservation of IFM and SSM while rendering cardioprotection against ischemia reperfusion (I/R). Rats were pre-treated with fisetin (20 mg/kg) intraperitoneally, and IRI was induced using Langendorff isolated heart perfusion technique. Hemodynamic parameters were recorded, and the cardiac injury was assessed using infarct size (IS), lactate dehydrogenase (LDH), and creatine kinase (CK) levels. Subpopulation-specific mitochondrial preservation was evaluated by electron transport chain (ETC), catalase, superoxide dismutase (SOD), and glutathione (GSH) activities. The bioavailability of fisetin in IFM and SSM was measured using the fluorescence method. The ability of fisetin to bind directly to the mitochondrial complex-1 and activating it through donating electrons to FMN was studied using molecular docking studies and further validated by in vitro rotenone sensitivity assay. Cardioprotective effects exhibited by fisetin were mainly mediated through IFM preservation. Mitochondrial bioavailability of fisetin is more in IFM than SSM in both ex vivo and in vitro conditions. Fisetin increased mitochondrial ATP production in I/R insult hearts by activating ETC complex 1. Inhibition of complex 1 prevents the ATP-producing capacity of fisetin. Our results provide evidence that fisetin plays a protective role in myocardial IRI, possibly by preserving the functional activities of IFM.

Keywords Ischemia-reperfusion · Mitochondria · Electron transport chain · Reactive oxygen species · Fisetin · Cardioprotection

Introduction

Ischemia-reperfusion injury (IRI) is an unavoidable cellular injury that occurs during revascularization in the treatment of an ischemic or infarcted heart [2]. IRI is characterized by a multifactorial pathology and mediated through a complex chain of events that involves depletion of energy substrates, alteration of ionic homeostasis, production of reactive oxygen species, and cell death by apoptosis and necrosis. There is increasing evidence that mitochondria play a vital role in this process. Defective recovery of cardiac physiology in the ischemia-reperfusion (I/R) challenged heart is associated with the dysfunction of myocardial contractile units that are regulated by mitochondria [3]. An effective drug to manage this important medical condition is still lacking. Fisetin

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(3,3',4',7-Tetrahydroxyflavone, Supplementary Fig. 1), a dietary flavonoid, present in several fruits and vegetables such as strawberry (*Fragaria ananassa*), apple (*Malus domestica*), persimmon (*Diospyros kaki*), grape (*Vitis vinifera*), onion (*Allium cepa*), and cucumber (*Cucumis sativus*) at concentrations in the range of 2–160 µg/g. Fisetin has multiple biological functions like free radical scavenging, antioxidant, anti-inflammatory, antiproliferative, anti-platelet aggregation, antiapoptotic, neuroprotective, cardioprotective, and anticancer properties [4, 5].

Its ability to work via multiple pathways has resulted in its broad application or suggestion to use in the management of various diseases. Liu and his group demonstrated that fisetin regulated AMPK/NF-κB p65 and p38MAPK/smads3 signaling pathways and improved the cardiac function of myocardial infarcted rat hearts by reducing atrial inflammation and fibrosis [6]. A study by Althunibat and his co-workers recently reported the fisetin's ability to mediate cardioprotection in diabetes-induced cardiac injury [7]. Another study by Garg and his group identified that fisetin could attenuate isoproterenol-induced cardiac injury by down-regulating RAGE and NF-κB [8]. Its ability to scavenge free radicals, prevent apoptosis, activate PI3K signaling pathway (key cardioprotective signaling cascade), inhibit GSK-3β, and reverse the mitochondrial dysfunction, the key player responsible for I/R injury, makes it an effective drug to treat I/R [1].

Many investigators have demonstrated physiologically distinct types of mitochondria located at different regions in cardiac tissue, namely, interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) [9, 10]. IFM located between the myofibrils were aligned in longitudinal rows and had mainly the tubular structure. SSM was positioned mainly beneath the subsarcolemmal and had a lamelliform structure [11]. They showed distinct respiratory rate (IFM exhibited 1.5 times faster than SSM), different metabolic enzyme activity (succinate dehydrogenase and citrate synthase activity is higher in IFM), morphological structure, and lipid content (higher ceramide content in IFM) [12]. Depending upon the energy status, the superoxide radical production also varies with the subpopulations (In glutamate plus malate energy source, IFM release more superoxide radicals) [13].

The heart requires a constant supply of energy for myocyte contraction. Being located in the myofibrils, the IFM fraction of the mitochondria is believed to provide the bulk of energy for cardiomyocyte contraction. This assumption was supported by the early findings that suggest IFM possesses a higher rate of substrate oxidation (about 1.5 times) than the SSM fraction [12]. Consistent with these observations, investigators have shown higher sensitivity to Ca²⁺ overload that can inhibit ATP synthesis [14]. To strengthen this assumption Dabkowski and his group

demonstrated severely impaired subsarcolemmal mitochondria in DB/DB hearts compared to interfibrillar mitochondria, which were unaffected, indicating the critical role played by SSM in metabolic disorder [15].

These differences between the subpopulations indeed affect the mitochondrial response in disease conditions. Our previous findings had shown that the sensitivity of IFM and SSM towards myocardial ischemia reperfusion injury were different, where IFM fractions were severely impaired than SSM [10]. Myocardial ischemia reperfusion injury is a bioenergetics disorder centered at cardiac mitochondria due to its ability to produce ATP and control different metabolic flux and its involvement in redox biology and signaling. Since the pathology behind ischemia-reperfusion is not only confined to the lack of ATP generation but also the disturbances in the cellular metabolism, this emphasizes the need and importance of mitochondrial subpopulation in determining the pathology and treatment efficacy of I/R injury.

Previous studies from our lab had shown the protective effect of fisetin on mitochondria (reducing oxidative stress) that render cardioprotection against ischemia reperfusion injury [1]. However, in this study, the impact of fisetin on mitochondria was measured cumulative rather distinct in different subpopulations. Considering the importance of the subpopulations in determining the pathology of myocardial ischemia reperfusion injury, the therapeutic agent explored for its efficacy should be evaluated at the subpopulation level, and the evidence in this regard for fisetin is absent. Hence revisiting the mechanism of action of fisetin in ameliorating ischemia reperfusion injury at the level of mitochondrial subpopulation becomes important and inevitable. Such investigations are a prerequisite for the translation of fisetin from the bench to bedside as well as to utilize it as a nutraceutical. Moreover, the previous study has shown that resveratrol, a natural phenolic compound, was reported to bind compound-complex I nucleotide-binding site of mitochondria which may stimulate or inhibit its activity [16], and fisetin belongs to the class of phenolic compounds.

Therefore, the aim of this study was to investigate the role of fisetin in the subpopulation-specific mitochondrial preservation of IFM and SSM while demonstrating cardioprotection against I/R.

Materials and Methods

Chemicals

Fisetin was purchased from TOCRIS Bioscience, Bristol, UK. All the other chemicals used were bought from Sigma Aldrich.

Animals

All the experimental animal protocols followed in this research were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), SASTRA University, Thanjavur, India (CPCSEA approval number: 552/SASTRA/IAEC/RPP). 8-week-old male Wistar rats ($n = 24$) were purchased from the central animal facility and maintained in ventilated polycarbonate cages under a 12 h light/dark schedule with ad libitum water and feed supply.

Ex Vivo Perfusion Model of Ischemia Reperfusion Injury

Rats were randomly divided into four groups: (1) Control (N); (2) Fisetin-control (FC) (fisetin 20 mg/kg, injected intraperitoneally 1 h before the experiment protocol) (fisetin procured from TOCRIS Bioscience, Bristol, UK); (3) Ischemia-Reperfusion (IR); (4) Fisetin-I/R (FIR) (fisetin 20 mg/kg was injected intraperitoneally 1 h before the induction of ischemia) and I/R was induced using Langendorff isolated heart perfusion technique as described previously [17]. Fisetin was solubilized in 1% sodium bicarbonate to a final concentration of 14 mM (1 mg in 250 μ l of 1% Na₂CO₃), and the final concentrations were made up by diluting in buffer [18]. Briefly, the I/R protocol consisted of 30 min of ischemia-induced by stopping the buffer flow, followed by 60 min of reperfusion-induced by resuming the flow. Throughout the experiment, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and rate pressure product (RPP) were recorded and calculated using LabChart physiological data analysis software (ADIInstrumentsInc, Sydney, Australia). Perfusates were collected during the end of the reperfusion and used for further biochemical analysis. At the end of the experiment, the hearts were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

Injury Assessment

The infarct size (IS) was measured using 2,3,5-triphenyl tetrazolium chloride (TTC) staining as described previously [19]. The images were acquired using a stereo zoom microscope. The percentage of infarcted tissue was analyzed using image processing and analysis software ImageJ (NIH-USA). The levels of cell death were estimated using LDH, CK, and caspase-3 activities in cardiac tissues and perfusate using our previously described method [20].

