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In vitro effect of resveratrol against oxidative injury of human coronary artery endothelial cells

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Aim: Reactive oxygen species induce oxidative stress that may contribute to endothelial dysfunction. In addition to endogen antioxidants, adding antioxidants to diet has been used to reduce oxidative-stress-induced endothelial dysfunction. Resveratrol is a natural phytoalexin which has many effects including antioxidant activity. The aim of this study was to investigate whether resveratrol could protect human coronary artery endothelial cells against in vitro oxidative-stress-mediated injury.

Materials and methods: Human coronary artery endothelial cells were treated with varying concentrations of hydrogen peroxide for different durations and hydrogen peroxide plus varying concentrations of resveratrol. Then cell death was measured using lactate dehydrogenase release. Human coronary artery endothelial cells were exposed to hydrogen peroxide and resveratrol plus hydrogen peroxide for 15 min, 60 min, and 6 h. Reactive oxygen species was measured using a fluorometric assay.

Results: Preincubation of cells with 10 and 50 µM resveratrol for different periods caused a significantly decreased hydrogen-peroxide-induced cell death. Preincubation with resveratrol caused a significant decrease in reactive oxygen species production.

Conclusion: Our results demonstrate that resveratrol protects human coronary artery endothelial cells, in vitro, against oxidative-stress-induced injury by decreasing cell death and reactive oxygen species production.

Key words: Resveratrol, human coronary artery endothelial cell, oxidative stress

Resveratrolün insan koroner arter endotel hücrelerinde oksidatif hücre hasarina karşı in vitro etkisi

Amaç: Reaktif oksijen ürünleri endotel fonksiyon kaybına katkıda bulunabilen oksidatif stresi indüklemektedir. Endojen antioksidanların yanı sıra, diyetle antioksidanların alımı, endotel fonksiyon kaybını indükleyen oksidatif stresi azaltmaktadır. Resveratrol antioksidan aktiviteyi kapsayan birçok etkilere sahip doğal bir fitoaleksindir. Bu çalışmanın amacı resveratrolün in vitro oksidatif stresin aracılık ettiği hasara karşı insan koroner arter endotel hücrelerini koruyucu etkisi olup olmadığının araştırılmasıdır.

Yöntem ve gereç: İnsan koroner arter endotel hücreleri farklı süre ve konsantrasyonlarda hidrojen peroksitle ve hidrojen peroksit + resveratrol ile muamele edildi. Daha sonra hücre ölümü laktat dehidrogenaz salınımı ile ölçüldü. İnsan koroner arter endotel hücreleri hidrojen peroksite ve hidrojen peroksit + resveratrole maruz bırakıldı. 15., 60. dakikada ve 6. saatte reaktif oksijen ürünleri florometrik ölçümle değerlendirildi.

Bulgular: 10 ve 50 µM resveratrol ile farklı zaman noktalarında preinkübasyon, hidrojen peroksit ile indüklenen hücre ölümünün anlamlı olarak azalmasına neden olmaktadır. Resveratrol reaktif oksijen ürünleri oluşumunda anlamlı azalmaya neden olmaktadır.

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Sonuç: Bulgularımız, resveratrolün, in vitro insan koroner arter endotel hücrelerinde reaktif oksijen ürünleri oluşumunu ve hücre ölümünü azaltarak oksidatif stresin indüklediği hücre hasarına karşı koruyucu etkisinin olduğunu göstermektedir.

Anahtar sözcükler: Resveratrol, insan koroner arter endotel hücresi, oksidatif stres

Introduction

Endothelial cells, which are located on the inner surface of blood vessel walls, play an important role in cardiovascular homeostasis, such as vascular tone regulation, myocardial function, and inflammatory responses (1). Endothelial dysfunction has been implicated in the pathophysiology of cardiovascular diseases such as hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure (2-5).

Reactive oxygen species (ROS) produced during general cell metabolism play many roles in cellular function (6). Increased production of ROS changes the cellular signaling system and causes damage to lipids, proteins, and DNA (7). Enhanced activity of oxidant enzymes and/or reduced activity of antioxidant enzymes cause oxidative stress (8). Oxidative stress is characterized by increased endogenous production of ROS such as superoxide anion and hydrogen peroxide. Hydrogen peroxide (H_2O_2) is produced by vascular and inflammatory cells and induces oxidative stress which may cause endothelial dysfunction and cellular injury. Because H_2O_2 does not have an unpaired electron it is less reactive than ROS. It can cause endothelial cell injury by inducing mitochondrial dysfunction (9,10). Oxidative stress contributes to mitochondrially mediated cellular injury. Mitochondrial permeability transition is a mechanism causing mitochondrial failure. Mitochondrial permeability transition can cause necrosis due to ATP depletion (11).

