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Original Article

Cardioprotective effects of gallic acid in a rat ischemia-reperfusion model: role of apoptosis, inflammation, and antioxidant defense

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Abstract

Background and purpose: Heart disease is a major global health problem. Gallic acid (GA) possesses cardioprotective properties. This study aimed to evaluate the therapeutic effects of GA pretreatment in ischemia-reperfusion (I/R) and elucidate its underlying mechanisms.
Experimental approach: Forty adult male Wistar rats were subjected to this experiment. GA was given to the rats through gavage at doses of 15 and 30 mg/kg/day, 10 days before the induction of ischemia. To induce I/R, the left anterior descending coronary artery was occluded for 30 min, and reperfusion continued for 24 h. Malondialdehyde (MDA) levels, antioxidant enzyme activity, and inflammatory cytokines were assessed using kits. Myocardial injury markers were analyzed by ELISA, and infarct size was assessed through 2,3,5-triphenyltetrazolium chloride staining. Real-time polymerase chain reaction was utilized to quantify the relative gene expression of *Bax* and *Bcl-2*.
Findings/Results: The findings indicated that pretreatment with GA led to significant improvement in inflammatory cytokines, antioxidant enzyme activity, and a decrease in MDA levels. GA also decreased infarct size and myocardial injury markers significantly. Moreover, pretreatment with GA revealed a significant increase in the expression of the *Bcl-2* gene, while the expression of the *Bax* gene decreased.
Conclusion and implications: Inclusively, the results suggested that GA may hold significant potential as a therapeutic agent for reducing myocardial injury in the context of I/R, with 30 mg/kg/day proving more effective than 15 mg/kg/day, offering a promising path for further investigation.

Keywords: Apoptosis; Gallic acid; Inflammation; Ischemia-reperfusion; Myocardial infarction; Oxidative stress.

INTRODUCTION

Myocardial disease is regarded as one of the top causes of death globally. Myocardial ischemia happens when the blood supply to the heart tissue is inadequate or restricted (1). Although reperfusion enhances heart function and reduces the infarct size, it later leads to an increase in reactive oxygen species (ROS). This results in the peroxidation of membrane lipid and production of cytotoxic products like malondialdehyde (MDA) due to the interaction of membrane lipids with ROS.

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The membranes are thereby damaged; myocardial cells are injured, leading to heart failure and irrecoverable tissue damage. These changes have been associated with reduced levels of endogenous antioxidant compounds, including glutathione system (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) (2). On the other hand, the inflammatory responses associated with ischemia-reperfusion (I/R) are one of the vital features of myocardial I/R injury (3). The inflammatory response is characterized by the release of various cytokines, including tumor necrosis factor (TNF-α) and interleukins (such as IL-1β) (4). Previous studies have suggested that timely inhibition of TNF-α following I/R may improve hemodynamic parameters, prevent adverse cardiac remodeling, and protect cardiac function (5,6). In addition, apoptosis of cardiomyocytes is implicated in ischemic and non-ischemic heart diseases and myocardial infarction (MI). The *Bcl-2* gene family was the first gene family recognized to regulate apoptosis. This includes a variety of structurally related molecules, such as the pro-apoptotic gene, *Bax*, and the anti-apoptotic gene, *Bcl-2*, both of which are integral to the cell death pathway (7). Additionally, elevated levels of cardiac enzymes, particularly cardiac troponin-I (cTn-I), lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), and creatine phosphokinase (CPK), signify injury and necrosis of myocardial cells (8).

Gallic acid (GA), or 3,4,5-trihydroxybenzoic acid, is a natural low molecular weight secondary metabolite found widely in plants. It is utilized in medicine for its antioxidant and anti-inflammatory properties. Furthermore, GA protects against cardiac injury by enhancing the capacity of the endogenous antioxidant in an isolated heart animal model of ischemia/reperfusion injury (9). Likewise, previous studies have shown possible anti-oxidative and cardioprotective effects of GA (10,11). Mechanistically, GA has been shown to modulate signaling pathways associated with oxidative stress and inflammation, including the inhibition of nuclear factor kappa B (NF-κB) activation and downregulation of pro-inflammatory cytokines (12). Moreover, GA may modulate apoptosis by regulating the expression of *Bcl-2* family proteins and preserving mitochondrial integrity (13,14). Due to these multifaceted protective effects, GA is considered a promising therapeutic agent in cardiovascular research.