Mitochondrial Subpopulation Isolation

Differential centrifugation technique was used for the isolation of mitochondrial subpopulations [10]. The cardiac tissue was finely minced, and a homogenate (10%) was prepared in isolation buffer (pH-7.4). Later, the tissue homogenate was subjected to centrifugation at 600 \times g for 10 min, and the supernatant was subjected to centrifugation at 6000 \times g for 10 min, followed by two centrifugations at 8000 \times g for 10 min, to yield the subsarcolemmal mitochondria enriched pellet. For the isolation of interfibrillar mitochondria, the pellet from the first centrifugation step that contains unbroken tissue will be further treated with 0.5 mg/g tissue of trypsin for 10 min. After 10 min, diluted the samples with isolation buffer and subjected to centrifugation at 600 \times g for 10 min and proceeded with the supernatant as explained above. Finally, the mitochondrial pellet was dissolved in storage buffer, and protein concentration was determined by Bradford reagent (BioRad) using bovine serum albumin as standard. The mitochondrial purity was assessed using oxygraph, and the mitochondrial yield obtained was around 2.5 mg/g cardiac ventricle tissue.

Mitochondrial Oxidative Phosphorylation

Clarke-type oxygen electrode (Hansatek, UK) was used to measure the mitochondrial oxygen consumption at 37°C in respiration buffer with glutamate/malate (GM) (5 mM/2.5 mM) and succinate (5 mM) as substrates [17]. State 3 and state 4 respiration rates were measured after the addition of 2.5 mM ADP and 2,4-dinitrophenol, respectively. The oxygen consumption control by phosphorylation was measured via respiratory control ratio (RCR), which was calculated as a ratio of state 3 to state 4 respiration. The ADP/O ratio measured the efficiency of oxidative phosphorylation.

Mitochondrial Electron Transport Chain Analysis

Mitochondrial oxidative phosphorylation system (OXPHOS) was assessed by ETC enzyme activities in mitochondria. Spectrophotometry-based ETC assay analysis was carried out using a specific donor-acceptor [21]. Activities of rotenone-sensitive NADH-oxidoreductase (NQR), succinate decylubiquinone DCPIP reductase (SQR), Ubiquinol cytochrome c reductase (QCR), and cytochrome c oxidase (COX) were measured and normalized to citrate synthase activity.

Mitochondrial Antioxidant Status and Free Radical Scavenging Activity

The antioxidant status of IFM and SSM was evaluated by measuring GSH and GSSH levels using Ellman's reagent as reported by [22], superoxide dismutase (SOD) as reported by [23] and the catalase activity as reported by [24]. The free radical scavenging potential of fisetin was measured using superoxide, hydroxyl, and nitric oxide scavenging activity assays, as reported by us previously [25].

In Vitro Induction of I/R in Isolated Mitochondria

IFM and SSM isolated from control rat hearts were divided into four groups: (1) Control (C): The IFM and SSM fractions were resuspended in respiration buffer (0.3 M Mannitol, 10 mM KCl, 5 mM MgCl₂, 20 mM KH₂PO₄, 20 mM Hepes, 1 mM EGTA, 0.1% BSA) and incubated for 50 min at 37 °C; (2) Fisetin control (FC): IFM and SSM resuspended in respiration buffer and incubated at 37 °C for 10 min and then treated with fisetin at various concentrations (25 μM, 50 μM, 100 μM, 200 μM, and 400 μM) for another 10 min, centrifuged and resuspended in respiration buffer for another 30 min; (3) Ischemia-reperfusion (I/R): IFM and SSM fractions equilibrated at 37 °C in respiration buffer for 20 mins and incubated at 37 °C in hypoxia buffer purged with nitrogen gas to induce ischemia for 15 min. For reperfusion, the mitochondria were again resuspended in respiration buffer for another 15 min; (4) Fisetin-I/R (FIR): The IFM and SSM kept for 10 min in respiration buffer and treated with fisetin for another 10 min and subjected to I/R protocol as described previously. After the respective treatments, the mitochondrial pellet resuspended in respiration buffer, stored at –80 °C until use.

Adenosine Triphosphate in Isolated Mitochondria

Levels of ATP and ATP producing capacity of mitochondria were determined using a luminescence ATP detection assay system (ATPlite) (PerkinElmer) according to the manufacturer's protocol. ATP producing ability was evaluated by incubating isolated mitochondria in the non-energized medium for about 10 min before proceeding to the analysis.

Reactive Oxygen Species in Isolated Mitochondria

The total ROS level in the mitochondria was analyzed by fluorescence method using 2',7'-dichlorofluorescein diacetate (DCHFDA, Cat No. D6883, Sigma Aldrich, USA) as reported in [26], and the fluorescence was measured at Ex/Em = 485/530 nm.

Determination of Fisetin Concentration in Isolated Mitochondria

The bioavailability of mitochondrial fisetin was analyzed by the fluorescence method. Briefly, the mitochondrial pellet was lysed by giving an osmotic shock in a hypotonic medium (25 mM K₂PO₄, 5 mM MgCl₂ pH 7.2), and then the fluorescence was measured at Ex/Em = 400/500 nm. The estimation was done using a standard curve of known concentrations of fisetin.

Molecular Docking of Fisetin with Mitochondrial Complex 1

The docking simulation was carried out using Schrödinger Release 2020-1. Docking calculations were carried out on *Thermusthermophilus* NADH-quinone oxidoreductase subunit 1 (PDB: 3IAM), a structural homologous of *Homo sapiens*. The protein was prepared using a protein preparation wizard (Schrödinger, LLC, New York, NY, 2020). The structure of fisetin was drawn using MarvinSketch (V17.29) (www.chemaxon.com) and prepared using LigPrep, (Schrödinger, LLC, New York, NY, 2020). A receptor grid was generated using receptor grid generation wizard, and the grid box was kept centered on the NADH binding site. Fisetin was docked in complex 1 NADH binding domain using extra precision (XP) docking algorithm implemented in GLIDE (Schrödinger, LLC, New York, NY, 2020). The docked poses and interactions were analyzed using UCSF Chimera [27] and PyMOL Molecular Graphics System (Version 2.0, Schrödinger, LLC). Gaussian 09 (Revision A.02) was used to calculate the single electron transfer (SET) and bond dissociation energy (BDE).

