Adropin is involved in the protective effect of ellagic acid against high-glucose induced injury in human umbilical venous endothelial Cells

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Research Article

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Abstract

Background*:* There is escalating evidence suggesting the beneficial effects of ellagic acid (EA) on the cardiovascular system. The present study aimed to investigate the protective effect of EA in human umbilical vein endothelial cells (HUVECs) against high glucose (HG)- induced endothelial dysfunction and the potential roles of adropin and NO in this regard.

Methods and Results*:* The experimental groups consisted of normal and HG (30 mM, 48 h) treated *HUVECs* incubated without or with 5 or 10 μM of EA (6 groups of at least 6 replicates, each). The cell count and metabolism were studied. Moreover, the markers of redox state including malondialdehyde (MDA), the activities of superoxide dismutase (SOD) and catalase enzymes, as well as ferric reducing antioxidant power (FRAP) were assayed. The levels of adropin and eNOS gene expression were also studied using qPCR. A high concentration of glucose reduced cell count and caused lipid peroxidation, reduced antioxidant capacity of the cells, decreased NO levels, and downregulated the expression of *NOS3* (encoding eNOS) and *ENHO* (encoding Adropin) genes. Ellagic acid reversed all these effects.

Conclusions*:* These results suggest a significant protective effect for EA against HG-induced injury in HUVECs. The improved redox state and upregulation of *NOS3* and *ENHO* genes seem to play critical roles in this regard.

1. Introduction

Cardiovascular diseases (CVDs) including peripheral artery disease, coronary artery disease, myocardial infarction, and stroke are the most important causes of mortality worldwide [1]. In 2016, there were approximately 17.9 million deaths due to CVDs, that is 31%, of all-cause global deaths [2]. This annual mortality has been expected to escalate to 23.6 million by 2030 [3]. A variety of risk factors are involved in the morbidity and mortality of CVDs including but not limited to obesity, hypertension, dyslipidemia, and diabetes [4]. Among these, type II diabetes (T2D) is the most important one and accounts for two-thirds of all CVD-caused mortalities [5].

Diabetes mellitus (DM) is defined by a hyperglycemic status. The occurrence of DM is increasing considerably worldwide. An estimate of 592 million people, worldwide, is expected to have DM by 2035, as predicted by the International Diabetes Federation [6, 7]. Diabetes mellitus is a metabolic disease that is recognized as an independent predisposing factor for CVDs. It shows microvascular manifestations such as retinopathy, nephropathy, neuropathy, and macrovascular manifestations such as myocardial infarction, stroke, and coronary artery disease. The chance of macrovascular complications is two to four times higher in patients with type 2 DM (T2DM), compared to patients without T2DM [7].

Endothelial cell (EC) dysfunction has an important role in the pathogenesis of both diabetes and CVDs [8]. Endothelial damage is characterized by the incapability of endothelium to modulate vascular homeostasis, the physiological balance of vasoconstriction, pro-thrombotic and pro-inflammatory effects [9] that promote atherosclerosis and coronary heart disease. Several studies have demonstrated that high

levels of reactive oxygen species (ROS), decreased bioavailability of nitric oxide (NO), and modification of endothelial permeability are associated with hyperglycemia in endothelial damage [10, 11].

Studies of diseased human coronary arteries showed that about 60% of total vascular ROS is produced by NADPH oxidase (NOX), and NOX4 has the most important role in ROS generation in HUVECs [12, 13].

Nitric oxide is a key determinant of vascular homeostasis, and it has an antiatherogenic role [14]. NO has two distinct pathways to regulate cardiovascular function: stimulation of PKG by activating soluble guanylate cyclase, and direct S nitrosylation of proteins [15]. Nitric oxide is produced from its precursor, L-arginine, by endothelial nitric oxide synthase (eNOS) [11].

Adropin is a peptide hormone with an endogenous biologically active substance encoded by the energy homeostasis associated (*ENHO*) gene that was discovered in 2008 [16]. Kidney, pancreas, liver, brain, heart, coronary artery endothelial cells, and HUVECs express adropin [17]. Adropin levels change in response to different physiological and pathophysiological stimuli. This protein is a key modulator in the homeostasis of glucose, fatty acid, and energy. It improves insulin sensitivity, and it may be involved in the pathogenesis of T2DM [18]. Adropin has a beneficial effect on endothelial cells and has been recognized as a novel regulator for these cells [19]. It is a key modulator of eNOS gene expression and regulates the bioavailability of NO in coronary arteries [20].

