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RESEARCH ARTICLE

Studying the Protective Effect of Ellagic Acid Against High Glucose-Associated Toxicity in H9C2 Cardiomyocytes

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SUMMARY

Diabetes mellitus leads to an increased risk factor for cardiovascular diseases. Accumulating evidence has demonstrated that high glucose (HG) can promote massive apoptosis in cardiomyocytes. Oxidative stress has been known as main factor responsible for HG-induced apoptosis. Ellagic acid, a natural phenolic compound, exhibits anti-inflammatory, anti-atherogenic, and antioxidant effects. This study was carried out to evaluate the effects of ellagic acid on HG-induced oxidative damage in H9C2 cells. The effect of ellagic acid on the viability of cells was evaluated by the MTT method. The oxidative stress parameters, including levels of malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (TAC), and superoxide dismutase (SOD) activity were also measured. The results indicated that ellagic acid (10 μM and 20 μM) could remarkably enhance the cell viability of H9C2 cells exposed to HG. In addition, ellagic acid significantly improved the levels of intracellular GSH, TAC, and SOD, whereas the levels of MDA were attenuated. These results revealed a protective effect of ellagic acid on HG-induced cytotoxicity, at least partially, by increasing antioxidant activity and preventing oxidative stress.

Key Words: *H9C2 cells, high glucose, ellagic acid, cardiotoxicity, oxidative stress*

H9C2 Kardiyomiyositlerde Yüksek Glikoz Bağlantılı Toksisiteye Karşı Elajik Asitin Koruyucu Etkisinin İncelenmesi

ÖZ

Diabetes mellitus, kardiyovasküler hastalıklar için riskin artmasına neden olan bir faktördür. Elde edilen veriler, yüksek glikozun (HG) kardiyomiyositlerde yaygın apoptozu teşvik edebileceğini göstermiştir. Oksidatif stres, HG'nin neden olduğu apoptozdan sorumlu ana faktör olarak bilinmektedir. Doğal bir fenolik bileşik olan elajik asit, anti-inflamatuar, anti-aterojenik ve anti-oksidan etkiler göstermektedir. Bu çalışma, elajik asidin H9C2 hücrelerinde HG'nin neden olduğu oksidatif hasar üzerindeki etkilerini değerlendirmek için yapılmıştır. Elajik asitin hücre canlılığı üzerindeki etkisi MTT yöntemi ile değerlendirilmiştir. Malondialdehit (MDA), glutatyon (GSH), toplam antioksidan kapasite (TAC) ve süperoksit dismutaz (SOD) aktivitesini içeren oksidatif stres parametreleri de ölçülmüştür. Sonuçlar, elajik asitin (10 uM ve 20 uM), HG'ye maruz kalan H9C2 hücrelerinin hücre canlılığını önemli ölçüde artırabildiğini göstermiştir. Ek olarak, elajik asit, hücre içi GSH, TAC ve SOD düzeylerini önemli ölçüde artırırken, MDA düzeylerini azaltmıştır. Birlikte ele alındığında, bu sonuçlar, elajik asitin, en azından kısmen, antioksidan aktiviteyi artırarak ve oksidatif stresi önleyerek, HG'nin neden olduğu sitotoksisite üzerinde koruyucu bir etkisini ortaya koymuştur.

Anahtar Kelimeler: *H9C2 hücreleri, yüksek glukoz, ellajik asit, kardiyotoksisite, oksidatif stres*