Rotenone Sensitive Activity of Complex 1 in Isolated Mitochondrial Subpopulation Challenged to I/R

Mitochondrial ETC Complex 1 activity was assayed as follows. The IFM and SSM were divided into 4 groups: (1) Control (C): IFM and SSM fractions were resuspended in respiration buffer and incubated for 50 min at 37 °C; (2) Fisetin control (FC): IFM and SSM resuspended in respiration buffer and incubated at 37 °C for 10 min and then treated with 200 μM fisetin for another 10 min, centrifuged and resuspended in respiration buffer for another 30 min; (3) Rotenone control (RC): IFM and SSM resuspended in respiration buffer and incubated at 37 °C for 10 min and then treated with 4 μM rotenone for another 10 min, centrifuged and resuspended in respiration buffer for another 30 min; (4) Rotenone-fisetin (RF): IFM and SSM resuspended in respiration buffer and incubated at 37 °C for 10 min and then treated with 4 μM rotenone for

Table 1 Hemodynamics measurement at the end of reperfusion

Hemodynamic parameter	Normal	IR	Fisetin-control	Fisetin-IR
LVDP × 10 (mmHg)	11.1 ± 2.0	4.0 ± 0.1*	9.3 ± 0.3	7.6 ± 0.9
HR (bpm)	300 ± 37	273 ± 18	314 ± 20	327 ± 16
RPP × 10 ⁴ (mmHg × bpm)	3.3 ± 0.19	1.09 ± 0.43*	2.94 ± 0.87	2.49 ± 0.18*
-dp/dt × 10 (mmHg)	82.2 ± 10.1	4.3 ± 0.6*	71.5 ± 5.9	73.6 ± 7.3
+dp/dt × 10 (mmHg)	109.9 ± 17.2	3.6 ± 0.7*	99.7 ± 8.9	88.6 ± 10.9

Data were represented as mean ± SD of 6 individual experiments. Left ventricular developed pressure (LVDP), heart rate (HR), rate pressure product (RPP). Rate of rise of left ventricular pressure (dp/dt)

*p < 0.05 vs control

another 10 min. Mitochondria were again pelleted and resuspended in 200 μM fisetin for another 10 min followed by respiration buffer for another 20 min. After the respective treatments, the rotenone sensitive complex 1 activity was assayed as previously described [21].

Statistical Analysis

Statistical analysis was carried out by GraphPad Prism (version 8.00) for Macintosh, GraphPad Software, La Jolla California, USA (www.graphpad.com). Experimental results were expressed as mean ± SD. Comparison between groups was made using analysis of variance (ANOVA) with post-hoc test. ANOVA was performed for multiple comparisons between groups using two-way comparison of means by Dunnett test, and p value < 0.05 was considered statistically significant.

Results

Fisetin Recovered Hemodynamics and Attenuated Cardiac Injury by Preserving Interfibrillar Mitochondrial Function

The changes in the cardiac function of different experimental groups are depicted in Table 1. Accordingly, comparing to control, the isolated rat heart showed reduced cardiac performance and increased cardiac injury when subjected to I/R, which is evident from the low left ventricular developed pressure (LVDP) and rate pressure product (RPP) (63 & 66 %, respectively) as shown in Table 1. Compared to sham control, the increased infarct size (IS) (Fig. 1B), elevated caspase-3 activity (29%) (Fig. 2E) and increased creatine kinase (CK) (47%) and lactate dehydrogenase (LDH) (44%) activities in the perfusate (Fig. 2B and D) confirmed the cardiac injury imposed on rat heart subjected to I/R. But interestingly, fisetin treatment significantly improved the recovery of cardiac physiological functions (Table 1) and subsequently reduced the IS (67%), thereby decreasing the levels of LDH (52%), CK (48%), and caspase-3 activities (19%) in I/R challenged heart (Fig. 2).

The effect of fisetin on ADP-induced changes in the level of oxygen consumption in experimental groups and the calculated respiratory control ratio (RCR) are given in Fig. 3. Statistical analysis revealed that there were significant differences in mitochondrial RCR ($p < 0.05$) and ADP/O ($p < 0.05$) of FIR group from the IR in both mitochondrial subpopulations, where the latter group showed a prominent decline in RCR and ADP/O ratio ($p < 0.05$) from the control group when the mitochondria were incubated in energized medium containing glutamate and malate as the substrate. Similar results were observed in both the subpopulations when the mitochondria were incubated in energized medium containing succinate as the substrate. Even though insignificant changes were found between mitochondrial subpopulations, in GM enriched medium ADP/O ratio of IFM was higher than SSM, but with succinate as the energized medium, SSM exhibited a better ADP/O ratio.

Mitochondrial electron transport chain (ETC) enzyme activities in IFM and SSM were measured and found to be improved in fisetin treated I/R challenged heart (Fig. 4). The decreased proximal ETC enzymes, rotenone-sensitive NADH-oxidoreductase (NQR), and succinate decylubiquinone DCPIP reductase (SQR) activities were effectively reversed by fisetin treatment, but the similar impact was not exhibited by cyclooxygenase (COX) and ubiquinol cytochrome c reductase (QCR), the distal ETC enzymes. Fisetin administration has improved the ETC activities more prominently in IFM of I/R, evident by 55, 58, and 68% improvement in SQR, QCR, and COX activities in IFM from I/R (Fisetin untreated group), while only 22, 50, and 58% improvement was observed in SSM correspondingly from I/R (Fisetin untreated group). Among the mitochondrial subpopulation, I/R-induced mitochondrial dysfunction was more prominent in IFM and was recovered effectively by fisetin.

Fisetin Improved Antioxidant Enzyme Activities and Reduced Oxidative Stress in a Mitochondrial Subpopulation

As the effect of fisetin on the ETC enzyme activity was significantly higher in IFM than SSM, we further studied

Fig. 1 Representative infarcts of different groups stained with TTC. **A** Control (C) ($n = 3$). **B** Ischemia-reperfusion (IR) ($n = 3$). **C** Fisetin control (FC) ($n = 3$) and **D** Fisetin-IR (FIR) ($n = 3$)



whether ETC enzymatic activity was catalyzed through a reduction in the oxidative stress enacted by fisetin's antioxidant property. The potential of fisetin in modulating oxidative status was evaluated by endogenous antioxidant systems, including catalase, SOD, and GSH: GSSG ratio. It was observed that the treatment of fisetin significantly increased the catalase, SOD, and GSH: GSSG activities in the I/R heart (Fig. 5). Furthermore, fisetin also functioned as a potential free radical scavenger by reducing ROS and RNS during I/R. As shown in Fig. 6 the superoxide, hydroxyl, and nitric oxide radical levels in the tissue homogenate of fisetin treated groups are significantly lesser than the I/R group. These results showed that the fisetin's potential to contract oxidative stress could be attributed to its potential to increase the antioxidant enzyme activities through its effective free radicals scavenging capacity.

Levels of Fisetin in the Mitochondrial Subpopulations

To understand whether the higher functional preservation of IFM by fisetin is associated with the mitochondrial subpopulation-specific bioavailability of fisetin, we estimated the concentration of fisetin in IFM and SSM using the fluorescence method. As shown in Table 2, the

bioavailability of fisetin in the IFM of fisetin-control and fisetin-I/R treated groups are (41% and 97%) higher compared to SSM. These results indicate that the higher preservation of IFM is associated with the higher uptake of fisetin by IFM.

In Vitro Incubation of Isolated Mitochondria with Fisetin Indicate Superior Uptake of Fisetin by IFM

To rule out the possibilities of the spatial location of IFM and SSM in fisetin uptake, we exposed the isolated mitochondrial subpopulation with 200 μ M of fisetin and evaluated the fisetin uptake using the fluorescence method. After 10 min exposure to fisetin, we observed a significantly higher concentration of fisetin uptake in IFM than SSM, as shown in Table 2.

Higher Uptake of Fisetin Leads to Improved Preservation of IFM

We observed that in vitro exposure of IFM and SSM with varying concentrations (25 μ M, 50 μ M, 100 μ M, 200 μ M, 400 μ M) of fisetin improved the ATP producing capacity of IFM than SSM as shown in (Fig. 7A). Further evaluation of the total oxidative stress in mitochondria treated with fisetin exhibits decreased oxidative stress in

