Protective Effect of Total Flavones of Rhododendra on Ischemic Myocardial Injury in Rabbits

Li-Ping Yuan, Zhi-Wu Chen, Fan Li and Liu-Yi Dong
Department of Pharmacology, *Anhui Medical University*, *Hefei*, *China 230032*

Fei-Hu Chen
College of Pharmacy, *Anhui Medical University*, *Hefei*, *China 230032*

Abstract: This study was to investigate the effect of total flavones of rhododendra (TFR) on ischemic myocardial injury in rabbits. Rabbit ischemic myocardial injury was induced by occluding the anterior descent of the left artery (LAD). The ECG was recorded; the plasma creatine kinase (CK), nitric oxide (NO) and endothelin-1 (ET-1) levels were measured using spectrophotometry, Griess method and radioimmunoassay, respectively. The myocardial ischemic size and infarction size were determined by dual staining with Evan's blue and Nitroblue tetrazolium reductionest (N-BT). A typical ECG S-T segment elevation and an increase of plasma CK activity were observed 6 and 24 hours after the induction of ischemia. These changes were inhibited in rabbits treated with either TFR (30, 60 mg/kg) or ginkgo biloba extract (EGB) for 7 days, indicating a protective effect of TFR on ischemic myocardial injury. The myocardial ischemic size and infarction size were $40.7 \pm 3.6\%$ and $36.8 \pm 3.6\%$ respectively in the control group, while TFR (60 mg/kg) pretreatment for 7 days significantly reduced both myocardial ischemic size ($32.40 \pm 5.38\%$, $p < 0.05$) and infarction size ($28.7 \pm 5.8\%$, $p < 0.05$). In addition, the occlusion of LAD resulted in an increase of ET-1 and a decrease of NO levels in the plasma, effects that were inhibited by TFR treatment, suggesting a possible mechanism for the protective effect of TFR against myocardial ishchemic injury.

Keywords: Total Flavones of Rhododendra (TFR); Ischemic Myocardial Injury; Myocardial Infarction; Creatine Kinase (CK); Nitric Oxide (NO); Endothelin (ET).

Correspondence to: Dr. Zhi-Wu Chen, Department of Pharmacology, Anhui Medical University, Hefei, China 230032. Tel: (+86) 551-516-1133, E-mail: wzcxiong@mail. hf.ah.cn

Introduction

Coronary artery disease, a very common clinical disease that often leads to myocardial infarction, is one of the main concerns of public health, especially in developed countries (Tendera, 2004; Stone, 2004). Finding more effective drugs with fewer risks has been a challenge. Some traditional Chinese medicines have been proven to be effective in alleviating symptoms that are similar to those induced by coronary heart disease. It was found that some traditional Chinese medicines, especially the flavones found in many plants, have protective effects against myocardial ischemic injury. Rhododendra is one of the plants that is rich in flavones, such as quercetin, hyperin and rutin (Dai *et al*., 2004; Wang *et al*., 2002), and has been used for treating patients with bronchitis for hundreds of years. Recent studies (Knekt *et al*., 2002; Ross *et al*., 2002) have shown that most flavones have protective effects against myocardial ischemic injury, such as in canine model of myocardial ischemia-reperfusion and in ischemia-reperfused rabbit hearts (Maulik *et al*., 1999; Ning *et al*., 1993). Also, hyperin has been shown to possess a protective effect against rabbit myocardial ischemia-reperfusion injury (Wang *et al*. 1996). However, there is very limited information about the protective effect of total flavones of Rhododendra (TFR) on animal models of myocardial ischemic injury. By using a rabbit model of myocardial ischemia, we set out to investigate the effect of TFR on myocardial ischemic indexes (ECG S-T segment, plasma CK level, ischemic risk size, myocardial infarction size) and its mechanism.

Materials and Methods

Drugs and Reagents

TFR (content of flavones over 60%) was provided by the Institute of Nature Medicine, Anhui Medical University. Ginkgo biloba extract (EGB) was provided by the herb production factory of Ning-bo City (Ningbo, R.O.C,). Evans Blue (EB) and Nitroblue tetrazolium reductionest (N-BT) were purchased from Sigma (St. Louis, MO, USA). CK and NO test kits were purchased from Nanjing Jiancheng Biological Company (Nanjing, R.O.C.). ET test kit was purchased from Dongya Institute of Immunity Technology (Beijing, R.O.C.).

