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Investigating the role and mechanisms of tricin in ischemia-reperfusion-induced myocardial injury in LDLr -/- MICE

Feilong Sun[1], Xue Ma[2], Yu Wang[3] and Fang Liu[1]*

[1]Department of Cardiology I, Xinhua Hospital Affiliated to Dalian University, Dalian, China
[2]Department of Circulation 27, Zhongshan Hospital Affiliated to Dalian University, Dalian, China
[3]Department of Renal Internal Medicine I, Xinhua Hospital Affiliated to Dalian University, Dalian, China

Abstract: The objective of this study was to investigate the function and biological mechanisms of tricin in *in-vivo* damage to the myocardium produced by ischemia-reperfusion in LDLr -/- mice. The hypercholesterolemia animal model employed was male LDLr -/- mice. Coronary artery occlusion in mice resulted in the detection of oxidative stress and inflammatory pathology. In mice with coronary artery blockage, tricin reduced oxidative burden in the cardiac tissue and inflammatory mediators. Additionally, the ST segment of the animals receiving tricin was resumed. Tricine could dramatically lessen myocardial damage, according to pathological examination and triphenyltetrazolium chloride (TTC) staining. As a result of the research described above, the protective effects of tricin on myocardial injury have been explored, and the influence of inflammation and oxidative assaults in the ischemia-reperfusion injury (I/R) model of the heart has been demonstrated.

Keywords: Tricin, ischemia-reperfusion, myocardial, LDLr -/- mice, oxidative stress and inflammatory.

INTRODUCTION

The development of pharmacological tools to reduce deaths and incidence resulting from ischemic heart disease is the subject of active worldwide investigation Reperfusion injury has been found to be a technique that is commonly used to induce the formation of preclinical myocardial injury in studies that were conducted barely a decade ago. Ischemia/reperfusion (I/R)-induced myocardial dysfunction and structural destruction of tissue are prevalent drawbacks for individuals with certain cardiac conditions. In addition to coronary bypass surgery, organ donation, percutaneous coronary angioplasty, and thrombolysis, I/R can also occur in other circumstances. I/R damage is the term used to characterize the extra harm caused by reperfusion in organs and tissues that have undergone ischemia (Zeng *et al.,* 2009; Granado *et al.,* 2022). Since cardiomyocytes generate too many reactive oxygen substances during reperfusion, oxidative distress is a significant factor in I/R injury. Protective effects of antioxidant enzymes have been demonstrated in several investigations to prevent the post ischemic myocardium injury (Wang *et al.,* 2014).

The production of vasoconstrictor substances, nonreperfusion, severe inflammatory responses, cell death via necrosis and apoptosis are only a few of the mechanisms that interact to cause reperfusion injury (Weinreuter *et al.,* 2014; Davidson *et al.,* 2020 Interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are two examples of pro-inflammatory cytokines that have been shown to be produced following reperfusion. Both of these cytokines are responsible for inducing an excessive amount of localized inflammation. Numerous investigations have demonstrated that reducing severe inflammatory response can alleviate cardiac malfunction brought on by I/R injury and lower the size of infarction (Hua *et al.,* 2015). The primary reason for mortality is myocardial infarction, usually results in ischemia necrosis of the cardiac muscle. Pro-inflammatory cells, particularly polymorpho nuclear leukocytes/monocytes, are quickly drawn in the cardiovascular system after infarction. Inflammation, oxidative stress and the unregulated deposition of extra cellular matrix components are triggered by intruding neutrophils and macrophages (Lin *et al.,* 2007).

A natural compound tricin exists as a flavonoid called an O-methylated flavone. Sugarcane and rice bran both contain it. Flavonoids have known antioxidant effects, as well as other advantages that could be helpful in reducing cardiac ischemia-reperfusion damage. It is generally known that flavonoids have a role in cardiovascular protection and their pharmacological actions. The primary mechanisms behind flavonoid pharmacological effects on I/R damage include antioxidant, anti-inflammatory, anti-platelet aggregation, anti-apoptosis and myocardial function regulatory actions (Jai *et al.,* 2020). The nature of the anti-inflammatory effect of tricin isolated from Njavara rice bran has already been shown in experimental models employing human peripheral blood mononuclear cells (in the manifestation of bacterial lipopolysaccharide) and carrageenan-induced paw inflammation in rodents (Shalini *et al.,* 2015). Therefore, we are slated to utilize the LDL receptor-deficient (i.e., LDLr -/-) mouse as an animal model of hypercholesterolemia to examine the role of Tricin in ischemia-reperfusion-induced cardiac impairment.

