Eugenol protects the transplanted heart against ischemia/reperfusion injury in rats by inhibiting the inflammatory response and apoptosis

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Abstract. The aim of the present study was to investigate the protective effect of eugenol on the transplanted heart and explore its mechanisms of action. Male Sprague-Dawley rats were randomly divided into a sham group (n=10), a eugenol group (n=10 pairs, donors and recipients) and a control group (n=10 pairs, donors and recipients). The recipients in the eugenol group received an intraperitoneal injection of eugenol (20 mg/kg/day). The sham group and the control group received equal volumes of physiological saline by intraperitoneal injection. After 15 days the recipients in the control and eugenol groups underwent abdominal heterotopic heart transplantation, while the sham group received only a coeliotomy. The orthotopic hearts in the sham group and the heterotopic hearts in the eugenol and control groups, as well as the peripheral blood samples from all three groups were taken 3 h post operation for biochemical, histopathological, molecular and apoptosis analyses. Compared with the control group, the eugenol treatment significantly reduced the myocardial malondialdehyde content, serum cardiac troponin I, creatine kinase-MB, tumor necrosis factor-α and interleukin-6 levels (P<0.05) and significantly alleviated myocardial injury. Western blot analysis demonstrated that the protein expression of cleaved Poly (ADP-ribose) polymerase 1, BAX and active caspase-3 in the eugenol group were significantly decreased, while B-cell lymphoma 2 expression was significantly increased compared with the control group (P<0.05). The myocardial apoptosis rate of the eugenol group was significantly decreased compared with the control group (P<0.05).

In conclusion eugenol treatment significantly reduced myocardial injury and demonstrated protective effects for the transplanted heart.

Introduction

A recent investigation by the China National Center for Cardiovascular Diseases demonstrated that over 4.5 million patients have heart failure, and this number will only trend upward in the next 10 years (1). For patients with end-stage heart failure, heart transplantation has proven to be a successful therapeutic procedure (2,3) that results in a dramatic improvement in patient survival. A great development in this technique has been made over the past half century; however, the field of heart transplantation is still facing some serious challenges, such as the shortage of donor organs, cardiac allograft vasculopathy and malignancy, and ischemia/reperfusion injury (I/R) injury. These challenges can limit the application and the success of heart transplantation (4,5). As an independent risk factor, I/R injury is associated with early and late survival as well as the subsequent delayed cardiac allograft vasculopathy (6,7). Therefore, the development of more effective protection to prevent the myocardium from I/R injury is required to play a pivotal role in increasing the success of heart transplantation.

Eugenol (4-allyl-2-methoxyphenol), mainly derived from the essential oils of cloves, is a natural phenolic compound with various desirable pharmacological functions (8). The mixture of zinc oxide-eugenol has been applied as a temporary filling material in dentistry due to its anesthetic and antimicrobial properties (9). Antiproliferative and pro-apoptotic effects of eugenol have been reported on malignant melanoma cells where eugenol was shown to have selective action on tumor cells but did not interfere with normal cell growth (10). Other previous studies have concentrated on the anti-diabetic and anti-hypertension effects of eugenol in diabetic rats (11,12). In different experimental models, the anti-inflammatory and anti-oxidant properties of eugenol have been explored (13-15). Eugenol exhibits a protective effect against I/R-induced liver damage and exhibits anti-ischemic properties in isoproterenol-induced myocardial infarction in rat models (16,17).
Thus, identifying the protective role of eugenol against donor heart injury after heterotopic heart transplantation in rats will provide direction for heart disease research in the future.

Materials and methods

Animals. Male SD rats weighing 250-300 g were obtained from the Department of Experimental Animal Center, Third Xiangya Hospital of Central South University (Changsha, China). The weight of rats in the three groups showed in Table I. Animals were maintained in laminar flow cages in a specific pathogen-free animal facility and provided ad libitum access to standard rodent chow diet and filtered water. The protocol was approved by the Animal Ethics Committee of Central South University. All rat experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Experimental design

Animal grouping. Fifty male SD rats were randomly divided into a sham group (n=10), a eugenol group (n=10 pairs, donor and recipient) and a control group (n=10 pairs, donor and recipient). The weight of 5 subgroups had no significantly statistical differences. The recipients of the eugenol group received an intraperitoneal injection of 20 mg/kg eugenol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 15 days, and a corresponding volume of physiological saline was applied in the sham group and the control group for 15 days. Then, all rats were fasted for 12 h, and water was taken freely before the operation.

