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Original Article

Ellagic acid promotes ventricular remodeling after myocardial infarction through inhibiting inflammatory response

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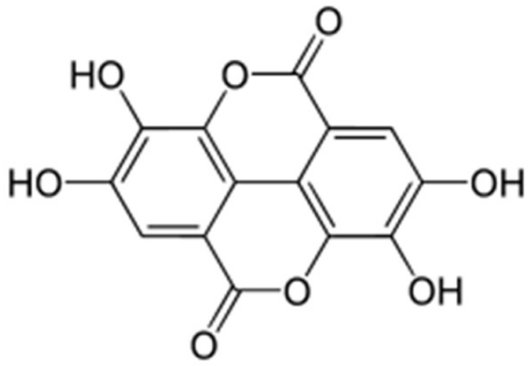
Abstract: *Objective:* Ellagic acid has anti-proliferative and antioxidant properties, which can treat some heart diseases, including myocardial infarction. The purpose of this study is to explore the effects of ellagic acid on ventricular remodeling after myocardial infarction. *Methods*: Rat myocardial infarction was established, and treated with different concentration of ellagic acid, histological examination was performed by H&E staining and cardiac function assessment was evaluated. In addition, the inflammatory responses were performed by immunofluorescence staining and RT-PCR. Finally, the possible mechanism was explored by Western blot. *Results:* In present study, we primarily found that ellagic acid was likely to alleviate the severity of myocardial infarction and improve the cardiac function in rats. The follow-up experiments demonstrated that ellagic acid potently decreased the infiltration of dendritic cell and reduced the inflammatory cytokines secretion including TNF-α, IL-1β and TGF-β1. During the anti-inflammatory process, exogenous ellagic acid could markedly down-regulated the increased the expression of TLR4 and the phosphorylation level of p65, IκB and ERK1/2 in rat with myocardial infarction, revealing that TLR4/ERK/NF-κB signaling pathway may be the vital downstream targets of ellagic acid. *Conclusion:* These findings show that ellagic acid targets TLR4/ERK/NF-κB signaling pathway, which may have potential clinical value for modulation myocardial infarction.

Keywords: Myocardial infarction, ellagic acid, anti-inflammation, TLR4, NF-κB

Introduction

Myocardial infarction (MI), commonly known as a heart attack, occurs when blood flow stops to a part of the heart, causing damage to the heart muscle [1]. The World Health Organization indicates that 12.2% of worldwide deaths were from ischemic heart disease [2]. The most common symptom is chest pain or discomfort which may travel into the shoulder, arm, back, neck, or jaw. Often it is in the center or left side of the chest and lasts for more than a few minutes. About 30% of people have atypical symptoms [3], with women more likely than men to present atypically [4]. Among those over 75 years old, about 5% have had an MI with little or no history of symptoms [5]. An MI may cause heart failure, an irregular heartbeat, or cardiac arrest. It already causes a serious threat to the safe of people's life and health, and burdens the health system of governments. Thus an effective therapeutic drug is under urgent needed.

Myocardial infarction is associated with an inflammatory reaction [6-8]. The previous studies indicated that inflammation can extend myocardial injury came as the result of implementing anti-inflammatory strategies in animal models of myocardial ischemia and reperfusion. The systemic administration of corticosteroids was shown to decrease infarct size in a canine model of experimental myocardial infarction [9]. Subsequent investigations suggested that corticosteroids inhibit the inflammatory process decreasing the number of infiltrating leukocytes [10]. It indicates that in the early stage of MI, reasonable reducing the inflammation is good for reducing the infarct size.



Schematic 1. The chemical structure of ellagic acid.

Ellagic acid (**Schematic 1**), also known as gallogen, is a natural phenol antioxidant found in numerous fruits and vegetables. The antiproliferative and antioxidant properties of ellagic acid have prompted research into its potential health benefits against cancer, heart disease, and other medical problems [11, 12]. In this study, we used ellagic acid to treat the rats with myocardial infarction, then to investigate the myocardial protection effects of ellagic acid, and the relationship between ellagic acid and inflammation.