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INTRODUCTION

Diabetes mellitus (DM) is a leading cause of many chronic conditions, including nephropathy, retinopathy, neuropathy, and cardiovascular diseases (Lin, 2014; Chawla, 2016). High glucose (HG), the most important feature of DM, can cause the development of cardiovascular diseases (Martín-Timón, 2014). It has been shown that HG stimulates myocardial apoptosis through oxidative stress and inflammatory reactions (Yan, 2011). It has been recently indicated that HG leads to the overproduction of reactive oxygen species (ROS), ultimately resulting in the activation of the apoptotic pathway (Kumar, 2012). ROS are chemically reactive chemical species containing oxygen. Oxidative stress is a condition characterized by increased intracellular levels of ROS. It is known that oxidative stress has an essential role in the pathogenesis of cardiovascular complications of diabetes (Haidara, 2006). Oxidative stress can result in the reduction of antioxidant defense proteins such as glutathione (Cnubben, 2001). Furthermore, free radicals cause modifications in proinflammatory mediators such as nuclear factor kappa B (NF-κB) and suppress nuclear factor erythroid 2-related factor 2 (Nrf2), which are major transcription factors implicated in antioxidant capacity (Videla, 2009). These events ultimately lead to ROS attack on proteins, lipid membranes, and DNA, causing cardiac cell damage and apoptosis (Datta, 2000). Therefore, natural antioxidant scavengers such as polyphenol compounds can be useful in reducing the free radicals in cardiac cells. Studies have shown that polyphenol compounds have beneficial effects on cardiovascular diseases (Quiñones, 2013). Ellagic acid is a natural polyphenolic compound found in blackberry, strawberry, bayberry, pineapple, and pomegranate (Amakura, 2000). It is reported to have important biological properties including antimicrobial, anti-inflammatory, anti-atherogenic, and anti-mutagenic effects (Rogerio, 2006; Abuelsaad, 2013). Ellagic acid has been shown to have potent antioxidant activity that effectively scavenges ROS (Devipriya, 2007). Moreover, studies have shown that ellagic acid exhibits beneficial effects in the treatment of hypertension and prevention of nephrotoxicity (Al-Kharusi, 2013; Berkban, 2015). Studies have indicated that the antioxidant activity of polyphenols plays an important role against oxidative stress-induced injury in cardiomyocytes (Mattera, 2017). Trolox, a vitamin E analog, inhibits oxidative damage to H9C2 cells under HG condition (Davargaon, 2019). Interestingly, one study reported that ellagic acid provided more effective protection than vitamin E against oxidative stress damage in embryonic and placental tissues of C57BL/6J mice (Hassoun, 1997). H9C2 cells are proper *in vitro* model for cardiac research studies (Zordoky & El-Kadi, 2007). Therefore, the present study aimed to examine the protective effects of ellagic acid on HG-induced oxidative injury in H9C2 cells with a focus on its antioxidant properties.

MATERIALS AND METHODS

Cell culture

The H9C2 cells were purchased from the National Cell Bank of Iran (NCBI code: C585, Pasteur Institute of Iran, Tehran, Iran). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA, USA) containing 4 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 1000 mg/L glucose. The culture medium was supplemented with 1% penicillin-streptomycin (Gibco, USA) and 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA). The cells were maintained in 5% CO2 and 37°C.

Determination of cell viability

The effect of ellagic acid on HG toxicity was measured via the analysis of viable cells using MTT colorimetric assay. Briefly, 1×10^4 H9C2 cells were seeded into each well of a 96-well plate. After 24 h incubation, the cells were treated with different glucose concentrations (20, 35, 50, and 65 mM) for 24 h. The level of 50 mM glucose was chosen for the next steps. Then, the cells were pretreated with various concentrations of ellagic acid (5, 10, 20, and 30 μM, Sigma Aldrich, St Louis, MO, USA) for 24 h and next, the cells were

treated with HG (50 mM) for 24 h. After the above mentioned time, 10 µM of MTT (5 mg/ml, Sigma, USA) was added to each well and the plates were incubated for 3-4 h at 37°C. Then, the medium was removed and 100 µM of DMSO was added to each well and the absorbance was measured with an ELISA plate reader at 570 nm.

Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA), a three-carbon low molecular weight aldehyde, is one of the end products of lipid peroxidation (LPO) in the cells. MDA level has been implicated as a marker of oxidative stress and antioxidant status (Gaweł, 2004). After treatment, the H9C2 cell lines (3×10^6 cells) were washed twice with PBS and then cells were centrifuged at 3000 g for 5 min. Then, 200 µl of the sample was mixed with 500 µl of 20% trichloroacetic acid (Sigma, USA), 400 µl of thiobarbituric acid (TBA, Sigma, USA), and 500 µl of sulfuric acid (Merck Company, Darmstadt, Germany). Then it was heated for 30 min in a boiling water bath. After cooling, samples were mixed with 0.8 ml of n-butanol (Merck, Germany), next centrifuged at 4100 g for 12 min. The quantification of MDA levels in the samples was measured in the absorbance of 532 nm.