Animals

This investigation conforms to the regulations stipulated by Anhui Medical University Animal Care Committee which follows the protocol outlined in *The Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH publication No. 85-23, revised 1996).

Total of 36 healthy rabbits (male to female: 1:1) were kept under standard conditions with a 12-hour light–dark cycle and given free access to food and tap water until their body weight reached 2.0 ± 0.5 kg. The rabbits were randomly divided into the following 6 groups: sham, control, TFR 15 mg/kg, TFR 30 mg/kg, TFR 60 mg/kg and

EGB 100 mg/kg. TFR or EGB were administered by intragastric injection (ig) for 7 days. Rabbit ischemic myocardial injury was induced at day 7 after treatment.

Extraction of Total Flavones from Rhododendra

Briefly, after being marinated in 75% alcohol for 8 hours, the dried flowers of rhododendra (500 g × 3) were boiled under reflux for 2 hours. The boiled fluid was filtered and concentrated. The concentrates were chromatographed on polyamide thread columns (25 × 18 cm; Wako Pure Chemical Industry Co., Osaka, Japan) to obtain crude total flavones. The crude total flavones were further purified by ethyl acetate and ethanol (V:V 76:38). A final lyophilized yellow powder of the total flavones was obtained and used for this study. The content of TFR was determined by UV-spectrophotometry.

Rabbit Ischemic Myocardial Injury Model

Seven days after TFR or EGB treatment, rabbits were anesthetized with 20% uretharum (1 g/kg, iv). Under sterile conditions, they were intubated and ventilated with room air supplemented with a low flow of oxygen by a mechanical ventilator (tidal volume, 20–30 ml, respiratory rates, 20–30/minutes). A left thoracotomy was performed, the pericardium was opened, and a 4-0 silk ligature was passed twice around the left anterior descending coronary artery 5 mm from its origin, which supplies most of the left ventricle with blood (Elizabeth *et al.*, 1999). This ligature was left untied until the beginning of the experimental protocol (sham-operated animals were also operated, but the coronary ligature was not tied). Standard lead II of ECG was recorded. After all surgical procedures were completed, the rabbits were allowed to stabilize for 10 minutes and then the ligature around the left anterior descending coronary artery was tied to completely occlude the vessel. After surgical operation, the chest was closed and the rabbits were allowed to recover from anesthesia for 24 hours. Additional anesthesia was given when ECG was recorded at 3 hours, 6 hours and 24 hours. At 24 hours of ischemia, the rabbits were injected with the Evan's Blue Dye. The hearts were harvested 5 minutes after the Evan's Blue injection. The ischemic size and infarction size were determined by dual staining of Evan's Blue and N-BT.

Determination of Plasma Endothelin-1

Blood samples were collected directly into prechilled test tubes containing an inhibitor solution (2 mg EDTA and 1000 kallikrein inhibiting units of aprotinin per milliliter of blood). The blood samples were centrifuged at 2000 g for 15 minutes at 4°C. Plasma was stored at −70°C and assayed within 2 weeks. ET-1 was measured by using an ET-1-21 specific (^{125}I) radioimmunoassay kit from Beijing Dongya Institute of Immunity Technology.

Measurement of NO Level and CK Activity

Blood was collected into heparinized test tubes and centrifuged for 5 minutes at 2000 g. The plasma fraction was transferred into two other clean tubes. NO level was measured at 550 nm by Griess method and CK activity was detected at 340 nm by spectrophotometry.

Determination of Infarction Size

At the indicated time point of myocardial ischemia, 5 ml of 20% Evans Blue Dye was injected through the marginal ear vein and allowed to circulate for 5 minutes. The left ventricle was isolated from the rest of the heart. The area at risk (unstained) was isolated from the normal myocardium (stained blue), weighed and then cut into 2-mm-thick transverse slices. The area at risk was placed in a 37ºC solution of 0.2% N-BT for 30 minutes. NBT stains the viable tissue dark blue, and leaves the non-viable zone pale. N-BT blue (non-infarction) tissue was isolated from the N-BT pale (infarction) tissue and each area was weighed (Chen *et al*., 1999). The ischemic size was calculated as the following: weight of the left ventricular area at risk/weight of the left ventricle; the infarction size was calculated as follows: weight of the infarction area /weight of the left ventricular area at risk.

