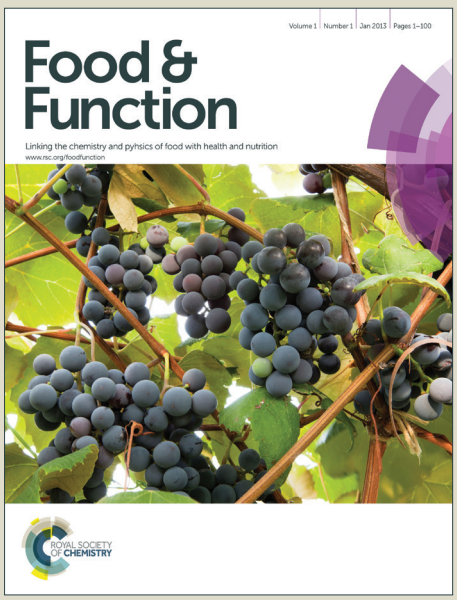
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

δ-Tocopherol prevents methylglyoxal-induced apoptosis by reducing ROS generation and inhibiting apoptotic signaling cascades in human umbilical vein endothelial cells

Moon ho Do[a], Su nam Kim[b], Seung-Yong Seo[a], Eui-Ju Yeo [c], and Sun Yeou Kim[a, d, e *]

[a]College of Pharmacy, Gachon University, Incheon, Republic of Korea

[b]Natural Medicine Center, Korea Institute of Science and Technology, Gangneung 210-340, Republic of Korea

[c]Department of Biochemistry, Gachon University School of Medicine, Incheon, Republic of Korea

[d]Gachon Institute of Pharmaceutical Science, Gachon University, Incheon, Republic of Korea

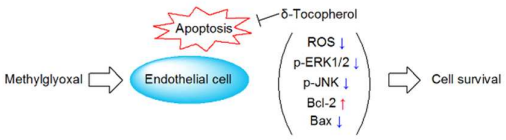
[e]Gachon Medical Research Institute, Gil Medical Center, Inchon, Republic of Korea

*Correspondence: Sun Yeou Kim

College of Pharmacy, Gachon University, #191, Hambakmoero, Yeonsu-gu, Incheon 406-799, Republic of Korea.

Tel: +82-32-899-6411, Fax: +82-32-899-8962, E-mail: sunnykim@gachon.ac.kr

Table of Contents



δ-Tocopherol protects HUVECs against apoptotic activity induced by methylglyoxal.

Abstract

Methylglyoxal (MGO) is a highly reactive metabolite of glucose, which is known to cause damage and induce apoptosis in endothelial cells. Endothelial cell damage is implicated in the progression of diabetes-associated complications and atherosclerosis. Nuts are high in vitamin E. Consumption of nuts has been recommended for the prevention of cardiovascular disease. However, different nuts contain different forms of vitamin E, which can have different effects on endothelial cells. In this work, we investigated the protective effect of different isoforms of vitamin E on MGO-induced apoptosis in human umbilical vein endothelial cells (HUVECs). Among all forms of vitamin E, δ-tocopherol showed the highest effect on apoptosis of HUVECs. We also compared the anti-apoptotic activity of δ-tocopherol to that of α-tocopherol in MGO-treated HUVECs. Pretreatment with α- or δ-tocopherol significantly inhibited MGO-induced changes in cell morphology, cell death, and production of intracellular reactive oxygen species. δ-Tocopherol prevented MGO-induced apoptosis in HUVECs by increasing Bcl-2 expression and decreasing Bax expression. Interestingly, α-tocopherol also inhibited these factors but to a lesser extent than δ-tocopherol. MGO was found to activate mitogen-activated protein kinases (MAPKs). Compared to pretreatment with α-tocopherol, pretreatment with δ-tocopherol more strongly inhibited the activation of MAPKs, such as JNK and ERK1/2. These findings suggest that δ-tocopherol may be a more effective regulator of MGO-induced apoptosis than α-tocopherol.

Keywords : Advanced glycation end products, Methylglyoxal, HUVECs, Tocopherol, Reactive oxidative species, Apoptosis

1. Introduction

Advanced glycation end-products (AGEs) are automatically generated by a non-enzymatic reaction between the reducing sugars and free amine groups of proteins. The formation and accumulation of AGEs has been known to occur at an accelerated rate in diabetes patients, and their role in endothelial dysfunction is now well-known [1]. In endothelial cells, AGEs cause mitochondrial dysfunction, cellular dysfunction and, ultimately, cell death [2]. AGEs also increase the production of pro-inflammatory mediators and the generation of reactive oxygen species (ROS) [3]. AGEs have been reported to activate mitogen-activated protein kinase (MAPK) pathways such as c-Jun N terminal kinase (JNK) and p38 [4].

Methylglyoxal (MGO) is a highly reactive metabolite of glucose and a precursor to AGEs. It is formed by the non-enzymatic fragmentation of triose phosphates or products of the Amadori rearrangement [5]. Increased MGO levels have been observed in vascular endothelial cells that were cultured in media with a high glucose content [6]. MGO levels are particularly high in patients with either type 1 or type 2 diabetes [7]. MGO mediates the inflammation and apoptosis of vascular endothelial cells, the generation of ROS and impairs endothelial function [8, 9]. MGO has also been reported to induce phosphorylation of JNK, p38 MAPKs and extracellular signal-regulated kinase (ERK1/2) [10].

For the past few years, there has been an increased focus on the role of vitamin E in preventing chronic damage to endothelial cells–a widely known cause of cardiovascular disease [11]. It has also been reported that the consumption of nuts that are rich in vitamin E may prevent cardiovascular disease [12]. Nuts contain various

forms of vitamin E and their relative proportions depend on the types of nuts. For example, almonds and hazelnuts contain large amounts of α-tocopherol whereas pistachios and walnuts contain a greater proportion of γ- and δ-tocopherols [13].

Vitamin E is a potent antioxidant and has eight different forms: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols. It has been reported that vitamin E prevents protein glycation *in vitro* by inhibiting the formation of the lipid peroxidation product malondialdehyde and that its antioxidant nature reduces AGE-mediated apoptosis [14, 15]. Furthermore, recent studies showed that α-tocopherol reduced MGO-induced oxidative stress in human umbilical vein endothelial cells (HUVECs) and that γ-tocopherol prevented serum MGO increases in patients with diabetes [16, 17]. MGO and AGEs are known to be reduced by α- or γ-vitamin E form; however the effect of δ-tocopherol on MGO has yet to be reported. This study may be vital in proving it to be an effective and potent limiter of MGO and AGEs.

Although δ-tocopherol is not the predominant form of vitamin E, its important roles in cell function are widely known. Recently, the antioxidant and anti-inflammatory activities of γ- and δ-tocopherols are superior to those of α-tocopherol [18]. δ-Tocopherol is found in various plant seeds and mushrooms such as *Canavalia gladiata* and *Cordyceps militaris* [19, 20]. *Cordyceps militaris* is itself recognized as the most common edible, medically beneficial mushroom. Chu H-L *et al*. reported that *Cordyceps militaris* has a protective effect on oxidative stress induced by increased glucose in HUVECs [21]. δ-Tocopherol is the only form of vitamin E detected in *Cordyceps militaris* [20]. δ-Tocopherol is a potent antioxidant with anti-inflammatory activity [22]. Onshima Y *et al.* reported that low level of δ-tocopherol is associated with deep white

matter lesions in women [23] and that δ-tocopherol quenches peroxyl radicals more efficiently than α-tocopherol [24].