**Corresponding author:* e-mail: kathyliufang@sina.com

MATERIALS AND METHODS

Reagents

Tricin (USA) was purchased from Sigma-Aldrich. Jiancheng Bioengineering Institute (Nanjing, China) was contacted to acquire ELISA kits for interleukin- (IL-) 6, IL-1β and TNF-α, as well as myocardial enzymes (CK), lactate dehydrogenase (LDH), superoxide dismutase (SOD), and malondialdehyde (MDA). Cell Signaling Technology (Danvers, USA) resources the antibodies.

Experimental animals

The hypercholesterolemia animal model employed was male LDLr -/- mice. Mice (two per cage) weighed between 30 and 35g and resided in cages with suitable bedding. Staff members actively engaged in the experimental method and routinely handled the animals, and all cages were apparent from the room. The temperature and humidity were precisely controlled at a pleasant 21°±1°C and 55%±5%, respectively. The animals were housed in a 12h light and 12h dark cycle with free access to water and rodent food.

Every study followed the guidelines for handling and using experimental animals. The Ethics Committee for the Care and Use of Laboratory Animals at Xinhua Hospital, Dalian 116000, China (Ref. No. XHADU/2022/001) authorized all animal experiments and analyses utilized in this study. Good Laboratory Practice and guidelines for the humane treatment of animals were also strictly adhered to throughout all of the experiments that were conducted.

Important criteria

The future planning of the study design is a crucial factor. In other words, randomization for appropriate control versus treatment, blinding of investigators (to the extent feasible for a given experimental procedure and the subsequent data analysis) and use of adequate statistics are crucial for replication of all experimental models of myocardial I/R and infarction.

Anesthesia

Anesthesia was administered in a Perspex chamber using 4% isoflurane for induction and 2% isoflurane and 0.5 L/min oxygen for maintenance of the anesthetic effect. Isoflurane was used because it allows for a speedy induction and easy withdrawal from anesthesia without impacting the animal's cardiovascular or respiratory parameters.

Surgical preparation

Each researcher donned a hair cover, disposable clothing, gloves, and shoes. To prevent stray infectious airborne particles from entering the surgical workplace, an extractor fan was suspended above it. Before surgery, the rat skin, heating pad, needle, electrodes and operating table were cleaned with didecil-dimetilammonium chloride 0.175%. Additionally, a hot glass dry bead sterilizer was used to clean the surgical instruments. The body temperatures of the animals were continuously monitored using a rectal temperature probe after they were positioned on a rigid thermostatic rat/mouse pad.

Experimental protocol I/R

The following groups of LDLr -/- mice were created: sham group, I/R group, I/R+ diltiazem (Dil, 20mg/kg) group, and I/R+ Tricin (Tricin, 50mg/kg) group. Each group consisted of 10 LDLr, -/- mice. The sham surgery group had concurrent normal perfusion rather than ischemia-reperfusion. Normal saline was administered by intraperitoneal route to LDLr -/- mice in the sham surgery group and ischemia-reperfusion group for a period of four weeks. In the Dil and Tricin treatment groups, LDLr -/- mice received oral doses of Dil (20mg/kg) or Tricin (50 mg/kg) via intraperitoneal route daily morning 9:00 to 10:00 am for 4 weeks before undergoing ischemia-reperfusion (Ishibashi *et al.,* 1993; Breslow, 1996; Chen *et al.,* 2021; Heusch, 2020).

LDLr -/- animals were given thiopentone sodium (30 mg/kg) via intraperitoneal route to produce anesthesia. Following tracheotomy surgery, an animal respirator called a Techno positive pressure respirator was used to allow to breathe indoor air were used in the experiment (Crompton Parkinson Ltd., Great Britain). Using a silk thread, the left anterior descending coronary artery (LAD) was ligated underneath the base of the left circumflex artery after being dissected free above the first diagonal branch during a left thoracotomy and pericardiotomy (4-0). A kink or knot blocked the artery for 30 minutes. Two knot releasers were used to cut the silk thread after 30 minutes, allowing the heart to continue to receive blood for the following 4 hours. The whole surgery operation was performed on the mock control animals, but the LAD coronary artery was not ligated. A lead II electrocardiogram (ECG) was recorded during the study employing a Cardiart 408 (BPL) with level of sensitivity of 20 mm per mV and a paper speed of 50 mm per second.

