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ORIGINAL CONTRIBUTION

Akt/GSK-3β/eNOS phosphorylation arbitrates safranal-induced myocardial protection against ischemia–reperfusion injury in rats

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Abstract
Purpose Traditional medicine has been appropriately identified as the most productive soil for the cultivation and harvesting of modern medicines. Herein, we postulate that safranal, an active constituent of *Crocus sativus*, owing to its strong antioxidant and anti-apoptotic potential, could be a valuable molecule in alleviating myocardial ischemia–reperfusion (IR) injury.
Methods To evaluate this hypothesis, safranal (0.1–0.5 mL/kg/day, i.p.) or saline were administered to rats for 14 days, and on 15th day, one-stage ligation of left anterior descending coronary artery for 45 min was performed, followed by 60 min reperfusion.
Results We concluded that safranal not only significantly decreased infarct size, but also improved left ventricular functions and the overall hemodynamic status of the myocardium. Interestingly, safranal enhanced phosphorylation of Akt/GSK-3β/eNOS and suppressed IKK-β/NF-κB protein expressions in IR-challenged myocardium. Our findings also imply that safranal exhibits strong anti-apoptotic potential, as evidenced by upregulated Bcl-2 expression and downregulated Bax and caspase3 expression with decreased TUNEL positivity. Moreover, safranal dose-dependently normalized myocardial antioxidant and nitrotyrosine levels, cardiac injury markers (LDH and CK-MB), and decreased TNF-α level in IR-insulted myocardium. Histopathological and ultrastructural findings correlated with the functional and biochemical outcomes showing preserved myocardial architecture and decreased inflammatory cells and edema.
Conclusions Taken together, these results provide convincing evidence of safranal as an invaluable molecule in myocardial IR setting probably due to its fortified antioxidant and anti-apoptotic potential.

Keywords Safranal · Myocardial ischemia–reperfusion injury · Akt/GSK-3β/eNOS · Oxidative stress · Apoptosis

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Introduction

Acute myocardial infarction (MI) is a clinical entity caused by diminished coronary blood flow, usually a consequence of coronary arterial occlusion by an atherosclerotic plaque, thereby culminating in myocardial necrosis and cardiomyocyte demise. So, in order to protect the myocardium from otherwise lethal conditions, various cytoprotective proteins are upregulated that shield the ischemia–reperfused (IR)-damaged heart from further insult [1]. Perhaps, the most active and widely studied protein in this regard is glycogen synthase kinase-3β (GSK-3β). Accumulating evidence suggests that numerous drugs have been shown to converge on GSK-3β to exert their cardioprotective effects. GSK-3β being a ubiquitously expressed serine threonine/kinase has been shown to negatively regulate cardiac hypertrophy and subsequent heart failure [2, 3]. The phosphorylation and inhibition of GSK-3β at Ser9 lead to

the shutting down of the central inflammatory transcriptional pathway and apoptosis [4]. Moreover, various protein kinases including protein kinase A, B (Akt), and C participate in GSK-3β phosphorylation (Ser9) and subsequent inactivation. Therefore, GSK-3β being a direct substrate of Akt mediates various cellular processes including calcium signaling, cardiac homeostasis, myocardial growth and development, carbohydrate metabolism, and gene transcription [5].

Despite the substantial strides achieved in investigating new molecules and elucidating signaling mechanisms involved in myocardial IR injury, there is a significant decline in number of new drugs reaching the market [6]. At the same time, it has been fortunately recognized that active constituents derived from traditional medicinal products play a significant role in the prevention and progression of various cardiovascular diseases including acute MI [7, 8]. Safranal (2,6,6-trimethyl-1,3- cyclohexadiene-1-carboxaldehyde) is one of the organic compounds isolated from saffron (*Crocus sativus*), which has recently shown promising results in various in vivo models of IR injury [9, 10]. This beneficial response was primarily attributed to its strong free radical scavenging and anti-apoptotic properties. Hitherto, its effect on myocardial IR injury has not been studied conclusively. In view of the above facts, we undertook the present study (1) to decipher whether safranal attenuates myocardial IR injury in rats, and if so (2) what are its effects on Akt/GSK-3β and associated inflammatory, oxidative stress, and apoptotic pathways.

Materials and methods

Animals

The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of All India Institute of Medical Sciences (AIIMS), New Delhi, India (No. 499/IAEC/2009), and conforms with the Indian National Science Academy (INSA) Guidelines for the use and care of experimental animals in biomedical research.

Drugs and chemicals

Safranal was procured from SAFC Supply Solutions, St. Louis, USA. All primary and secondary antibodies were procured from Santa Cruz Biotechnology, USA. Rat tumor necrosis factor-alpha (TNF-α) (Diaclone Tepnel Company, UK), creatine kinase isoenzyme-MB (CK-MB) (Spinreact, Spain), and lactate dehydrogenase (LDH) isoenzyme (Logotech, Delhi, India) kits were used.