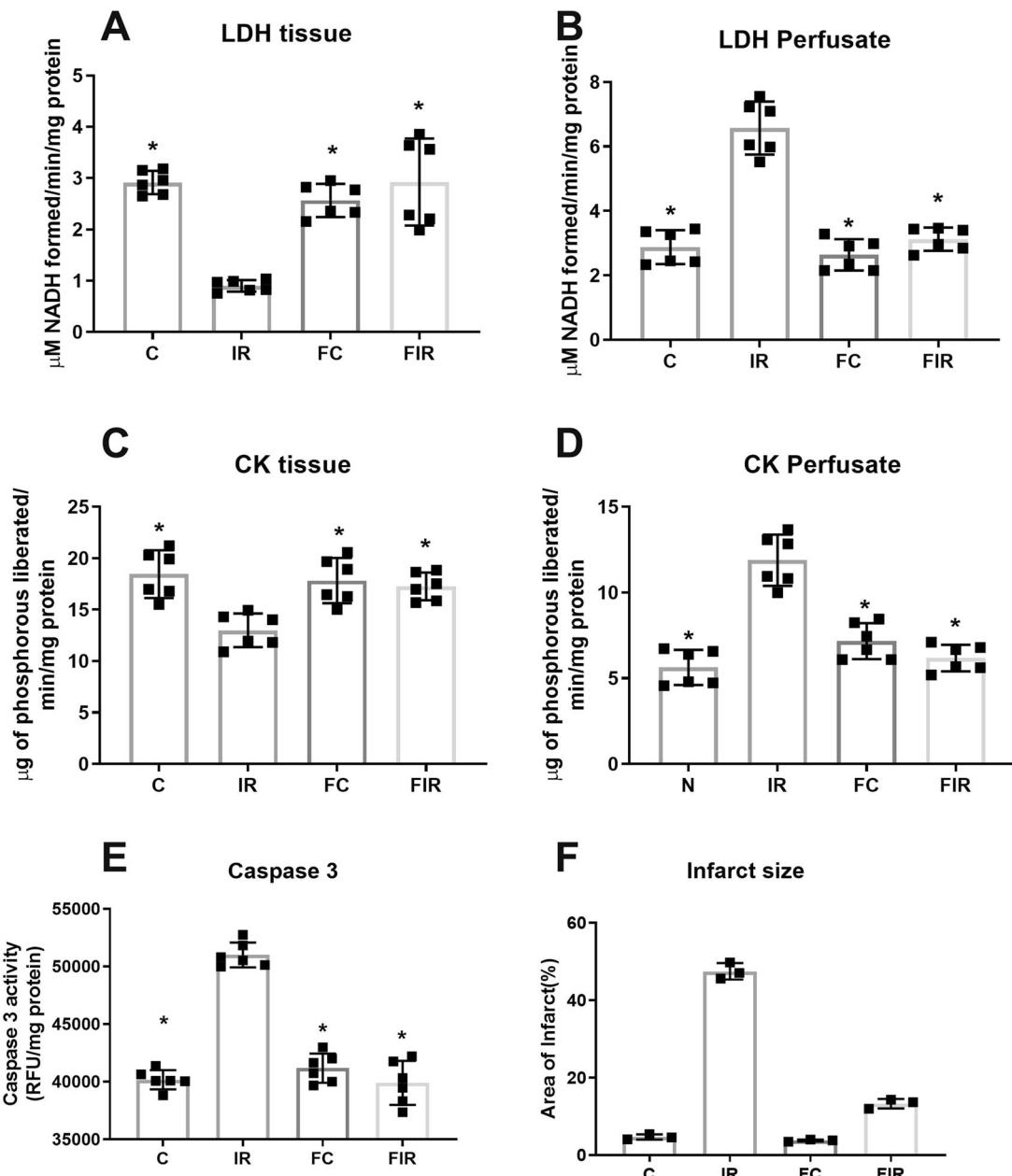


Fig. 2 Depicts the effect of fisetin treatment on IRI. Biochemical analysis of (A) LDH activity in the tissue; (B) LDH activity in the perfusate and (C) CK activity in the tissue; (D) CK activity in the perfusate. E Caspase-3 activity in the tissue. (Groups: C ($n = 6$), IR (n

= 6), FC ($n = 6$), FIR ($n = 6$)). F Area of infarct ($n = 3$). C Control, IR Ischemia-reperfusion, FC fisetin-control, FIR fisetin-IR. Data were represented as mean \pm SD. * $p < 0.05$ vs IR

IFM even at lower concentrations, whereas the fisetin offers protection to SSM only at a higher concentration (Fig. 7B). Next, we reconfirm the above results in the I/R model. Accordingly, the isolated IFM and SSM were subjected to ischemia reperfusion after 10 min of fisetin treatment. Mitochondrial functional analysis by ATP and ROS measurement was carried out, and the results were in coherence with the previous observations (Fig. 7C and D).

Activation of Complex 1 by Fisetin: An In Vitro Analysis

As the exposure of fisetin treatment improved the ATP production capacity of IFM, to explain the possible mechanism by which fisetin increased the ATP production in an in vitro condition, we docked fisetin in the NADH binding site of complex 1. The estimated binding energy of fisetin to complex 1 was -7.9 kcal/mol. Interestingly fisetin

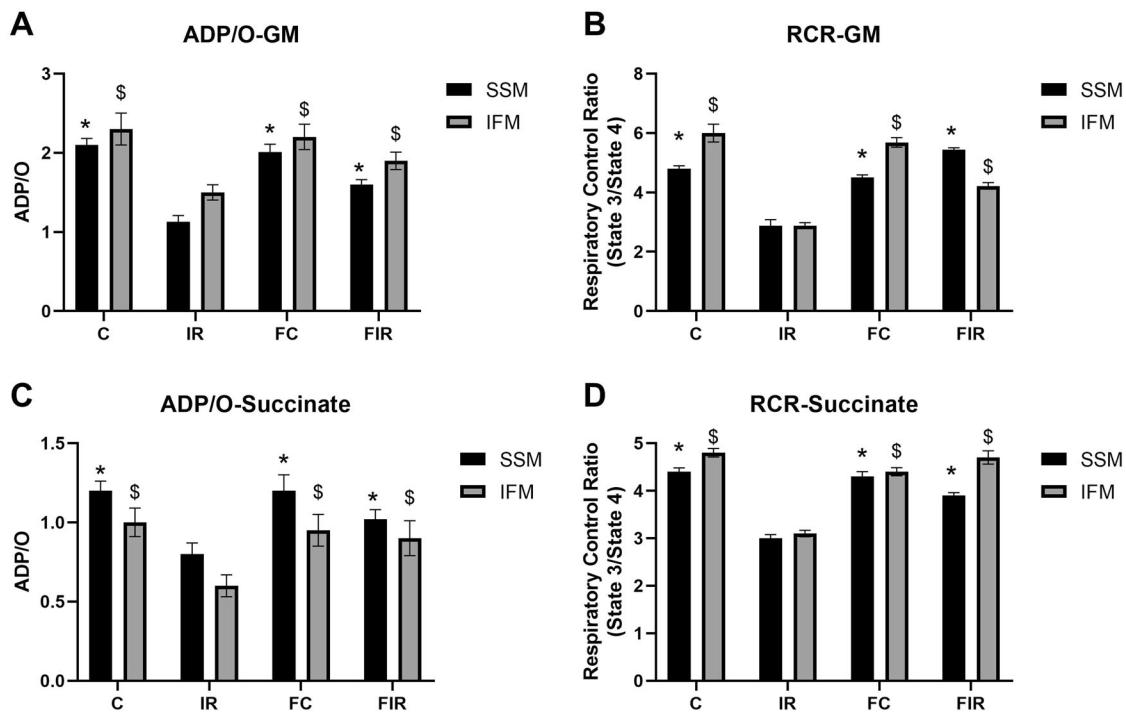


Fig. 3 The mitochondrial respiratory status between IFM and SSM measured via oxygraph (**A**) ADP/O ratio in the Glutamate malate (GM) medium, (**B**) Respiratory control ratio (RCR) in GM medium, (**C**) ADP/O ratio in the succinate medium and (**D**) RCR in succinate medium in the mitochondrial samples isolated from heart tissues.