Based on these observations, we hypothesized that δ-tocopherol may be more active than α-tocopherol in reducing MGO-induced apoptosis in HUVECs. In this study, we investigated the anti-apoptotic effects of δ-tocopherol on HUVECs, compared its inhibitory activity with that of α-tocopherol and related this to its effects on the MAPK signaling pathways.

2. Materials and Methods

2.1. Materials

MGO, RRR-α-tocopherol, rac-β-tocopherol, (+)-δ-tocopherol, tubulin, and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). (+)-γ-Tocopherol was perchased from Acros Organics (Morris Plains, NJ, USA). EGM-2 medium was obtained from Lonza (Walkersville, MD, USA). p38, phospho-p38 (P-p38), ERK1/2, phospho-ERK1/2 (P-ERK1/2), JNK and phospho-JNK (P-JNK) were obtained from Cell Signaling Technology (Danvers, Ma, USA). Bcl-2 and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Walnuts were purchased from Agriculture, Forestry and Farming Association Gapyeong (Gapyeong, Korea), pistachios were purchased from NUTSVILLE (Seoul, Korea), hazelnuts were purchased from garunara (Seoul, Korea) and almonds were purchased from raonorganic (Gimpo, Korea).

2.2. Preparation of tocopherols standards and nut extracts

The standard stock solutions of tocopherols were prepared by dissolving 1 mg of each compound in 1 ml acetone and stored at −20 °C. Walnuts, pistachios, hazelnuts and almonds

were extracted at 50 g in 100 ml acetone and then treated in an ultrasonic bath for 2 h. Afterwards the extract was filtered and evaporated. The extract was dissolved in acetone at a concentration of 10 mg/ml.

2.3. Cell culture

HUVECs were purchased from the American Type Culture Collection (Lot # 60319874, ATCC, VA, USA). HUVECs were cultured in EGM-2 supplemented with 2% FBS. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. The passage number of all the cells used was between 5 and 8.

2.4. Cell viability analysis and morphological examination

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HUVECs were seeded at 1×10^5 cells/well in 24-well plates and incubated for 24 h at 37 °C. The cells were then pretreated with testing materials including tocopherols and nut extracts for 1 h, followed by MGO treatment for 24 h. MTT solution was added with a final concentration of 0.1 mg/ml. This was followed by a 2-h incubation in the CO_2 incubator at 37 °C. The medium was gently removed and the reduced MTT was dissolved in 200 μl/well dimethyl sulfoxide. The absorbance at 570 nm was determined using a microplate reader (Molecular Devices, CA, USA). The morphological changes in the HUVECs were observed with an IncuCyte ZOOM imaging system (Essen Bioscience, MI, USA).

2.5. Western blotting

Changes in the levels of proteins related to MAPKs and apoptosis in the HUVECs were

evaluated with Western blotting experiments. After harvesting, cells were homogenized and lysed in a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. They were then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was collected and assayed for protein concentration using the Bradford assay. Equal amounts of proteins were resolved on SDS-PAGE and transferred to a nitrocellulose membrane. The blots were blocked with an aqueous solution of skimmed milk powder for 1 h at room temperature and then probed with the primary antibodies against tubulin, p38, P-p38, ERK1/2, P-ERK1/2, JNK, P-JNK, Bcl-2 and Bax overnight at 4 °C. Proteins were detected using a ChemiDoc XRS+ imaging system (Bio-Rad, CA, USA).

2.6. Cell apoptosis assay

To determine the effect of δ-tocopherol on MGO-induced apoptosis in HUVECs, an annexin V apoptosis detection kit (Santa Cruz Biotechnology, CA, USA) was used. Briefly, 3.0×10^5 cells were seeded in a 6-well plate and incubated overnight at 37 °C. The cells were then treated with MGO and α- or δ- tocopherol for 24 h. Afterwards, cells were washed with PBS and resuspended in binding buffer with annexin V-FITC and propidium iodide (PI) at room temperature for 15 min and then analyzed by flow cytometry (FACSCalibur flow cytometer; Becton Dickinson, San Jose, CA).

2.7. Detection of intracellular ROS

Cells were seeded in a 12-well plate and incubated overnight at 37 °C. After 24 h, cells were pretreated with α- or δ- tocopherol for 30 min, followed by MGO treatment for 60 min. Cells were washed with PBS and EGM-2 media after which 20 μM DCF-DA was added. The cells were then incubated for 30 min at 37 °C before being washed with PBS. Cells were

photographed using a JuLI live-cell imaging system (NanoEnTek, Seoul, Korea).

2.8. HPLC analysis

Analysis was carried out on a Waters system (Waters Corp., Milford, MA, USA), consisting of separation module (e2695) with a photodiode array detector (2998). UV absorbance was monitored from 200 to 400 nm. Qualitative analysis was carried out by 292 nm and Column temperature was maintained at 30 ℃. Separation was carried out using an INNO column (150×4.6 mm; particle size, 5 μm; Young Jin Biochrom, Seongnam, korea) with methanol/water (92/8, v/v) as the mobile phase. The flow rate was 1 ml/min.

2.9. Statistical analysis

Values are given as mean ± S.D. Statistical analysis of results was performed using one-way ANOVA followed by Bonferroni's test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of tocopherols on MGO-induced reduction of cell viability in HUVECs

The structures of tocopherols are shown in Fig. 1A. Although α-tocopherol was shown to reduce MGO-induced oxidative stress in HUVECs[16]. The effect of δ-tocopherol on MGO-induced oxidative stress and cell death has yet to be reported. Therefore, we first investigated the morphological changes that took place in HUVECs after treatment with tocopherols and MGO. As shown in Fig. 1B, MGO treatment led to an apparent reduction in cell density, a loss of confluence and an increase in the number

of floating cell fragments; treatment with tocopherols reduced these morphological changes. The effect of tocopherols on the cell viability of HUVECs was investigated using an MTT assay. HUVECs were pretreated with 50 μM α-, β-, γ- and δ-tocopherols for 1 h and then exposed to 500 μM MGO for 24 h. The cell viability of HUVECs was reduced markedly after MGO treatment although this effect was ameliorated by pretreatment with α- and δ-tocopherols (Fig. 1C). Moreover, pretreatment with α- or δ-tocopherol (10–100 μM) increased cell viability in a dose-dependent manner (Fig. 1D).

3.2. Effects of δ-tocopherol on MGO-induced apoptosis in HUVECs

To examine whether MGO-induced cell death is related to apoptosis, we used a FACS analysis based on annexin V-FITC and PI double staining. As shown in Fig. 2, treatment of the HUVECs with MGO led to an increase in the number of early apoptotic and late apoptotic cells. However, this increase in early apoptotic and late apoptotic activity induced by MGO was decreased by pretreatment of HUVECs with α-tocopherol. The anti-apoptotic effect of δ-tocopherol was similar to that of α-tocopherol.