Experimental groups

Male Wistar albino rats (150–170 g, $n = 72$) were randomly assigned to six different experimental groups.

Group 1 ($n = 12$): sham

Rats were administered normal saline (0.5 mL/kg/day, i.p.) for 14 days, and on 15th day, the animals were surgically operated and LAD coronary artery was exposed and suture was passed beneath it but not subjected to ligation and reperfusion.

Group 2 ($n = 12$): IR control

Rats were administered normal saline (0.5 mL/kg/day, i.p.) for 14 days, and on 15th day, they were subjected to LAD coronary artery ligation for 45 min and reperfusion for 60 min.

Groups 3–5 ($n = 12$ each): safranal 0.1 + IR, safranal 0.25 + IR, and safranal 0.5 + IR, respectively

Rats were administered safranal (0.1, 0.25 and 0.5 mL/kg/day, i.p., respectively) for 14 days, and on 15th day, they were subjected to LAD coronary artery ligation for 45 min and reperfusion of 60 min.

Group 6: safranal per se ($n = 12$)

Rats were administered safranal (0.5 mL/kg/day, i.p.) for 14 days, and on 15th day, the animals were surgically operated and LAD coronary artery was exposed and suture was passed beneath it but was not subjected to ligation and reperfusion.

Surgical procedure and assessment of hemodynamic functions

The animals were anesthetized with pentobarbitone sodium (60 mg/kg, i.p.), and myocardial ischemia was produced via one-stage occlusion of the LAD coronary artery for 45 min followed by 60 min of reperfusion by means of the surgical procedure as described previously [11]. After 15 min stabilization period (baseline), hemodynamic variables were monitored and recorded at an interval of 15 min. The parameters which were recorded are mean arterial pressure (MAP), heart rate (HR), left ventricular end-diastolic pressure (LVEDP), inotropic state (+LVdP/dt_{max}), and lusitropic state (−LVdP/dt_{max}) of the heart.

At the end of the reperfusion period, blood samples were withdrawn via cardiac puncture and serum was separated by centrifugation (Heraeus Biofuge, Germany) at

3,000×*g* for 5 min and analyzed for CK-MB activity and TNF-α level. Subsequently, all the animals were killed and their hearts were excised and processed for biochemical, morphological, and molecular studies.

Determination of the infarct size

At the end of the reperfusion period, monastral blue (0.5 mL/kg) was injected into the left atrium over 30 s to determine the in vivo area at risk. Thereafter, animals were killed and their heart was excised and left ventricle was separated and kept at −20 °C for 30 min for uniform sectioning. The ventricle was sliced into 5-mm thick transverse sections and allowed to thaw at room temperature. From every slice, tissue on either side of the line of demarcation established by monastral blue dye was dissected under magnifying glass (10×) specifically to include only tissue stained with the dye or tissue unstained with the dye. In order to visualize the infarction, both masses (unstained and stained with blue dye) of the slices were incubated separately in buffered (Phosphate buffer, pH 8.5), 1% triphenyl tetrazolium chloride (TTC) (SIGMA Chemical Co., MO, USA) for 20 min at 37 °C. TTC staining discriminated infarcted, i.e., unstained and appearing white or pale yellow, from non-infarcted myocardium, i.e., the portion stained deep red [12].

Biochemical studies

A 10% heart homogenate was prepared in ice-cold 0.1 M phosphate buffer (pH 7.4) and used to measure malondialdehyde (TBARS) level [13]. The homogenate was centrifuged at 3,000×*g* for 20 min at 4 °C, and the supernatant was used for the estimation of total protein, LDH, and glutathione peroxidase (GSHPx) [14]. Furthermore, CK-MB and TNF-α level were estimated spectrophotometrically in serum using commercial kit.

Western blot analysis

Heart tissues (50 μg protein samples) were separated by Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (MDI, Ambala, India), which was blocked for 2 h with 5% bovine serum albumin and incubated for 24 h at 4 °C with primary antibody. The primary antibodies (dilution) used were as follows: e-NOS (1:1,000), P-eNOS (Ser1177) (1:1,000), GSK-3*β* (1:1,000), P-GSK-3*β* (Ser9) (1:1,000), nitrotyrosine (NT) (1:2,500), IKK-*β* (Ser180) (1:1,500), NF-κBp65 (Ser536) (1:1,500), MnSOD (1:2,500), Nox4 (1:1,000), Akt (1:1,500), P-Akt (Ser473) (1:1,000), Bcl2 (1:1,500), Bax (1:1,000), Caspase3 (1:1,000), and *β*-actin (1:2,500). The primary antibody was detected with horse radish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (1:10,000). The antibody–antigen complexes were visualized using diaminobenzidine (DAB), and immunoreactive bands were quantified by Bio-Rad Quantity One 4.4.0 software (BIO-RAD, Hercules, CA, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assays were performed using an in situ cell death detection kit, POD (Roche, Germany) following the manufacturer's instructions. Total cell counts and TUNEL-positive cells were analyzed in a drop of PBS using excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green). Quantitative assessment of TUNEL-positive cells was done by counting a minimum of 200 cells. The number of positive cells out of the total counted cells was expressed as percentage positivity.

