Cardioprotective potential of sesamol against ischemia/reperfusion injury induced oxidative myocardial damage.

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Abstract

Sesamol, an active component of sesame oil exhibits wide array of biological properties. The present was carried out to scrutinize the protective role of sesamol in myocardial ischemia reperfusion (M I/R) oxidative cardiac injury in a murine model. Pretreatment of Sesamol (50 mg/kg) was done for 7 days before the surgery period. M I/R injury were surgically induced by left anterior descending (LAD) ligation for 45 min followed by 3 h of reperfusion. The cardioprotective efficacy of sesamol was authenticated by measuring biochemical markers, protein expression of apoptotic related genes and inflammatory gene expression. In the present study, M I/R rats displayed elevated infarct size, serum cardiac markers (CK, LDH and cTnI) MDA and MPO activity. Meanwhile, the level of antioxidants (SOD, CAT and GSH) was significantly reduced in ischemic tissues due to oxidative stress. Further, serum (TNF-α, IL-1β) and mRNA (TNF-α, IL-1β and NF-ƙB) level of inflammatory mediators were significantly elevated M I/R rats. Protein expression of apoptotic related genes (Bcl-2, Bax and Caspase-3) were significantly altered in the event of M I/R injury. However, sesamol treatment significantly reduced the infarct size, resorted the cardiac markers, reduced the lipid peroxidation, neutrophil infiltration and increased the antioxidants level. Furthermore, sesamol administration elicited significant downregulation of inflammatory genes, Bax, Caspase-3 apoptotic proteins and upregulation of anti-apoptotic Bcl-2 protein. In conclusion, our study demonstrated that sesamol exerts cardioprotective property against M I/R injury mediated through antioxidant, anti-inflammatory and anti-apoptotic mechanism.

Introduction

Globally, cardiovascular related diseases are the prominent causes of fatality and acute myocardial infarction is the serious cardiovascular event which elicits higher rate of morbidity and mortality. Therapeutic intervention like reperfusion of ischemic myocardial tissue is vital to ameliorate the devastating consequences of an infarcted heart [1]. Albeit, the patient may recover substantially however, it leads to myocardial ischemia-reperfusion (I/R) injury. Thus, myocardial I/R injury elicit increase in the size of myocardial infarct and diminished cardiac function [2].

Reports suggest that several bio mechanisms have been implicated in the pathology of myocardial I/R injury. In the myocardial ischemic area, rampant release of reactive oxygen species (ROS) and thus directly detoriate the cell membrane leading to cell toxicity and death [3]. Further, the generated ROS triggers the signal transduction pathways to activate inflammatory cytokines network which are the mainstay in the regulation of cell viability and apoptosis [3]. Meanwhile, rampant release of inflammatory mediators such as tumor necrosis factor-a (TNF-a) and IL-1β enhances the production of ROS to cause oxidative myocardial injury [4,5]. In this scenario, therapeutic agent which can limit ROS, diminishing myocardial apoptosis, inflammation and restoring heart functions have been implicated to ameliorate myocardial damage following I/R.

Sesamol, an active phenolic compound of sesame oil exhibits potent antioxidant and free radical scavenging activity. Previous preclinical reports underscore that sesamol exhibits wide array of biological activities like radioprotective [6], anti-mutagenic [7], anti-ulcer [8], neuroprotective [9] and anti-platelet activity [10]. Previous reports substantiate the protective role of sesamol against the isoproterenol and doxorubicin provoked myocardial injury [11,12]. In the light of above credible evidences, the current study was undertaken to evaluate the protective role of sesamol on ischemia/reperfusion induced oxidative myocardial injury.

Materials and Methods

Animals

Sprague Dawley male rats (200-250 g) were purchased from Wuhan University, China. The rats were caged in a well maintained animal room with a 12 h light/dark cycle and with central air conditioning (25°C, 70% humidity). The animals were allowed free access to tap water and standard rodent diet.

Chemicals

Sesamol and sodium thiopental were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals used were analytical grade.