3.3. Effects of δ-tocopherol on the levels of Bax and Bcl-2

We used western blotting to investigate whether MGO could affect the level of Bcl-2 and Bax proteins in HUVECs. As shown in Fig. 3, cells treated with MGO showed lower Bcl-2 protein level and higher Bax protein level than did the control cells. Treatment with α- and δ-tocopherols had a reverse effect: the level of Bax decreased, whereas that of Bcl-2 increased.

3.4. Effects of δ-tocopherol on MGO-induced ROS generation

It is known that an increased level of intracellular ROS may induce apoptosis. With this in mind, we examined whether an increased formation of ROS is associated with MGO-induced apoptosis in HUVECs after DCF-DA staining and observation using a JuLI live-cell imaging system. We also assessed the antioxidative effect of α- and δ-tocopherols in MGO-induced apoptosis. As shown in Fig. 4, MGO treatment for 1 h significantly increased the level of intracellular ROS in HUVECs and pretreatment with α- or δ-tocopherol for 30 min significantly decreased ROS generation.

3.5. Effects of δ-tocopherol on MAPK activation

Phosphorylation-induced activation of MAPK is a vital step in the process of MGO-induced apoptosis. As shown in Fig. 5, the phosphorylation of JNK, ERK1/2 and p38 in MGO-treated HUVECs was observed by western blot analysis using antibodies against JNK, P-JNK, ERK1/2, P-ERK1/2, p38, P-p38, and tubulin as an internal control. Treatment of HUVECs with MGO caused an increase in the phosphorylation of p38, ERK1/2 and JNK. Pretreatment with α- and δ-tocopherols blocked the phosphorylation of ERK1/2 and JNK. However, α- and δ-tocopherols made no significant difference in the extent of the phosphorylation of p38 protein.

3.6. Contents of tocopherols in different forms of nuts

Using the RP-HPLC, we confirmed that nuts contained tocopherols. RP-HPLC methods are generally not considered to separate β- and γ-tocopherols [39]. Therefore, the content of β- and γ-tocopherols was totally calculated. Fig. 6A shows a chromatogram obtained for a standard mixture of tocopherols. The linearity of each tocopherol was calculated based on the concentrations in three peaks: a-c. The tocopherol content in different forms of nuts is shown

237 in Table 1. This result shows that α-tocopherol is the main form in almonds and hazelnuts,
238 whereas β- and γ-tocopherols are the main forms in pistachios. δ-tocopherol is mainly found
239 in walnuts at the highest concentration compared to other types of nuts.
240
241 3.7. Effect of different forms of nuts on MGO-induced cell death
242 The effect of nuts on the cell viability of HUVECs was investigated using an MTT assay.
243 HUVECs were pretreated with 50 μg/mL nut extracts for 1 h and then exposed to 500 μM
244 MGO for 24 h. The cell viability of HUVECs was increased markedly by pretreatment with
245 all nut extracts (Fig. 7). This result shows that walnuts extract is the most effective on MGO-
246 induced cell death, compared to the extracts from other forms of nuts.
247
248 **4. Discussion**
249 MGO is known to be a highly reactive metabolite of glucose, and it induces cellular injury
250 and apoptosis in endothelial cells. In this study, we confirmed that δ-tocopherol, one of the
251 vitamin E forms, could protect against MGO-induced apoptosis and oxidative damage. To
252 compare the antiapoptotic effect of α-, β-, γ- and δ-tocopherols, we pretreated cells with each
253 tocopherol at the concentration of 50 μM for 1 h. We found that, out of all the forms, δ-
254 tocopherol had the highest effect on MGO-induced morphological changes and cell death
255 (Fig. 1B, C). We carried out the experiments using different concentrations of tocopherols
256 and MGO in the same experimental methods (Fig 1D, S1). And we also compared the
257 potency of δ-tocopherol and α-tocopherol on cells pretreated with MGO for 1 h (Fig S2). As
258 expected, these results also showed that δ-tocopherol was more effective than α-tocopherol
259 even at lower concentrations and reduced MGO-induced cell death to a statistically
260 significant extent (Fig. 1D). Tocopherols and MGO Cotreatment reduced MGO-induced

apoptosis, came up similar results. However, post-treatment of tocopherols in MGO-treated HUVEC cells did not show any effect on their survival (Fig S2). Since serum free media was used for the experiments, cell survival lasted around 24hrs. Since then, cell death was induced. So, cell survival data could not be obtained after treatment longer than 24 hours (data not shown).

Difference between the potential of α-tocopherol and δ-tocopherol at 50uM concentration on cell viability is clear in the Fig. 1D and S1 where statistical analysis shows that for α-tocopherol, $p>0.01$ whereas for δ-tocopherol $p>0.001$. This finding was supported by the data shown in the Fig. 5 where western blot analysis for MAPK indicates δ-tocopherol as more potent protective molecule against MGO induced damage.

Annexin V-FITC/PI double staining indicated that MGO treatment increased apoptosis in HUVECs and that α- and δ-tocopherols protected MGO-induced apoptotic cells (Fig. 2). We observed that δ-tocopherol is more effective in early apoptosis than α-tocopherol. Although tocopherols are related to both the early and late stages of apoptosis, it seems that δ-tocopherol plays a crucial role in the early stages of apoptosis.

Bcl-2 and Bax proteins have been shown to play an important role in the modulation of cell apoptosis [25]. Pretreatment with α- or δ-tocopherol inhibited MGO-induced apoptosis by decreasing the level of Bax and by increasing the level of Bcl-2, respectively (Fig. 3B, C). δ-tocopherol showed higher activity than α-tocopherol in modulating the level of Bcl-2 and Bax. These data support the fact that δ-tocopherol is more potent than α-tocopherol in HUVECs.

Several studies have reported that MGO can increase the expression of ROS generation, and ROS generation may play a role in AGE-RAGE formation [26, 27]. To

investigate this further, we endeavored to find out whether δ-tocopherol reduces MGO-induced ROS generation. Using DCF-DA, we detected an increase in the generation of intracellular ROS in MGO-treated cells. This data is in agreement with previous studies showing increased ROS generation concurrent with MGO-induced apoptosis [8, 9, 28]. In our results, ROS generation was reduced by treating the cells with α- or δ-tocopherol (Fig. 4). We therefore propose that pretreatment of δ-tocopherol could inhibit ROS generation in MGO-treated HUVECs. Inhibition of ROS generation in MGO-treated HUVECs by δ-tocopherol is superior to that achieved by α-tocopherol. Structurally, α-tocopherol contains three methyl groups on position 5, 7 and 8, whereas δ-tocopherol contains only one methyl group on position 8. The superiority of δ-form might be due to two substituents on *ortho*-positions of the chromanol nucleus as the reaction rate and oxidation mechanism are considered to depend on the number of methyl groups on the nucleus [29, 30]. However, this discussion may not be definitive and call for more research.