Histological analysis

Left ventricular heart tissues fixed in buffered paraformaldehyde were embedded in paraffin, and serial sections (3 μm thick) were cut using microtome (Leica RM 2125, Germany). Several sections were stained with H&E for any histopathological changes.

Ultrastructural analysis by transmission electron microscope (TEM)

The Karnovsky's fixed tissues were washed in phosphate buffer (0.1 M, pH 7.4) and post fixed for 2 h in 1% osmium tetraoxide in the same buffer at 4 °C. The specimens were then embedded in araldite CY212 to make tissue blocks and ultrathin sections (70–80 nm). The sections obtained were stained with uranyl acetate and lead acetate and examined under a transmission electron microscope (Morgagni 268D, Fei Co., 60 kV, the Netherlands). The pathologist performing the morphological studies was blinded to the treatment protocol [11, 12].

Statistical analysis

The data have been presented as mean ± SD. The repeated ANOVA test followed by post hoc Bonferroni test was used for hemodynamic and western blotting parameters. In case of biochemical parameters, one-way ANOVA followed by post hoc Bonferroni test was used. The histopathological grading was expressed as median score, and Kruskal–Wallis test was employed to compare the groups. The $P < 0.05$ has been considered statistically significant.

Results

Mortality

An overall mortality of 9.37% was observed during the study period, which was either due to excessive bleeding or ligation of the coronary artery during the course of the surgery.

Hemodynamic parameters

Figure 1a–e depicts the effect of safranal on hemodynamic functions during IR-induced MI in rats. IR control rats exhibited significantly ($P < 0.01$) decreased MAP, HR, $\pm$LVdP/dt_{max}, and increased LVEDP as compared to sham group. Intriguingly, safranal at 0.25 and 0.5 mL/kg/day doses showed almost similar effects in normalizing MAP as compared to IR control group. This improved cardiac performance at two doses (0.25 and 0.5 mL/kg/day) was also associated ($P < 0.01$) in significantly normalizing $\pm$LVdP/dt_{max} and LVEDP, which was quite obvious with the progression of the reperfusion period. However, there was no significant change in HR value in safranal-treated groups as compared to normal treated rats.

Biochemical parameters

Table 1 represents the effect of safranal on the activities of antioxidant enzymes and lipid peroxidation markers. IR control rats exhibited significant ($P < 0.01$) depletion in the activities of GSHPx, along with enhanced TBARS levels as compared to sham group. Interestingly, pretreatment with safranal for 14 days dose dependently upregulated the endogenous antioxidant defense system. Moreover, this preventive effect was associated with significantly ($P < 0.01$) decreased TBARS levels.