Induction of myocardial I/R injury

The myocardial I/R injury was experimentally done by left anterior descending (LAD) ligation for 45 min followed by 3 h of reperfusion [13]. Briefly, the overnight fasted rats were anesthetized with sodium pentobarbital (40 mg/kg; i.p). Once the mechanical ventilation was done, the heart rate and rhythm was measured using limb lead II of the ECG apparatus. The trachea was removed and the rats were ventilated with room air by rodent pressure ventilator (Crompton Parkinson Ltd., England) by maintaining the followings conditions (50 strokes/ min; tidal volume of 15 ml/kg). A left thoracic incision was done exposing the pericardium and then using 4-0 sterilized silk thread a slip knot at the distal 1/3 of the LAD origin was performed. The animals were monitored for myocardial ischemia associated cardiac functions after 5-15 min of ischemia and then underwent 45 min of ischemia. After the ischemic period, the slip knot was removed gently and the myocardium was reperfused for 3 h. After the surgery period, the chest cavity was closed and post-operative analgesic buprenorphine (0.1 mg/kg s.c.) was administered.

Experimental design

The rats were randomly categorized into three groups (n=10) as follows,

Group 1: Sham operated group (Sham): The silk thread was inserted underneath the LAD but the ligation was not performed.

Group 2: Myocardial ischemic group (M I/R): The LAD was ligated 45 min followed by 3 h reperfusion and treated with vehicle (0.9% NaCl; i.v).

Group 3: M I/R + Sesamol group: The rats received sesamol (50 mg/kg b.wt; p.o) daily for 7 days.

Evaluation of myocardial infarct area

Twenty four hour after the I/R period, the whole heart was carefully removed, dissected into transverse slices of 2 mm thick sections and incubated in a 1% solution 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, USA) for 15 min at 37°C. The infarct areas shows (white) and the ischemic area remain stained (red). The infarct size was measured in terms of as the ratio of the infarct area to the whole ventricular.

Estimation serum clinical markers of myocardial injury

After the reperfusion period, the blood was withdrawn by cardiac puncture, kept at 37°C for 30 min, and centrifuged at 4°C, 3000 g for 10 min. Then the separated serum was stored at -80°C for various biochemical analyses. The severity of cardiac injury was assessed by the estimation of lactate dehydrogenase (LDH), creatine kinase (CK) and cardiac troponin I (cTnI) in serum. LDH and CK levels were analysed by spectrophotometric methods using commercially available diagnostic kits (Biosino Bio-Technology and Science Inc., Beijing, China). ELISA technique was used for measuring the serum cTnI levels as per the manufacturer step wise instruction (Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China).

Evaluation of antioxidants

After reperfusion, the myocardial tissue samples were homogenized (IKA, Staufen, Germany) in 10 ml of phosphate buffer at 48°C. Further, the homogenate was centrifuged at 300 g for 15 min. The level of antioxidants, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), reduced glutathione (GSH) in the supernatant was measured by spectrophotometer using commercially available kits. The protein content was measured using the Bradford method [14].

Evaluation of lipid peroxidation and Myeloperoxidase

The myocardial tissue level of malondialdehyde (MDA), an effective marker of lipid peroxidation and myeloperoxidase (MPO) activity were estimated using standard kits (Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China).

Serum TNF-α and IL-1β concentrations

The serum level of proinflammatory cytokines (TNF-α and IL-1β) were detected by ELISA technique based on the protocol instructed by the manufactures (Huijia Biotechnology Company, Xiamen, China).

Detection of apoptotic proteins markers by western blotting

The ischemic myocardial tissue were isolated and homogenized in ice chilled RIPA buffer conditions (Applygen Technologies Inc., China). Then 20 µg of protein samples were resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for Bcl-2, Bax and Caspase-3. The primary antibodies used in the study were as follows, Bcl-2, 1:750; Bax, 1:1,000; Caspase-3, 1:1,000 (Abcam, USA). The blot was thoroughly washed and incubated with HRP conjugated goat anti-mouse IgG (Abcam, USA). β-actin (1:5,000, Abcam, USA) was served as control. The binding of antibody was detected by ECL detection kit (Applygen

Technologies Inc, China) and the protein bands were visualized using Gel Doc XR system (Bio-Rad, USA).