MAPKs play a major role in cell differentiation and cell apoptosis [31, 32]. ERK1/2, JNK and p38 are major proteins in MAPK group. Although ERK1/2 is related to proliferation and cell progression in certain cell systems [33], JNK and p38, as well as ERK1/2 are also implicated in apoptosis [34-36]. In this study, we observed that, among all MGO-activated MAPKs, pretreatment with α- or δ-tocopherol most dramatically inhibited the activation of JNK and ERK1/2 (Fig. 5B, C). The inhibition of apoptosis by δ-tocopherol was accompanied by the inhibition of MAPK activation suggesting that δ-tocopherol could modulate the MAPK signaling pathways in MGO-treated HUVECs. The MAPKs can be activated independently and they are involved in apoptosis. Several studies in recent years have also suggested that MGO-induced cytotoxicity is associated with the activation of members of the

MAPK family, including JNK, p38, and ERK1/2 [37]. The results of the present study found that, in HUVECs, δ-tocopherol significantly decreased the activation of JNK and ERK1/2, but not that of p38. Moreover, δ-tocopherol was more effective in inhibiting the activation of JNK and ERK1/2 than α-tocopherol. These data thus support the fact that δ-tocopherol is more potent than α-tocopherol in HUVECs.

In recent studies vitamin E have been reported to regulate many processes including inflammation, carcinogenesis, and the antioxidant pathways [38]. In addition, numerous studies have demonstrated that tocopherols can be either antiapoptotic or proapoptotic, depending upon cell types. Dimethyl and monomethyl tocopherols might be a pro-apoptotic [39-41]. Susan *et al.* reported that γ-tocotrienol upregulated the expression of anti-apoptotic genes to promote intestinal cell survival [42]. Our data also showed that HUVECs treated for 24 h with α- and δ-tocopherols alone did not cause cell death (Fig. S1). However, pretreatment with α- and δ-tocopherols prevents MGO-induced apoptosis by inhibiting apoptotic signaling cascades (Figs. 3 and 5).

Elisia *et al.* showed that δ-tocopherol induced inflammation by modulating NF-κB and Nrf2, an oxidative stress response in FHs 74 Int cell line [43]. However, Li *et al.* demonstrated that δ-tocopherol showed antioxidant and anti-inflammatory activities[44]. In the present study, it was observed that δ-tocopherol reduced oxidative stress in HUVECs (Fig. 4). The results suggest that δ-tocopherol might function as antioxidant at least in certain cell types.

Although the concentrations of tocopherols are, considering the concentrations of cultured cells, much higher than physiological conditions. In our research, the protective effects of the vitamin E on the MGO-toxicity were investigated at various concentrations. A high concentration of MGO was used to induce cellular injury and cytoxicity, including

apoptosis in HUVECs screening models. The screening system we used involved high concentration of MGO therefore a higher concentration of sample is needed for recovery of cells. As a result, our results indicated that only a highly concentration of vitamin E significantly protects cell toxicity in HUVECs. Therefore, it would seem that higher concentration of vitamin E is needed for recovery of cells by MGO-induced toxicity. In addition, some similar previous studies reported that vitamin E played a role in cell protection particularly with high concentration [45, 46, 47]. Again our study is to confirm that Vitamin E is not a drug candidate rather it is a nutrient found in food material and being consumed for long times therefore optimal time period and frequency of its treatment to the cells.

Of the eight forms of vitamin E, only α-tocopherol is used clinically as a human dietary supplement alongside the other dietary antioxidants found in fruits, vegetables, and nuts [48]. Cardiovascular disease can be prevented by consuming nuts. And it is thought that nuts are used as resources as bioavailable antioxidants such as tocopherols, directly gives cardioprotective effect [49]. Since increased vitamin E intake is related to a reduced risk of heart disease and hypertension [50], it seems prudent for the population to increase their consumption of foods rich in vitamin E. In the present study, we confirmed that different forms of nuts have different amounts of vitamin E. Although walnuts contains high ratio of β- and γ-tocopherols among tocopherol types (Table 1) but our cell viability assay data suggest that the most effective component of the walnuts may be δ-tocopherol because cell viability is the highest in δ-tocopherol-treated group (Fig. 1). There's also the possibility that tocopherols can coexist as the optimum ratio in walnuts. Under our present data, we are forced to propose that positive effect of walnuts in cell survival may be due to δ-tocopherol.

Li *et al.* reported that δ-Tocopherol is a powerful antioxidant and more active than α-, γ-tocopherol in *in vivo* models [51]. Our results indicate that δ-tocopherol is the most beneficial

form of vitamin E. It can be surmised that increased consumption of walnuts would be an effective means of preventing cardiovascular diseases. Sesso *et al.* has reported that vitamin E might have no effect on the prevention of cardiovascular disease in aged men [52]. Also, α-tocopherol may be implicated in the reduction in the levels of other forms of vitamin E, especially γ-and δ-tocopherols, in serum [53]. When all these facts are considered, we can expect that in the future the use of walnut as a dietary resource for δ-tocopherol will be increased. More research is needed, however, to determine the protective effects of crude extract of walnuts on MGO-induced apoptosis in HUVEC cells.

5. Conclusion

δ-Tocopherol plays a protective role in HUVECs by reducing MGO-induced apoptosis. We also observed that δ-tocopherol could prevent MGO-induced apoptosis in HUVECs by reducing ROS generation and the downstream apoptotic signaling cascades associated with ROS generation. Interestingly, δ-tocopherol was found to be more active than α-tocopherol in preventing MGO-induced apoptosis in HUVECs. Although this data is promising, *in vivo* investigations are also required.

Acknowledgement

This research was supported by the Bio & Medical Technology Development Program of the NRF funded by the Korean government, MSIP (NRF-2014M3A9B6069338)

Reference

1. N. Ahmed, *Diabetes Res Clin Pract*, 2005, **67**, 3-21.
2. B. Y. Li, X. L. Li, Q. Cai, H. Q. Gao, M. Cheng, J. H. Zhang, J. F. Wang, F. Yu and R. H. Zhou, *Apoptosis : an international journal on programmed cell death*, 2011, **16**, 732-745.
3. A. Goldin, J. A. Beckman, A. M. Schmidt and M. A. Creager, *Circulation*, 2006, **114**, 597-605.
4. M. Alikhani, C. M. Maclellan, M. Raptis, S. Vora, P. C. Trackman and D. T. Graves, *American journal of physiology. Cell physiology*, 2007, **292**, C850-856.
5. M. Takeuchi, Z. Makita, R. Bucala, T. Suzuki, T. Koike and Y. Kameda, *Mol Med*, 2000, **6**, 114-125.
6. M. Mukohda, M. Okada, Y. Hara and H. Yamawaki, *J Pharmacol Sci*, 2012, **118**, 303-310.
7. N. M. Hanssen, C. D. Stehouwer and C. G. Schalkwijk, *Biochem Soc Trans*, 2014, **42**, 443-449.
8. M. G. Rosca, T. G. Mustata, M. T. Kinter, A. M. Ozdemir, T. S. Kern, L. I. Szweda, M. Brownlee, V. M. Monnier and M. F. Weiss, *Am J Physiol Renal Physiol*, 2005, **289**, F420-430.
9. J. Kim, J. W. Son, J. A. Lee, Y. S. Oh and S. H. Shinn, *Journal of Korean medical science*, 2004, **19**, 95-100.
10. H. Yamawaki, K. Saito, M. Okada and Y. Hara, *American journal of physiology. Cell physiology*, 2008, **295**, C1510-1517.
11. Z. Cordero, D. Drogan, C. Weikert and H. Boeing, *Critical reviews in food science and nutrition*, 2010, **50**, 420-440.
12. C. E. O'Neil, D. R. Keast, T. A. Nicklas and V. L. Fulgoni, 3rd, *Journal of the*

American College of Nutrition, 2011, **30**, 502-510.