Materials and methods

Materials

Ellagic acid, protein block solution were ordered from Sigma-Aldrich (MO, USA), Nylon suture was purchased from Kangning Industrial Co., Ltd. (Anhui, China). Pentobarbital sodium, paraformaldehyde, dimethylbenzene and paraffin were obtained from Aladdin (Shanghai, China). TRIzol solution was ordered from Invitrogen Life Technologies. The primers of TNF-α, IL-1β, TGF-β1 were obtained from Funengbio Co. (Shanghai, China). Nitrocellulose membrane and enhanced chemiluminescence kit were obtained from Millipore (Billerica, MA, USA). Primary antibodies: CD11c, p-IκB-α, total-IκB-α, TLR4 were purchased from ThermoFisher Scientific; p-P65, total-P65, p-ERK1/2, total-ERK1/2 were obtained from Santa Cruz; GAPDH was obtained from Sigma-Aldrich.

Animals

Seventy-five 2-month-aged male Sprague & Dawley rats (220-260 g) were purchased from the Laboratory Animal Center of Shanghai Chinese Academy of Sciences. These rats were divided into 5 groups, the control group with sham operation, the MI group with myocardial infarction treatment, MI rats treated with 10 mg/kg/day ellagic acid (EA-L group), MI rats treated with 50 mg/kg/day ellagic acid (EA-M group), MI rats treated with 100 mg/kg/day ellagic acid (EA-H group). After receiving one treatment, each mouse was individually housed and fed ad libitum.

Myocardial infarction model

Thoracotomy and heart exposition were performed as reference described [13, 14]. Once the heart was exposed, a 7-0 nylon suture was used to ligate left anterior descending artery (LAD) at a site about 2-3 mm from LAD origin. The sham group underwent the same thoracotomy procedure except for the LAD ligation.

Cardiac function assessment

Echocardiography was performed for the measurement of cardiac dimensions and function. After operation, these rats received an intraperitoneal injection of 0.01 mL 1% pentobarbital sodium per gram of body weight and two-dimensional long axis images were record from the left caudal (apical) view, using a Philips CX30 ultrasound system couple with a L15 high-frequency probe. Two-dimensional guided M-mode images at chordae tendineae level were evaluated. M-mode measurements of left ventricle end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) were performed by using the leading-edge method of the American Society of Echocardiograph. For estimation of each parameter, the average of three measurements from three different cycles in an image was obtained. Left ventricular end-diastolic and systolic volumes (LVEDV and LVESV, respectively) were calculated by the biplane method of disks (modified Simpson's rule) [15]. Ejection fraction (EF) was determined by using (LVEDV-LVESV/LVEDV) × 100%, and fractional shortening (FS) was calculated from the M-mode echocardiography images as (LVEDD-LVESD/LVEDD) × 100%.

TTC staining

For TTC staining, rats were euthanized by injecting overdose pentobarbital sodium. Then hearts were harvested 24 hours after MI and

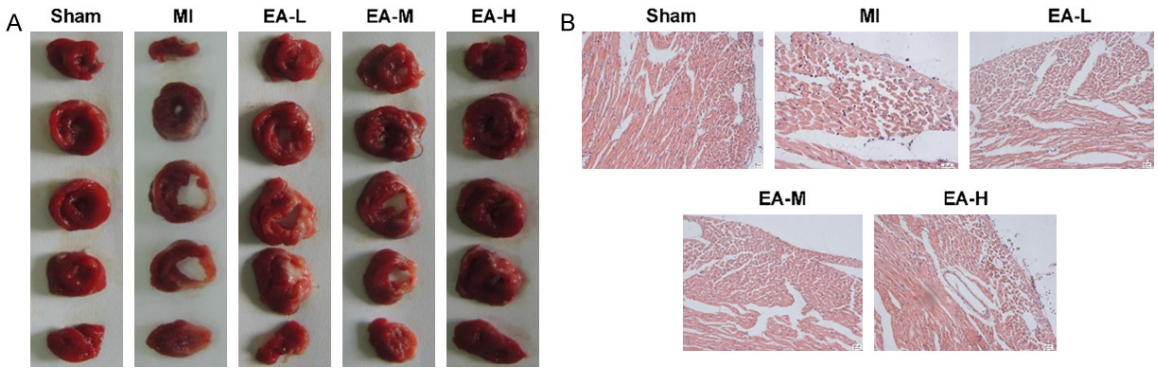


Figure 1. Ellagic acid relieves the myocardial infarction. A: Effect of ellagic acid on infarct size of rat with myocardial infarction. B: Light microscopy photomicrographs depicting sections from myocardium of rat with operation.

sectioned into five 1.2 mm-thick slices that were perpendicular to the long axis of the heart. The slices were then incubated in 1% freshly-made TTC solution at 37°C for 15 min and then digitally photographed.