RT-PCR detection of inflammatory markers

From the frozen rat ischemic myocardial sample the RNA was isolated and purified using RNAase (Sigma, USA) according to the manufacturer's protocol. The cDNAs was synthesized from 1 μg of the purified RNA sample. The oligonucleotide primers used in the study were shown in Table 1. The reaction was done for 30 cycles at using the following steps: 30 s denaturing at 95°C followed by a 30 s annealing step at 57°C. Finally the extension step was carried out for 1 min at 72°C. The densities were analyzed with computer based image analysis (Motic Images Advanced 3.2).

Table 1. List of primers used in the RT-PCR analysis.

S.No	Gene	Primer Sequence
1	NF-kB	Forward: 5'- CCTAGCTTTCTCTGAACTGCAAA-3'
		Reverse: 5'- GGGTCAGAGGCCAATAGAGA-3'
2	IL-1β	Forward: 5'- GAGGCTGACAGACCCCAAAAGAT -3'
		Reverse: 5'- GCACGAGGCATTTTTGTTGTTCA-3'
3	TNF-α	Forward: 5'-ATGAGCACGGAAAGCATGATCCGA-3'
		Reverse: 5'-CCAAAGTAGACCTGCCCGGACTC-3'
4	β-actin	Forward: 5'GCACCACACCTTCTACAATG-3'
		Reverse: 5'-TGCTTGCTGATCCACATCTG-3'
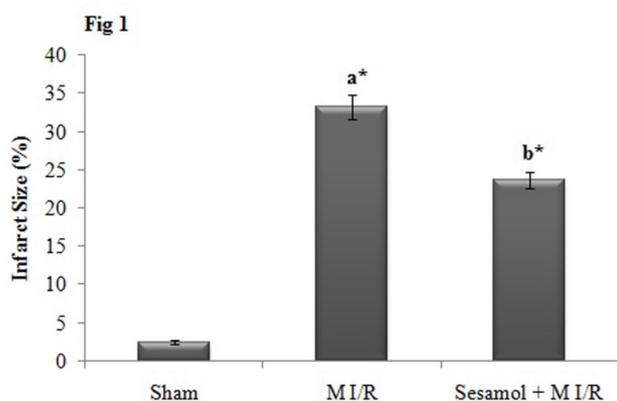
Figure 1. Effect of sesamol on the myocardial infarction induced by M I/R. Measurement of Infarct area was measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. The results were shown as mean ± SEM (n=7). $^{a*}p<0.05$, compared to Sham; $^{b*}p<0.05$, compared to M I/R.

Statistics

The biochemical data are shown as mean ± SEM. The data were analysed using SPSS software v 17. The differences between the measured parameters among groups were done by one-way ANOVA. The Tukey's test was applied for multiple comparisons. P values<0.05 were considered as statistically significant.

Table 2. Effect of Sesamol and myocardial I/R injury on serum cardiac markers.

Groups	LDH (IU/L)	CK (IU/L)	cTnI (μg/ml)
Sham	145.34 ± 5.43	103.76 ± 4.32	1.23 ± 0.23
MI/R	567.98 ± 10.76^{a*}	456.32 ± 9.76^{a*}	3.43 ± 0.56^{a*}
Sesamol + MI/R	176.65 ± 4.67^{b*}	112.43 ± 6.78^{b*}	1.41 ± 0.35^{b*}

All values expressed as mean ± SEM (n=7). $^{a*}p<0.05$, compared to Sham; $^{b*}p<0.05$, compared to MI/R. LDH, Lactate Dehydrogenase; CK, creatine kinase; cTnI, Cardiac Troponin T.

Results

Effect of sesamol on serum clinical markers of myocardial ischemia

The M I/R animals displayed marked elevation of LDH, CK and cardiac troponin I levels in the serum (p<0.05). By contrast, sesamol treatment significantly decreases the clinical markers level in the serum, compared with that of the M I/R group (p<0.05). The results were shown in Table 2.