13. S. Lux, D. Scharlau, W. Schlormann, M. Birringer and M. Glei, *The British journal of nutrition*, 2012, **108**, 1177-1186.

14. S. K. Jain and M. Palmer, *Free radical biology & medicine*, 1997, **22**, 593-596.

15. J. C. Mamputu and G. Renier, *J Leukoc Biol*, 2004, **75**, 1062-1069.

16. C. Masterjohn, E. Mah, Y. Guo, S. I. Koo and R. S. Bruno, *The Journal of nutritional biochemistry*, 2012, **23**, 292-298.

17. T.-Y. Hu, C.-L. Liu, J.-Y. Chen and M.-L. Hu, *Journal of Functional Foods*, 2013, **5**, 745-754.

18. Q. Jiang, *Free radical biology & medicine*, 2014, **72**, 76-90.

19. H. Yoshida, N. Yoshida, I. Kuriyama, Y. Tomiyama-Sakamoto and Y. Mizushina, *Food Chemistry*, 2013, **136**, 807-812.

20. F. S. Reis, L. Barros, R. C. Calhelha, A. Ciric, L. J. van Griensven, M. Sokovic and I. C. Ferreira, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2013, **62**, 91-98.

21. H.-L. Chu, J.-C. Chien and P.-D. Duh, *Food Chemistry*, 2011, **129**, 871-876.

22. Q. Jiang, *Free Radical Biology and Medicine*, 2014, **72**, 76-90.

23. Y. Ohshima, T. Mizuno, K. Yamada, S. Matsumoto, Y. Nagakane, M. Kondo, N. Kuriyama, T. Miyazaki, K. Takeda, T. Nishimura, M. Nakagawa, K. Ozasa and Y. Watanabe, *J Nutr Health Aging*, 2013, **17**, 456-460.

24. V. P. Singh, J. F. Poon and L. Engman, *Organic letters*, 2013, **15**, 6274-6277.

25. F. Li, G. Ambrosini, E. Y. Chu, J. Plescia, S. Tognin, P. C. Marchisio and D. C. Altieri, *Nature*, 1998, **396**, 580-584.

26. W. H. Chan and H. J. Wu, *Journal of cellular biochemistry*, 2008, **103**, 1144-1157.

27. H. Ha and H. B. Lee, *Current diabetes reports*, 2001, **1**, 282-287.

28. S. Phalitakul, M. Okada, Y. Hara and H. Yamawaki, *Acta physiologica (Oxford, England)*, 2013, **209**, 212-219.

29. A. Kamal-Eldin and L. A. Appelqvist, *Lipids*, 1996, **31**, 671-701.

30. L. Packer, S. U. Weber and G. Rimbach, *The Journal of nutrition*, 2001, **131**, 369s-373s.

31. J. Ravindran, N. Gupta, M. Agrawal, A. S. Bala Bhaskar and P. V. Lakshmana Rao, *Apoptosis : an international journal on programmed cell death*, 2011, **16**, 145-161.

32. R. Seger and E. G. Krebs, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 1995, **9**, 726-735.

33. P. Lenormand, G. Pages, C. Sardet, G. L'Allemain, S. Meloche and J. Pouyssegur, *Advances in second messenger and phosphoprotein research*, 1993, **28**, 237-244.

34. R. J. Davis, *Cell*, 2000, **103**, 239-252.

35. A. Y. Choi, J. H. Choi, H. Yoon, K. Y. Hwang, M. H. Noh, W. Choe, K. S. Yoon, J. Ha, E. J. Yeo and I. Kang, *European journal of pharmacology*, 2011, **668**, 115-126.

36. G. Yang, W. Yang, L. Wu and R. Wang, *The Journal of biological chemistry*, 2007, **282**, 16567-16576.

37. A. A. Akhand, K. Hossain, H. Mitsui, M. Kato, T. Miyata, R. Inagi, J. Du, K. Takeda, Y. Kawamoto, H. Suzuki, K. Kurokawa and I. Nakashima, *Free radical biology & medicine*, 2001, **31**, 20-30.

38. F. Galli and A. Azzi, *BioFactors (Oxford, England)*, 2010, **36**, 33-42.

39. V. Viola, S. Ciffolilli, S. Legnaioli, M. Piroddi, M. Betti, F. Mazzini, E. Pierpaoli, M. Provinciali and F. Galli, *BioFactors (Oxford, England)*, 2013, **39**, 485-493.

40. M. Betti, A. Minelli, B. Canonico, P. Castaldo, S. Magi, M. C. Aisa, M. Piroddi, V. Di

Tomaso and F. Galli, *Free radical biology & medicine*, 2006, **41**, 464-472.

41. H. Huang, Y. He, X. X. Cui, S. Goodin, H. Wang, Z. Y. Du, D. Li, K. Zhang, A. N. Tony Kong, R. S. DiPaola, C. S. Yang, A. H. Conney and X. Zheng, *Journal of agricultural and food chemistry*, 2014, **62**, 10752-10758.

42. S. Suman, K. Datta, K. Chakraborty, S. S. Kulkarni, K. Doiron, A. J. Fornace, Jr., K. Sree Kumar, M. Hauer-Jensen and S. P. Ghosh, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2013, **60**, 488-496.

43. I. Elisia and D. D. Kitts, *American journal of physiology. Gastrointestinal and liver physiology*, 2013, **305**, G940-949.

44. G. Li, M.-J. Lee, A. B. Liu, Z. Yang, Y. Lin, W. J. Shih and C. S. Yang, *Free Radical Biology and Medicine*, 2012, **52**, 1151-1158.

45. J. C. Mamputu and G. Renier, *Journal of leukocyte biology*, 2004, **75**, 1062-1069.

46. T.-Y. Hu, C.-L. Liu, J.-Y. Chen and M.-L. Hu, *Journal of Functional Foods*, 2013, **5**, 745-754.

47. R. Pazdro and J. R. Burgess, *Biochimica et biophysica acta*, 2012, **1822**, 550-556.

48. M. Meydani, *Annals of the New York Academy of Sciences*, 2004, **1031**, 271-279.

49. R. Blomhoff, M. H. Carlsen, L. F. Andersen and D. R. Jacobs, Jr., *The British journal of nutrition*, 2006, **96 Suppl 2**, S52-60.