ROS play an important role in the pathogenesis of disease, and taking antioxidant nutrients through diet, as well as relying on endogenic antioxidants, forms the defense system for decreasing ROS-induced endothelial dysfunction (7). Enzymatic antioxidants involve the enzymes superoxide dismutase, glutathione peroxidase, and catalase; the non-enzymatic antioxidants are ascorbic acid, α-tocopherol, glutathione, carotenoids, flavonoids, and stilbenes (12).

Resveratrol is a polyphenolic compound belonging to the stilbene family found in grapes, peanuts, mulberries, and red wine (13). In France a negative relationship has been found between red wine consumption and the prevalence of cardiovascular disease (French paradox) (14,15). In 1982 Arichi et al. (15) determined that stilbene components are found in the roots of *Polygonum cuspidatum*, which has been used in traditional Japanese and Chinese medicine to treat diseases of the heart, liver, and skin.

Resveratrol is a molecule with anti-inflammatory, anti-oxidant, cytoprotective, anti-carcinogenic, neuroprotective, cardioprotective, anti-platelet aggregation, anti-atherogenic, estrogen-like growth-promoting, and immunomodulatory effects (6,16).

The anti-oxidant properties of resveratrol are dependent upon the redox properties of its phenolic hydroxyl groups and on the potential for the delocalization of electrons through its chemical structure (17). Rubiolo et al. (18) have shown that resveratrol increases the activity of antioxidant enzymes in primary rat hepatocyte cultures and thus protects these cells from oxidative-stress-induced damage. It has been shown that resveratrol induces endogenic antioxidants and phase 2 enzymes (e.g. glutathione transferase and NAD(P)H:quinone oxidoreductase-1) in cultured aortic smooth muscle cells and cardiomyocytes. Therefore, this compound increases resistance to electrophilic and oxidative injury (19,20).

Human coronary artery endothelial cells (HCAECs) are significant for the elucidation of the cardioprotective effects of resveratrol. No data have been found in the literature regarding the in vitro effects of resveratrol on the death and ROS injury of these cells. The aim of this study was to investigate the possible effect of resveratrol on the in vitro oxidative-stress-mediated injury of HCAECs. For this purpose, incubation with H_2O_2 was used as an in vitro oxidative stress model, and the cell injury was assessed using LDH release and ROS production.

Materials and methods

RSV was purchased from Sigma, and H_2O_2 was purchased from Riedel-de Haen and Merck. The LDH cytotoxicity detection kit was purchased from Roche Diagnostics GmbH. The 3'-(p-hydroxyphenyl) fluorescein (HPF; Molecular Probes) was purchased from Invitrogen (USA). HCAECs were obtained from Cambrex Bio Science Walkersville, Inc. HCAECs were grown in culture medium (EGM-2 MV SingleQuots). The reagent pack was purchased from Cambrex (one reagent pack containing: trypsin/EDTA, trypsin neutralizing solution, and HEPES buffered saline solution), and the cell culture was performed according to the manufacturer's instructions. Seeding density was 5000 cells/cm^2 in HCAECs. HCAECs were maintained in endothelial growth medium supplemented with 25% fetal bovine serum, hydrocortisone, hFGF-B, VEGF, R3 –IGF-1, ascorbic acid, hEGF, and GA-1000 (Clonetics). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every other day and passaged every 5-9 days. HCAECs were used at passages 5-7 in all experiments.

Effect of resveratrol on HCAECs (control study)

In order to test the possible effect of resveratrol on intact cells, HCAECs were incubated for 1, 12, and 24 h only with resveratrol (10, 50, and 100 µM). At the end of each incubation period the medium was collected and the effect of resveratrol on HCAECs was determined by LDH release into the medium. Lactate dehydrogenase—a cytoplasmic enzyme which is released from dead or plasma-membrane-damaged cells into the cultured medium—was determined spectrophotometrically. At the end of each incubation period the medium was removed from 96 well plates, and the LDH activity was measured using an LDH assay kit according to the manufacturer's instructions.