Therefore, the present study aimed to comprehensively evaluate the cardioprotective effects of GA as a pre-treatment strategy in a rat model of myocardial I/R injury. Specifically, we investigated its impact on oxidative stress markers, inflammatory cytokines, apoptotic gene expression, cardiac injury biomarkers, and histopathological outcomes to elucidate the potential mechanisms underlying its protective actions.

MATERIALS AND METHODS

Drugs and chemicals

The ELISA kits were acquired from Zellbio (Germany). Sigma Co. (St. Louis, USA) supplied the GA and 2,3,5-triphenyltetrazolium chloride (TTC) used in this study.

Study design

The research used 40 adult male Wistar rats, with weights ranging between 250-300 g, provided from the animal house of Lorestan University of Medical Sciences. One week before starting the experiment, the rats were housed under temperature-controlled (22 ± 2 °C) conditions with constant access to water and food in a 12-h dark/light cycle. This preparation period provided optimal acclimatization. The experimental protocols received the necessary approvals from the Research Ethics Committee of Lorestan University of Medical Sciences (ID: IR.LUMS. REC.1403.296, dated: 2024-11-19) as well as the laboratory animal house and Cardiovascular Research Center (Khorramabad, Iran). The animals were randomly allocated into 4 groups (n = 10) as follows: 1. sham-operated group as negative control (sham), 2. I/R group treated with a vehicle as positive control (I/R), 3. group receiving GA at the dose of 30 mg/kg/day for 10 days followed by I/R (G1), 4. group receiving GA at the dose of 15 mg/kg/day for 10 days followed by I/R (G2). This allocation ensured balance and randomness of the experimental design. GA dissolved in 0.9% saline solution as a vehicle was administered at 1 mL of volume per animal using a gavage tube (9).

Surgical procedure

To anesthetize the rats, xylazine (5 mg/kg, Alfasan Co, Holland) and ketamine (60 mg/kg, Alfasan Co, Holland) were administered intraperitoneally (15). Ischemia was induced by ligating the left anterior descending (LAD) artery according to a protocol described in a previous study (15). Briefly, a left thoracic incision was made to open the chest and expose the heart. To set off MI, the left LAD was identified, and a 6-0 silk slipknot was located about 1 mm inferior to the auricle of the left atrium. The ends of the slipknot were threaded through a polyethylene tube to form a snare. Ischemia was performed for 30 min, then recirculation was allowed by removing the slipknot and continued for 24 h. The rats were monitored during surgery.

Electrocardiogram parameters

Twenty-four hours after reperfusion, the heart activity of the animal was monitored with standard limb leads. Pressure transducer signals were acquired and analyzed using a PowerLab system (AD Instruments, Australia). An electrocardiogram (ECG) was recorded in lead II, and ST segments and RR intervals were determined. The ECG was obtained using bio-amplifier devices in conjunction with the PowerLab system (16).

Evaluation of cardiac markers

Following a period of 24 h after reperfusion, blood specimens (1.5-2 mL) from the tail vein of rats were collected to determine 4 cardiac enzyme markers, including LDH, cTn-I, CPK, and CK-MB. The concentrations of these biomolecules were measured using the methods recommended in the kit manual, through an ELISA reader (17).

Percentage of infarct size

At the end of the experiment, heart tissue was harvested, quickly frozen at -20 °C, and then maintained at that temperature for 2 h. To prepare 1-mm-thick slices, the heart tissue was divided; the segments were then submerged in the TTC solution at a temperature of 37 °C for 10 min. To enhance the contrast between infarcted and non-infarcted regions, the sections were placed in 10% buffered formalin overnight. The infarcted region was measured using ImageJ, software engineered by the National Institutes of Health (Bethesda, USA), by calculating the percentage of the infarcted area compared to the total area of the bilateral slices (15).

Assessment of inflammatory cytokines

Inflammatory cytokines were assessed by measuring tissue levels of TNF-α and IL-1β as proinflammatory biomarkers, and IL-10 as an anti-inflammatory biomarker. At the end of the experiment, cardiac tissues were homogenized, and after centrifugation, the concentrations of inflammatory biomarkers in the supernatants were assessed with an ELISA reader (Biotek, USA) according to the kit guidelines (9).