Myocardial infarct size estimation

The literature states that the following procedures were carried out: (a) separate the rat heart, (b) remove blood stains using ice-cold saline water, (c) eliminate just above ligation, unnecessary tissues with the help of a cutting tool and (d) for the preoid of 30 minutes, place the heart in a -20°C refrigerator before putting it in a 1% TTC (2,3,5-triphenyl tetrazolium chloride) solution and letting it stand in 37°C water. Myocardial infarction to risk area ratio expressed as the percentage of risk area is known as the “myocardial infarction area” (infarction area/risk area).

Inflammatory cytokines in serum

Serum IL-6, IL-1 and TNF- concentrations have been assessed using ELISA kits, with the instructions provided by the manufacturer being strictly adhered to.

Finding of cardiac tissue enzymes

The presence of myocardial enzymes indicates myocardial necrosis. Blood was drawn from the left common carotid artery and after that, it was spun in a centrifuge at 3500 revolutions per minute for 10 minutes. After that, the CK and LDH kits were used to conduct an analysis of the supernatant that had been collected earlier.

Estimation of serum antioxidants

After drawing a blood sample from the left common carotid artery, the sample was centrifuged for t10 minutes at a speed of 3,500 rpm; the supernatant was then removed and quantified and the MDA and SOD kit procedures were then followed.

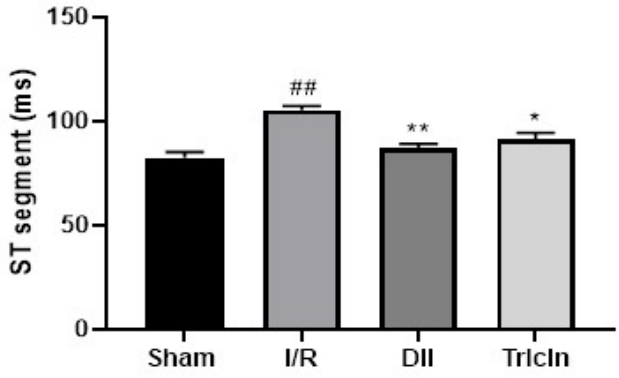
STATISTICAL ANALYSIS

In the study different experiments data were expressed by graph pad prism software (Version 8). The Tukey's multiple comparison test was employed to statistically judge against the experimental groups. Statistics were judged important at a value of $P<0.05$.

RESULTS

Changes in ECG parameters in myocardial infarcted LDLr -/- mice

Fig. 1 shows how the ST segment was markedly raised in the I/R group. In addition, we discovered that the I/R group's R amplitude compared to the control group was lower. The outcomes showed how well-established the I/R damage model was. The above-mentioned situations were evidently improved by Tricin and Dil, which partially demonstrated the preventive benefits.

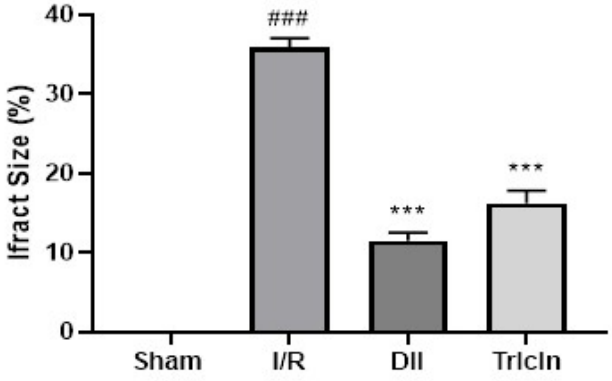


The data are articulated as mean ±SEM. ## $P<0.01$ when compared to the control group and ** $P<0.01$ and * $P<0.05$ compared to the I/R group.