Therapeutic approaches that prevent high glucose (HG) induced oxidative stress may decrease the risk of cardiovascular-diabetic complications [10]. Anti-oxidant therapies are the new approach to defeat endothelial dysfunction by decreasing ROS generation, improving oxidative balance [8], and increasing the production of endothelial NO. Recently, different antioxidants have been found to activate and increase the synthesis of endothelial NO [21]. Flavonols have a wide range of biological activities, such as vasorelaxant, antioxidant, and anti-inflammatory effects in addition to inhibiting some kinases. As a result, there is a strong positive correlation between the dietary consumption of high levels of flavonols and reduced risk of cardiovascular diseases [22, 23]. Flavonols can readily enter the cells and show their biological activities due to their small, lipid-soluble molecules [22].

Ellagic acid (EA) is a phenolic compound that can be found in the form of ellagitannin in fruits such as pomegranate, blackberry, strawberry, and raspberry [24]. Several studies have demonstrated that EA can suppress oxidative stress [12, 25]. It is capable of exerting protective effects against oxidative stress in the aorta of diabetic mice [26]. Furthermore, many studies have shown anti-inflammatory, anti-cancer, and anti-atherosclerotic properties for EA [27, 28]. In this study, we verified the potential beneficial effect of ellagic acid on HG-induced endothelial dysfunction in HUVECs. We also investigated the possible role of some key mediators in this regard.

2. Materials And Methods

2.1. Culture of HUVECs and treatment

Human umbilical vein endothelial cells (Iran University of Medical Sciences, Tehran-Iran) were cultured in Dulbecco's Modified Eagle Medium (DMEM) F-12 (Gibco Life Technologies Ltd, UK) mixed with penicillin (100 IU/mL, Biowest, France), streptomycin (100 µg/mL, Biowest, France), and fetal bovine serum (10%, Gibco). The cells were cultured in a mixture of air atmosphere and CO2 (95%:5%, respectively) at 37°C. To avoid variations among experiments, isogenic cell lines were derived and used. Cells had a cobblestone appearance and large dark nuclei. Upon ~ 70% confluency, the cells were dissociated using trypsin-EDTA. The HUVECs in different experimental groups were incubated using either 5.5 mM (physiologic concentration) or 30 mM of glucose (Sigma-Aldrich, G-7021, USA), in the presence (5, 10, 20 µM) or absence of ellagic acid (Sigma-Aldrich, Germany) for 48 h. To make the stock solution (10mM), ellagic acid was dissolved in dimethyl sulfoxide (DMSO). The control groups received a similar concentration of DMSO as placebo.

2.2. MTT cell metabolism assay and cell counting

The rate of cell metabolism of HUVECs in response to different concentrations of ellagic acid was measured by 3-[4, 5-dimethylthylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT, Sigma-Aldrich, UK) assay. The assay is based on the reduction of MTT to formazan by mitochondrial dehydrogenases, leading to the formation of purple formazan crystals which can be dissolved in DMSO, and their optical density to be measured as an indicator of cell metabolism by a spectrophotometer at 550 nm [29].

To calculate the number of viable cells, the HUVECs were counted under a light microscope following staining with trypan blue.

2.3. Determination of endothelial nitric-oxide production

The level of NO released from endothelial cells is a key regulator of blood vessel function. Nitric oxide has an extremely short half-life and quickly converts to nitrite and nitrate metabolites [14]. To assess the level of nitric oxide, nitrate was initially reduced to nitrite using vanadium chloride. The nitrite level was then assayed using the Griess method [30]. Briefly, 50 µl of sample/nitrite standards were mixed with 50 µl of freshly prepared vanadium chloride solution (200 mg in 25 ml of 1 M HCl) and then 50 µl of Griess reagent (Sigma) containing N-(1-naphthyl) ethylenediamine dihydrochloride (1%) in deionized water and sulphanilamide (2%) in hydrochloric acid (1.47 M). After 30 minutes of incubation at 37◦C, the optical density was determined using a microplate reader (EPOC 2) at 550 nm. Sodium nitrite was used as a standard.

2.4. Lipid peroxidation assay

ROS accumulation, which was caused by HG-induced oxidative damage in the cells, can lead to lipid peroxidation [31]. Lipid peroxidation was evaluated spectrophotometrically by measuring malondialdehyde (MDA) in cell lysate based on Lata & Pari protocol [32]. Briefly, samples (100 µl) were mixed with 2 ml of thiobarbituric acid reactive substances (TBARS) solution (containing equal volumes of 15% trichloroacetic acid, 0.25 M HCl, and 37% thiobarbituric acid) before being incubated at 96°C for 30 min. The samples were left to cool to the room temperature, before being centrifuged at 10000 g for

10 minutes. The absorbance of supernatants was detected at 532 nm. The amount of lipid peroxidation was expressed as equivalent nanomoles of malondialdehyde (MDA).

2.5. Antioxidant enzyme activity

HUVECs were scraped off the wells and harvested for the detection of antioxidant enzyme activity. Following 30 seconds of sonication in cold PBS buffer, the cells were centrifuged at 15,000 g (15 min, 4°C). The supernatant was separated and was used for antioxidant enzyme assays.