Statistical Analysis

The data were expressed as mean ± SD, and analyzed by one-way repeated-measure ANOVA and Student's t test for comparisons between groups. $p < 0.05$ was considered statistically significant.

Results

Effect of TFR on the Changes of Segment S-T of ECG

The S-T segment of ECG in the untreated rabbits of the control group rose significantly at 1 hour, 2 hours, 3 hours, 6 hours and 24 hours of ischemia compared with the sham group ($p < 0.01$ or $p < 0.05$). The administration of TFR (30 or 60 mg/kg) for 7 days markedly decreased the elevation of S-T segments at 6 hours and 24 hours of ischemia ($p < 0.01$, $p < 0.05$) compared to respective untreated group. Administration of 100 mg/kg EGB had a similar effect as that seen in the TFR treated group (Fig. 1).

Effect of TFR on the Ischemic and Infarction Size

The occlusion of LAD induced severe myocardial ischemic injury as indicated by the measurement of the ischemic size and the infarction size in the left ventricle at 24 hours of ischemia in untreated rabbits of the control group. TFR treatment for 7 days markedly decreased the ischemic size in 30 mg/kg group (33.4 ± 4.5%) and in 60 mg/kg group
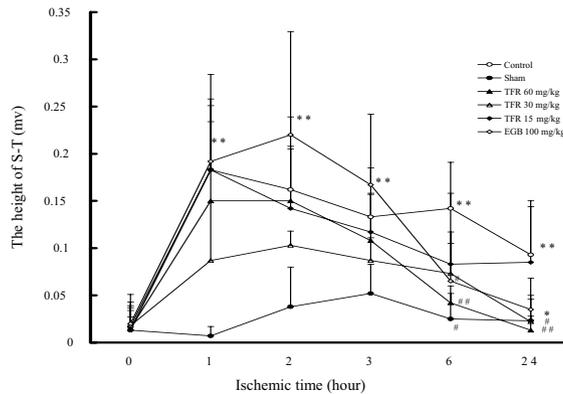
Figure 1. Effects of TFR on the alteration of S-T segment of ECG (Mean ± SD, n = 6). Standard lead II of ECG was recorded before and at different time points after the occlusion of LAD. $^*p < 0.05$, $^{**}p < 0.01$ compared to the sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the control.

Table 1. Effects of TFR on the Ischemic Size and Infarct Size in Rabbits

Group	Dose mg/kg	The Size at Risk %	The Infarction Size %
Control		40.7 ± 3.7	36.8 ± 3.6
TFR	60	32.4 ± 5.4*	28.7 ± 5.8*
	30	33.4 ± 4.5*	30.4 ± 6.1
	15	36.7 ± 1.1	34.2 ± 7.0
EGB	100	33.4 ± 3.6*	29.9 ± 6.6*

mean ± SD, n = 6. Rabbits were given TFR or EGB at the indicated dose daily. At day 7, myocardial ischemic injury was induced by occluding LAD for 24 hours. The ischemic size and the infarction size were determined by Even's Blue and N-BT double staining. $^*p < 0.05$ compared to the control.

(32.4 ± 5.4%) compared with the control group (40.7 ± 3.7%, p < 0.05). TFR (60 mg/kg) markedly decreased the infarction size (28.7 ± 5.8%) compared to the control rabbits (36.8 ± 3.6%, p < 0.05) also. Treatment with 100 mg/kg EGB had a similar effect in reducing ischemic size and infarction size (Table 1).