Measurement of total thiol groups

The total thiol groups were determined by the HU method (Hu, 1994). 5, 5'-dithiobis-(2-nitrobenzoic acid), also called DTNB or Ellman's reagent, is a versatile water-soluble compound for evaluating sulfhydryl groups in the solution. Briefly, the cell lysates were added to Ellman's reagent (5 mM, Sigma, USA), and centrifuged for 10 min. The absorbance of the mixture was measured at 412 nm.

Measurement of total antioxidant capacity (TAC) levels

The TAC levels were determined by the ferric reducing antioxidant power (FRAP) assay. This method was based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) in the presence of tripyridyltriazine (TPTZ) by the colorimetric method. Briefly, the FRAP reagent containing TPTZ (10 mM, Sigma, USA), HCl (40 mM, Merck, Germany), $FeCl_3$ (20 mM, Merck, Germany), and acetate buffer was freshly prepared and added to the samples. The mixture was incubated for 5 min at 37°C, and the absorbance at 593 nm was recorded using an ELISA plate reader.

Measurement of superoxide dismutase (SOD)

SOD is an essential antioxidant defense against oxidative stress in the body. SOD is the first line of physiological defense and most strong antioxidant in the cells (Tsai, 2015). To assay for SOD activity, 610 µl of PBS and 90 µl of pyrogallol (Sigma, USA) were added to the supernatant. The absorbance was measured at 420 nm (Marklund & Marklund, 1974).

Statistical methods

All data were represented as the mean ± S.D in GraphPad Prism 5.0 software (San Diego, CA, USA). Statistical analyses were evaluated using the one-way ANOVA followed by Tukey's post hoc test. A *P* value less than 0.05 was assumed to be statistically significant.

RESULTS

Effect of high glucose on cell viability

To identify the effect of HG on cell viability, MTT reduction assay was used. H9C2 cells were exposed to different glucose concentrations (20, 35, 50, and 65 mM) for 24 h. The results showed that HG (35, 50, and 65 mM) decreased cell viability in H9C2 cells in a concentration-dependent manner. HG in the concentration of 50 mM was selected for experiments ($P < 0.001$) (Figure 1).

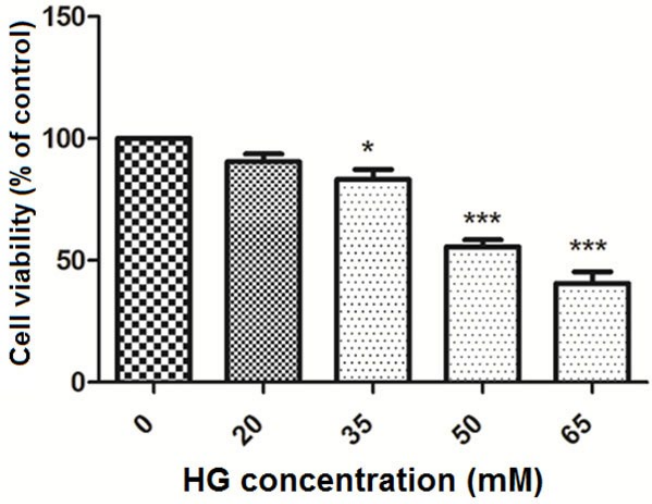

Figure 1. Cell viability of H9C2 cells after treatment with various concentrations of glucose. The values are expressed by mean ± S.D; n=5. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the control group.

Ellagic acid attenuated HG-induced loss of cell viability in H9C2 cells

The H9C2 cell lines were pre-incubated with ellagic acid followed by exposure to HG. The results showed that ellagic acid significantly increased cell viability as compared with the HG group. Because ellagic acid at concentrations of 10 μM and 20 μM produced significant protective effects, these concentrations were chosen for use in this study ($P < 0.001$ for both concentrations) (Figure 2).