H&E staining

The hearts were fixed in 4% paraformaldehyde, dehydrated with graded ethanol, cleared in dimethylbenzene and embedded in paraffin. Paraffin sections (5 μm) were prepared using a Leica histotome (Leica Microsystems, Wetzlar, Germany) and deparaffinized with immersion in dimethylbenzene prior to rehydration. HE staining was subsequently performed according to standard procedures.

Immunofluorescent assay

For immunofluorescence staining, mouse heart cross sections were fixed with 4% PFA, blocked/permeabilized with Protein Block Solution containing 1% saponin, and then stained with anti-rat mononuclear phagocyte CD11c antibodies. FITC secondary antibodies were obtained from Abcam and used in conjunction with these primary antibodies.

RT-PCR

Total RNA was extracted from infarcted heart tissue using TRIzol and its concentration was determined. Total RNA (0.5 μg) was used as a template to prepare cDNA. The mRNA expression of target genes was quantified using SYBR Premix EX Taq on the ABI 7500 sequence detection system (Advanced Biosystem, Thermo Fisher Scientific, Waltham, MA, USA). PCR was performed with the following thermocycling conditions: An initial 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The thermocycler used in the present study was the StepOnePlus™ Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers were obtained from Funengbio Co. (Shanghai, China), and the sequences were as follows: TNF-α, forward 5'-caagagcccttgccctaa-3', reverse 5'-cagagcaagactccaaagta-3'; IL-1β, forward 5' atggcaactgtccctgaactcaact-3' and reverse 5'-atggcaactgtccctgaactcaact-3'; TGF-β1, forward 5'-gcctccgcatcccacctttg-3' and reverse 5'-gcgggtgacttctttggcgt-3'. Housekeeping gene β-actin was used as an internal reference to normalize the results. All experiments were performed in triplicate.

Western blotting

Western blotting was performed according to standard protocols. Briefly, the total protein from infarcted heart tissue was extracted using radioimmunoprecipitation lysis buffer containing 1 mM phenyl methane sulfonyl fluoride and the protein concentration was determined using the Bradford method (Beyotime Institute of Biotechnology, Nantong, China) according to the manufacturer's instructions. 30 μg total protein sample was separated by 10% SDS-PAGE and transferred into a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline containing Tween 20 (PBST) with 5% non-fat milk for 1 h at 4°C. The membrane was then incubated with the following primary antibodies for 12 h at 4°C, including p-P65, total-P65, p-IκB-α, total-IκB-α, p-ERK1/2, total-ERK1/2, TLR4 and GAPDH, and followed by three washes with PBST and