Fig. 1: Tricin effects on the ST segment in LDLr -/- mice

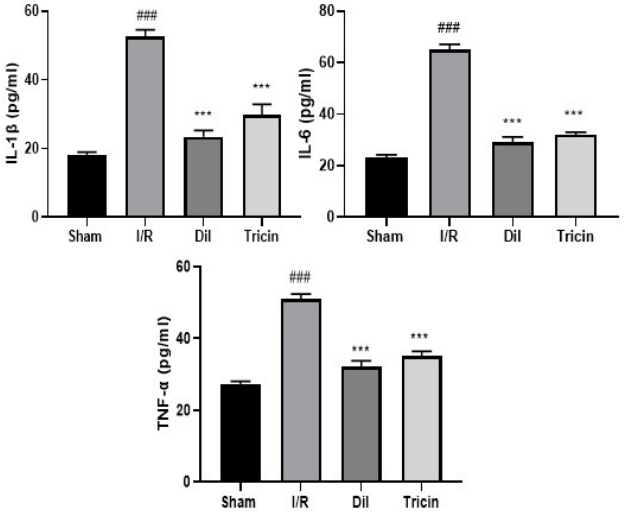
Myocardial infarct size estimation

Fig. 2 shows that compared to the control group of LDLr -/- mice, the I/R group had infarcts that were much larger than those in the control group. On the other hand, Dil (20 mg/kg) and Tricin (50mg/kg) therapy significantly decreased the infarct size.



The data are expressed as mean ±SE. ### $P<0.001$ when compared to the control group and *** $P<0.001$ compared to the I/R group.

Fig. 2: Myocardial infarct size in different groups of animals



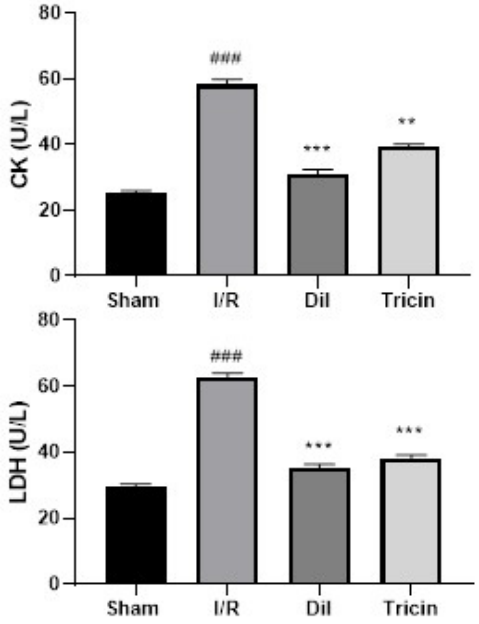
Values are shown as means ± SEM. ### $P<0.001$ compared with the control group; *** $P<0.001$ in comparison to the I/R group.

Fig. 3: Action of Tricin on pro-inflammatory cytokines on I/R LDLr -/- mice

Inflammatory cytokines in serum

Myocardial dysfunction brought on by myocardial infarction, serious congestive heart failure and sepsis may be mediated by proinflammatory cytokines. A rising amount of research points to the possibility that inflammatory factors are key players in ischemia-reperfusion damage. Additionally, ischemia-reperfusion causes tissue invasion by inflammatory cells in addition to the local transcriptional and translational elevation of cytokines. These inflammatory cells produce a range of

cytokines that might be fatal for the cardiac myocytes. In this investigation, we measured the TNF-α, IL-6 and IL-1 serum levels in I/R LDLr -/- mice. It was established that the I/R group's serum levels of TNF-α, IL-6 and IL-1 significantly increased. After being treated with Dil or Tricin, the circulating levels of TNF-α, IL-6 and IL-1 were all significantly decreased (fig. 3).



Data have been expressed as mean ± SEM. ### P<0.001 compared to the control group; ***P<0.001 as opposed to the I/R group; **P<0.01.