The activities of catalase and superoxide dismutase (SOD) enzymes were determined using commercial assay kits (ZellBio, Germany) following up the protocols of the manufacturer.

2.6. FRAP assay

The ferric reducing antioxidant power (FRAP) assay was employed to verify the in vitro antioxidant capacity of ellagic acid [33]. Ten µl of the cell lysate was supplemented with 180 µl of the fresh FRAP reagent containing FeCl3 (5 ml, 20 mM), sodium acetate (50 ml, 300 mM, pH 3.6), and 2,4,6-tripyridyl-s-triazine (5 ml, 10 mM). FeSO4 solution was used for the standard curve. The optical density was detected at 593 nm using a microplate reader (EPOCH 2, BioTek Instruments Inc., USA) after 40 minutes of heating at 40°C. The FRAP values of samples were estimated using the standard curve of FeSO4 and reported as µM of FeII equivalents.

2.7. Reverse transcription-quantitative polymerase chain reaction (RT qPCR)

Total RNA from HUVECs in all experimental groups was isolated using the Total RNA Isolation Kit (DENAzist Asia Co., Iran). The extracted RNA samples had an absorbance ratio of 1.8 to 2.0 at 260/280 nm. The cDNA was synthesized from 5 µg of total RNA using random hexamer primers and MMLV reverse transcriptase (Thermo Fisher Scientific, USA). The qPCR was performed in a Rotor-Gene Q real-time thermocycler (Qiagen, USA) to quantify transcripts of *ENHO* and *NOS3*, and *ACTB* genes, and. The reaction mixture contained 10 pmol of each primer, 2 µl template cDNA, and 10 µl of 2X SYBR® qPCR master mix (Amplicon, Denmark). The amplification program included the following steps: 95°C for 15 min, followed by 45 cycles of 95°C for 30 s, 30 s annealing at 68°C for *ENHO*, 66.2°C for *NOS3*, and 60°C for *ACTB*, and extension at 72°C for 30 seconds. Emission from each sample was recorded during thermal cycling, and the threshold cycle (Ct) value was calculated from raw fluorescence data using Rotor-Gene Q software. The following primer sequences were used for qPCR: *ENHO* forward, 5'-caggctcccaagccttagtcg-3' and reverse, 5'- gtggagatgtctacctgcagtc-3', *NOS3* forward, 5'-ggatgagtatgacgtggtgtcc-3'and reverse, 5'- agatgctgttgaagcggatctta-3'; *ACTB* forward, tgcagaaggagatcactg and reverse, cttgctgatccacatctg. The copy number for the target genes were calculated based on the relevant standard curves and were normalized by the copy number of the reference gene (*ACTB*).

2.8. Statistics

Data are represented as means ± SD. Statistical analyses and drawing the graphs were performed and prepared using the GraphPad Prism version 9 (GraphPad Software, USA). Inter-group comparisons were analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni/Dunnett tests. Differences with p-values < 0.05 were considered statistically significant.

3. Results

3.1. Ellagic acid (EA) did not affect cell count and metabolism at low concentrations

Initially, we determined the effect of EA on cell count and metabolism of HUVECs under both normal and HG conditions. As assessed by the MTT test, cell metabolism was not altered by EA at 0–10 μM following 48 h of incubation, regardless of glucose concentration. However, EA at 20 μM decreased the cell metabolism in both normal (5.5 mM) and high-glucose (30 mM) groups (Fig. 1A). On the other hand, 30 mM glucose and 20 μM EA significantly reduced the cell count (Fig. 1B and 1C). These results showed that cell count and metabolism remained stable at low EA concentrations (5, 10 μM) (Fig. 1). Therefore, we chose 5 and 10 µM concentrations of EA for the rest of the study.

3.2. Ellagic acid increases NO levels in endothelial cells exposed to HG condition

Decreased endothelial production of NO is a critical factor to investigate atherosclerosis in diabetic patients [34]. To determine whether HG treatment decreases NO levels in endothelial cells, we measured nitrite levels in the supernatant of cultured cells by the Griess method. As shown in Fig. 2, the HG medium decreased the levels of nitrite which is the metabolite of NO. However, this alteration induced by HG was reversed by 10 µM of ellagic acid.

3.3. Ellagic acid improves redox status in HG-treated HUVECs

Oxidative stress plays a critical role in cellular injury due to hyperglycemia. Oxidative stress can induce lipid peroxidation and disrupt endogenous antioxidants in diabetes [31]. The effect of EA on lipid peroxidation of HUVECs was studied by measuring MDA levels. As shown in Fig. 3A, there was a significant increase in MDA levels in HUVECs treated with a high concentration of glucose. This effect was significantly inhibited by EA at 10 μM. The activity of SOD significantly increased in HG-treated groups (Fig. 3B). Treatment with 10µM of EA caused a further increase in SOD activity. On the other hand, catalase activity decreased due to HG treatment (Fig. 3C). The activity of this enzyme significantly increased in HUVECs treated with EA, regardless of glucose concentration. Consistent results were observed using FRAP assay as an indicator of the antioxidant capacity of cultured cells (Fig. 3D).