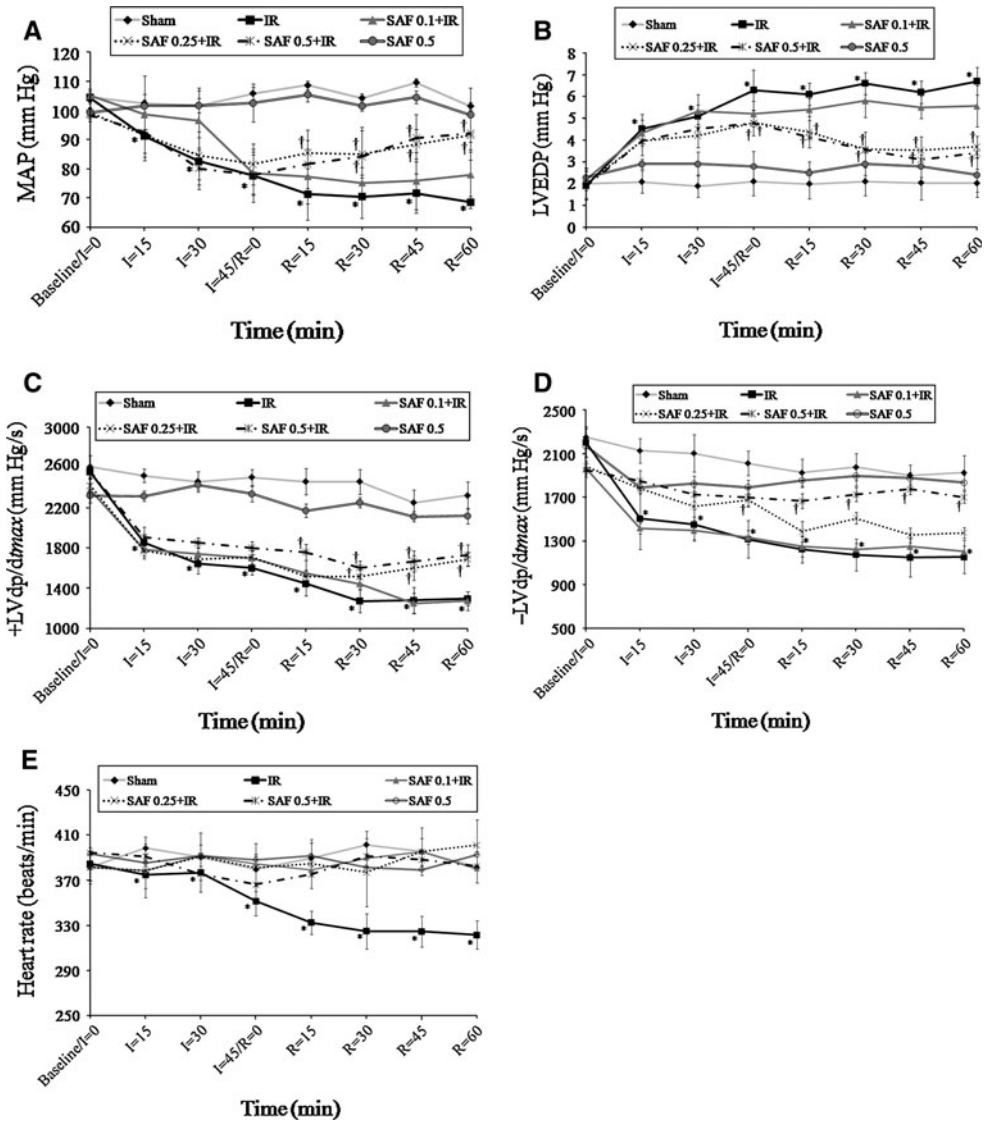

Fig. 1 Effect of safranal on **a** Mean arterial pressure (MAP), **b** Left ventricular end-diastolic pressure (LVEDP), **c** Maximal positive rate of left ventricular pressure (+LVdP/dt_{max}), **d** Maximal negative rate of left ventricular pressure (−LVdP/dt_{max}), and **e** Heart rate (HR) following IR in rats. *$P < 0.01$ versus sham, †$P < 0.01$ versus IR control

Table 1 Effect of safranal on biochemical parameters

Groups	TBARS (nmol/g)	GSHPx (U/mg protein)	LDH (U/mg protein)	CK-MB (U/L)	TNF-α (pg/mL)	TUNEL positivity (%)	Infarct size (%)
Sham	55.4 ± 3.1	0.79 ± 0.08	0.09 ± 0.01	221 ± 18	3.9 ± 0.5	1.3 ± 0.2	–
IR	129.1 ± 4.1*	0.21 ± 0.09*	0.03 ± 0.004*	601 ± 39*	56.1 ± 5.1*	35.7 ± 1.5*	39.0 ± 2.6*
SAF 0.1 + IR	105.7 ± 5.7	0.37 ± 0.03†	0.04 ± 0.01	521 ± 45†	44.5 ± 5.7	29.2 ± 4.4	32.9 ± 3.1
SAF 0.25 + IR	76.1 ± 6.5†	0.44 ± 0.03†	0.06 ± 0.008†	378 ± 54§	31.9 ± 4.5†	21.9 ± 1.8†	24.8 ± 5.2†
SAF 0.5 + IR	42.5 ± 4.8§	0.59 ± 0.06§	0.06 ± 0.009†	333 ± 21§	31.1 ± 3.8†	20.1 ± 2.0†	23.9 ± 1.7†
SAF 0.5	57.1 ± 3.2	0.7 ± 0.1	0.09 ± 0.01	229 ± 32	6.4 ± 0.8	2.1 ± 0.5	–

TBARS thiobarbituric acid reactive substances, *GSHPx* glutathione peroxidase, *LDH* lactate dehydrogenase, *CK-MB* creatine kinase-MB isoenzyme, *TNF-α* tumor necrosis factor-alpha, *TUNEL* positivity and Infarct size

* $P < 0.01$ versus sham and † $P < 0.05$, § $P < 0.001$ versus IR control

Table 1 also represents the effect of safranal on the activities of myocardial injury markers. IR control rats exhibited significant ($P < 0.01$) elevation of CK-MB and TNF-α levels and decreased LDH activity as compared to sham group. Pretreatment with safranal for 14 days dose dependently prevented the leakage of cardiac injury markers from cardiomyocytes. Moreover, this preventive effect was associated with significantly ($P < 0.01$) decreased TNF-α levels in serum.