Fig. 4: Cardiac enzymes activity in Tricin treated I/R LDLr -/- mice

Finding of cardiac tissue enzymes

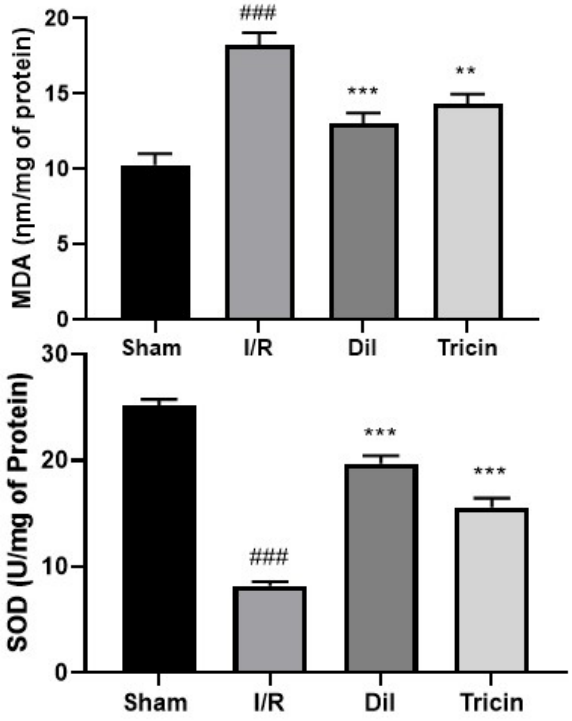
The release of a marker intracellular enzyme into the effluent allowed researchers to gauge the severity of reperfusion injury in each group of heart tissue. According to fig. 4, the I/R group experienced significantly more LDH and CK leakage than the other treatment groups. I/R produced an upsurge in LDH and CK expression in the rat heart, however, treatment with Dil or Tricin significantly abrogated this effect.

Determination of MDA and SOD

The I/R group's serum MDA levels were discovered to have grown by a level that was statistically meaningful (fig. 5). After administering Dil or Tricin, the MDA concentration in I/R animals lowered significantly. Additionally, I/R-challenged mice showed significantly reduced serum SOD levels. However, adding Dil or Tricin to the diet dramatically raised the serum SOD levels in the I/R animals.

DISCUSSION

In our investigation, we discovered that tricin decreased the amount of oxidative assault and inflammatory mediators in the heart tissue of mice with coronary artery blockage. The ST segment of the tricin-treated animals was also maintained. According to the pathological analysis, we found that tricine might significantly minimize myocardial damage. The aforementioned research clarified how tricin protects against myocardial damage and showed how inflammation and oxidative stress are engaged in the cardiac ischemia-reperfusion injury (I/R) model.



Data have been expressed as mean ±SEM. ### P<0.001 when compared with the control group and ***P<0.001 and **P<0.01 as opposed to the I/R group.

Fig. 5: Effects of Tricin on MDA and SOD in I/R LDLr -/- mice

By tampering with the genes that regulate blood cholesterol levels and lipoprotein metabolism a variety of transgenic mice have been developed recently. One of the earliest gene-targeted strains created for atherosclerosis investigations is the LDL receptor knockout (LDLr-/-) mouse. The LDLr -/- mouse has now been extensively used for scientific investigation of familial hypercholesterolemia and how that genetic change results in atherosclerosis. This animal closely reflects the disease of familial hypercholesterolemia in people. When given a high-fat diet, the LDLr -/- mouse develops severe hypercholesterolemia and arterial abrasions. For the research of the impact of hypercholesterolemia on MI, the LDLr -/- mouse thus seems to be the perfect animal model (Ishibashi *et al.,* 1993; Breslow, 1996; Henninger *et al.,* 1997). Consequently, the LDLr -/- mouse was the animal of choice for our research.

Thrombolysis and coronary artery bypass surgery are two of the several therapies available today for cardiovascular disorders. Although the mortality rate has decreased dramatically, the outcomes of these therapies are less than ideal due to the ischemia-reperfusion injury damage. Accordingly, ischemia-reperfusion cardiac damage has to be prevented and treated in medical studies. In the current study, Tricin administration amended ST, lowered the infarction zone of the heart, decreased CK-MB in addition to LDH, alleviated cardiac pathological alterations and dropped markers of inflammation as well as oxidative hassle in myocardial ischemia/reperfusion mice.

Myocardial ischemia can be mimicked on an electrocardiogram (ECG) by the presence of Q-waves, T-wave inversions, and ST-segment aberrations. Myocardial ischemia's early symptoms often include T waves and ST-segment deviations (Lin and Zheng, 2019). In this investigation, we came to the conclusion the ST segment was significantly elevated in the I/R cohort. In addition, we demonstrated that the I/R group's R amplitude was below that of the control group. The outcomes showed how well-established the I/R damage model was. Tricin and Diltiazem clearly made the scenarios listed supra better, which helped to illustrate the advantages of prevention.