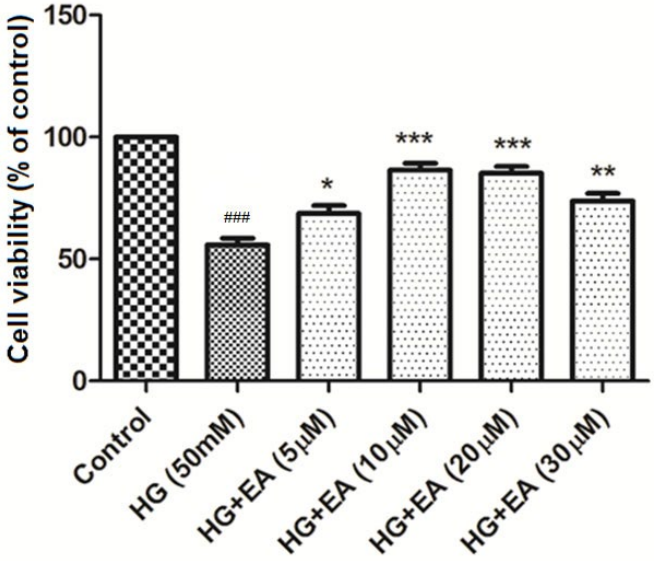

Figure 2. Effects of ellagic acid (EA) on cell viability in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=5. $^{###}P < 0.001$ compared with the control group; $^{*}P< 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were compared with the HG group.

Effect of ellagic acid on LPO in H9C2 cells

To evaluate whether ellagic acid is involved in HG-induced LPO, the cells were pre-incubated with ellagic acid and then treated with HG. Our results indicated that HG increased the level of MDA. Treatment of H9C2 cells with ellagic acid (10 μM and 20 μM) decreased MDA levels as compared with HG ($P < 0.001$ for both groups) (Figure 3a).

Ellagic acid restored HG-induced decrease in the activity of GSH

Our results showed that HG significantly reduced GSH levels compared with control ($P< 0.001$), whereas pretreatment with ellagic acid (10 μM and 20 μM) for 24 h, significantly increased the GSH levels compared with H9C2 cells exposed to HG ($P < 0.001$ for both groups) (Figure 3b).

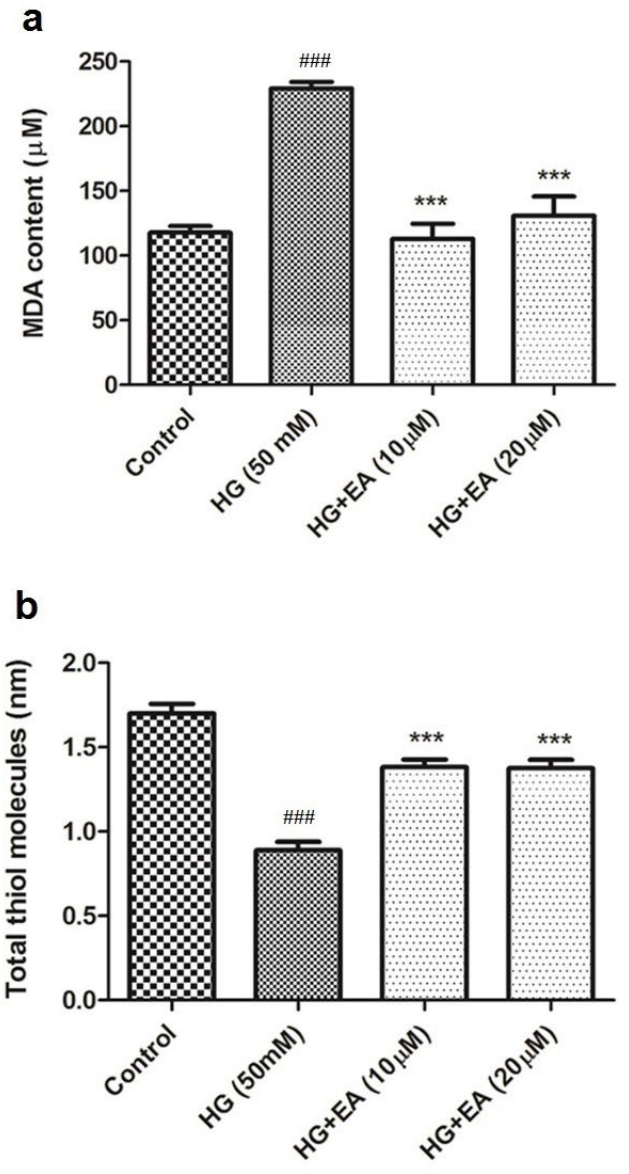

Figure 3. Effects of EA on MDA and GSH levels in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=3. $^{###}P < 0.001$ compared with the control group; $^{***}P < 0.001$ was compared with the HG group.