Measurement of MDA content

MDA levels (in cardiac tissues), which indicate one of the final products of lipid peroxidation, were measured by assessing thiobarbituric acid-reactive substances (TBARS) formation (18).

Measurement of the activity of antioxidant enzymes

To measure the activity of antioxidant enzymes, cardiac tissues were homogenized in ice-cold phosphate-buffered saline (pH ~ 7.4) and then centrifuged at 4000 rpm for 20 min at -4 °C. The resulting supernatants were collected to evaluate SOD, GPx, CAT, and total antioxidant capacity (TAC). All assessments were conducted using colorimetric methods and appropriate commercial kits, according to the manufacturer's protocols (9).

Real-time polymerase chain reaction

Frozen heart tissue samples were processed with an extraction kit (Ampliqon, Denmark) to isolate total mRNA for the genes of *Bax* and *Bcl-2*, as well as the housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The concentration and purity of extracted mRNA were assessed by spectrophotometry (S.N: D015, Eppendorf, Nanodrop Thermo Scientific, Germany), measuring absorbance at wavelengths of 260 and 280 nm. Then, cDNA was synthesized from

total mRNA following the manufacturer's instructions provided by the cDNA synthesis kit (Ampliqon, Denmark). The *Bax* and *Bcl-2* gene expression concentrations in cardiac tissues were determined through real-time polymerase chain reaction (RT-PCR) using a RunMei Q200 device (China). Specific primer sequences used in the analysis were obtained from Bioneer, based in Daejeon, South Korea (Table 1). The relative expression concentrations of the *Bax* and *Bcl-2* genes were quantified utilizing the comparative cycle threshold (Ct) method and the ΔΔCt method, with the assistance of RunMei QC 3.2 software (19).

Statistical analysis

The data were analyzed using SPSS, version 2.2, and displayed as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was subsequently applied to examine the normality of data. A one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test was executed for multiple comparisons. A significance level of $P < 0.05$ was defined to determine statistical significance.

RESULTS

ECG parameters

Based on the findings (Fig. 1A), the I/R group demonstrated a significant acute elevation of the ST segment compared to the sham group. Nevertheless, the group pretreated with GA at the dose of 30 mg/kg (G1 group) displayed a notably reduced ST segment when compared to the untreated I/R group. However, there was no significant difference between the G2 group and the I/R group.

The RR interval assessment showed a significant increase in the untreated I/R group compared to the sham group. However, pretreatment with GA in the G1 group resulted in a significant decrease compared to the I/R group, while no such reduction was observed in the G2 group (Fig. 1B).

Table 1. The sequence of specific primers utilized in real-time PCR.

Gene	Specific primer sequence
Bax	Forward: 5-CAACATGGAGCTGCAGAGGA-3
	Reverse: 5-GGAAAGGAGGCCATCCCAG-3
Bcl-2	Forward: 5-CTGGTGGACAACATCGCTCT-3
	Reverse: 5-GCATGCTGGGGCCATATAGT-3
GAPDH	Forward: 5-GCGAGATCCCGCTAACATCA-3
	Reverse: 5-ATTCGAGAGAAGGGAGGGCT-3

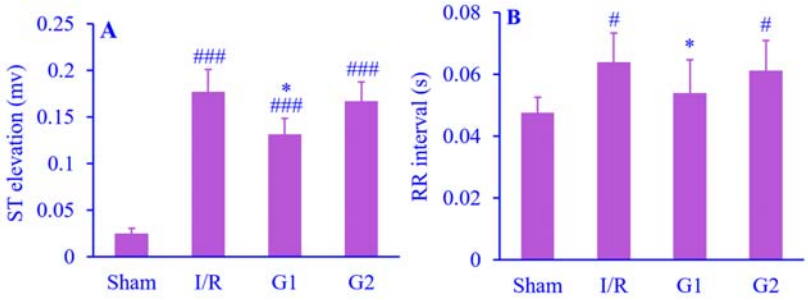

Fig. 1. The effect of gallic acid on electrocardiogram parameters in experimental groups. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ represent significant differences compared to the sham group; $^{*}P < 0.05$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R.

Cardiac markers

LDH activity showed a significant increase after 24 h of reperfusion compared to the sham group. In contrast, a significant decrease in LDH activity was observed in the G1 pretreatment group in comparison to the I/R group, whereas no significant alteration was found in the G2 group compared to the I/R group (Fig. 2A).