Heart harvest from donor rats. The donor rats of the eugenol and control groups were subjected to 40 mg of chloral hydrate (Sigma-Aldrich; Merck KGaA) per 100 g body weight of the rats by intraperitoneal administration and no signs of peritonitis were observed during the experiment, and then these rats were intravenously administered sodium heparin 100 U per 100 g as previously described (18,19) which did not show significant interference on the three groups of anesthetic effects [data not shown]. The rats were euthanized by terminal exsanguination and the aorta was identified, isolated, ligated and perfused slowly with histidine-tryptophan-ketoglutarate (HTK) solution (Custodiol; Dr Franz-Kohler Chemie GmbH, Hesse, Germany) at 4°C until the heart stopped beating. The ascending aorta, pulmonary artery and vena cava were transected, and the hearts were stored in HTK solution at 4°C for 6 h before transplantation.

Heterotopic heart transplantation. After receiving an intraperitoneal injection of eugenol or physiological saline for 1 h, the recipients of the eugenol and control groups were subjected to abdominal heterotopic heart transplantation (20,21), while the sham group was subjected only to coeliotomy without heterotopic heart transplantation. At sacrifice, rats were fully anaesthetized as described above 3 h after operation. The native hearts of the sham group and the transplanted hearts of the eugenol and control groups, along with 3 ml peripheral blood sample per rat of the three groups, were harvested for further analyses, and then 10-12 ml blood was obtained by terminal exsanguination in the three groups. Rat death was confirmed by the absence of corneal and pupillary reflexes and an apnea test.

Dosage determination of eugenol. In our preliminary experiment, recipient rats were randomly divided into four groups (5 rats/group) and received different eugenol treatment (0, 2, 20, 200 mg/kg/day, respectively) by intraperitoneal injection for 15 days. Heterotopic heart transplantation (20,21) was performed in eugenol and control groups. Results showed that rat weight of 200 mg/kg/day Eugenol treatment group was lower than that of 0, 2, 20 mg/kg/day Eugenol treatment groups (data not shown). The specific reasons are not very clear. Furthermore, 200 mg/kg/day Eugenol treatment did not show a stronger myocardial protection than 20 mg/kg/day Eugenol treatment (data not shown), research shows 100 mg/kg/day Eugenol treatment amplifies the injury via oxidant and inflammatory effects (17). Therefore, we chose the dosage of 20 mg/kg/day Eugenol treatment for our experiment.

Myocardial malondialdehyde (MDA) content. Myocardial tissue homogenate was harvested from 100 mg of myocardial tissue and centrifuged at 1,600 x g for 10 min at 4°C, and the supernatant was snap-frozen at -80°C until analyzed. The MDA concentrations of the liquid supernatants were detected by a Lipid Peroxidation MDA Assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions.

Serum levels of cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Before the hearts were collected, peripheral blood was sampled from the three groups and centrifuged at 12,000 x g for 20 min. The supernatant was snap-frozen at -80°C until analyzed. A commercial enzyme-linked immunosorbent assay (ELISA) kit was used to identify serum levels of cTnI, CK-MB, TNF-α and IL-6 (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blotting. Right and left ventricular myocardial tissues from each rat were separately harvested and lysed by RIPA buffer (CWbio, Beijing, China) containing 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF; Keygen, Nanjing, China) and 1X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined by a commercial BCA protein assay kit (Keygen). A total of 50 µg of protein was separated by 10% SDS-PAGE electrophoresis (Keygen), and samples were transferred to 0.45 µm polycryliclindene fluoride (PVDF) membrane (EMD Millipore, Billerica, USA). The blots were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies (diluted 1:1,000) against cleaved PARP1 (Cell Signaling Technology, Inc., Danvers, MA, USA), active Caspase-3 (ImmunoWay Biotechnology Company, Plano, TX, USA), BAX, BCL2 and GAPDH (Wuhan Sanying Biotechnology, Wuhan, China) overnight at 4°C. The membranes were washed with TBST and then incubated with appropriate horse radish peroxidase (HRP)-conjugated secondary antibody (diluted 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature, then washed again, and finally, the membrane was covered with ECL (Enhanced Chemiluminescence) hing substrate (Millipore) and exposed to X-ray films.
Results

Eugenol treatment decreases cTnI, CK-MB, TNF-α and IL-6 expression in serum. cTnI and CK-MB are sensitive and specific indicators of myocardial damage. Further, I/R injury leads to the early activation of inflammatory cytokines such as IL-6 and TNF-α. In comparison with the sham and control groups, heart transplantation treatment could significantly increase the level of serum cTnI, CK-MB, TNF-α and IL-6 in the eugenol and control groups (Fig. 1 and Table II). Serum cTnI and CK-MB levels of the eugenol group (5.89±0.77 and 20.16±2.07 ng/ml, respectively, n=3) were significantly lower than those of the control group (7.34±1.02 and 22.92±1.68 ng/ml, respectively, n=3) (Table II).