(Groups: C ($n = 6$), IR ($n = 6$), FC ($n = 6$), FIR ($n = 6$)). C Control, IR Ischemia-reperfusion, FC fisetin-control, FIR fisetin-IR. Data were represented as mean \pm SD. * $p < 0.05$ vs IR (SSM); \$ $p < 0.05$ vs IR (IFM)

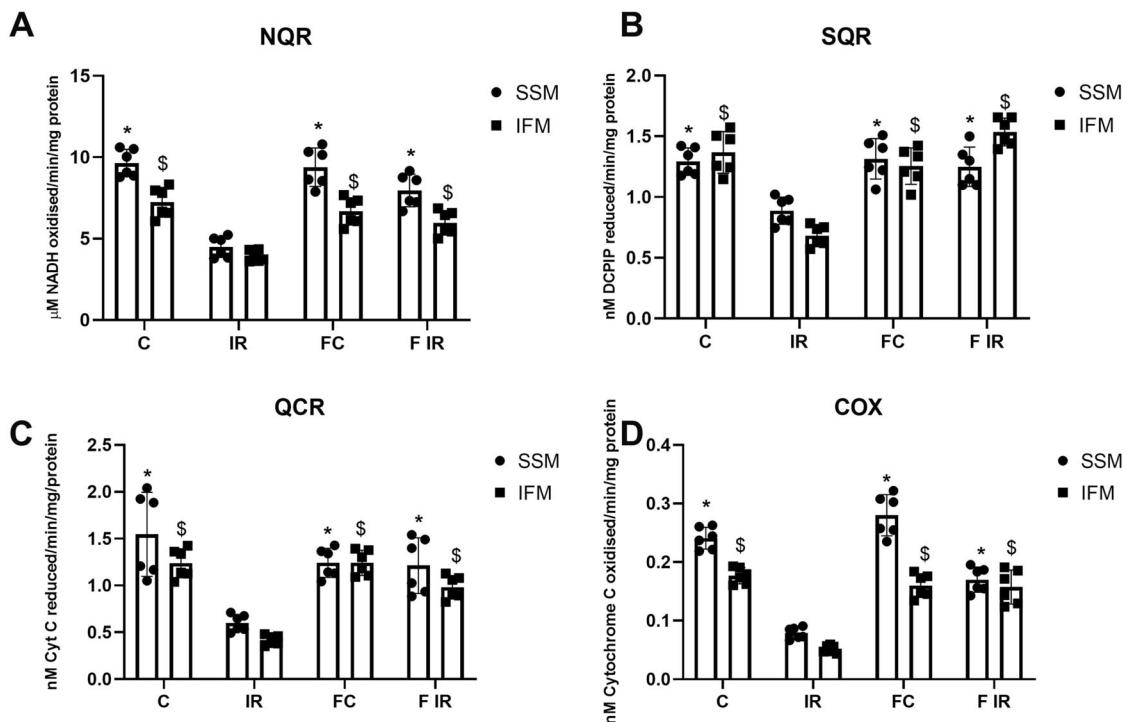


Fig. 4 Mitochondrial electron transport chain enzyme activities in cardiac mitochondrial subpopulations using spectrophotometry based analysis (A) NQR, (B) SQR, (C) QCR, and (D) COX activities in the mitochondrial samples isolated from heart tissues. (Groups: C ($n = 6$),

IR ($n = 6$), FC ($n = 6$), FIR ($n = 6$)). C Control, IR Ischemia-reperfusion, FC fisetin-control, FIR fisetin-IR. Data were represented as mean \pm SD. * $p < 0.05$ vs IR (SSM); \$ $p < 0.05$ vs IR (IFM)

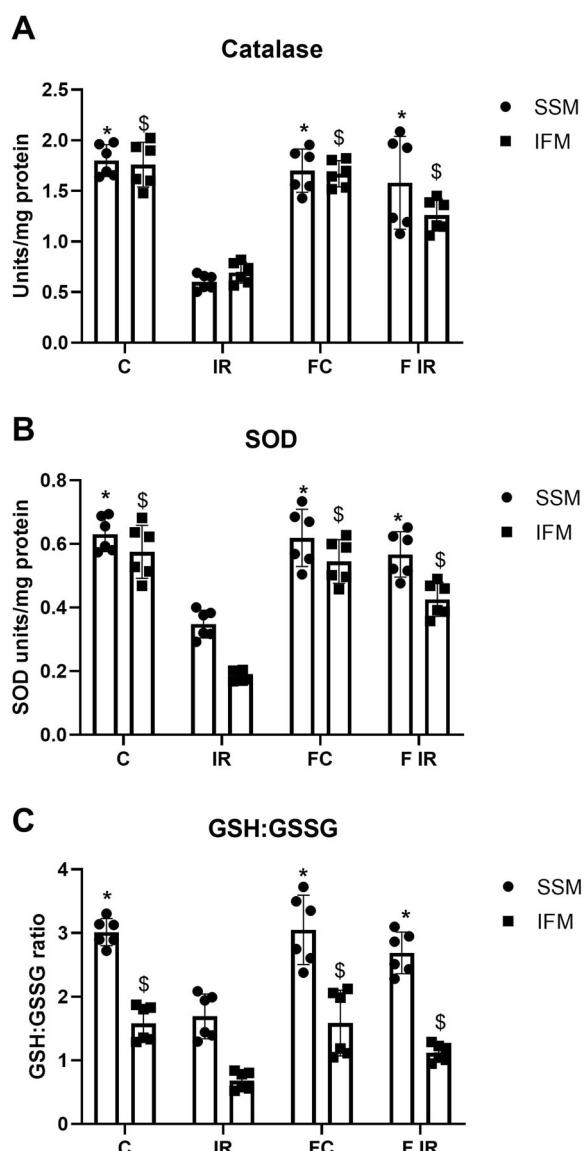


Fig. 5 Spectrophotometric analysis of oxidative stress and antioxidant defense system in mitochondrial subpopulations isolated from heart tissues. **(A)** Catalase activity, **(B)** SOD activity, **(C)** GSH: GSSG activity in the respective groups. (Groups: C ($n=6$), IR ($n=6$), FC ($n=6$)), FIR ($n=6$)). C Control, IR Ischemia-reperfusion, FC fisetin-control, FIR fisetin-IR. Data were represented as mean \pm SD. * $p < 0.05$ vs IR (SSM); \$ $p < 0.05$ vs IR (IFM)

binds exactly to the same site where NADH binds. As shown in (Fig. 8D), the binding orientation of fisetin is such that the two hydroxyl groups of fisetin (3' and 4') are in close proximity (10.7 Å and 10.8 Å) respectively to the electron receiving group (N5) of the flavin mononucleotide (FMN) molecule. Binding analysis showed that the side chains of three phenylalanine in the NADH binding site (F70, F78, and F205) formed aromatic stacking with the A, B, and C rings of the fisetin molecule and held it tightly within the binding site to facilitate the electron transfer (Fig. 8D).

ATP Producing Capacity of Fisetin by Inhibiting Complex 1 Activity

To validate the in silico hypothesis, whether fisetin activates complex 1 ETC enzyme directly to improvise the mitochondrial ATP production, we treated fisetin along with a complex 1 inhibitor rotenone. As shown in Fig. 9, fisetin treatment significantly increased ATP level in IFM compared with control. But when the complex 1 activity is inhibited using rotenone before the fisetin treatment, the ATP level drastically decreased in both IFM and SSM. Overall the result substantiates the ability of fisetin to increase ATP production by activating complex 1.

Discussion

Myocardial ischemia-reperfusion injury induced in an isolated rat heart via Langendorff perfusion system imparts functional and morphological alterations in the heart which closely resemble the pathological changes observed in the human heart undergoing revascularization procedures such as coronary artery bypass graft (CABG) and percutaneous transluminal coronary angioplasty (PTCA) [28]. During I/R, excessive release of free radicals and calcium overload leads to mitochondrial dysfunction. It has been implicated as one of the critical causative factors for the injury [29]. In the present study, we have demonstrated the protective effects of fisetin on mitochondria, particularly interfibrillar mitochondria, while rendering cardioprotection against I/R injury.