3.4. Ellagic acid upregulates the expression of *ENHO* and *NOS3* genes

Endothelial dysfunction is an early stage in the promotion of atherosclerosis, in which eNOS signaling is impaired [35]. Therefore, we determined the level of *ENHO* and *NOS3* transcripts by RT-qPCR. In HG-treated cells, the expression levels of mRNA for Adropin (encoded by *ENHO* gene) (Fig. 4A) and eNOS (encoded by *NOS3* gene) (Fig. 4B) significantly decreased. However, treatment of the cells with EA significantly increased these expression levels in both normal glucose- and HG-treated HUVECs.

4. Discussion

Endothelial dysfunction is an essential stage in the progression of cardiovascular implications in diabetes [13] which is triggered by endothelial cell dysfunction [8]. The generation of ROS as well as decreased NO production through endothelial cells impairs the function of blood vessels [36]. The present study demonstrated that HG levels can induce cell dysfunction through excessive cellular MDA, increased oxidative stress, and reduced NO generation subsequent to downregulation of adropin and eNOS in HUVECs. Ellagic acid prevented HG-mediated endothelial injury via scavenging ROS and upregulation of adropin and eNOS in HUVECs.

At the beginning of this research, we verified the effect of EA on the cell count and metabolism of HG-treated HUVECs. Although EA at 20 μM significantly decreased both parameters, they were not affected by lower concentrations of EA. High glucose concentration did not affect cell metabolism, but it slightly reduced the number of cells. These results were desirable since we could continue our study using lower concentrations of EA with minimal interference with cell count and metabolism.

The endothelium plays a critical role in preserving a balance between vasoconstrictor and vasodilator agents [14]. Endothelial dysfunction, characterized by impaired production of NO by endothelial cells, is the earliest event in the progression of cardiovascular disorders, such as diabetic cardiovascular disease, hypertension, and atherosclerosis [9]. In this research, we initially studied the effect of EA on the release of NO from HG-treated cells. We then pursued our studies to determine the possible mechanisms behind this effect.

In this study, the amount of NO significantly diminished as a result of HG concentration, and EA restored this decline in HUVECs. Consistently, a high concentration of glucose downregulated the expression of the eNOS gene, and EA reversed this effect. In agreement with our results, several studies have reported a decrease in NO level in HUVECs following 24 h or 48 h of HG-treatment [11, 37]. Similarly, both in vitro and in vivo models have revealed that eNOS expression is diminished due to high extracellular glucose concentration [11, 37, 38]. As far as the authors are aware, there is no report concerning the effect of ellagic acid on NO level or eNOS expression in HUVECs exposed to HG concentration. However, our group has previously shown that the anti-arrhythmic and cardioprotective effects of pomegranate, the main source of ellagic acid, are mainly mediated via NO [39, 40]. In another study, an EA-rich extract of Gunnera

tinctoria increased intracellular NO levels in HUVECs treated with a high extracellular concentration of glucose [21]. None of these studies addressed the status of expression or eNOS activity. Interestingly, Lee et al (2010) reported the potential clinical benefits of EA in the prevention of oxidized low-density lipoprotein (oxLDL)-associated atherogenic diseases [41]. Accordingly, EA has ameliorated oxLDL-impaired expression of eNOS in HUVECs via PI3K/Akt/eNOS signaling pathway [42]. Therefore, EA may be able to improve and augment PI3K/AKT/eNOS pathway which has been previously reduced by hyperglycemia.

A high level of glucose is associated with excess ROS generation which has an important role in endothelial dysfunction in diabetes [10, 11]. NADPH oxidases are the critical sources of ROS in vascular cells [13]. Reactive oxygen species reduce the ability of endothelial cells to produce NO via inhibition of eNOS [43]. In addition to reduced formation of NO, ROS decrease the level of NO via the formation of peroxynitrite. Nitric oxide may react with a superoxide anion to yield peroxynitrite. The resultant molecule is a highly reactive oxidant and nitrating agent. It leads to lipid peroxidation and damages vital biomolecules including proteins and nucleic acid and ends up in necrosis and apoptosis [44, 45]. In this study, excess glucose levels impaired the redox state of the cells and ellagic acid had a remarkable effect on improving the antioxidant capacity of the cultured cells. Consistently, the cell population and metabolism were adversely affected under HG conditions, and ellagic acid significantly reversed these effects. There is a large body of evidence regarding the overproduction of ROS in HG conditions. For instance, HUVECs incubated at 30 mM of glucose showed a significant rise in ROS generation as indicated by markers of oxidative stress [43, 46]. Ellagic acid, a polyphenol found in some fruits and nuts, performs a variety of biological activities including scavenging ROS [47]. In addition, it has an indirect effect on resisting oxidative stress by activating cellular antioxidant enzymes [27]. We did not find a similar study regarding the beneficial effects of EA on HG-treated HUVECs. However, the ellagic acid-enriched extract of G. tinctoria decreased cell death and the production of ROS [21]. Consistent results have been achieved using an in vivo model in which diabetic patients received oral supplements of EA [28]. Moreover, other natural antioxidants have been successfully used to ameliorate the oxidative damage in HUVECs exposed to excess glucose levels. As an example, resveratrol, a potent antioxidant found in red grapes, has improved the oxidant and antioxidant balance in HG-treated HUVECs [46].