50. J. E. Maras, O. I. Bermudez, N. Qiao, P. J. Bakun, E. L. Boody-Alter and K. L. Tucker, *Journal of the American Dietetic Association*, 2004, **104**, 567-575.

51. G. X. Li, M. J. Lee, A. B. Liu, Z. Yang, Y. Lin, W. J. Shih and C. S. Yang, *Cancer prevention research (Philadelphia, Pa.)*, 2011, **4**, 404-413.

52. H. D. Sesso, J. E. Buring, W. G. Christen, T. Kurth, C. Belanger, J. MacFadyen, V.

Bubes, J. E. Manson, R. J. Glynn and J. M. Gaziano, *JAMA : the journal of the American Medical Association*, 2008, **300**, 2123-2133.

53. H. Y. Huang and L. J. Appel, *The Journal of nutrition*, 2003, **133**, 3137-3140

481 Table 1. Contents of tocopherols in different nuts

Nuts	Concentration (mg/g)			
	Total tocopherol	α-tocopherol	β- and γ-tocopherol	δ-tocopherol
Almonds	1.188	1.153 ± 0.018	0.035 ± 0.001	-
Hazelnuts	2.688	2.117 ± 0.032	0.570 ± 0.003	0.001 ± 0.000
Pistachios	1.104	0.217 ± 0.001	0.886 ± 0.003	0.001 ± 0.000
Walnuts	1.485	0.234 ± 0.002	0.738 ± 0.004	0.513 ± 0.002

482

Figure legends

Fig. 1. Effects of tocopherols on MGO-induced reduction of cell viability in HUVECs. **A** Chemical structures of tocopherols. **B** Photomicrographs of MGO-treated HUVECs without (-) or with (+) α-, β, γ- and δ-tocopherols. **a** control; **b** 500 μM MGO; **c** MGO + α-tocopherol (50 μM); **d** MGO + β-tocopherol (50 μM); **e** MGO + γ-tocopherol (50 μM); **f** MGO + δ-tocopherol (50 μM). **C** Viability of HUVECs treated with MGO and α-, β, γ-, or δ-tocopherols and analyzed by MTT assay. **D** Viability of HUVECs treated with MGO and various concentrations of α- or δ-tocopherols and analyzed by MTT assay. The percent cell viabilities are presented as mean ± SD of eight independent experiments. (***$p < 0.001$ vs. control, ## $p < 0.01$ and ### $p < 0.001$ vs. 500 μM MGO treatment only and $ $p < 0.05$ vs. α-tocopherol)

Fig. 2. The effect of α- and δ-tocopherols on MGO-induced apoptosis in HUVECs. **A** Representative cytograms of annexin V-FITC and PI staining of MGO-stimulated HUVECs. Cells were pretreated with α- or δ-tocopherol for 1 h followed by 500 μM MGO treatment. After 24 h, cells were harvested and analyzed by flow cytometry. **a** control; **b** 500 μM MGO; **c** MGO + α-tocopherol (50 μM); **d** MGO + δ-tocopherol (50 μM). **B** Percentage of early and late apoptotic cells as analyzed by flow cytometry. (**$p < 0.01$ vs. control, # $p < 0.05$ and ### $p < 0.001$ vs. 500 μM MGO treatment only)

Fig. 3. Effects of α- and δ-tocopherols on Bax and Bcl-2 protein expression in MGO-treated HUVECs. Cells were pretreated without (-) or with (+) α- or δ-tocopherols for 1 h followed by 500 μM MGO treatment for 24 h. **A** Representative western blot of Bcl-2, Bax, and

tubulin as an internal control. **B** Relative band intensity of Bcl-2. **C** Relative band intensity of Bax. Bar values are presented as mean ±SD of three independent experiments. (***p < 0.001 vs. control, # p < 0.05, ## p < 0.01 and ### p < 0.001 vs. 500 μM MGO treatment only)

Fig. 4. Effect of tocopherols on MGO-induced ROS generation. HUVECs were pretreated with α- or δ-tocopherols for 30 min followed by 500 μM MGO treatment for 60 min. ROS generation was detected by staining with the fluorescent dye DCF-DA **A** control; **B** 500 μM MGO ; **C** MGO + α-tocopherol; **D** MGO + δ-tocopherol.

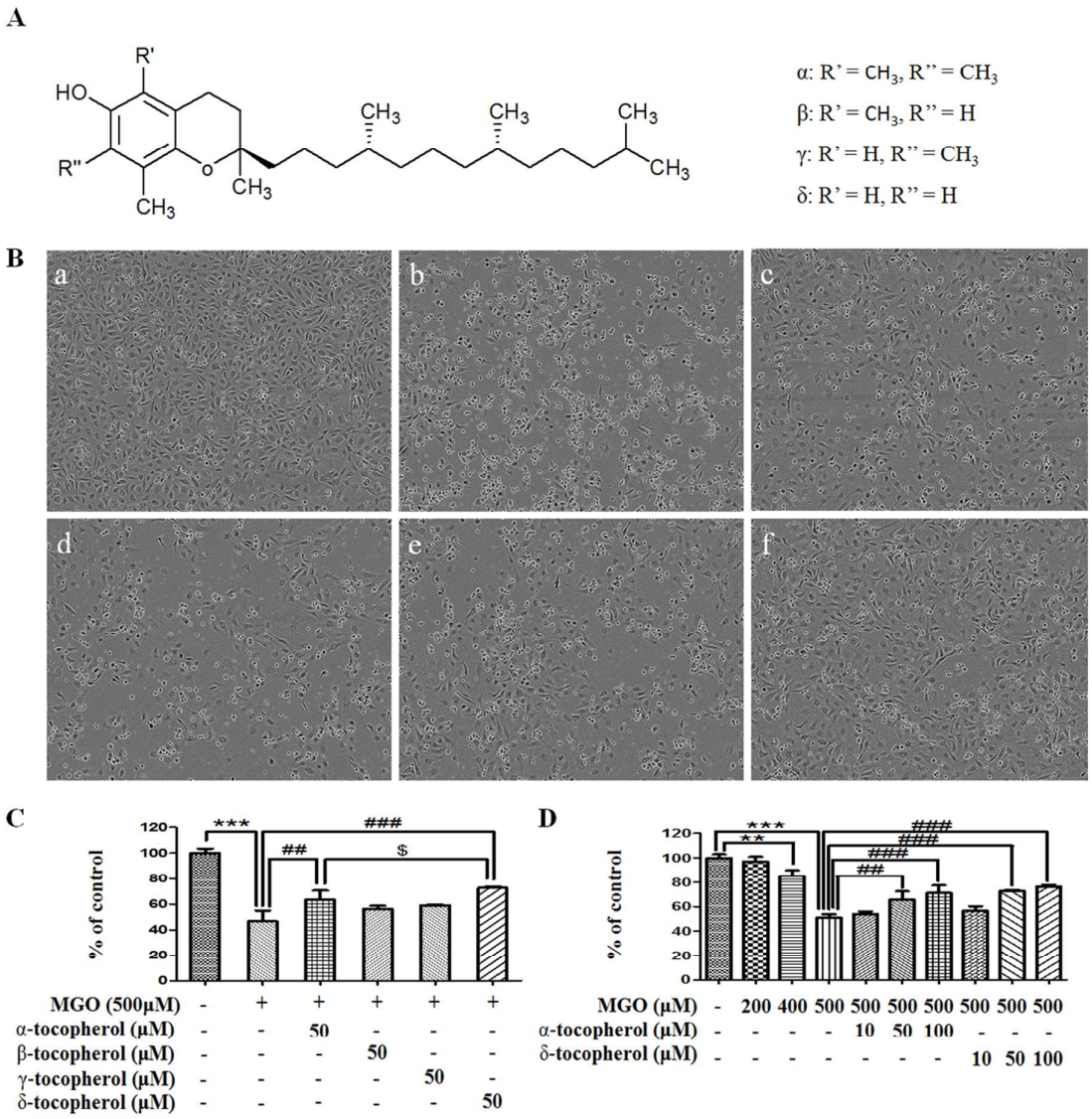
Fig. 5. Effects of δ-tocopherols on MAPK signaling pathways in HUVECs. Western blots of total and phosphorylated forms of MAPKs. Cells were pretreated without (-) or with (+) α- or δ-tocopherol for 1 h followed by 500 μM MGO treatment for 1 h. **A** Representative western blot of MAPKs. **B** Relative band intensity of P-JNK. **C** Relative band intensity of P-ERK1/2. **D** Relative band intensity of P-p38. Bar values are presented as mean ±SD of three independent experiments. (*p < 0.05 vs. control and # p < 0.05 vs. 500 μM MGO treatment only).