Myocardial ischemia reperfusion injury is a disorder developed with bioenergetics and metabolism during revascularization procedure [29]. Many flavonoids are reported to interfere with metabolic and bioenergetics dys-homeostasis; the recent addition to this class is fisetin [30]. In our early study, we had shown that fisetin-mediated cardioprotection was linked to the reduction in oxidative stress, apoptosis, and improved mitochondrial function [1]. Oxidative stress experienced by the I/R challenged myocardium is the secondary effect of ROS generated during the event via NADPH Oxidase, Complex I, and III enzymes in the mitochondria. Like other flavonoids, fisetin is reported to be a potent antioxidant. The free radical scavenging ability of fisetin identified in the present study supports the early findings (Fig. 6). Moreover, Zhang and his group demonstrated the potential of fisetin to activate NRF/ARE signaling, thereby augment the expressions of antioxidant genes [31]. Accordingly, we found the improved antioxidant enzymes activity in tissue treated with fisetin (Fig. 5). Fisetin is known to activate PI3K/Akt signaling in cardiomyocytes. A recent study from our group showed that cardioprotection against myocardial I/R is

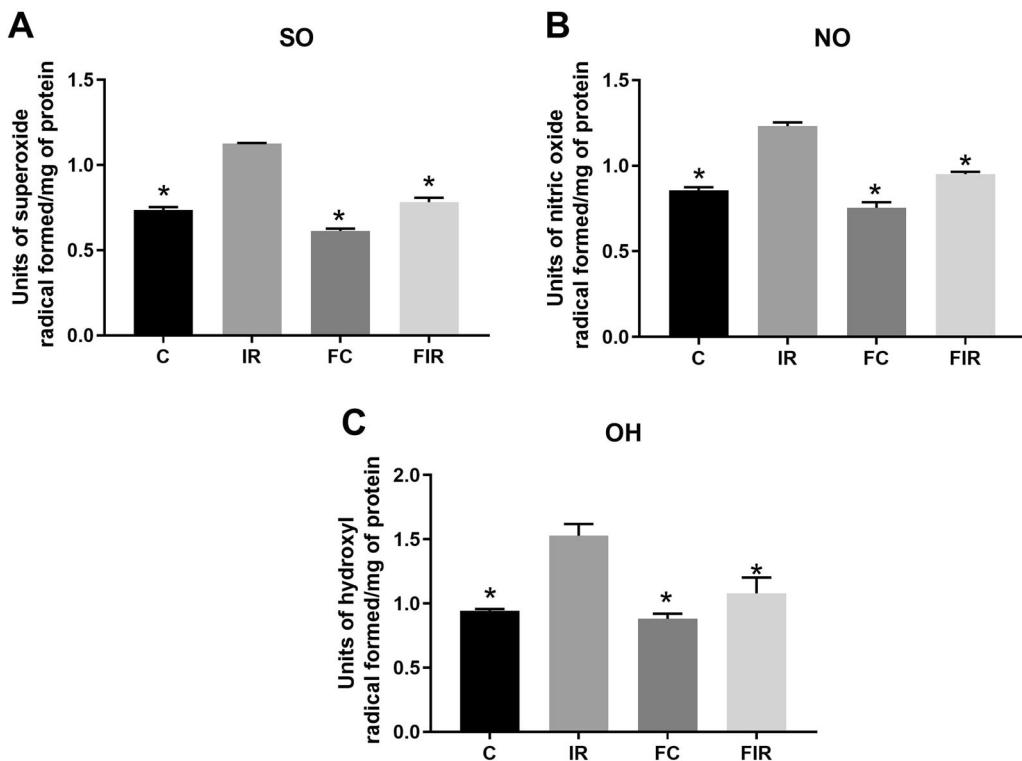


Fig. 6 Free radical scavenging activity of fisetin using biochemical based analysis (A) Superoxide, (B) Hydroxyl and (C) Nitric oxide radical. C ($n = 6$), IR ($n = 6$), FC ($n = 6$), FIR ($n = 6$). C Control, IR

Ischemia-reperfusion, FC fisetin-control, FIR fisetin-IR. Data were represented as mean \pm SD. * $p < 0.05$ vs IR

Table 2 Ex vivo and in vitro estimation of fisetin concentration in IFM and SSM

Groups	Conc. of fisetin (μM)/mg of SSM	Conc. of fisetin (μM)/mg of IFM
Ex vivo		
Normal	0	0
Ischemia-reperfusion (IR)	0	0
Fisetin-control	271 ± 7^a	$383 \pm 11^{b,c}$
Fisetin-IR	219 ± 3^a	$433 \pm 4^{b,c}$
In vivo		
Incubation of 200 μM fisetin to IFM and SSM	18 ± 5	155 ± 3^c

Data were represented as mean \pm SD of 6 individual experiments

^a $p < 0.05$ vs Normal (SSM)

^b $p < 0.05$ vs Normal (IFM)

^c $p < 0.05$ vs respective SSM

linked to the activation of PI3k/Akt signaling [32]. Accumulated evidence emphasizes the importance of PI3K, one of the major components of the RISK signaling pathway, that conferring the cardioprotection against I/R by stimulating the preservation of mitochondria via potassium ATP channel and thereby activating antiapoptotic event [33].

Recent developments in cardiovascular research suggest mitochondria as a therapeutic target to prevent cardiac I/R

injury [34]. Taking into account that mitochondria being the initiator of apoptosis and generator of free radicals during I/R, the dependency of fisetin on mitochondria in the event of its cardioprotection is not well explored. The previous reports have shown that fisetin improved the mitochondrial respiration and bioenergetics capacity of mitochondria from the I/R challenge and thereby enhanced the ATP level. Moreover, fisetin reduced the oxidative stress experienced by the mitochondria [32]. Despite the evidence that showed mitochondrial protection by fisetin, its impact on mitochondria is unclear due to the presence of distinct cardiac mitochondrial subpopulations; importantly, previous studies have testified the distinct response of mitochondrial subpopulations like IFM and SSM towards cardiac I/R injury. These subpopulations in the heart were different in shape, composition, and locations. This may be due to the multiple functions of mitochondria like energy production, acting as a metabolic site for most biochemical pathways linked to carbohydrate, lipid, protein, and nucleic acid metabolism, detoxification, autophagy. Considering the plethora of functions, mitochondria present in myofibrils where a constant supply of energy is required may be envisaged with mainly ATP production. The mitochondria beneath the sarcolemma may involve in metabolic regulation and coordination [12]. Not many studies are reported in the literature that distinct the pharmacological effect at the level

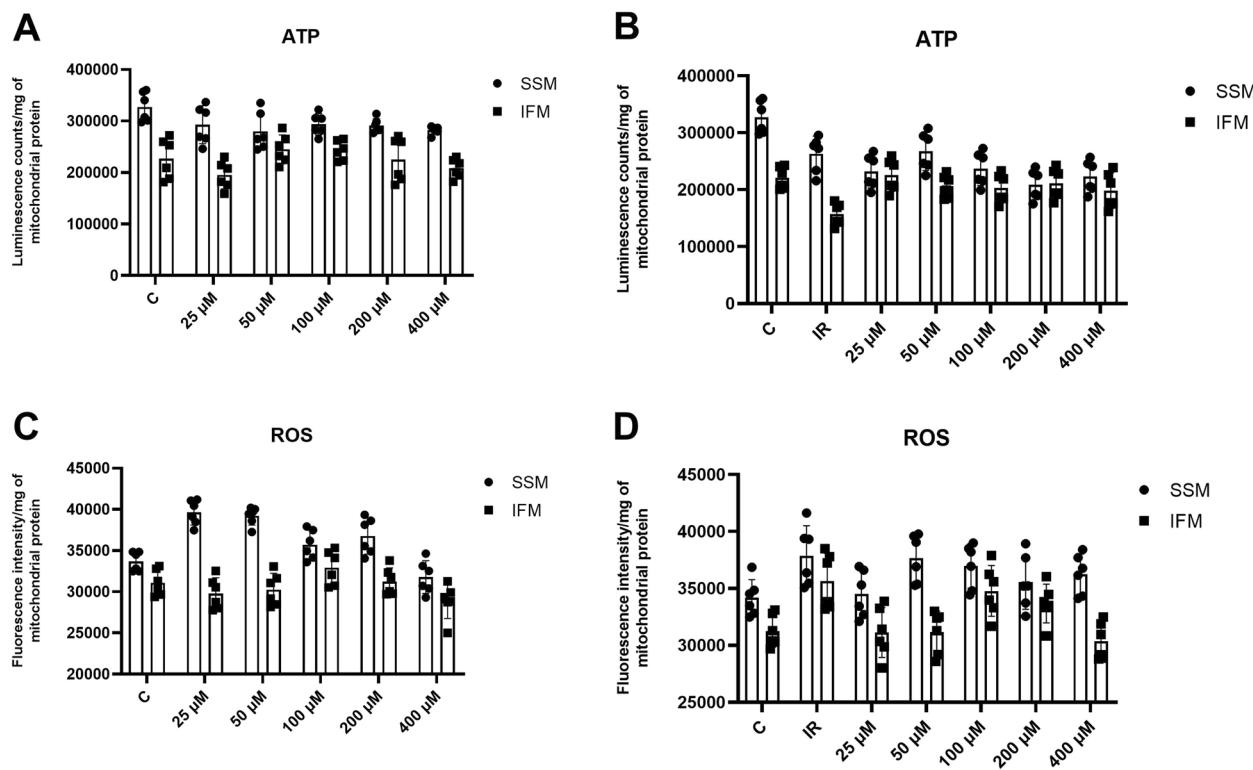


Fig. 7 Impact of fisetin uptake on ATP and ROS production in isolated mitochondria. Luminescence kit method was used to assess the (A) ATP production in Fisetin-control ($n = 6$), (B) ATP production in Fisetin-IR (FIR) ($n = 6$), (C) ROS production in Fisetin-control ($n = 6$) and DCFHDA based fluorescence assay used to estimate, (D) ROS