As shown in Fig. 2B, the CK-MB levels significantly increased in the I/R group in comparison to the sham group. In contrast, the group pretreated with GA at the dose of 30 mg/kg (G1 group) exhibited significantly lower CK-MB levels compared to the I/R group. Though no significant difference was found between the G2 and the I/R groups.

Figure 2C showed that cTn-I activity was significantly elevated in the I/R group compared to the sham group. However, the administration of GA in the G1 pretreatment group significantly decreased cTn-I activity compared to the I/R group. No significant difference was found in cTn-I activity in the G2 group compared to the I/R group (Fig. 2C).

The evaluation of CPK levels showed a significant rise in enzyme concentration in the I/R group in comparison to the sham group (Fig. 2D), and the administration of GA in the G2 pretreatment group indicated no significant difference compared to the I/R group. But the G1 pretreatment group had a significant decrease in CPK concentration compared to the I/R group (Fig. 2D).

Percentage of infarct size

After induction of ischemia, the size of the infarction area in the I/R group was significantly greater than that in the sham group, significantly. On the other hand, pretreatment with GA in the G1 group resulted in a substantial reduction of infarct size compared to the I/R group. Nonetheless, the infarct size in the G2 group was not significantly different from I/R group (Fig. 3).

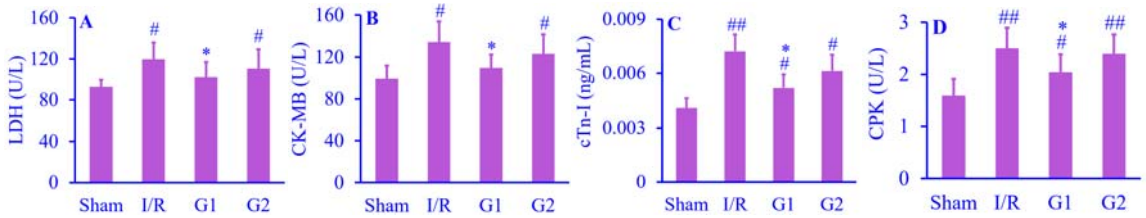

Fig. 2. The effect of gallic acid on cardiac enzymes in experimental groups. (A) LDH, (B) CK-MB, (C) cTn-I, (D) CPK. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ represent significant differences compared to the sham group; $^{*}P < 0.05$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R; LDH, lactate dehydrogenase; CK-MB, creatine kinase-MB; cTn-I, cardiac troponin-I; CPK, creatine phosphokinase.

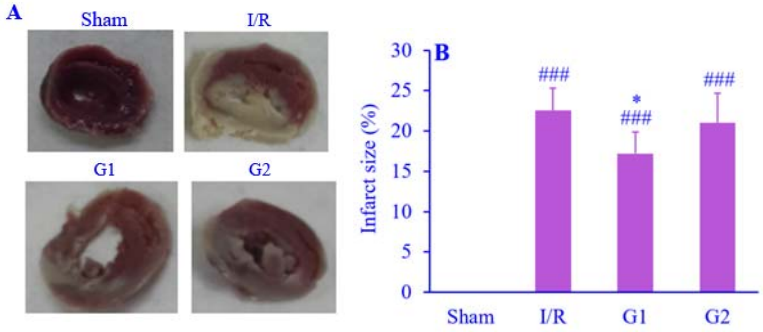

Fig. 3. The effect of gallic acid on the size of infarction in the experimental groups. (A) Photos of heart tissue slices and (B) bar graph representing the percentage of infarct size. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#\#\#}P < 0.001$ represent significant differences compared to the sham group; $^{*}P < 0.05$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R.

Inflammatory cytokines

Figure 4A showed that the IL-10 concentration in the I/R group was lower than that in the sham group. On the other hand, pretreatment with GA in the G1 group caused a significant increase in IL-10 concentration in comparison to the I/R group. But no change in L-10 concentration was observed in the G2 group compared to the IR group.

The levels of TNF-α in cardiac tissue increased significantly following I/R compared to the sham group (Fig. 4B). TNF-α concentration in the G1 group after pretreatment with GA significantly decreased compared to the I/R group. Despite this, no significant change was observed between G2 and I/R groups (Fig. 4B).