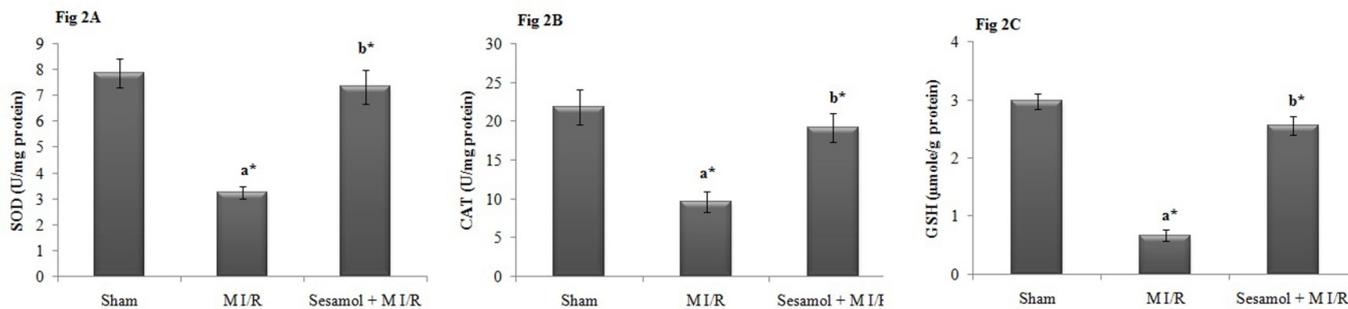### Treatment with sesamol reduced the myocardial infarct size

As when compared to the sham rats, the infarct size was significantly (p<0.05) increased in the M I/R group. Meanwhile, sesamol pretreatment significantly (p<0.05) reduced the infarct size percentage (Figure 1).

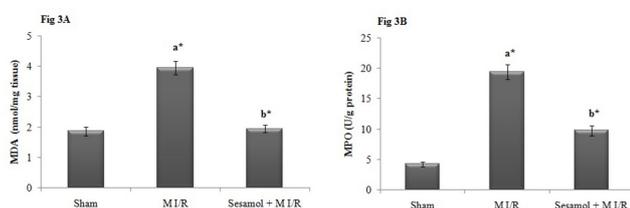### Effect of sesamol treatment on antioxidant status

As shown in Figure 2, myocardial levels of SOD, GPx and GSH were significantly elevated in the M I/R group, compared to sham operated group (P<0.05). Whilst, the inhibited antioxidant levels were significantly restored by sesamol treatment (P<0.05).

### Sesamol inhibited the oxidative stress during myocardial I/R injury

In our study, M I/R rats displayed significant MDA, a lipid peroxidation marker and MPO, an index of neutrophil infiltration in myocardial tissue (p<0.05). In contrary, sesamol treatment significantly (p<0.05) attenuated the elevated MDA and MPO activity and thus inhibited the oxidative stress (Figure 3).

Figure 2. *Effect of sesamol on antioxidants level in the myocardial tissue. The M I/R induced rats elicited depleted antioxidants level and sesamol treatment effectively attenuated the oxidative stress by restoring the antioxidant status to normal. (A) Superoxide dismutase (SOD) level; (B) Catalase (CAT) level; (C) Reduced Glutathione activity (GSH). The results were shown as mean $\pm$ SEM (n=7). $^{a*}p<0.05$, compared to Sham; $^{b*}p<0.05$, compared to M I/R.*

Figure 3. *Effect of sesamol on lipid peroxidation and neutrophil infiltration. As a result of oxidative stress induced by M I/R, there was significant elevation of MDA level and MPO activity in cardiac tissue. Sesamol treatment markedly reduced the oxidative stress by reducing the lipid peroxidation and neutrophil infiltration. (A) MDA; (B) MPO. The results were shown as mean $\pm$ SEM (n=7). $^{a*}p<0.05$, compared to Sham; $^{b*}p<0.05$, compared to M I/R.*
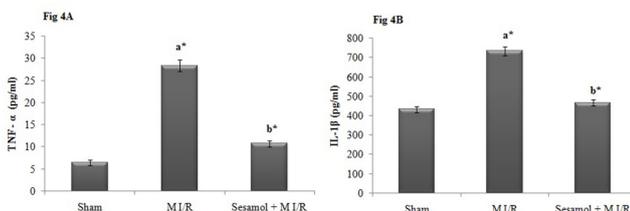
Figure 4. *Sesamol significantly reduced the serum proinflammatory cytokines level in M I/ R rats. (A) Tumor necrosis factor-α (TNF- α); (B) Interleukin - 1β (IL-1 β). The results were shown as mean $\pm$ SEM (n=7). $^{a*}p<0.05$, compared to Sham; $^{b*}p<0.05$, compared to M I/R.*

Sesamol reduced the serum level of TNF-α and IL-1β

As elicited in Figure 4, the level proinflammatory cytokines (TNF-α and IL-1β) were significantly elevated in the serum of M I/R rats (p<0.05). However, sesamol administered rats elicited substantial reduction of serum inflammatory markers to normal status (p<0.05).