Western blotting and TUNEL studies

Figure 2 illustrates the effect of safranal on various proteins expressions. Surprisingly, we did not find any significant change in the expression of Akt/GSK-3β/eNOS in either of the treatment groups. These findings prompted us to evaluate the extent of phosphorylation. Interestingly, IR control rats showed significant downregulation of phosphorylation of Akt/GSK-3β/eNOS, while treatment with safranal dose dependently upregulated their phosphorylation. Moreover, this increased phosphorylation by safranal was also associated with dose-dependent, reduced NT levels in IR-challenged myocardium.

To decipher the role of molecular mechanisms underlying anti-inflammatory and antioxidant activity of safranal, we next assessed IKK-β/NF-κB and Nox4 and MnSOD expression levels, respectively. As predicted, IR-challenged myocardium was associated with the increased IKK-β/NF-κB, Nox4 and decreased MnSOD expressions. Intriguingly, safranal treatment dose dependently attenuated the IKK-β/NF-κB and Nox4 and upregulated the MnSOD protein expression levels in IR-insulted myocardium.

Since inhibition of the apoptotic processes has shown to prevent the myocardial ischemia reperfusion injury, we next studied the effect of safranal on apoptotic markers including TUNEL positivity (Table 1) and Bax, Bcl2, and caspase3 expressions levels. IR control rats showed significantly ($P < 0.01$) upregulated TUNEL positivity and Bax and caspase 3 expressions along with decreased Bcl2 levels. On the other hand, safranal dose dependently attenuated the TUNEL positivity and normalized Bax, Bcl2, and caspase 3 expressions levels.

Irreversible ischemia–reperfusion injury assessment

TTC staining of myocardium revealed that IR injury strongly corroborated with the development and progression of irreversible ischemic injury of myocardium. IR-challenged myocardium resulted in significantly increased percent infarct size (39.0 ± 2.6) as compared to sham group. On the other hand, pretreatment with safranal 0.25 and 0.5 mL/kg/day significantly ($P < 0.01$) drastically reduced the infarct size (24.8 ± 5.2 and 23.9 ± 1.7, respectively) as compared to IR group (Table 1).

Histopathological and ultrastructural studies

Table 2 demonstrates the grading of histopathological damage incurred in the myocardium. Sham group showed normal architecture of myocardium, whereas IR resulted in significantly cardiomyocyte membrane damage with extensive edema, myonecrosis, and inflammatory cell infiltration. The safranal-treated rats (0.25 and 0.5 mL/kg/day) obviated cardiomyocyte membrane damage with occasional areas of myofiber loss and inflammatory cell infiltration and exhibited almost similar cellular integrity as compared to sham group (Fig. 3A1–A6).

Figure 3B1–B6 represents the ultrastructural changes in the myocardium. IR-challenged rats resulted in significant disorganization of mitochondria with swollen and disrupted cristae, chromatin condensation, cytoplasmic vacuoles, myofiber loss, and cardiomyocyte membrane damage as compared to the sham group, which exhibited normal architecture. Rats treated with safranal 0.25 and 0.5 mL/kg/day showed normal myofibrillar ultrastructure and mild