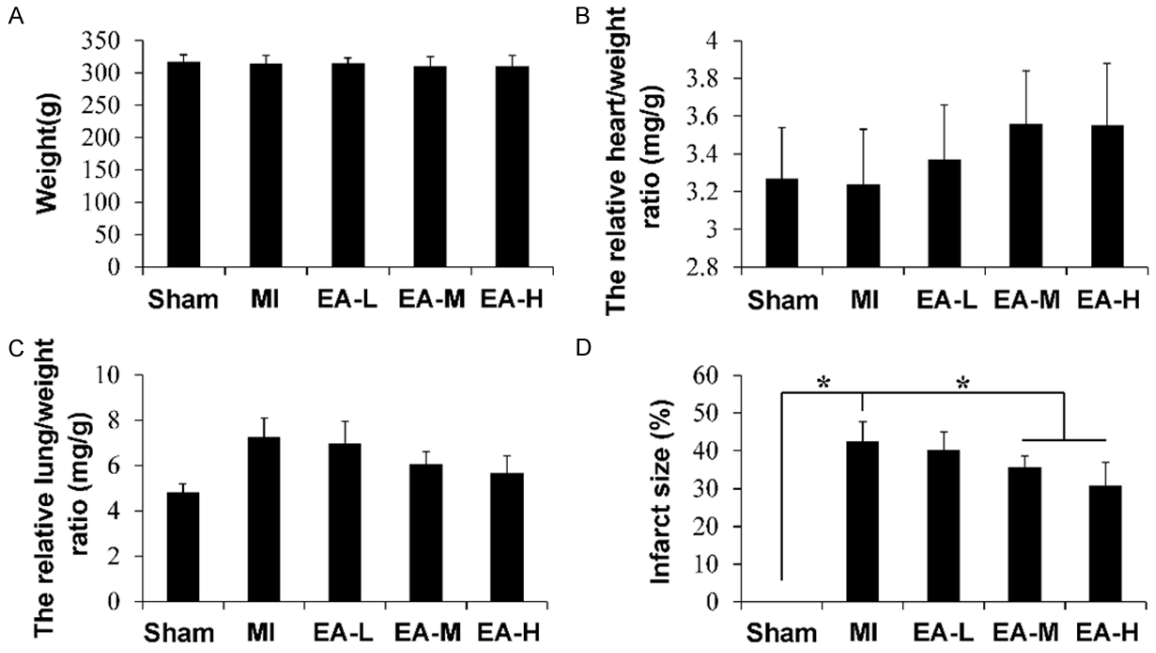


Figure 2. The body weight (A), relative heart/weight ratio (B), and relative Lund/weight ratio (C) were not differentiated in each groups, but infarct size (D) was reduced by the administrations of exogenous ellagic acid. Error bars ± SD, *P < 0.05.

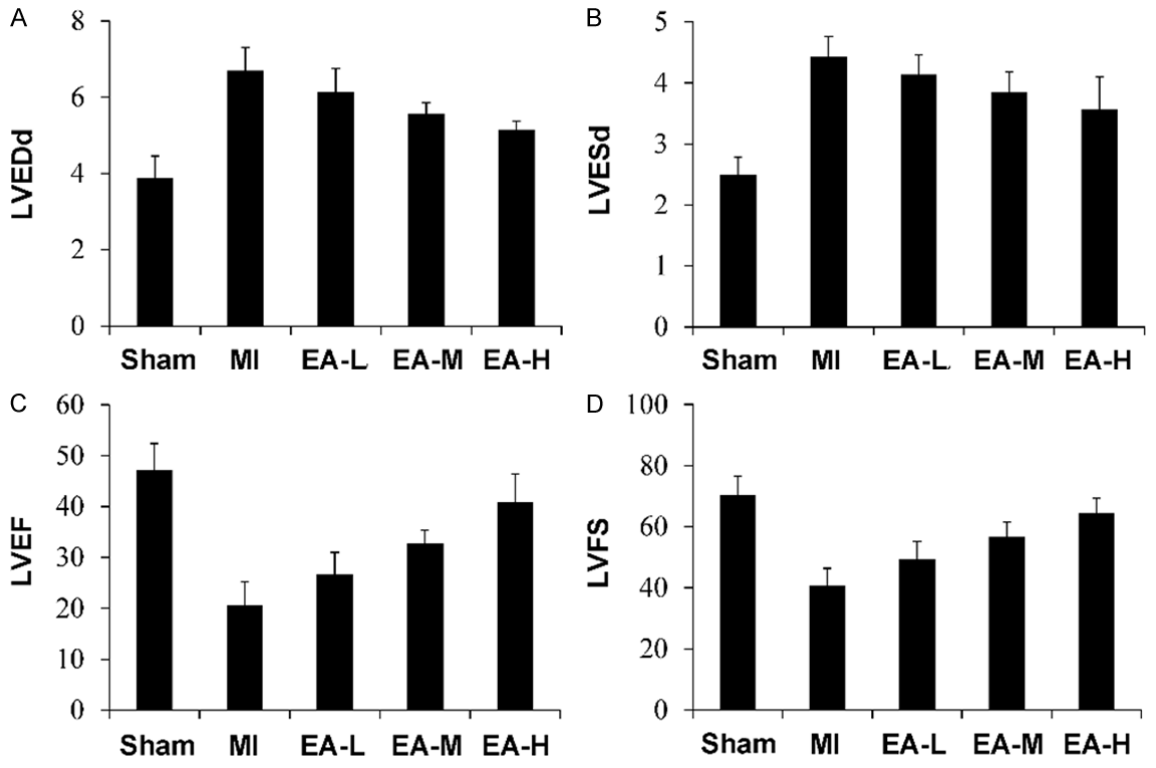


Figure 3. LVEDD (A), LVESD (B), LVEF (C), and LVFS (D) were measured in rat with myocardial infarction in absence and presence of ellagic acid. Error bars ± SD.