production in Fisetin-IR (FIR) ($n = 6$). Data were represented as mean \pm SD. * $p < 0.05$ vs Control (SSM); \$ $p < 0.05$ vs Control (IFM). In graphs (A) & (C) all values are statistically insignificant in comparison with control

of the mitochondrial subpopulation. Perhaps this may be one of the reasons for the failure of transition from pre-clinical to the clinical setting.

Thus, in this study, we evaluated the effect of fisetin on IFM and SSM in intact tissue from cardiac I/R animals and also in isolated mitochondria from cardiac tissue. Figures 4 and 5 shows fisetin imparted higher recovery of ETC enzymes in IFM than SSM from I/R challenged cardiac tissue. We asked ourselves why this difference exists, and our search found two conflicting reports in the literature. Few investigators explained the spatial location of IFM (in the myofibrils) and SSM (beneath sarcolemma) to be the reason for the distinct response of this subpopulation. But few other investigators suggested the difference between the structural compositions, especially the differences in their lipids content, mainly cardiolipins [12], as the reason. In the present study, we found a different level of fisetin concentration in IFM and SSM in myocardial tissue pre-treated with fisetin and checked whether the spatial location was responsible for the fisetin preference over IFM by initiating an *in vitro* experiment with isolated IFM and SSM from an untreated myocardium, where we incubated the subpopulations with fisetin in non-energized conditions and estimated the fisetin levels. Our result provides evidence for

better fisetin uptake by IFM than SSM suggest that the distinct response of IFM and SSM in fisetin-treated I/R myocardium is not because of special location but maybe with the difference in the membrane structural composition as suggested by other investigators. In this line of thought, previous studies have already reported structural differences between IFM and SSM via electron microscopic studies, which showed a distinct cristae morphology and density in IFM and SSM with high ATP producing capacity in IFM [12].

To further understand whether the preferential uptake of fisetin by IFM imparts any biological significance, we incubated the isolated mitochondrial subpopulations to hypoxia buffer for 15 min followed by normal respiration buffer for 15 min (to mimic ischemia-reperfusion) and then measured the ATP and ROS levels. Fisetin improved ATP level significantly in IFM than SSM. Another interesting observation we noted that even though the experiments were conducted in non-energized conditions, an elevated ATP production was found that raises the question whether fisetin can donate electrons like few other polyphenolic compounds (eg. Resveratrol). This assumption was made based on the study by Gueguen and his co-workers [16] in their investigation with resveratrol, which showed that

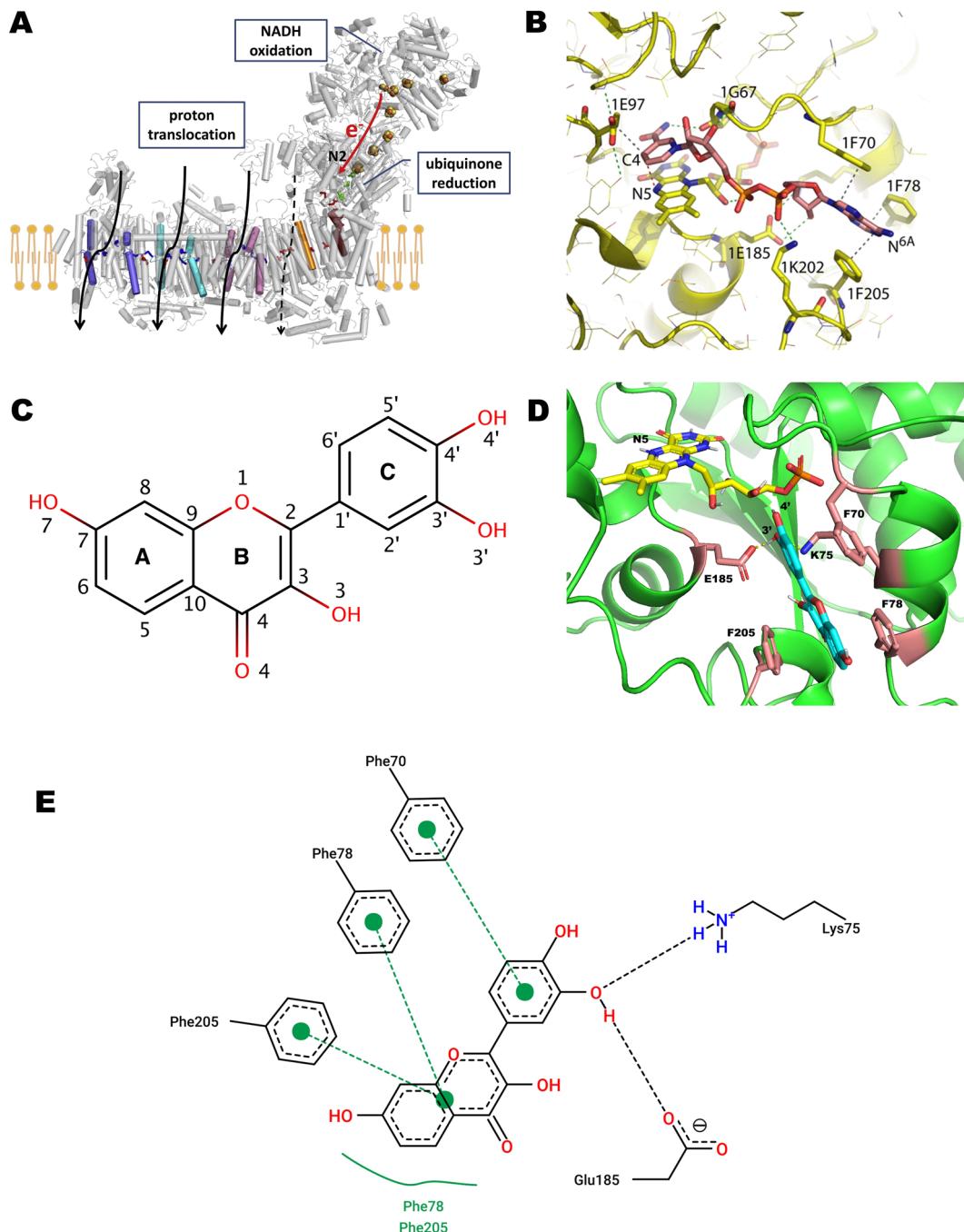


Fig. 8 Insilico docking simulation of fisetin with ETC complex 1. **A** Redox-linked proton translocation by complex 1. Electrons are transferred from the NADH oxidation site to the ubiquinone reduction site (adapted from Volker Zickermann et al., 2016), **(B)** Complex 1

binding site: NADH and FMN represented as yellow and pink sticks respectively (PDB: 3IAM). **C** 2D structure of fisetin. **D** Docking pose of fisetin on NADH binding site of complex 1. **E** 2D binding interaction map of fisetin and complex 1

resveratrol could bind to complex I of ETC and thereby increase respiration rate by donating electrons. In this direction, by using in silico methods, we evaluated the possibilities of fisetin to directly bind to ETC complex 1 and activate it for the enhanced ATP production. Our molecular docking simulation results showed that fisetin is capable of binding to complex 1 in a very similar way that NADH and

resveratrol bind to the NADH binding site of complex 1 (Fig. 8B and D). Interestingly fisetin interacts with the same key amino acid residues (F70, K75, F78, E185, and F205) in complex 1 to which NADH interacts while transferring its electron to the electron acceptor FMN in the crystal structure PDB:3IAM. Further, our docking results are in coherence with the results observed for resveratrol by