Adropin is an important marker of endothelial dysfunction in patients with DM [19]. It increases the production of NO through the VEGFR2-extracellular signal-regulated kinase 1/2 and VEGFR2-phosphatidylinositol 3-kinase-Akt pathways, thereby regulating the bioavailability of NO [20]. Inadequate adropin downregulates eNOS [48] which may lead to endothelial dysfunction. We hypothesized that EA may increase NO via the upregulation of adropin. Our results indicate that a high level of glucose downregulates adropin, and EA upregulates the expression of this hormone regardless of the levels of glucose. To the best of our knowledge, the effects of HG condition or EA on adropin in HUVECs have not been reported in previous studies. However, clinical evidence has shown that the serum concentrations of adropin are declined in patients with Chinese type 2 diabetes [18] and children with type 1 diabetes [49].

To sum up, the present study revealed that exposure to HG might cause dysfunction in HUVECs via oxidative damage and reduced NO levels. EA may be a valuable therapeutic choice to minimize cell injury through reducing oxidative stress and increasing the expressions of adropin and eNOS genes.

5. Conclusion

This study is the first to examine the relationship between EA, the expression of *ENHO*, the level of glucose in the culture medium, and endothelial dysfunction. Our results suggest that low levels of *ENHO* expression in HG-treated HUVECs may lead to the promotion of endothelial cell dysfunction. This could also be used as a novel biomarker of endothelial dysfunction in T2DM. In addition, this study provides further evidence regarding the potential prophylactic role of EA against atherosclerosis. However, further studies are required to functionally investigate the underlying mechanisms.

Abbreviations

EA	ellagic Acid
HUVECs	human umbilical vein endothelial cells
DM	diabetes mellitus
CVDs	cardiovascular diseases
T2DM	type II diabetes
NO	nitric Oxide
ENHO gene	energy homeostasis associated gene
eNOS	endothelial nitric oxide synthesis
DMSO	dimethyl sulfoxide
ED	endothelial dysfunction

Declarations

Data availability statments

The datasets analyzed in the present study are available from the corresponding author on reasonable request.

Conflict of Interest: The authors have no conflict of interest to report.

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Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Somayeh Sheikh], [Hesam Dehghani] and [Hamid Reza Kazerani]. [Somayeh Sheikh] performed experiments. The first draft of the manuscript was written by [Somayeh Sheikh]. All authors read and approved the final manuscript.