Effect of sesamol on myocardial apoptosis

In our study, myocardial I/R injury markedly affected the apoptosis process, which was evident from the increased protein expression of Caspase-3, Bax and decreased expression of Bcl-2 expression in myocardial tissue. Meanwhile, sesamol

treatment downregulated the Caspase-3, Bax protein expression and upregulated the Bcl-2 protein expression effectively and thus inhibited the apoptosis (Figure 5A). The Relative protein level of Caspase-3, Bax protein expression was significantly higher and Bcl-2 was decreased in the M I/R group, compared to the sham rats (p<0.05). Compared to the M I/R group sesamol treated rats displayed significant (p<0.05) reduction of Caspase-3, Bax protein expression and elevation of Bcl-2 protein levels (Figure 5B).

### Sesamol down-regulated inflammatory markers mRNA expression profile in M I/R group

The RT-PCR analysis elicited the up-regulated mRNA gene expression of inflammatory markers, TNF-α, IL-1β and NF-ƙB in the ischemic myocardial tissue. Whilst, administration of sesamol downregulated the mRNA gene expression of TNF-α, IL-1β and NF-ƙB (Figure 6A). The Relative mRNA expression of TNF-α, IL-1β and NF-ƙB was significantly higher in the M I/R group, compared to the sham rats (p<0.05). Compared to the M I/R group, sesamol treated rats displayed significant (p<0.05) reduction of TNF-α, IL-1β and NF-ƙB mRNA expression levels (Figure 6B).

Discussion

The present study reveals the protective role of phenolic antioxidant, sesamol in myocardial I/R injury induced oxidative myocardial damage. Preclinical reports substantiates that oxidative stress is a prime factor in the etiology of myocardial I/R insult. In the event of reperfusion of ischemic heart, rampant generation of free radicals occurs which overture to form oxidative myocardial injury [15]. The ROS generated during reoxygenation of ischemic heart lead to the turbulence in the myocardial membrane, thus alters the integrity which results in the leakage of cardiac marker enzymes LDH and CK in the serum. Elevated cardiac marker enzymes in the serum are a severe indicator of myocardial membrane integrity [16]. On the other hand, cardiac troponins are the proteins molecule, which mediate calcium dependent actin and myosin interaction. Thus, cardiac Troponin-1 (cTnI) serves as a reliable diagnostic marker to assess myocardial