MDA content. The myocardial MDA content of the sham group was 3.81±0.56 nmol per 1 mg protein (mg.prot), which was significantly higher than the levels of the eugenol and control groups (9.42±0.89 and 11.16±0.96 nmol/mg.prot, respectively, n=3) (Table I). Compared with the control group, myocardial MDA content in the eugenol group was significantly lower. These results serve as convincing evidence that eugenol decreased oxidative stress reaction and oxygen free radical injury after heart transplantation.

Histopathology. H&E-stained heart sections from the sham group revealed that there was no obvious myocardial damage, the cardiac muscle fibers were regular, and there was no edema between cells (Fig. 2). In contrast, the control group exhibited extensive myocardial injury, including irregularly arranged cardiac muscle fibers and interstitial edema with infiltration of inflammatory cells (Fig. 2). However, the severity of the injury in the eugenol group was significantly less than that of the control group (Fig. 2).

Eugenol treatment decreases the expression of apoptotic proteins cleaved PARP1, BAX and active Caspase-3 and increases the expression of anti-apoptotic protein BCL2. To test whether eugenol treatment can reduce myocardial cell apoptosis in a transplanted heart, we used the Western blot method to measure protein expression levels of apoptosis molecules in rat myocardial tissues, including cleaved PARP1, BCL2, BAX and active Caspase-3. Our results showed that the eugenol and control groups had significantly higher levels of cleaved PARP1, BAX and active Caspase-3 and lower levels of BCL2 compared with the sham group (Fig. 3). This suggests that the heart transplantation treatment induced early apoptosis of the cardiomyocytes. In comparison with the sham group, the expressions of cleaved PARP1, BAX and active Caspase-3 in the eugenol and control groups were significantly increased, and the expression of BCL2 was significantly decreased (Fig. 3), which suggests that the eugenol treatment inhibited cardiomyocyte apoptosis in heart transplantation.

Eugenol treatment reduces TUNEL-positive muscle cells. In addition to Western blot analysis, TUNEL staining was used to characterize apoptosis, which provided knowledge about myocardial damage based on DNA breaks. Compared with the sham group (apoptosis rate, 0.88±0.19%), heart transplantation treatment resulted in an increase in the percentage of TUNEL-positive cells in the eugenol (apoptosis rate, 8.95±1.35%) and control (apoptosis rate, 11.75±1.71%) groups (Fig. 4). In comparison with the sham group, the percentage of TUNEL-positive cells significantly increased in the eugenol and control groups (Fig. 4). The reduction of TUNEL-positive cells in the eugenol group compared with the control group demonstrated the effects of eugenol on apoptosis.

Discussion

Our present research provided obvious evidence that pretreatment with eugenol (20 mg/kg/day) in recipient rats before...
Heart transplantation protected the donor heart from myocardial injury. A similar study showed the protection of liver I/R injury by eugenol (17). However, our study focused on the protection of a transplanted heart and the dosage of eugenol (20 mg/Kg) which was higher than their effective dosage (10 mg/Kg) (17) showed significant protective effect for the donor heart. Most importantly, their experimental results showed the protective effect of eugenol on the autologous liver.

| Parameter          | Sham       | Eugenol    | Control    |
|--------------------|------------|------------|------------|
| cTnI (ng/ml)       | 1.47±0.17  | 5.89±0.77<sup>a</sup> | 7.34±1.02<sup>ab</sup> |
| CK-MB (ng/ml)      | 6.34±0.87  | 20.16±2.07<sup>a</sup> | 22.92±1.68<sup>ab</sup> |
| TNF-α (pg/ml)      | 25.30±6.29 | 115.48±16.13<sup>a</sup> | 145.09±20.50<sup>ab</sup> |
| IL-6 (pg/ml)       | 69.26±9.11 | 135.77±16.31<sup>a</sup> | 172.80±20.41<sup>ab</sup> |
| MDA (nmol/mg.prot) | 3.81±0.56  | 9.42±0.89<sup>a</sup> | 11.16±0.96<sup>ab</sup> |