The findings showed that IL-1β concentration in heart tissue increased greatly after induction of ischemia in the I/R group compared to the sham group (Fig. 4C). In contrast, pretreatment with GA significantly decreased IL-1β concentration in the G1 group compared to untreated I/R group, but this decrease was not observed in the G2 group compared to the untreated I/R group (Fig. 4C).

Activity of antioxidant enzymes and MDA content

The TAC assessment showed a significant reduction in activity in the I/R group in comparison to the sham group. Though the TAC concentration significantly increased in the G1 group compared to the untreated I/R group. Remarkably, the study findings found no significant variation in TAC concentration between the G2 and I/R groups (Fig. 5A).

The GPx concentration changes in response to I/R and pretreatment with GA were measured in heart tissue. The results showed that I/R caused a notable decrease in GPx concentration in the I/R group in comparison to the sham group. GA at the dose of 30 mg/kg caused a significant increase in the concentration of GPx in the G1 group compared to the I/R group. The findings of study did not find a statistically significant impact on GPx levels in the G2 group compared to the I/R group (Fig. 5B).

As depicted in Fig. 5C, CAT activity alterations in response to ischemia and GA were assessed. Ischemia caused a significant decrement in CAT activity in the I/R group compared to the sham group. But pretreatment with GA in the G1 group caused a notable increase in CAT activity compared to the I/R group. Interestingly, the administration of GA did not have a significant effect on CAT activity in the G2 group compared to the I/R group (Fig. 5C).

After the I/R procedure, SOD activity was notably decreased in the I/R group in comparison to the sham group. Nevertheless, pretreatment by GA in the G1 group significantly enhanced SOD activity compared to the untreated I/R group. Interestingly, there was no significant effect in SOD activity between the I/R and the G2 groups (Fig. 5D).

According to the results, the I/R group displayed a higher concentration of MDA compared to the sham group. Pretreatment with GA significantly decreased MDA content in the G1 group compared to the I/R group. Notably, no significant difference in MDA concentration emerged between the I/R and G2 groups (Fig. 5E).

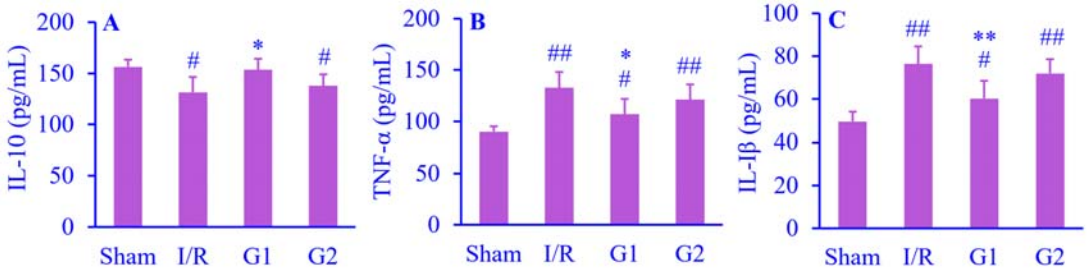

Fig. 4. The effect of gallic acid on inflammatory cytokines in the experimental groups. (A) IL-10, (B) TNF-α, (C) IL-1β. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ represent significant differences compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha; IL-1β, interlukin-1β.