Effect of TFR on plasma CK Activity

Elevation of plasma CK activity was detected as early as 1 hour after the occluding of LAD and gradually increased with time. Plasma CK activity reached maximum level at 24 hours of LAD occlusion. The elevation of plasma CK activity was significantly lower in TFR (30, 60 mg/kg) or EGB (100 mg/kg) treated rabbits than in the control group (Fig. 2).
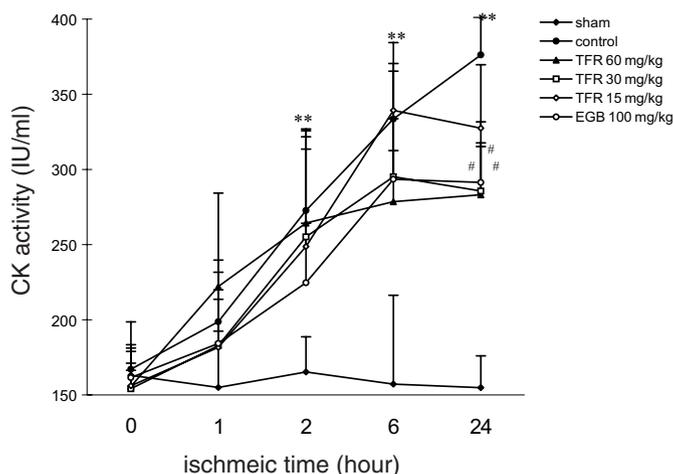
Figure 2. Effects of TFR on plasma CK activity of LAD-occluded rabbits (Mean ± SD, n = 6). Blood samples were collected before and at different time points after the occlusion of LAD. [**]$p < 0.01$, compared to the sham; [#]$p < 0.05$, compared to the control.

Effect of TFR on the Plasma Contents of ET and NO

There was a significant increase of plasma ET level at 24 hours of ischemia in the control group. The increase of ET-1 level in TFR treated (30, 60 mg/kg) rabbits was smaller (Fig. 3). A decrease of plasma NO content was observed at 3 hours of ischemia. Treatment with TFR (30, 60 mg/kg) inhibited the decrease of plasma NO content (Fig. 4). Similar to TFR groups, EGB (100 mg/kg) treated rabbits had lower plasma ET-1 and higher NO contents compared to the control group.

Discussion

In the present study, we observed a time dependent increase in the myocardial injury indexes (ECG S-T segment elevation, plasma CK activity, ischemic size and infarction size) in rabbits. Treatment with TFR (30 or 60 mg/kg) or EGB (100 mg/kg) significantly lowered the indexes. We also observed that plasma ET-1 level was increased and NO level was decreased in rabbits with myocardial ischemia. The myocardial ischemia induced changes of plasma ET-1 and NO levels were significantly obvious in TFR or EGB treated rabbits compared to the control group.

CK is a very important metabolic enzyme in myocardiocytes (Hillis, 1977) and is released into the blood stream by injured myocardiocytes. Thus, plasma CK level is a reliable index with which to evaluate myocardial ischemic injury. In this study, myocardial ischemic injury was detected as early as 1 hour after LAD occlusion and became more severe with time, as indicated by the increase in plasma CK activity and the elevation of ECG S-T segment, another myocardial injury index. The administration of TFR markedly
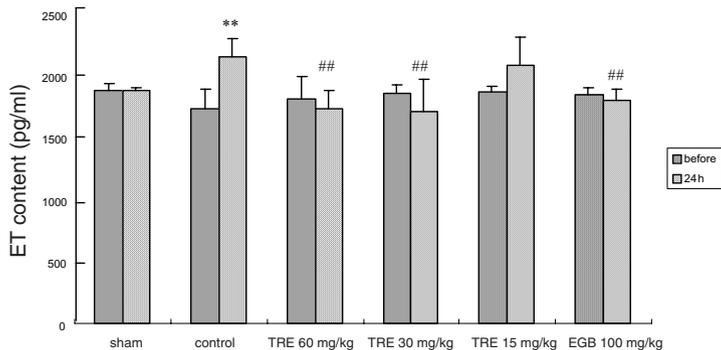
Figure 3. Effects of TFR on plasma ET-1 levels in LAD-occluded rabbits (Mean ± SD, n = 6). Blood samples were collected before and 24 hours after the occlusion of LAD. **$p < 0.01$ compared to the sham; ##$p < 0.01$, compared to the control.
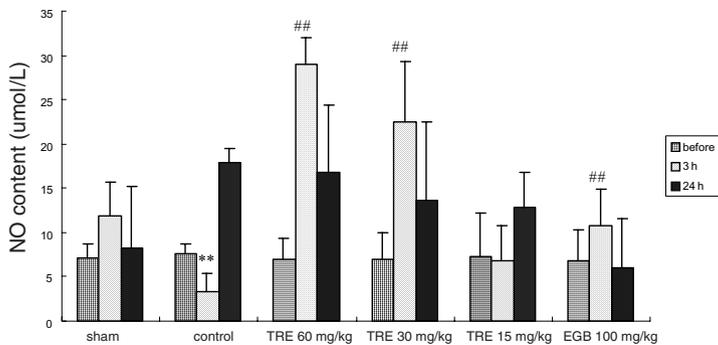