Ellagic acid attenuated HG-induced decrease in TAC levels

Effects of ellagic acid on TAC levels were studied by FRAP assay. As shown in Figure 4a, results illustrated that the HG condition markedly reduced TAC levels while ellagic acid (10 μM and 20 μM) significantly enhanced TAC levels ($P < 0.001$ and $P < 0.01$, respectively).

Ellagic acid increased HG-induced decrease in SOD activity

As shown in Figure 4b, SOD activity is markedly reduced in the HG-exposed group ($P < 0.001$). However, ellagic acid administration (10 μM and 20 μM) effectively increased SOD activity as compared with the HG group in H9C2 cells ($P < 0.001$ for both concentrations).

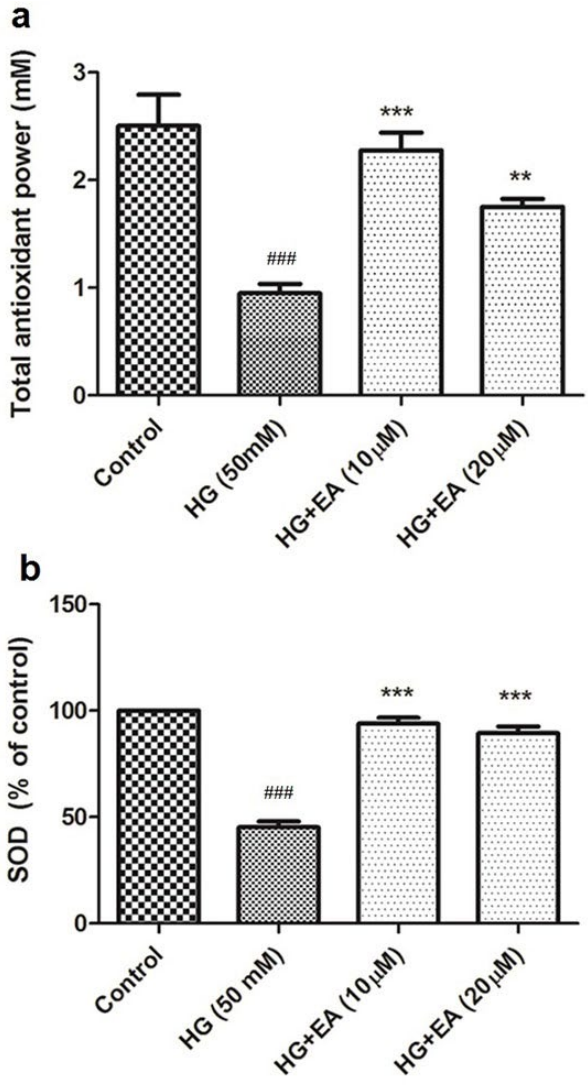

Figure 4. Effects of EA on total antioxidant capacity and SOD levels in HG-treated H9C2 cells. The values are expressed by mean ± S.D; n=3. $^{###}P < 0.001$ compared with the control group; $^{**}P < 0.01$ and $^{***}P < 0.001$ were compared with the HG group.

DISCUSSION

This study was designed to examine the cardioprotective effect of ellagic acid on HG-induced oxidative stress in H9C2 cardiac cells. We observed that ellagic acid prevented HG-induced cytotoxicity in H9C2 cells by decreasing the levels of MDA and increasing the levels of intracellular GSH, SOD, and TAC.