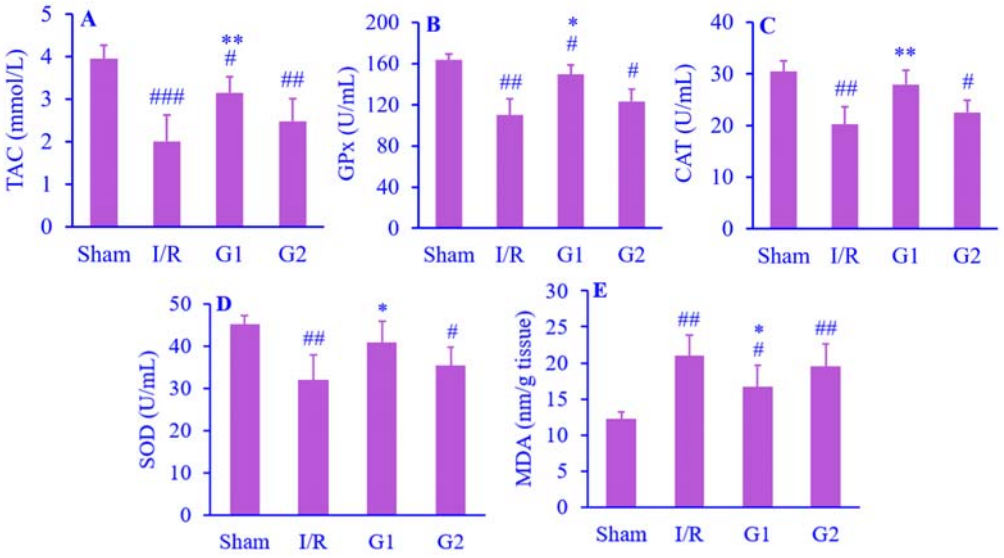

Fig. 5. The effect of gallic acid on the activity of antioxidant enzymes and the content of MDA in all experimental groups. (A) TAC, (B) GPx, (C) CAT, (D) SOD, (E) MDA. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ represent significant differences compared to the sham group; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R; TAC, total antioxidant capacity; GPx, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde.

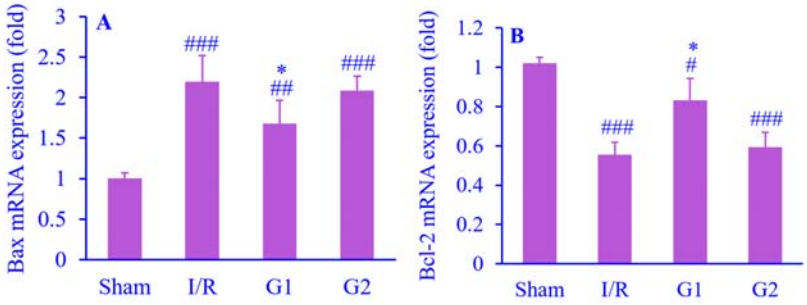

Fig. 6. The effect of gallic acid on apoptosis in all experimental groups. (A) *Bax* and (B) *Bcl-2* mRNA expression. G1 and G2 groups received gallic acid by gavage for 10 days, followed by I/R. The I/R group, as a positive control, received vehicle by gavage. The sham group was exposed to surgery without I/R induction. Data were expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ represent significant differences compared to the sham group; $^{*}P < 0.05$ versus I/R group. I/R, Ischemia/reperfusion; G1, gallic acid at the dose of 30 mg/kg/day + I/R; G2, gallic acid at the dose of 15 mg/kg/day + I/R.

Bax gene expression

The PCR results indicated a significant increase in *Bax* gene expression in the I/R group compared to the sham group. In contrast, pretreatment with GA in the G1 group substantially decreased *Bax* gene expression in comparison to the I/R group, whereas the G2 group showed no significant effect on the expression of the *Bax* gene than the I/R group (Fig. 6A).

Bcl-2 gene expression

The findings showed that the induction of I/R reduced *Bcl-2* expression compared to the sham group (Fig. 6B). However, no significant change was observed in *Bcl-2* gene expression in the G2 group compared to the I/R group. In contrast, pretreatment with GA significantly increased *Bcl-2* expression in the G1 group compared to the I/R group (Fig. 6B).