incubation for 30 min at 4°C with secondary antibody labeled with horseradish peroxidase. Finally, the membrane was washed three times with PBST and the protein bands were visual-

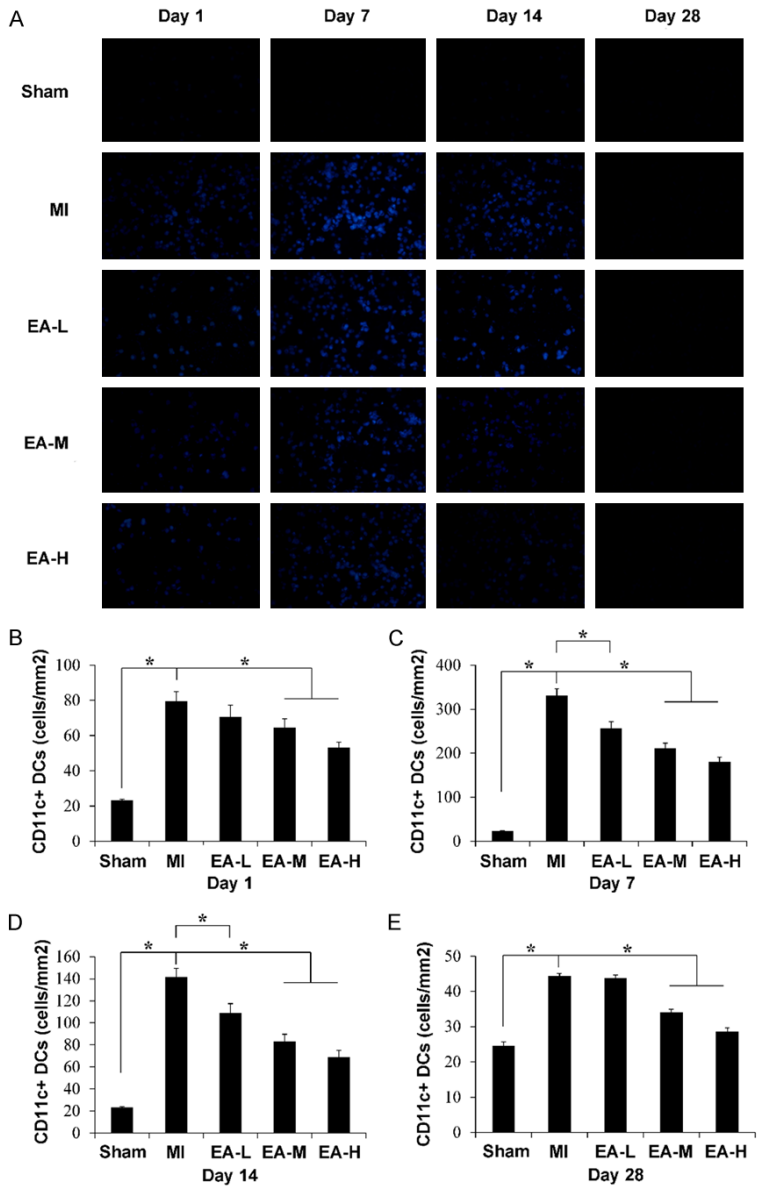


Figure 4. (A) Immunofluorescence detection of CD11c in rat with myocardial infarction in absence and presence of ellagic acid at different time point. Percentage of CD11c+ DC from (A) were presented as mean ± SD at Day 1 (B), Day7 (C), Day 14 (D) and Day 28 (E). *P < 0.05.

ized with Super Signal. Antibody binding was detected using an enhanced chemiluminescence kit (Millipore). The blots were then incubated in a commercial stripping solution (Pierce Biotechnology, Inc. Rockford, IL, USA) for 10 min.

Statistics analysis

Results are presented as mean ± SD and statistical analysis was performed using SPSS 19.0 software. Comparisons among groups were performed with one-way ANOVA. Differences were considered statistically significant when P < 0.05.