Fig. 6. HPLC chromatogram for the determination of tocopherols in different nuts. (1) δ-tocopherol, (2) β- and γ-tocopherol, (3) α-tocopherol with a mobile phase methanol/water (92/8, v/v) **A.** Chromatogram of standard tocopherols **B.** Chromatogram of almonds **C.** Chromatogram of hazelnuts, **D.** Chromatogram of pistachios, **E.** Chromatogram of walnuts.

Fig. 7. Effects of nuts on MGO-induced cell death in HUVECs. HUVECs were pretreated with various types of nut extracts for 1 h and then treated with 500 μM MGO for 24 h. The

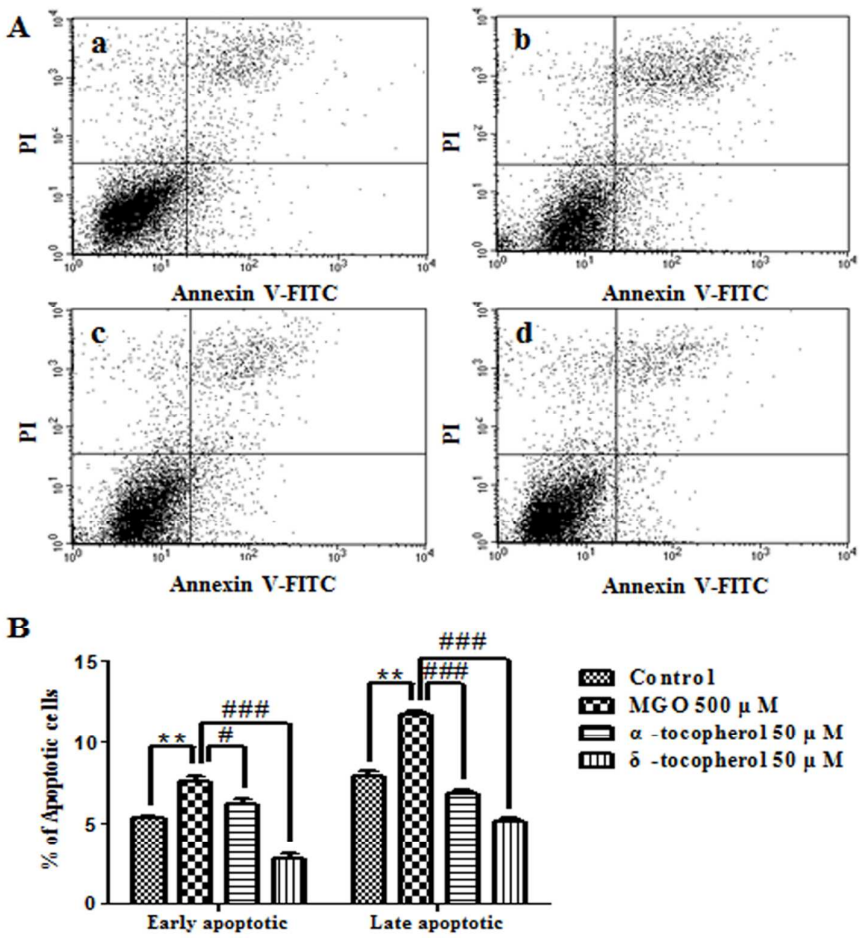
cell viability was analyzed by MTT assay. The percent cell viabilities are presented as mean ±SD of five independent experiments. (***$p < 0.001$ vs. control, # $p < 0.05$ and ### $p < 0.001$ vs. 500 μM MGO treatment only).