infarction [17]. Sesamol treatment effectively restricted the leakage of cardiac markers in serum and thus preserves the myocardial membrane integrity [12]. TTC staining is a reliable technique to assess the infarct size, which reflects the degree of myocardial necrosis in the event of M I/R. During TTC staining, it interacts with LDH in the viable myocardial tissue to form red formazan precipitate, whereas the infracted area stains white with TTC [18]. In the current study, M I/R rats displayed elevated myocardial infarct size with lack of TTC stain absorbing effect, thus reflecting a LDH leakage from the heart. Meanwhile, sesamol treatment effectively reduced the infarct size by preventing the LDH leakage [12].
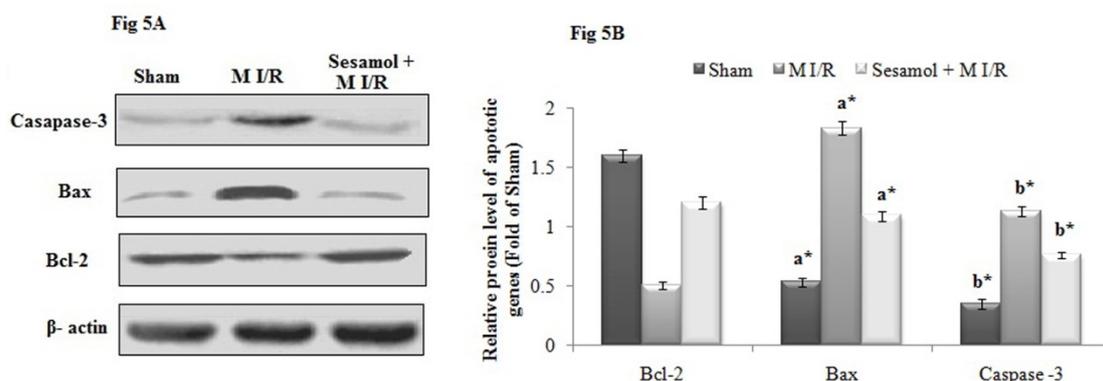
Figure 5. *Sesamol treatment effectively reduced the apoptosis in M I/R rats. (A) Corresponding western blots displays the expression levels of Bcl-2, Bax, Caspase-3 and β-actin. (B) Relative protein level of Bcl-2, Bax, Caspase-3 after normalization to β-actin. The results were shown as mean ± SEM (n=3).* $^{a*}p<0.05$, *compared to Sham;* $^{b*}p<0.05$, *compared to M I/R.*
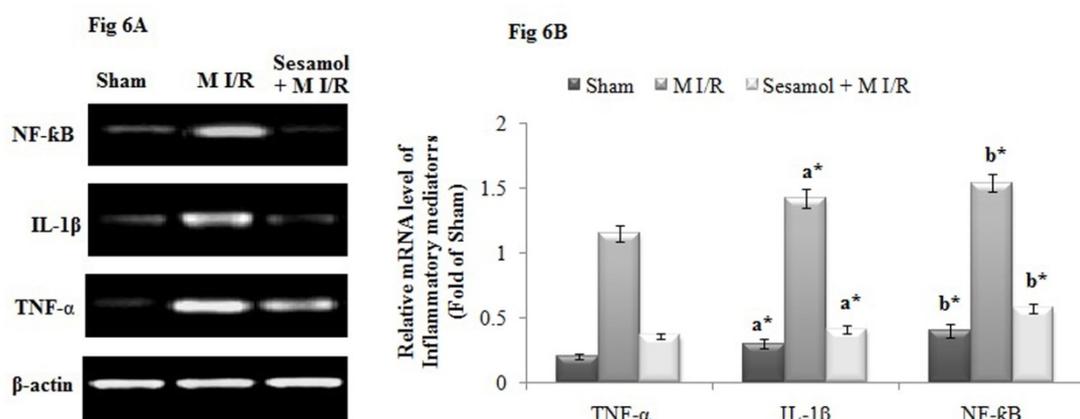
Figure 6. *Sesamol significantly attenuated the mRNA expression of inflammatory mediators. (A) RT-PCR analysis showed elevated mRNA expression of TNF-α , IL-1β and NF-ƙB in myocardial tissues. (B) Relative mRNA level of TNF-α , IL-1β and NF-ƙB after normalization to β-actin. The results were shown as mean ± SEM (n=3).* $^{a*}p<0.05$, *compared to Sham;* $^{b*}p<0.05$, *compared to M I/R.*

Evidences highlight that ischemic tissue generates excessive free radicals and other noxious products which adversely affects the vital biomolecules like lipids, proteins, carbohydrates and DNA, overture to oxidative myocardial damage [19]. The myocardial tissue is highly prone to oxidative attack due to the deprived state of antioxidants like SOD and CAT. This may lead to lipid peroxidation (LPO), distortion of membrane integrity and finally prelude to necrosis and cell death [20]. MDA is a toxic adduct formed during the LPO process and also serves as a reliable clinical indicator to asses LPO. Similarly in our study the myocardial MDA level was increased significantly in I/R rats. Whilst, the elevated MDA level was significantly attenuated by sesamol treatment mediated through its free radial scavenging ability [12].

During the initial period of reperfusion, rampant generation of superoxide and hydroxyl radicals are triggered in cardiac tissue. Whilst, in I/R conditions the antioxidants level are decreased which prone the myocardium to accelerated oxidative free radical attack [21,22]. The ROS orchestrate a predominate role in the myocardial I/R injury by substantially decreasing the body's natural antioxidant molecules like SOD, GPx and GSH [22]. Mounting evidences elicit that inhibition of ROS may be a viable clinical option to attenuate oxidative stress conditions [23]. Antioxidant protection during stress conditions, encompasses a complicated network in which each antioxidant are interlinked and thus exhibits synergistic action. In the current study, sesamol intervention displayed significant elevation of antioxidants SOD, GPx and GSH in myocardium, mediated through free radical quenching and anti-lipid

peroxidative effect. Array of preclinical reports underscore the antioxidant activity of sesamol in oxidative cardiac assault conditions [24,25].