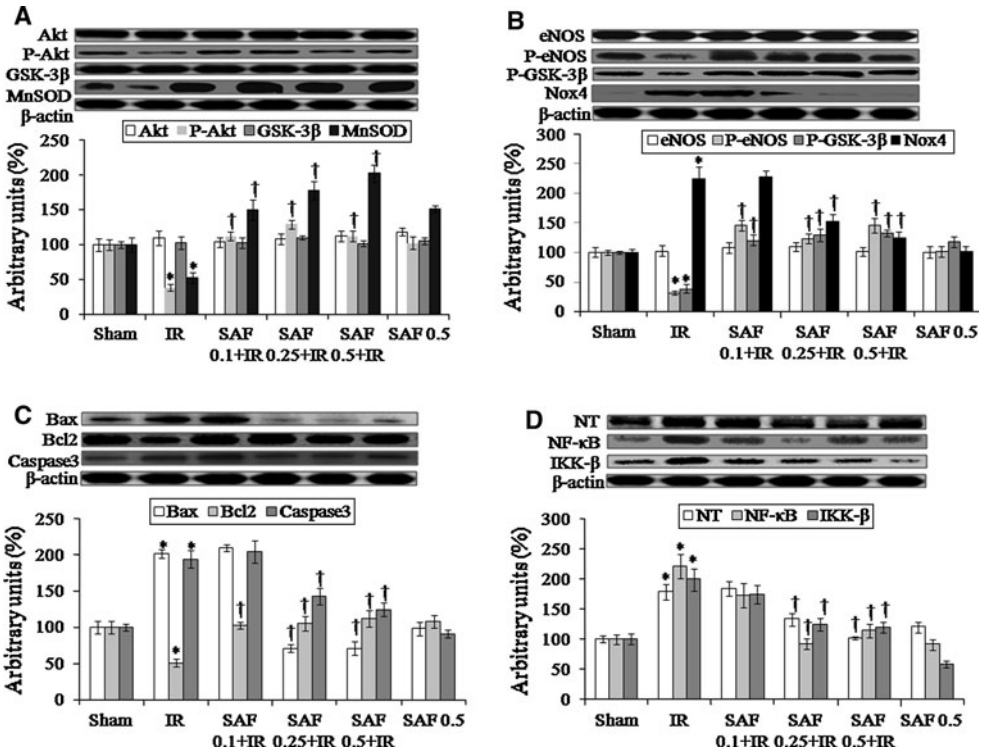

Fig. 2 Various protein expressions in heart homogenate in different experimental groups. **a** Akt, P-Akt, GSK-3β, and MnSOD **b** e-NOS, P-eNOS, P-GSK-3β, and Nox4 **c** Bax, Bcl2, and Caspase3 **d** NT, NF-κB p65, and IKK-β. Data are expressed as a ratio of normal control value (set to 100%). $^{*}P < 0.05$ versus sham, $^{\dagger}P < 0.05$ versus IR control

Table 2 Histological observations of heart tissue in experimental groups

Groups (score)	Coagulative necrosis (0–4)	Contraction band necrosis (0–4)	Loss of myofibers (0–4)	Edema (0–4)	Wavy fibers (0–4)	Inflammatory cells (0–4)	Total score (0–24)
Sham	0	0	0	0	0	0	0
IR	3	3	4	3	3	4	20
SAF 0.1 + IR	2	2	2	2.5	3	3	14.5
SAF 0.25 + IR	1	2	2	1.5	2	1.5	10*
SAF 0.5 + IR	1	1.5	1.5	1	1	1	7†
SAF 0.5	0	0	0	0.5	0	1	1.5

Score (0): representative of absence; Score (1): focal areas; Score (2): patchy areas; Score (3): confluent areas; Score (4): massive areas. * $P < 0.01$ versus sham and † $P < 0.05$ versus IR control

separation of the mitochondrial cristae without swelling and vacuolation.

Discussion

The present study reports a wide gamut of intriguing novel findings related to the signaling pathways by which safranal exerts its cardioprotective effect. We have shown here for the first time that safranal, an active constituent of *C. sativus*, prevents the development and progression of IR injury in rats. Such an improved cardiac performance in rats is primarily mediated through increased phosphorylation of Akt/GSK-3β/eNOS and inhibition of IKK-β/NF-κB pathways. Most impressively, our study also provides cogent evidence that safranal exerts potent anti-inflammatory and anti-apoptotic effects, as inferred by reduced TNF-α levels and TUNEL positivity and upregulation of anti-apoptotic markers. Furthermore, cardioprotective effect of safranal is also arbitrated by the upregulation of endogenous antioxidant defense system and reduced NT levels. Thus, these multiple pathways significantly appear to converge on limiting the infarct size by preserving myocardium in the ischemic–reperfused zone, which in turn leads to better inotropic reserves and contractile function.

The ramifications of IR injury are multifactorial, resulting from diminished contractile function to damage to multiple loci within the cell. The earlier outcome is represented by impaired contractility with concomitant