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Figures

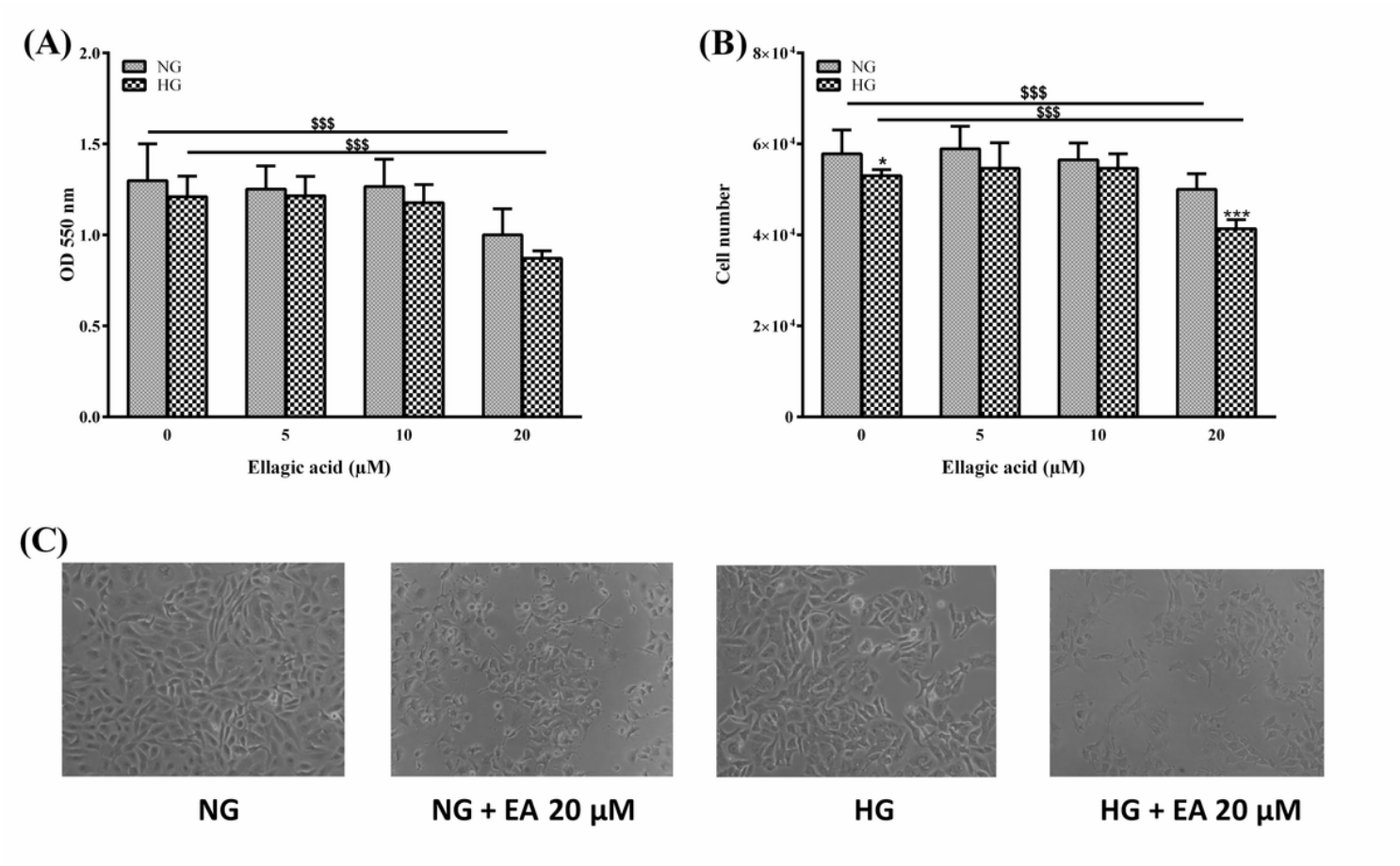

Figure 1

The effect of ellagic acid on MTT cell metabolism (A), cell count (B), and morphology (C) of human umbilical vein endothelial cells. The cells were incubated under normal glucose (NG) or high glucose (HG) concentrations for 48 h. Data are represented as mean + SD (n = 10). The asterisks indicate statistical significance compared to the adjacent normal glucose group (*: $p<0.05$, ***: $p<0.001$). $$$: $p<0.001$.