535 Fig. 1



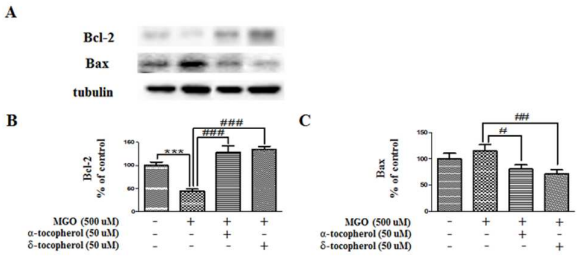
536

537 Fig. 2.



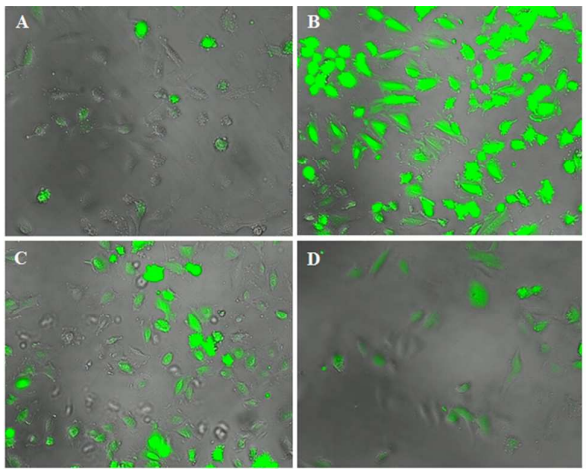
538

539 Fig. 3.

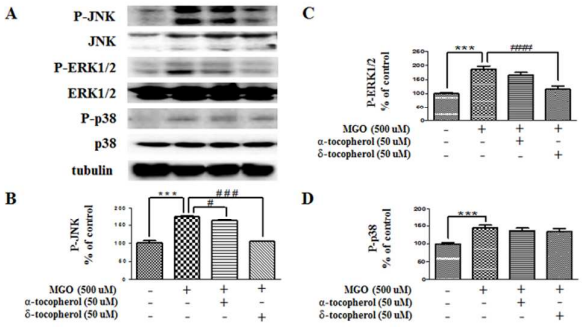


540

541 Fig. 4.

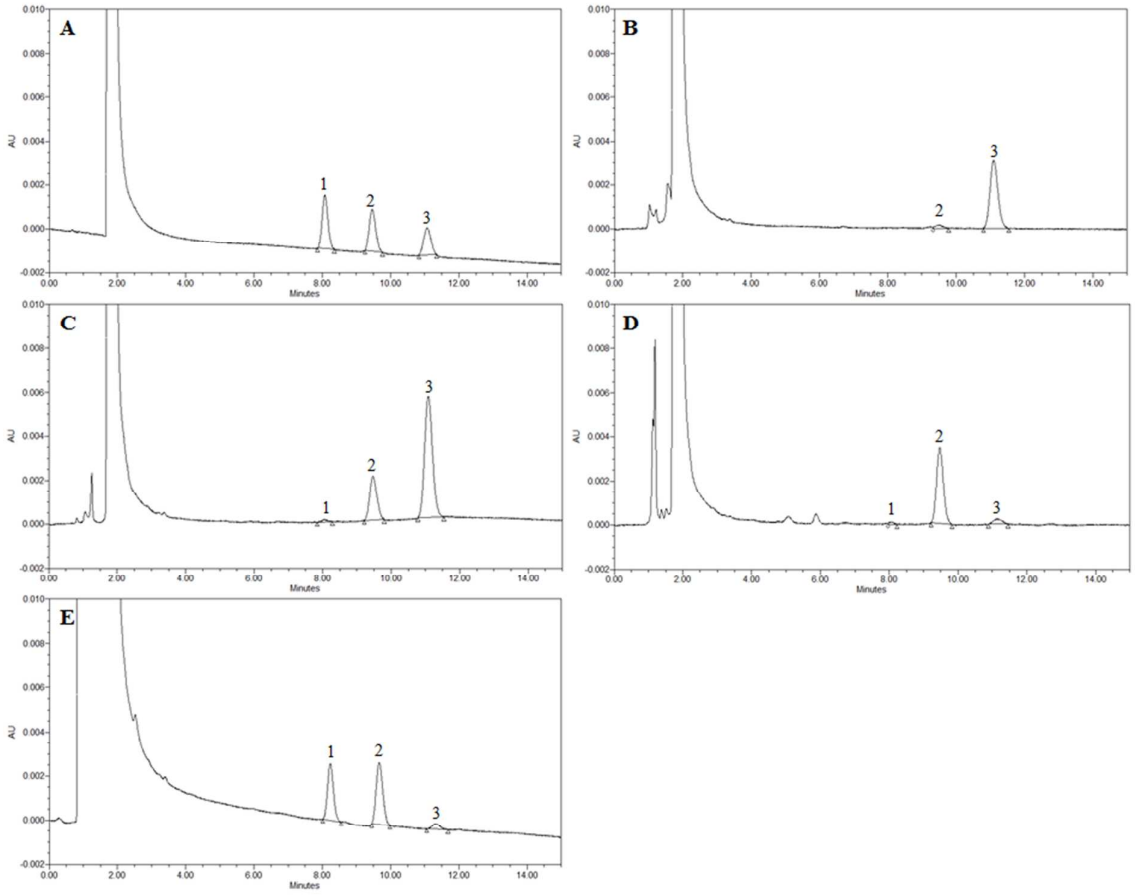
542 

543 Fig. 5.



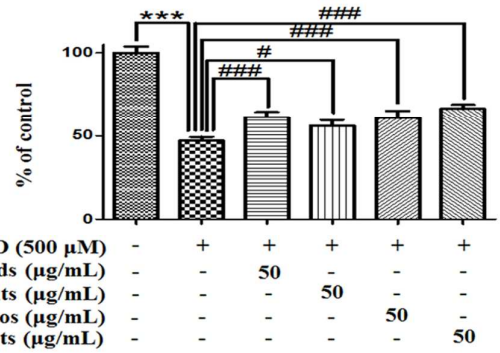
544

545 Fig. 6.



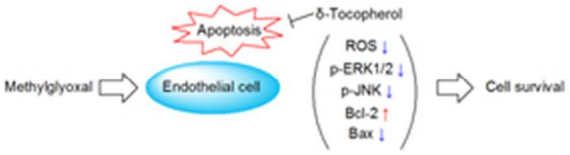
546
547

548 Fig. 7.



549

550



24x7mm (300 x 300 DPI)

Supplemental data

Fig. S1. The dose-dependent effects of tocopherols on MGO-induced cell death in HUVECs. Cells were pretreated without (-) or with (+) α- or δ-tocopherols for 1 h followed by 500 μM MGO treatment for 24 h. **A** Viability of HUVECs treated without (-) or with (+) MGO and various concentrations of α-tocopherol and analyzed by MTT assay. **B** Viability of HUVECs treated without (-) or with (+) MGO and various concentrations of δ-tocopherol and analyzed by MTT assay. The percent cell viabilities are presented as mean ± SD of three independent experiments. (***$p < 0.001$ vs. control, ## $p < 0.01$ and ### $p < 0.001$ vs. 500 μM MGO treatment only).

Fig. S2. The effects of tocopherol/MGO pretreatment on MGO-induced cell death in HUVECs. A Viability of HUVECs cotreated with MGO and α- or δ-tocopherols and analyzed by MTT assay. B Viability of HUVECs pretreated with MGO for 1 h and then exposed to α- or δ-tocopherol for 24 h and analyzed by MTT assay. The percent cell viabilities are presented as mean ± SD of three independent experiments. (***$p < 0.001$ vs. control and ### $p < 0.001$ vs. 500 μM MGO treatment only).

Fig. S1.

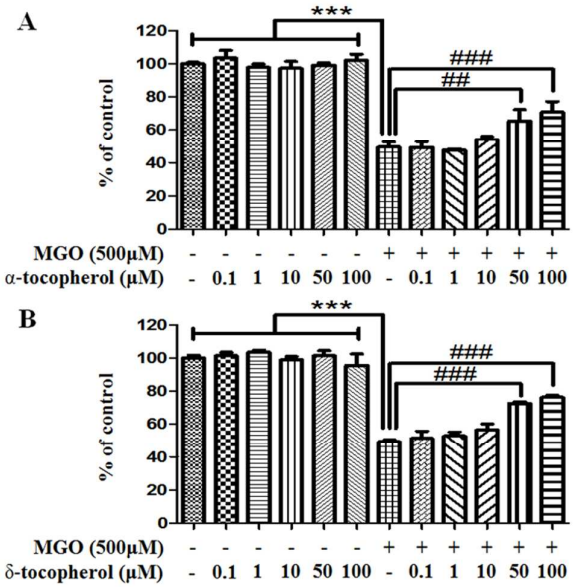


Fig. S2.