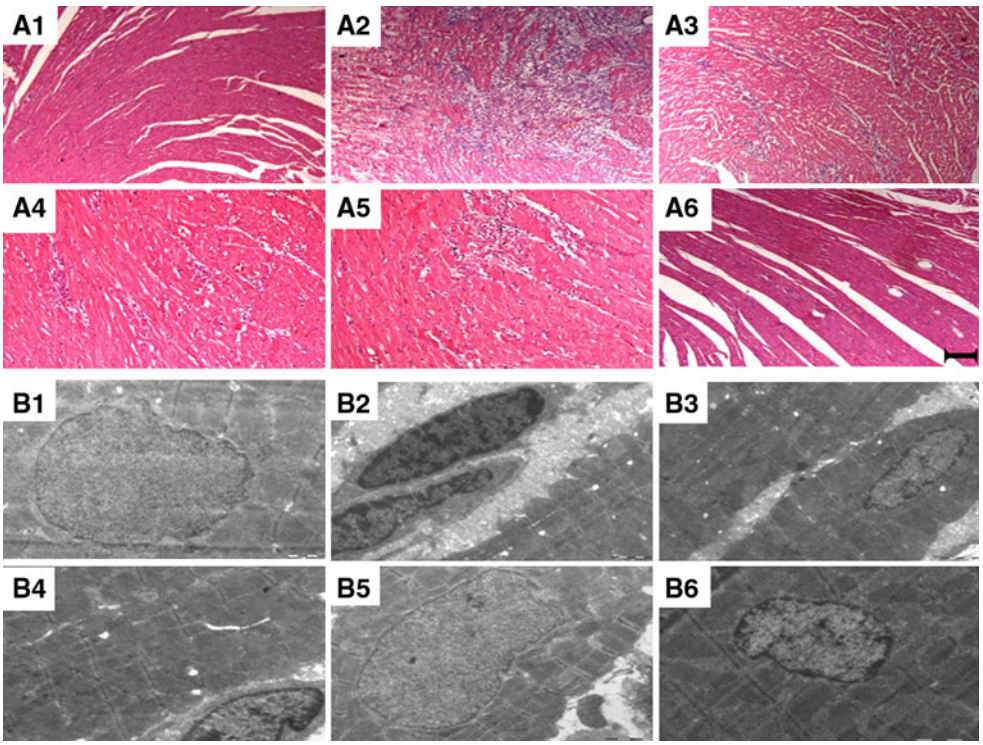

Fig. 3 Light microscopic study of cardiac tissue (*A1–A6*, 10×, *Scale bar* 50 μm) and electron microscopic study of myocytes (*B1–B6*, 4,000×, *Scale bar* 1 μm) in different experimental groups. (*A1* and *B1*) Sham group: (*A2* and *B2*), IR group: (*A3–A5* and *B3–B5*) IR + Safranal 0.1, 0.25, and 0.5 mL/kg/day, respectively and (*A6* and *B6*) safranal 0.5 mL/kg/day per se treated

inadequate ventricular emptying and reduced cardiac output, while the latter process ensues at the cellular level, accompanied by downregulation of various cytoprotective and cytoskeletal proteins. Additionally, proteolysis of actin filaments and their inability to interact with myosin cross-bridges during injury results in aberrations in excitation–contraction coupling, further contributing to the impairment in contractility [1]. In the present study, IR resulted in significantly decreased MAP, HR, $\pm LVdP/dt_{max}$ (impaired contractility and relaxation), and increased LVEDP as compared to sham group. This functional deterioration was associated with structural myocardial changes, characterized by coagulative necrosis of contraction bands and partial replacement of myofibrils by interfibrillar spaces. Moreover, this finding was further validated by subsequent increases in infarct size. Our data clearly indicate an increase in preload and afterload and the development of heart failure. Interestingly, safranal pretreatment for 14 days prevented this transition from diminished contractile function to heart failure and almost normalized the morphological architecture of the myocardium. Although the basic pharmacological mechanisms concerning safranal-mediated cardioprotection are not fully understood, our findings nevertheless allow us to postulate that phosphorylation of Akt/GSK-3β/eNOS and inhibition of TNF-α/IKK-β/NF-κB pathway by safranal could be the backbone of this effect. Enhanced phosphorylation of Akt/GSK-3β negatively regulates IR injury through actuating sarcoplasmic reticulum Ca^{2+}-ATPase2a (SERCA2a), leading to diminished cytosolic Ca^{2+} overload and improved contractile function [15–18]. Moreover, Woulfe and his coworkers have demonstrated that phosphorylation and subsequent inhibition of GSK-3β protect against post-MI remodeling and promote cardiomyocyte proliferation in the adult heart [19]. Consistently, inhibition of IKK-β/NF-κB/caspase3 as well as experimental induction of Bax and TNF-α deficiencies in mice has resulted in improved cardiac function and decreased infarct size following IR injury [1, 20, 21]. Additionally, other indirect roles of safranal might lie in the overexpression of Bcl-2, as augmented Bcl-2 expression also reduces Ca^{2+} overload [22]. Thus, the plausible mechanism of alleviating IR injury might be due to a combination of increased phosphorylation of Akt/GSK-3β/eNOS and suppression of TNF-α/IKK-β/NF-κB.