DISCUSSION

The present study demonstrated that GA exerted significant cardioprotective effects in a rat model of myocardial I/R injury. The findings indicated that pretreatment with GA, especially at the higher dose of 30 mg/kg/day, ameliorated myocardial injury by modulating oxidative stress, inflammation, apoptosis, and enzymatic markers of cardiac damage. The protective effects are consistent with and expand upon previous reports regarding the antioxidant and anti-inflammatory roles of GA in cardiovascular pathophysiology (9,10). Acute ST-segment elevation is a critical life-threatening factor identified on the ECG, which is one of the main medical tests utilized for detecting MI (20). In the current research, ST elevation was noted in the I/R group. However, in the G1 pretreatment group, ST elevation was significantly lower than in the I/R group, indicating an improvement in cardiac function following pretreatment. But the pretreatment with the dose of 15 mg/kg/day of GA in the G2 group had no effect on reducing ST elevation. This finding suggested that the dose of 30 mg/kg/day was likely more appropriate for achieving optimal therapeutic effects. Similarly, the pretreatment by GA reduced the level of RR interval in the G1 group. Consistent with this finding, several studies have indicated that the efficacy of GA at the dose of 30 mg/kg/day is superior to that of the 15 mg/kg/day dose (9,21). Evidence indicated that heightened oxidative stress from oxygen-free radicals significantly contributes to the growth of numerous diseases, such as MI (22). Thus, it is essential to quickly neutralize harmful free radicals to preserve cardiovascular health (23). The 3 antioxidant enzymes-GPx, SOD, and CAT-form the primary defense system of cells against oxygen-free radicals (24). Previous research indicated that ischemia led to reduced antioxidant levels in cardiac tissue, which were subsequently restored with GA therapy (9). This study findings demonstrated that the I/R group exhibited significantly reduced concentrations of antioxidant factors, including GPx, CAT, TAC, and SOD, and pretreatment with GA could improve the antioxidant enzymes. In agreement with previous research (9,25), the current findings suggested that GA, a potent natural antioxidant, could reduce the damaging effects of oxidative stress. Likewise, MDA is a commonly used biomarker for oxidative stress, particularly lipid peroxidation. High levels of MDA have been linked to severe coronary heart disease and acute MI (26). This study found elevated MDA levels in ischemic hearts, which is consistent with these observations. The current study also revealed that pretreating ischemic hearts with GA led to a noteworthy reduction in MDA contents. Additionally, there is growing evidence that dysregulation of immuno-inflammatory pathways is a major various cardiovascular disorders (27). This insight has the potential to pave the way for innovative therapeutic approaches to treating these conditions. In this experiment, rat heart tissue was utilized to measure the concentrations of IL-10, TNF-α, and IL-1β, to evaluate the balance between anti-inflammatory and proinflammatory responses. This study found that ischemic rats had higher concentrations of IL-1β and TNF-α, along with lower concentrations of IL-10, and that treatment with GA helped to mitigate these changes. These findings align with previous research (28,29), which has shown that ischemia triggers the production of proinflammatory factors, such as TNF-α and IL-1β, while reducing the production of anti-inflammatory factors, such as IL-10, following I/R. Furthermore, there are many specific enzymes that are present in cardiac muscles, which are very useful in the diagnosis of heart diseases. When the membrane of the cell is damaged, these factors are released and may be present in blood or surrounding fluid (30). Accordingly, in this study, the increased amount of CPK, CK-MB, cTn-I, and LDH was measured in the blood samples 24 h after reperfusion. Also shown earlier, there was a significant positive correlation between the level of increased cardiac enzymes and the infarct size (8,9), which confirmed the current results. The findings indicated a substantial rise in the infarct area and cardiac enzymes in ischemic hearts. On the other side, the previous research indicated that GA treatment significantly decreased the cardiac infarct size (9). In validation of this research, the data indicated that pretreatment with the dose of 30 mg/kg/day of GA significantly decreased the I/R injury-induced rise in enzyme levels and infarct volume. Moreover, the 2 typical members of the Bcl family, *Bax* and *Bcl-2*, exert a key function in inhibiting or promoting apoptosis, respectively. Both proteins play a vital role in the permeability and function of the mitochondrial membrane, as well as the release of

cytochrome c. The pro-apoptotic members of the Bcl family, like *Bax*, are mainly cytoplasmic, while *Bcl-2*, which is an anti-apoptotic member, is mainly present in the nucleus, mitochondria, and endoplasmic reticulum membrane (31). According to earlier reports (32,33), this study used *Bax* and *Bcl-2* indices to determine the rate of apoptosis. The results indicated an important rise in the concentrations of *Bax* and a sharp decline in the concentrations of *Bcl-2* in ischemic animals, which reflected the degree of apoptosis during I/R. Pretreatment with GA at the doses of 15 and 30 mg/kg/day showed the ineffectiveness of the 15 mg/kg/day dose on the levels of *Bax* and *Bcl-2*. The pretreatment at 30 mg/kg/day dosage significantly lowered *Bax* level while raising the expression of *Bcl-2*. Thus, the data indicated that GA at the 30 mg/kg/day dose was probably more appropriate than that at the dose of 15 mg/kg/day, confirmed also by a study previously performed (10).