Results

Ellagic acid reverses the myocardial infarction

We found that the infarct size of MI group was significantly smaller than Sham group (P < 0.05). However, the infarct size was gradually reduced when rats treated with increased concentration of ellagic acid (**Figure 1A**). The infarct size in EA-M and EA-H group was obviously smaller than MI group (P < 0.05, **Figure 2D**). Furthermore, the histology observations revealed that the tissue structure of Sham group was normal, while the tissue structure of MI group had already broken seriously. Interestingly, this kind of broken situation was a little better in EA-L group, and more better in EA-M and EA-H group (**Figure 1B**).

Cardiac function assessment

Each groups had no significant difference in body weight, the relative heart/weight ratio and the relative lung/weight ratio (**Figure 2A-C**). Next we examined the cardiac function in rats with myocardial infarction in absence or presence of ellagic acid. As shown in **Figure 3**, LVEDD, LVESD, LVEF and LVFS had differentially altered among each groups. In detail, after myocardial infarction, LV dilatation was evident both in untreated and ellagic acid-treated rat; however, rat stimulated with ellagic acid showed less ventricular dilatation. LVEDD and LVESD were smaller, and LVEF and LVFS were higher in ellagic acid-treated rat than in untreated rat after operation.

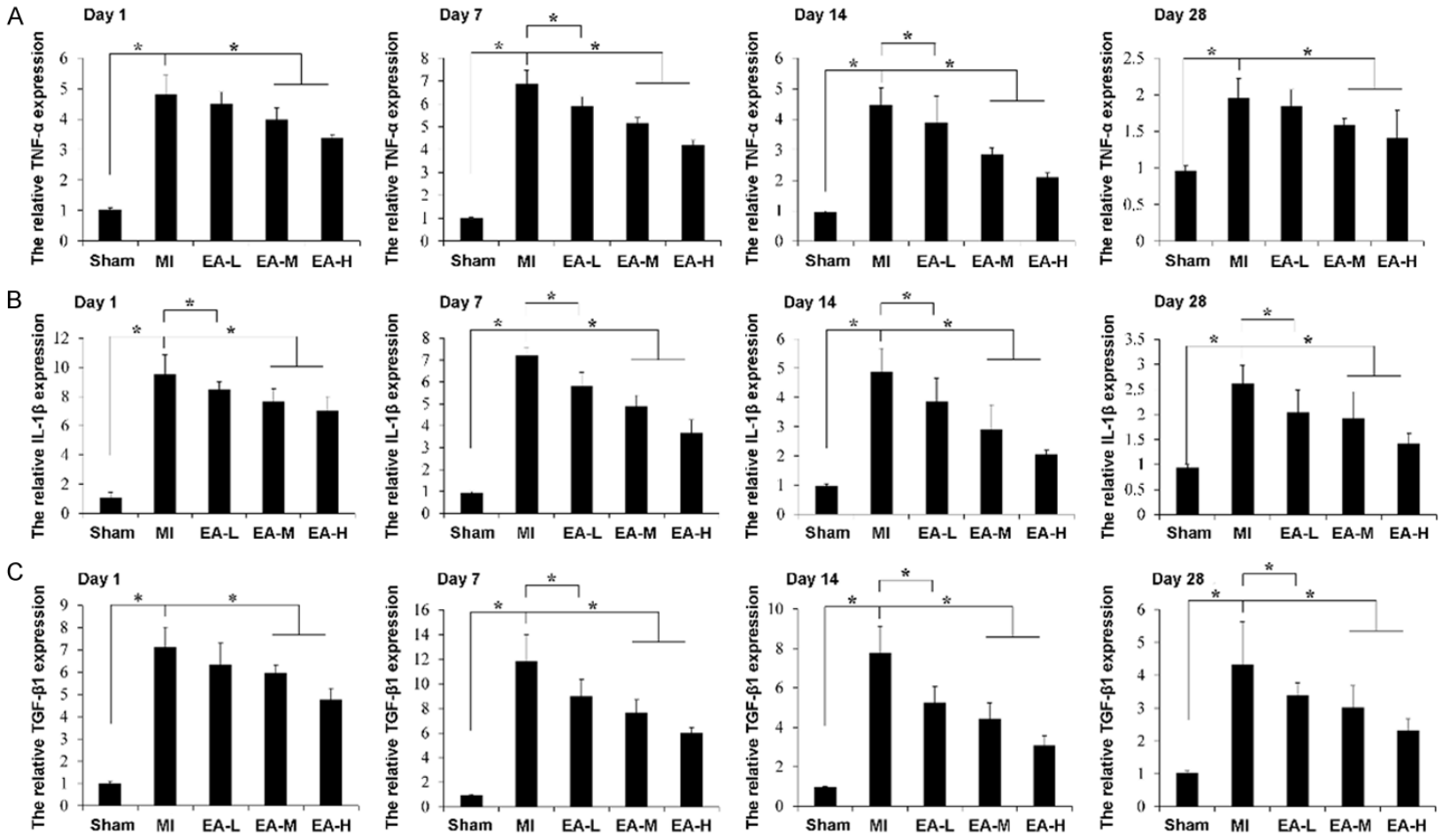


Figure 5. The expression analysis of TNF-α (A), IL-1β (B), TGF-β1 (C) in mRNA level in rats with myocardial infarction in absence and presence of ellagic acid at Day 1, Day 7, Day 14 and Day 28 by real time PCR. Data were presented as mean ± SD, *P < 0.05.

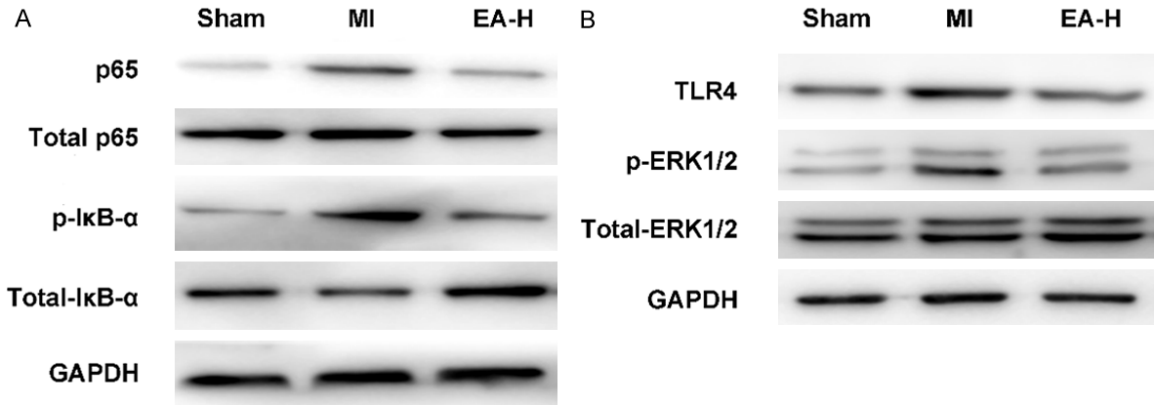