Clinical studies have shown that cardiovascular disease (CVD) is a major cause of death in patients with diabetes (Martín-Timón, 2014). It has been shown that oxidative stress has a crucial role in CVD development (Haidara, 2006). Previous studies demonstrated that HG stimulated apoptosis in vascular endothelial cells via the accumulation of ROS levels (Kumar, 2012; Aminzadeh & Bashiri, 2020). Furthermore, studies have shown that cardiac myocyte apoptosis under pathological conditions plays a major role in CVD. Experimental and clinical diabetic models have shown that oxidative stress mediated heart injury (Haidara, 2006). Excessive oxidative stress is an imbalance between ROS production and antioxidant defense systems, which plays an essential role in inducing apoptosis. Several biomarkers are used to evaluate oxidative stress conditions, such as levels of lipid peroxidation, SOD, catalase, and GSH.

Antioxidants organize the primary defense system that limits the toxicity associated with oxidative stress. Some studies have previously shown that antioxidant natural substances such as diallyl trisulfide could prevent HG-induced apoptosis in cardiac cells. It was shown that antioxidants could improve heart disease in diabetic conditions after the administration of streptozotocin (Kumar, 2013). It is also indicated that polyphenolic compounds have protective roles on different noxious stimuli, including rat model of Parkinson's disease and lipopolysaccharide-induced inflammation in RAW264 cells (Baluchnejadmojarad, 2017; Du, 2019; Wang, 2019).

In this research, we demonstrated that HG induced H9C2 cells injury while ellagic acid could prevent oxidative stress production in cardiac cells. H9C2 cells were pre-incubated with various concentrations of ellagic acid (5-30 μM) and the effect of ellagic acid on viability was determined by MTT assay. Our results showed that cell viability decreased when H9C2 cells were incubated with HG. Pre-incubation with ellagic acid successfully increased cell viability. This result is in agreement with previous findings indicating that ellagic acid enhanced cell viability and also provided cytoprotection against oxidized LDL-induced apoptosis in primary human umbilical vein endothelial cells (Ou, 2010).

Increasing documents in experimental and clinical studies propose that free radicals lead to increased lipid peroxidation and MDA content. The MDA level has been recognized as a circuitous parameter of oxidative stress in cells. Lipid peroxidation has been involved in the pathogenesis of various disorders such as diabetes, atherosclerosis, fibrosis, and cancer (Negre-Salvayre, 2010; Aminzadeh & Salarinejad, 2019). Lipid peroxidation can either directly degrade enzymes by free radicals, or by chemical changes in end products (Halliwell & Chirico, 1993). Our findings revealed that HG enhanced the levels of MDA in cardiac cells. A similar study reported that elevated level of MDA in diabetic model was induced by low dose of streptozoticin in rats (Yu, 2012). Moreover, this study showed that ellagic acid prevents the increased in MDA levels in HG condition. In line with current results, evidence indicates that ellagic acid inhibited LPO and attenuated the high-fat diet/low dose streptozotocin-induced diabetic nephropathy in rats (Ahad, 2014).

GSH is a common molecule found in many tissues and cells. GSH, a crucial regulator of intracellular oxidative balance, has an important role in cell death principally by binding to cysteine residues in proteins (Anderson, 1998). In the present study, our results showed that HG decreased GSH levels in H9C2 cells. We observed that pretreatment with ellagic acid increased GSH levels as compared with the HG group. Our findings are consistent with reports indicating that ellagic acid restored GSH levels on H_2O_2-induced apoptosis in PC12 cells (Pavlica & Gebhardt, 2005).

SOD is one of the most essential enzymes in the antioxidant defense system (Yim, 1990). In the present study, we observed that treatment of H9C2 cells with HG decreased SOD activity, while ellagic acid effectively increased SOD activity as compared with the HG group. The results of the present study are in agreement with studies indicating that ellagic acid enhances the SOD levels to protect against 2, 2-diphenyl-1-picrylhydrazyl (DPPH)-induced toxicity in V79-4 cells (Han, 2006).

CONCLUSION

Overall, the present study revealed a protective role of ellagic acid against HG-induced cardiotoxicity in H9C2 cells *in vitro.* Our results demonstrated that pretreatment of H9C2 cells with ellagic acid reduced the oxidative stress in HG condition. The antioxidant role of ellagic acid could be responsible for this activity.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis, literature research, analysis and interpretation of the data, reviewing the text (Aminzadeh, A.), experimenting, preparing the study text, analysis and interpretation of the data (Keshavarzi, E.)

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