TNF-, IL-6, and IL-1β are only a few of the cytokines that can be expressed during cardiac ischemia/reperfusion. An increase in cell permeability can accelerates the caspase cascade, which could result in the death of cardiac cells. The primary source of TNF-α secretion is the macrophage, which may trigger a series of inflammatory reactions by triggering the production of various pro-inflammatory cytokines with influencing neutrophil recruitment. IL-6, a key cytokine in inflammation, is crucial in the inflammation brought on by I/R. In addition to the up-regulation of cell adhesion factors and promoting neutrophil adherence to endothelial cells, IL-1 β may lead to the secretion of additional inflammatory mediators (Deten *et al.,* 2002; Mann, 2003). In the current investigation, we discovered that I/R LDLr -/- mice had serum levels of TNF-α and IL-6 plus IL-1β. It was established that the I/R group's serum levels of TNF-α, IL-6 as well as IL-1β substantially increased. The serum levels of TNF-α and IL-6 in addition to IL-1β were all reduced dramatically after administration with Dil or Tricin.

Free radicals produced by cardiac reperfusion injury can harm heart cells and resemble the clinical signs of ischemia-reperfusion destruction. According to earlier studies, I/R followed by antioxidant therapy helped repair the myocardium's damage from ROS (Du *et al.,* 2007). Superoxide dismutase and catalase (CAT), two antioxidant enzymes, can shield heart tissue against the detrimental effects of ROS. Antioxidants that neutralize ROS have been shown to be useful in treating cardiac damage brought on by I/R in the past. Therefore, to reverse the cardiac tissue damage and dysfunction brought on by I/R, it is required to discover and identify appropriate antioxidant therapies (Swaminathan *et al.,* 2010; Maulik *et al.,* 1996; Shan *et al.,* 2019). The macromolecules that are most vulnerable to oxidative stress are lipids.

Free radicals cause polyunsaturated lipids in the cell membranes to peroxidize, changing both the structure and functionality of the membrane. One of the several by-products of lipid peroxidation is MDA (Ozer *et al.,* 2005; Ghyasi *et al.,* 2012). In the existing research, we discovered that serum MDA levels in the control group were significantly up-regulated than those in the sham control group and that the size of their infarcts was considerably larger as well. These findings unmistakably demonstrated that the myocardium had suffered from ischemia-reperfusion injury.

Antioxidants that are present in nature may help with oxidative stress. Compared to control rats we found that tricin treatment dramatically reduced the infarct size and also lowered the overall elevated MDA level. O-methylated flavones, a kind of flavonoid present in rice bran and sugarcane, include tricin. It is generally known that flavonoids have special antioxidant characteristics that have long been recognized and they also possess additional qualities that may be significant to heart ischemia-reperfusion. They can limit the production of oxidants (for instance, by inhibiting xanthine oxidase and chelating transition metals), deter oxidants from attacking cell membrane targets (for instance, by donating electrons and engaging in antioxidative activities), block the spread of redox stress (by engaging in chain-breaking antioxidant activity) and boost cellular antioxidant capacity (through saving consequences for different cell reinforcements and prompting expression of endogenous oxidant scavenging agents). Through blocking pertinent enzymes and signaling pathways, flavonoids also have anti-inflammatory and anti-platelet aggregation actions. As a result, there is less oxidant generation and greater blood flow restoration in the ischemic zone. The last mechanism by which flavonoids cause vasodilation is probably interaction with ion channels. Numerous functions of flavonoids make them more useful as potential therapeutic therapies to lessen ischemia-reperfusion injury (Jia *et al.,* 2021; Rababa'h *et al.,* 2021).

CONCLUSION

According to our outcomes, Tricin protected LDLr -/- mice's hearts against the myocardial damage triggered by I/R. The antioxidant action and inhibition of pro-inflammatory cytokines are responsible for the observed positive results of this investigation. However, the present study was carried out only in male mice and thus, there is

also need of carrying out myocardial ischemia in female mice to find out if there is any gender specific differences of tricine against I/R-induced myocardial damage in LDLr -/- mice. Besides, we must also estimate the m-RNA expression of inflammatory cytokines, endogenous antioxidants and apoptotic factors to establish the mechanisms of tricine against myocardial infraction.

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