Figure 6. Ellagic acid regulated the expressions of TLR4, and the phosphorylation of p65, IκB, ERK1/2 in rat with myocardial infarction. A: The phosphorylation level of p65 and IκB were activated by myocardial infarction but inhibited by the additional administration of ellagic acid. B: The expression of TLR4 and phosphorylation level of ERK1/2 were enhanced by myocardial infarction but inhibited by the additional administration of ellagic acid.

Ellagic acid reduces the infiltration of dendritic cells

As shown in **Figure 4A**, the expression of CD11c positive cells (dendritic cells) was increased from day 1, and reached a maximum value on day 7, then decreased from day 7 to day 28 in all groups. As shown in **Figure 4B**, on day 1, the expression of CD11c positive cells in MI group was dramatically higher than Sham group ($P < 0.05$), while the expression of CD11c positive cells in EA-M and EA-H groups was lower than MI group ($P < 0.05$). Similarly, the expression of CD11c positive cells in EA-L, EA-M and EA-H groups was lower than MI group ($P < 0.05$) on both day 7 and day 14 (**Figure 4C** and **4D**). And the expression of CD11c positive cells in EA-M and EA-H groups was lower than MI group ($P < 0.05$) on day 28 (**Figure 4E**).

Ellagic acid decreases the inflammatory cytokines secretion

We also explored the expression of inflammatory cytokines, including TNF-α, IL-1β, TGF-β1 in each group after operation. As shown in **Figure 5**, the expression of TNF-α, IL-1β, TGF-β1 sharply increased in MI group when compared with Sham group ($P < 0.05$) at each indicated time point. After these infarcted rats received ellagic acid treatments, the expression of TNF-α, IL-1β, TGF-β1 was reduced, which suggested that ellagic acid was able to inhibit the inflammatory response after myocardial infarction. Moreover, this inhibitory effects could be enhanced with an increased concentration of ellagic acid.