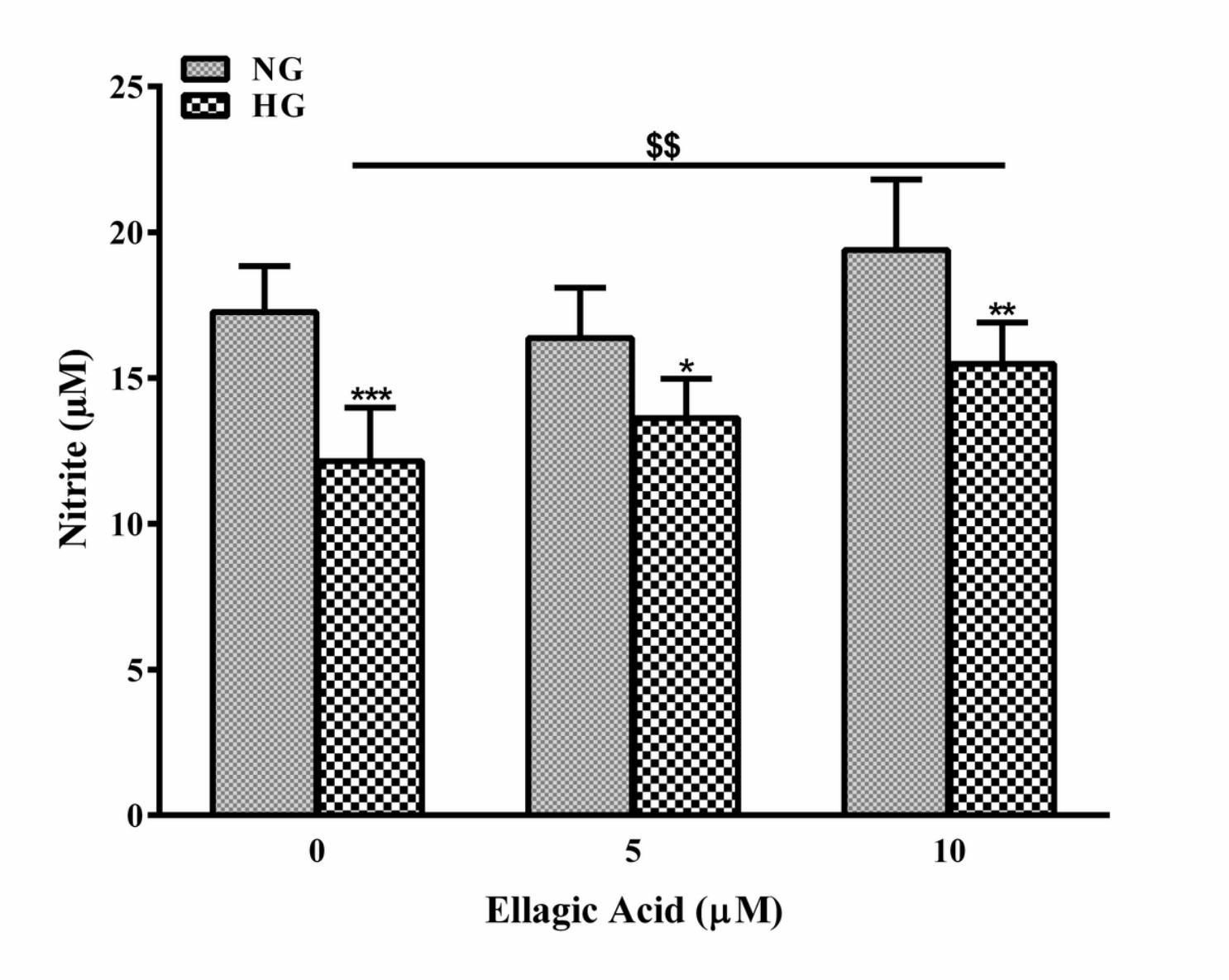

Figure 2

The effect of ellagic acid on the nitrite level of human umbilical vein endothelial cells. The cells were cultured using normal glucose (NG) or high glucose (HG) concentrations for 48 h. Data are shown as mean + SD (n = 6). The asterisks indicate statistical difference compared to the bordering normal glucose group (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). $$: $p<0.01$.