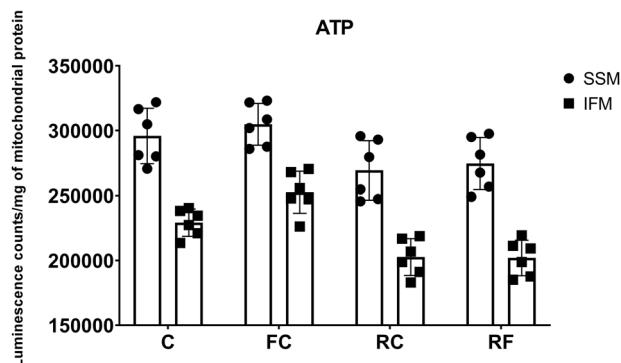


Fig. 9 ATP content in isolated SSM and IFM upon Fisetin treatment measured using luminescence kit method in presence and absence of complex 1 inhibitor (rotenone) ($n = 6$). C Control, FC Fisetin-control, RC Rotenone-control, RF Rotenone-Fisetin. Data were represented as mean \pm SD. * $p < 0.05$ vs Control (SSM); \$ $p < 0.05$ vs Control (IFM)

(Gueguen et al. 2015) in their study. Binding analysis showed that the two hydroxyl groups (3' -OH and 4' -OH) (Fig. 8C and D) in the fisetin are oriented very close (10.7 Å and 10.8 Å) to the electron receiving groups of FMN. Also, one of the recent studies showed that the electron transfer rate is maximum when donor-acceptor distances are at 30 Å [25]. Quantum mechanical calculations substantiate that single electron transfer (SET) and bond dissociation energy (BDE) of these two OH (3' and 4') groups are highly favorable for donating their electrons to FMN, thereby activating complex 1. Hence, we hypothesize that fisetin can bind directly to complex 1 and donate its electrons to FMN and activate the ETC enzymes to enhance ATP production. To further validate the complex 1 activation by fisetin, we inhibited complex I by rotenone prior to fisetin treatment to isolated mitochondria in an *in vitro* experimental condition and fisetin failed to increase the ATP

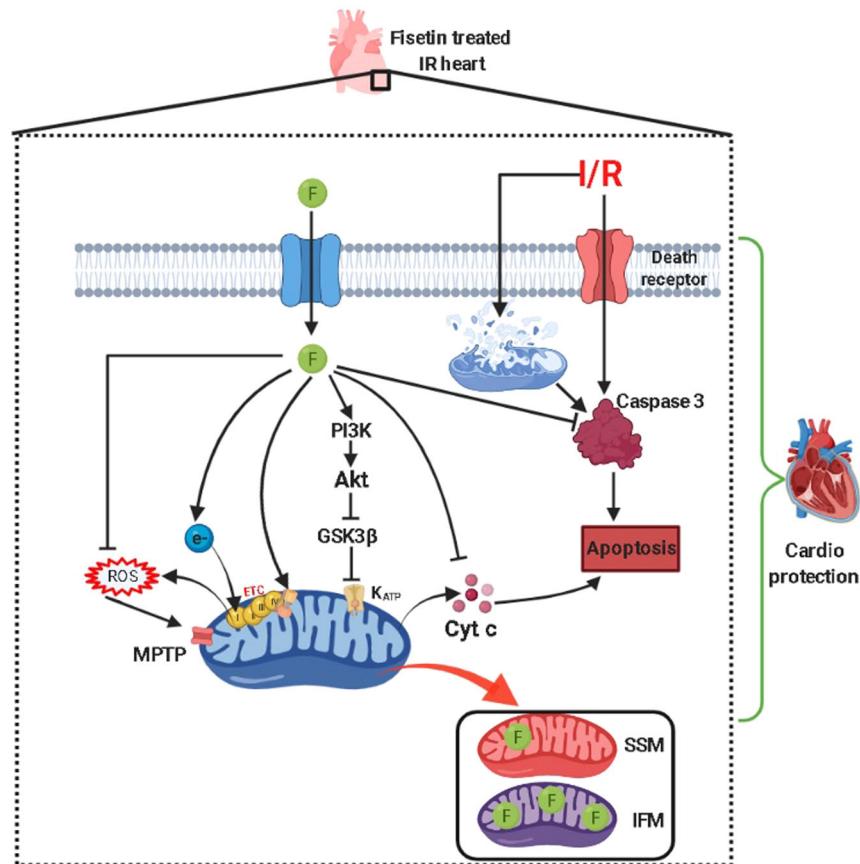


Fig. 10 Schematic diagram illustrating the mechanism of action of fisetin against myocardial ischemia reperfusion injury. Fisetin attenuate myocardial ischemia reperfusion injury by preventing apoptosis, suppress oxidative stress by scavenging free radicals and augment enzymatic and non-enzymatic antioxidants and preserving mitochondrial function, especially interfibrillar mitochondria. Further it donates electrons to the mitochondrial ubiquinone pool to maintain membrane potential and thereby prevent mPTP pore opening that control I/R

mediated cell death.. In addition, according to the previous report, it activate PI3K/Akt signaling involved in pro-survival pathway that protect cardiomyocyte from I/R injury. F Fisetin, I/R Ischemia-reperfusion, PI3K phosphatidylinositol 3-kinase, Akt protein kinase B, GSK3 β glycogen synthase kinase 3 beta, Cyt c Cytochrome C, SSM Subsarcolemmal mitochondria, IFM Interfibrillar mitochondria, MPTP Mitochondrial permeability transition pore, K_{ATP} ATP sensitive potassium channel

production in the presence of the complex 1 inhibitor (Fig. 9).

All these results confirmed that in fisetin-mediated cardioprotection, mitochondria act as the critical player. Fisetin restored the bioenergetics function of mitochondria and reduced the oxidative stress by attenuating the free radical production from mitochondrial redox complex proteins, scavenge the free radical, and recovered the antioxidant status of the mitochondria. Fisetin's ability to prevent oxidative stress can also be linked to the reduction in the endoplasmic reticulum (ER) stress and maintenance of redox homeostasis since the studies suggest the production of ROS in the ER and mitochondria as well, due to ER stress [35]. Furthermore, by activating PI3K signaling pathway, fisetin modulates mitochondrial function by regulating potassium ATP channel opening that controls mPTP transition and prevents the release of cytochrome c, the apoptotic triggering molecule. Besides, according to the current study, fisetin showed its preferential uptake to IFM over SSM, thereby maintaining the energy supply to the contractile unit of the heart. All the events in the fisetin-mediated mitochondrial protection are summarized in Fig. 10.

Furthermore, we demonstrated that mitochondria entrapped fisetin could modulate the ETC enzyme activities by probable complex 1 tethering and activate it for the enhanced ATP production. But more molecular studies are required to explain the mechanism by which fisetin preferentially enters IFM than SSM. Notably, this information pertains to IFM and SSM may help the investigators to target particular mitochondrial subpopulations for site-directed drug therapy, which may be applicable to another field of research.

Conclusion

Our data provide evidence that fisetin plays a protective role in I/R injury, possibly by preserving the functional activities of IFM, thereby maintaining the contractile function of the myocardium that render physiological recovery from I/R challenge heart.

Data availability

All data generated or analyzed during the present study are included in this published article.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Ethics Approval and Consent to Participate The present study was performed at SASTRA Deemed University. All the animal experiment procedures performed in this research were approved by the Institutional Animal Ethical Committee (IAEC), SASTRA University held on July 28th 2018 (CPCSEA approval number: 552/SASTRA/IAEC/RPP).

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