Values are presented as the mean ± standard deviation. <sup>a</sup>P<0.05 vs. the sham group. <sup>b</sup>P<0.05 vs. the eugenol group. IL, interleukin; TNF, tumor necrosis factor; MDA, malondialdehyde; CK-MB, creatine kinase-MB; cTnI, cardiac troponin I.
which had been pretreated with eugenol for 15 days (17), but our experiments showed the protective effect of eugenol on the allogeneic heart which had not been pretreated with eugenol. In clinical practice, it is difficult to use eugenol to pretreat the donor for a long-term which is not in accordance with ethical standards. What's more, our study not only provided sufficient evidence to demonstrate that eugenol can inhibit the lipid peroxidation and inflammatory reaction as previous studies have shown (13-15) but also for the first time demonstrated that eugenol can significantly inhibit the production of markers of myocardial injury and myocardial apoptosis in the transplanted heart.

Serum CK-MB and cTnI are biochemical markers of myocardial injury with notable specificity and sensitivity (25,26). These markers significantly increased after heart transplantation in previous studies (24,27,28). The same results were observed in our study, as serum CK-MB and cTnI levels of the eugenol and control groups were obviously higher than those of the sham group. Eugenol pretreatment led to decreases in CK-MB and cTnI levels in serum. These data might reflect that eugenol pretreatment could effectively reduce myocardial injury after heart transplantation. The mechanism behind this outcome might be related to the anti-inflammatory and anti-oxidative stress effects of eugenol as reported by other studies (14,15,17). Myocardial ischemia and hypoxia can increase the expression of inflammatory cytokine IL-6 (29,30) and subsequently activate various immune-related receptors to release TNF-α from cardiac mast cells, macrophages and endothelial cells (31,32). Warm reperfusion of grafts followed by cold ischemia is a signal not only to recruit neutrophils but also to increase their activation to release more inflammatory cytokines (including IL-6 and TNF-α), which in turn augments the myocardial tissue injury (28,33). Our study also revealed the infiltration of inflammatory cells into the myocardial tissue and the up-regulation of serum inflammatory cytokines 3 h after operation in the eugenol and control groups. This finding was also reported by several studies that noted inflammatory cytokine production and mononuclear cell infiltration in different heart transplantation models (24,34,35). The speed and strength of lipid peroxidation could be directly reflected by measuring the MDA content, which is the end product of lipid peroxidation metabolism (26). Compared with the control group, eugenol significantly down-regulated myocardial MDA content. These results indicated that pretreatment with eugenol could reduce oxidative stress reaction and oxygen free radical damage as reported by other studies (15,17).

Compared with the method of saline pretreatment, we observed that pretreatment with intraperitoneal injection...
of eugenol for recipients remarkably decreased the number of TUNEL-positive cells in the sectioned left ventricular myocardium of transplanted hearts. Furthermore, the level of apoptosis, as assessed by protein expression detection of cleaved PARP1, Bel-2, Bax and active Caspase-3, was significantly inhibited in hearts that were transplanted into recipients and received eugenol pretreatment. Consequently, the fact that eugenol could effectively inhibit myocardial apoptosis further confirmed that eugenol exhibits a protection effect against myocardial injury. According to our study, the mechanism of myocardial apoptosis inhibition by eugenol might be related to the fact that eugenol reduced the expression of inflammatory cytokines and eliminated the active oxygen radicals.

Because we observed down-regulation of myocardial injury markers and inflammatory cytokines as well as a reduction in cardiac cell apoptosis, our data supported the protection effect of eugenol against transplanted heart injury in the process of heart transplantation. However, there were still some limitations in our study. We only studied myocardial injury and apoptosis after eugenol treatment, and other parameters such as pharmacokinetics, myocardial function, immune rejection or other clinical events in eugenol treatment need to be tested in the future.

In conclusion, according to our present study, eugenol exerts protective effects on the transplanted heart in rat heterotopic heart transplantation through alleviating myocardial edema, down-regulating the myocardial inflammatory response and inhibiting myocardial apoptosis.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
DW and WF conceived and designed the study. WF, CL, YJ and LY performed the experiments. WF and ZJ wrote the paper. LJ, QX and LH wrote the paper, revised the manuscript and given final approval of the version to be published. All authors read and approved the manuscript.

Ethics approval and consent to participate
The study protocol was approved by the Animal Ethics Committee of Central South University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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