Altogether, the current findings demonstrated that GA exerted multi-mechanistic cardioprotection, including:

I. Antioxidant action: restoring enzymatic defense (SOD, GPx, CAT, and TAC) and reducing lipid peroxidation (MDA).

II. Anti-inflammatory effects: downregulating TNF-α and IL-1β, while upregulating IL-10.

III. Anti-apoptotic modulation: decreasing *Bax* and increasing *Bcl-2* expression.

IV. Reduction of infarct size and normalization of cardiac markers.

Notably, the protective effects were dose-dependent, with 30 mg/kg/day showing significant benefits across all parameters, whereas 15 mg/kg/day did not yield statistically significant results in most assessments. This suggests that a higher threshold of GA concentration is necessary to engage its protective mechanisms effectively.

Although the present findings showed that the higher dose of GA (30 mg/kg/day) produced more pronounced cardioprotective effects than the lower dose (15 mg/kg/day), the study design, which included only 2 doses of GA, did not allow for the establishment of a complete dose-response curve. Our results should therefore be interpreted as indicating a dose-related trend rather than definitive dose-dependence. Further research, including at least 3 graded doses, preferably with an intermediate concentration, will be essential to confirm the dose-response relationship and to determine the optimal therapeutic range for GA in myocardial I/R injury.

There were some strengths and limitations in this study. One of the main strengths of this study lies in its comprehensive approach to evaluating the cardioprotective effects of GA across multiple mechanistic levels. By integrating biochemical, molecular, electrophysiological, and histopathological analyses, we provided a robust evaluation of GA's protective potential in a well-established in vivo model of myocardial I/R injury. Another strength was the dose-comparison design, which allowed us to determine the dose-dependency of GA effects, revealing that the 30 mg/kg/day dosage was significantly more effective than the 15 mg/kg/day dose. Additionally, the use of multiple validated biomarkers, such as MDA, cardiac enzymes, antioxidant enzymes, cytokines, and apoptosis-related genes (*Bax* and *Bcl-2*), strengthened the reliability and translational relevance of our findings.

However, several limitations should be acknowledged. First, the follow-up period was restricted to 24 h post-reperfusion, which did not provide insight into the long-term effects of GA on cardiac remodeling, survival, or functional recovery. Second, while gene expression of *Bax* and *Bcl-2* was quantified, protein-level validation (Western blot or immunohistochemistry) was not performed, which would have strengthened the conclusions regarding apoptosis. Third, although the rat model is widely accepted for studying myocardial infarction, its extrapolation to human physiology requires caution. The study also lacked mechanistic interventions (pathway inhibitors or knockdown models) to pinpoint the exact molecular targets of GA. Furthermore, the use of only 2 doses (15 and 30 mg/kg/day) limited the ability to conclusively determine a dose-response relationship. While a trend toward greater efficacy at the higher dose was observed, confirmation of dose-dependence will require future studies incorporating at least 3 different dose levels. Finally, we did not assess potential side effects or toxicity associated with GA at the administered doses, which was crucial for future clinical application. Future studies should also explore GA pharmacokinetics, optimal administration timing, and potential synergy with existing cardioprotective therapies.

CONCLUSION

Finally, the results of the study confirmed that GA exerted anti-inflammatory action as well as significantly modulated oxidative stress in a rat model of myocardial infarction. GA reduced the infarct size and myocardial injury markers, which helped in the recovery of endogenous antioxidant defenses after I/R by the downregulation of proinflammatory factors and the upregulation of anti-inflammatory factors. It could also control apoptosis through *Bax* inhibition and *Bcl-2* regeneration. The results indicated that the efficacy of GA at the dose of 30 mg/kg/day was superior to that at the dose of 15 mg/kg/day.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

A. Nazari, F. Souri, V. Ghoranzadeh, M. Sedighi, F. Fathollahi Shoorabeh, and N. Khojasteh Kalansara contributed to the study conception and design, material preparation, data collection, and analysis; F. Souri and A. Nazari wrote the first draft of the manuscript; A. Nazari, F. Souri, V. Ghoranzadeh, M. Sedighi, F. Fathollahi Shoorabeh, and N. Khojasteh Kalansara studied and commented on previous versions of the manuscript. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

AI declaration

During the preparation of this work, the authors used ChatGPT to improve readability and language. After using this tool, the authors reviewed and edited the content and take full responsibility for the content of the publication.

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