Figure 4. Effect of TFR on plasma NO content in LAD-occluded rabbits (Mean ± SD, n = 6). Blood samples were collected before and 24 hours after the occlusion of LAD. **$p < 0.01$, compared to the sham; ##$p < 0.01$ compared to the control.

improved the injury indexes and reduced the myocardial ischemic size and the infarction size indicating a protective effect of TFR against LAD occlusion induced myocardial injury.

Several mechanisms might be involved in myocardial ischemia injury. Ischemia induced damages occurred not only directly in myocardiocytes but also in vascular endothelial cells, the latter may have an indirect detrimental effect on myocardiocytes. Therefore, the protection of vascular endothelial function may have a beneficial effect on myocardial ischemia. One of the mechanisms involved in cardioprotective effects of TFR may stem from its ability to protect endothelium cells. Endothelium plays an important role in modulating vascular tone by releasing either endothelium-derived constricting factors (EDCFs) or endothelium-derived relaxing factors (EDRFs). Endothelin-1 is one

of the most important EDCFs. Endothelin-1 has both vasoconstrictor and mitogenic properties, and is believed to play an important role in the pathogenesis of acute coronary syndromes and coronary atherosclerosis (Jakob *et al*., 1999; Kruger *et al*., 1999; Pernow *et al*., 2004). When exposed to conditions like oxidative stress, endothelial cells produce a great quantity of oxygen free radicals which results in cellular membrane damages to endothelial cells and thus leads to endothelin-1 release. More reports (Cannon, 1998) have shown that endothelin-1 continuously, strongly constricts the coronary artery and even stops the circulating of blood. Thereby, the increase in plasma endothelin-1 seen in untreated rabbits may contribute to ischemia induced myocardial injury.

NO (Gonon *et al*., 2004), one of the important EDRFs, has a wide range of biological functions including modulation of vascular tone, regulation of local cell growth, and protection of the vessel from injurious consequences of platelets and cells circulating in blood. NO is produced in endothelial cells while a constitutive NO synthase (NOS) converts L-arginine to citrulline (Gonon *et al*., 2004). A growing list of conditions, many of them risk factors for myocardial infarction, are associated with diminished release of NO into the arterial wall either because of impaired synthesis or excessive oxidative degradation. Diminished NO bioactivity may cause constriction of coronary arteries during exercise or during mental stress and contribute to provocation of myocardial ischemia in patients with coronary artery disease. Additionally, diminished NO bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, the precursor of the atherosclerotic plaque. Numerous therapies have been investigated to assess the possibility of reversing endothelial dysfunction by enhancing the release of NO from the endothelium, either through stimulation of NO synthesis or by protection of NO from oxidative inactivation and conversion to toxic molecules such as peroxynitrite.

The normal coronary vascular endothelium also releases NO. It has been shown (Schmetterer *et al*., 1997) that myocardium ischemia inhibits NOS expression at the level of transcription and translation and thus the overall production. Flavones have been shown to increase endothelial NOS activity and NO production (Delaflotte *et al*., 1984; Harris *et al*., 1997). In this study, we observed a decrease in plasma NO content after occlusion of LAD in untreated rabbits, while TFR treatment for 7 days before the occlusion of LAD reduced the drop in plasma NO content. Our results suggest that TFR inhibits ET release and promote NO release during ischemia. Because NO can be released from both myocardiocytes and endothelial cells, the precise mechanism of TFR on NO release is not clarified in the present study. However, another study we conducted showed that TFR induced relaxation of the rat aortic ring *in vitro* was markedly inhibited by the NOS inhibitor N-monomethyl-L-arginine methylester (L-NAME) (data not shown). This suggest that the stimulation of NO release from the endothelial cells may be, at least partially, involved in the protective mechanisms of TFR against myocardial ischemic injury.

In summary, the present study was the first to show that TFR has a protective effect against myocardial ischemic injury in rabbits. TFR treatment inhibited ET-1 release and stimulated NO release during ischemia, which might be indicative of the mechanism whereby TFR exerts its protective effect.

Acknowledgment

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