Acute MI is more often than not, preceded by chronic inflammation, epitomized by the activation of inflammatory signaling network (IKK-β/NF-κB), abnormal cytokine production (TNF-α, prototype downstream effector of NF-κB), and apoptosis [1]. As predicted, in our model of IR injury, we also observed an amplified TNF-α level that corroborated with increased translational activity of IKK-β/NF-κB/Bax/caspase3, myocardial TUNEL-positive cells,

and decreased expression of Bcl-2. Intriguingly, safranal-pretreated rats exhibited exactly the contradictory findings, implicating that the protective effect of safranal in the ischemic and infarcted myocardium is governed through inhibition of inflammation and/or apoptosis. It has been suggested that the feed-forward signaling of TNF-α and NF-κB via IKK-β pathway results in increased apoptosis and ROS production and contributes to the development of MI [23–25]. Moreover, Bcl-2 upregulation has been shown to regulate the activity of mitochondrial transition pore, thus inhibiting calcium overload and subsequently attenuating apoptotic and necrotic cell death pathways [22]. Besides, phosphorylation of Akt/GSK-3β/eNOS also inhibits IKK-β/NF-κB/Bax/caspase3 signaling pathway and therefore augmenting recruitment of Bcl-2 [5, 26]. Therefore, the mechanisms underlying strong anti-inflammatory and anti-apoptotic potential of safranal might be a direct suppression of IKK-β/NF-κB/Bax/caspase3/TNF-α or upregulation of Bcl2 expression. The observed effects are in accordance with the previous reports showing that the abrogation of inflammation by safranal results in amelioration of IR injury [9, 10].

The present study revealed, for the first time, that the myocardial salvaging effect of safranal is also mediated through bolstering the endogenous antioxidant defense system. Oxidative stress has been recognized as the central causal component in the progression and in the development of MI [1]. In the present study, we observed that IR challenge in rats resulted in a downregulation of antioxidant proteins (GSHPx and MnSOD) and upregulation of pro-oxidant proteins (Nox4) and lipid peroxidation (TBARS) in the myocardium. It has been postulated that IR-induced activation of IKK-β/NF-κB in heart amplifies oxidative stress (increased Nox4 and decreased MnSOD) and ROS production leading to enhanced cell membrane lipid peroxidation and disruption of sarcolemmal integrity [27–30]. On the other hand, safranal counteracted oxidative stress and lipid peroxidation, which could be due to its direct antioxidant activity or indirectly due to the inhibition of IKK-β/NF-κB and/or phosphorylation of Akt/GSK-3β, thereby resulting in decreased superoxide/ROS production and/or amplification of myocardial antioxidant defense system in IR heart. Hence, this phenomenon can potentially explain the prevention of oxidative stress in myocardium in safranal-treated rats.

Apart from hemodynamic to biochemical changes, several diagnostic markers like LDH and CK-MB have been used as predictors for pathological changes following IR injury. IR insult releases these enzymes into the extracellular fluid, ensuing in significant amplified levels of these enzymes in extracellular fluid and reduced levels in myocardium [1, 11, 12]. In agreement with this, we observed similar findings in IR control group indicating necrotic damage of the myocardial membrane with the decreased activity of LDH in myocardium and augmented activity of CK-MB in the serum. Interestingly, we observed that administration of safranal significantly normalized LDH and CK-MB activities in IR-challenged myocardium. The cardioprotection offered by safranal might be due to the reduction of myocardial damage by the preservation of cell membrane integrity and stability and thus restricting the leakage of LDH and CK-MB isoenzyme.

Furthermore, to establish the role of NO in the myocardium, we next assessed P-eNOS (activated form of eNOS) and NT levels in the myocardium. Notably, the primary source of NO in the heart is P-eNOS, which actively participates in the regulation of myocardial contractility and vasodilation [26, 31, 32]. Besides, it has also been recognized that Akt phosphorylates and activates eNOS [5]. Moreover, oxidative stress has been documented to augment the IR injury by promoting functional NO deficiency. Increased superoxide/ROS formation by IR injury combines with NO/tyrosine residues to produce nitrotyrosine (NT), which culminates in tissue damage. Of note, this enhanced NT formation has been recognized as a surrogate marker for ROS-arbitrated NO oxidation [33, 34]. Surprisingly, decreased NT level by safranal was well correlated with increased P-eNOS level in IR myocardium, which might be due to reduced formation of superoxide radical or enhanced P-Akt levels. Therefore, one of the mechanisms by which safranal ameliorated IR injury is through Akt phosphorylation and decreased NT levels.

In conclusion, these preliminary findings provide the first direct evidence that Akt/GSK-3β/eNOS phosphorylation, increased NO bioavailability and suppression of IKK-β/NF-κB pathway are the primary mechanisms responsible for the cardioprotective effect of safranal. Thus, our results clearly indicate that safranal can potentially emerge as an effective therapeutic strategy in the treatment for myocardial IR injury, although further experimental and clinical studies are invariably required to decipher the additional mechanisms involved and to establish its clinical efficacy.

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Conflict of interest None.

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