Ellagic acid reduces the inflammation through inhibiting TLR4/ERK/NF-κB signaling pathway

Finally, we further explored the possible mechanism of ellagic acid suppressing the inflammation. As shown in **Figure 6A**, the expression of p65 was significantly increased in MI group when compared with sham group, while the expression of p65 was decreased when the rats treated with high dose of ellagic acid. In addition, the expression of IκB-α was also reduced in EA-H group. Thus ellagic acid prohibited NF-κB signaling mainly through inhibiting IκB-α expression. Moreover, we also explored MAPK signaling expression, the up-stream signaling of NF-κB. As shown in **Figure 6B**, the expression of ERK1/2 was obviously enhanced in MI group when compared with Sham group; however, it decreased after treated with ellagic acid. Similarly, the expression of TLR4 was significantly enhanced in MI group when compared with Sham group; however, the expression of TLR4 was reduced in EA-H group when compared with MI group.

Discussion

Myocardial infarction induces ventricular remodeling, which leads to cardiac dysfunction and mortality [16, 17]. Previous study has demonstrated that inflammatory response is part of the endogenous pathological mechanism during myocardial infarction [6-8]. Thus the rational therapeutic intervention in myocardial infarction may be anti-inflammation. Our studies demonstrated the crucial role of ellagic acid

in myocardial infarction. Furthermore, we found that the effect of ellagic acid on myocardial infarction is related to its anti-inflammatory action because ellagic acid promotes the resolution of inflammation. In addition, we also introduced the potentially mechanistic link between ellagic acid and its anti-inflammatory function.

The core finding in our study was that ellagic acid alleviates the myocardial infarction through decreasing the infiltration of dendritic cell, an important inflammatory cell of the mammalian immune system, and reducing the inflammatory cytokines secretion. This is a novel discovery, because previous studies did not reveal how ellagic acid mediates the inflammatory response of myocardial infarction. In current, we further showed that the expression of TLR4 and of phosphorylation level of NF-κB, IκB and ERK1/2 were overall down-regulated by the exogenous ellagic acid in rat with myocardial infarction, thereby providing the plausible anti-inflammatory mechanism associated with TLR4/ERK/NF-κB signaling pathway. The TLR4/ERK/NF-κB signaling pathway regulated in myocardial infarction has been extensively documented in the literature [18-23]. However, the ability of ellagic acid to suppress the expression of these inflammation-related proteins and in turn relieves the myocardial infarction in rat was previously unrecognized.

TLR4 is a key member of the Toll-like receptors (TLRs) family that may be activated by lipopolysaccharide and nonbacterial agonists such as fatty acids [24-26], and is localized on the cell surface to mediate the transmembrane signaling transduction [27]. TLR4 triggers activation of MAPK/NF-κB signaling pathways to induce the release of cytokines and inflammatory mediators [28]. There are three parallel MAPK signaling pathways in mammalian cells, the P38 signaling pathway, the extracellular signal-regulated kinase (ERK) signaling pathway and the c-Jun N-terminal kinase (JNK) signaling pathway [29]. NF-κB, a common transcription factor, regulates various genes encoding inflammatory mediators and acts an important downstream target of MAPK signaling pathways in inflammatory and immune responses [30, 31]. In myocardial infarction models, NF-κB activation has been associated with the remodeling and dysfunction of the myocardium [22, 23]. In this study, exogenous ellagic acid could significantly decline the increased expression of TLR4 and the phosphorylation level of p65, IκB and ERK1/2 in rat with myocardial infarction, indicating that TLR4/ERK/NF-κB signaling pathway may be the downstream targets of ellagic acid. Certainly, the molecular mechanisms involved in ellagic acid-regulated NF-κB, IκB and ERK1/2 need to be further elucidated in future.

In summary, we provided the strong evidence that ellagic acid can limit the severity of myocardial infarction through the resolution of inflammation by influencing the expression of TLR4 and the phosphorylation level of p65, IκB and ERK1/2. Our study provides the new clues and valuable information towards the development of a complete understanding of the pathogenesis and development of the myocardial infarction. This raises the possibility that ellagic acid and targeting TLR4/ERK/NF-κB signaling pathway may have potential therapeutic value for patients with myocardial infarction.

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Disclosure of conflict of interest

None.

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