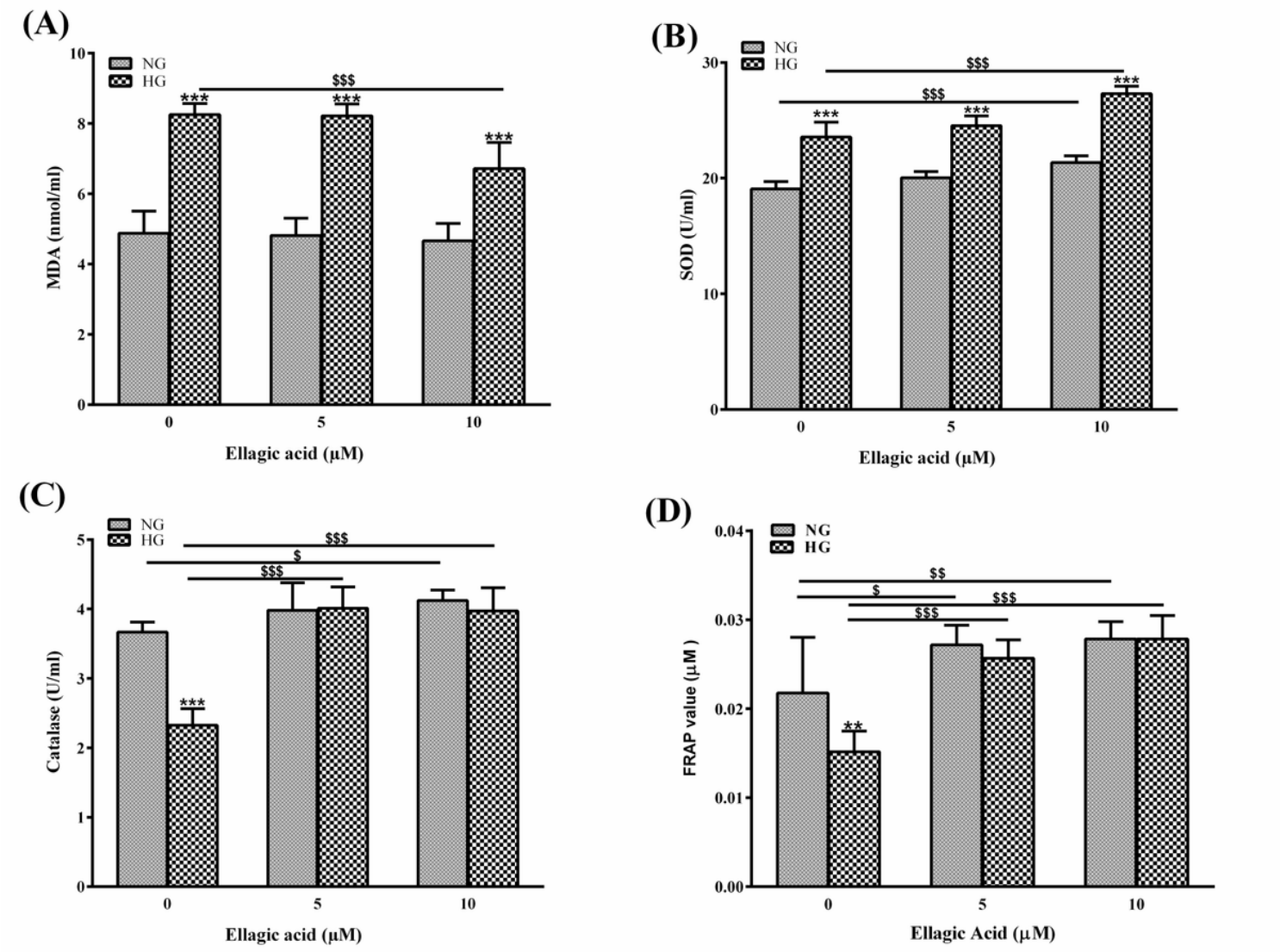

Figure 3

The effect of ellagic acid on redox status of human umbilical vein endothelial cells. The cells were exposed to normal glucose (NG) or high glucose (HG) concentrations for 48 h. A: the level of malondialdehyde (MDA) as biomarker of lipid peroxidation, B: superoxide dismutase (SOD) activity, C: catalase activity, D: ferric reducing antioxidant power (FRAP). Data are represented as mean + SD. The asterisks show significant difference compared to adjacent normal glucose group (**: p<0.01, ***: p<0.001). $: p<0.05,

$$: p < 0.01,$$

$: p<0.001.

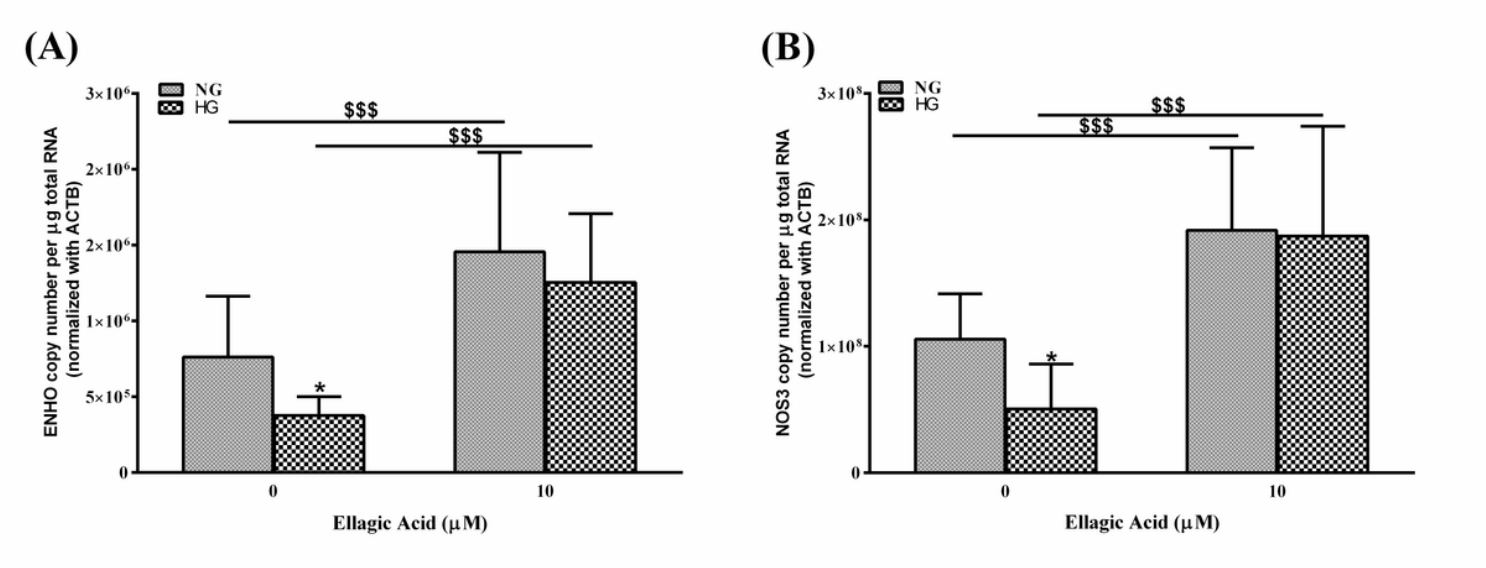

Figure 4

The effect of ellagic acid on the gene expression of *ENHO* (A) and *NOS3* (B). Human umbilical vein endothelial cells were incubated under normal glucose (NG) or high glucose (HG) conditions for 48 h. Data are shown as mean + SD. The asterisks indicate significant differences with the contiguous normal glucose group (*: $p<0.05$). $$$: $p<0.001$.