Inflammation mediates a cardinal factor in the progression of myocardial I/R injury [26]. Increased release of pro inflammatory cytokines, inflammatory cells aggregation and infiltration are the noxious process in the inflammatory reactions [27]. Chemokines mediated neutrophils attraction towards the infarct zone after 6 h of reperfusion is the hallmark reaction during myocardial I/R injury. The attracted neutrophils overture to cause vascular spasm and further aggravate the release of ROS, and other toxic enzymes [28]. TNF-α is a key inflammatory cytokine secreted during inflammation, which kindle the inflammatory cascade by elevating the level of other inflammatory molecules like Interleukin-1β (IL-1β) and Interleukin-6 (IL-6) [29]. TNF-α, is the major cytokine involved in the initiation and progression of myocardial I/R injury and also worsen the conditions by upregulation the expression of cell adhesion factor. Furthermore, TNF-α blocks the cardiac contractility through negative inotropic effect and also elicits hypotensive activity. On the other hand, TNF-α induces myocardial apoptosis and involved in the cardiac remodelling [30]. Results of the earlier research studies show a significant interlink betwixt the neutrophil and I/R injury. So, mitigation of neutrophil activity may reduce the adverse events during myocardial I/R injury [31,32]. In the present study, myocardial I/R rats displayed elevated serum level of TNF-α, IL-1 β and tissue activity of MPO, a marker for neutrophil infiltration. However, sesamol pretreated M I/R rats displayed reduced level of TNF-α, IL-1 β and MPO as a result of significant anti-inflammatory effect. Mounting experimental evidences portrays the anti-inflammatory potential of sesamol in various pathological conditions.

Previous reports suggest that NF-κB act as an upstream signal transduction molecule and regulate the expression of genes involved in the apoptosis [33]. Thus, NF-κB plays a predominant role in early inflammatory process during myocardial I/R injury [34]. Further, activation of NF-κB leads to upregulated expression of TNF-α and other inflammatory mediators, neutrophil infiltration, loss of myocardial contractility, and activation of myocardial apoptosis leading to I/R injury [35,36]. In our study, NF-κB expression was significantly downregulated by sesamol treatment when compared myocardial I/R group. Previous reports suggest that sesamol effectively inhibits NF-κB activation in various pathological conditions [37,38].

Bcl-2 is associated with the Bcl family anti apoptotic proteins, which is majorly in the r impediment of cell death and escalation of cell generation and growth [39]. Bcl-2 in sync with Bax proteins orchestrates equilibrium between cell development and cell death [40]. Reports highlights that the Bcl-2 and Bax level have a predominant role in the protection or triggering of myocardial apoptosis in the event of I/R injury [41]. Caspase-3 is the cardinal death protease in the apoptotic process since it acts downstream molecule for other caspases and death-initiating proteins involved in the apoptotic cascade.

The apoptotic effect of Caspase-3 is mainly rendered by chromatin condensation chromatin in nucleus and by DNA fragmentation process [42,43]. Thus, Caspase-3 serves as an effective indicator to study the degree cellular apoptosis. In this study, myocardial I/R rats showed upregulated expression of Bax, Caspase-3 and downregulated expression of Bcl-2. Meanwhile, sesamol treated rats displayed significant anti-apoptotic effect by reducing the expression of Bax, Casapase-3 and increasing the expression of Bcl-2. The anti-apoptotic effect of sesamol is widely reported in array of experimental studies [44-47].

In conclusion, the current research study exemplifies that sesamol mitigates myocardial ischemia/reperfusion injury. The protective mechanism is highly attributed due to infarct size reduction, prevention of myocardial membrane damage, blockade of lipid peroxidation and elevation of antioxidants. Further, the efficacy of sesamol may be due to inhibition of inflammation, neutrophil infiltration and apoptosis. However, further molecular and mechanistic perspective studies are warranted for the clinical utility of sesamol in ischemic conditions.

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