H_2O_2-induced oxidative stress, evaluation of cell death, and determination of the effect of resveratrol

The oxidative stress model used in this study was the incubation of cells with H_2O_2. For this purpose, HCAECs were seeded in a 96-well plate at 1×10^4 cells/well and allowed to grow to confluence. The cells were then incubated for 1, 3, and 6 h (21-23) with the oxidative-stress- inducing agent H_2O_2 (100, 250, 500, 750, and 1000 µM) (22,24). Non-H_2O_2-treated cells (control group) were incubated under the same conditions. At the end of each incubation period the medium was collected, and the cell death was evaluated with the LDH assay. As will be seen in Figure 1, the % LDH release was observed to be between 30% and 40%, which is considered an optimal cell death rate, corresponding to a H_2O_2 concentration of 750 µM and an incubation period of 1 h.

In order to determine the effect of resveratrol, the cells were preincubated with resveratrol at concentrations of 10, 50, and 100 µM (25) for 1 h, 12 h, and 24 h. After the preincubation the medium was changed, and then the cells were exposed to the selected concentration of 750 µM H_2O_2 for 1 h. After this period the medium was collected, and the cell death was evaluated by LDH release into the medium. The LDH activity was measured using a LDH assay kit [LDH Cytotoxicity Detection Kit (Roche Diagnostics GmbH)] according to the manufacturer's instructions.

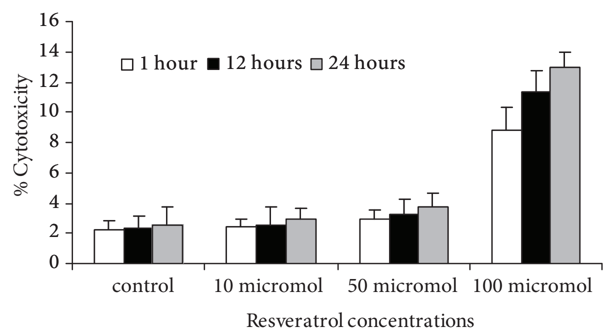

Figure 1. LDH release to the medium for HCAEC treated with resveratrol (10, 50, and 100 µM for 1, 12, and 24 h). Significant differences were found between control and 100 µM resveratrol groups. Results are expressed as mean ± SD (P = 0.020).

Evaluation of cell injury by measuring ROS generation and determination of the effects of resveratrol

To evaluate the ROS production due to injury 3'-(p-hydroxyphenyl) fluorescein (HPF) was used. Cells were exposed for different periods (15 min,

60 min, and 6 h) (26) with various concentrations of H_2O_2 (100, 250, 500, 750, and 1000 µM), and oxidative stress was produced. ROS generation was determined spectrofluorometrically using the HPF probe. Then, to determine the effect of resveratrol on the ROS generation, the cells were preincubated with resveratrol (10, 50, and 100 µM; 24 h) and then exposed to oxidative stress (750 µM H_2O_2). After treatment with H_2O_2, ROS formation was evaluated with HPF at 15 min, 1 h, and 6 h intervals, these time lines being predetermined in the optimization work with the ROS assay. The ROS generation was measured using an HPF probe according to the manufacturer's instructions.

Statistical analysis

All experiments were repeated 3 times with similar results. All data were expressed as mean ± standard deviation (SD). Variables were compared with the Mann-Whitney U test and a value of $P < 0.05$ was considered significant.

Results

The evaluation of cell death for HCAECs treated with resveratrol

The effect of resveratrol on the cell death of HCAECs is shown in Figure 1. When the cells were incubated with 10 and 50 µM resveratrol, significant cell death was not observed for all periods. However, a significantly high (15%) LDH liberation was observed for 100 µM resveratrol compared to the control (P = 0.020).

The inhibitory effect of resveratrol on cell death induced by H_2O_2 in HCAECs

Oxidative-stress-induced cell death was measured by the LDH release into the medium in HCAEC cultures. The HCAEC cultures were exposed to 100, 250, 500, 750, and 1000 µM H_2O_2 for 1, 3, and 6 h, and the 1 h treatment with 750 µM H_2O_2 significantly increased cell death, as shown in Figure 2 (P = 0.020). This concentration was selected for further studies with resveratrol. To determine the effect of resveratrol on cell death HCAECs were pretreated with 10, 50, and 100 µM resveratrol for 1, 12, and 24 h. Then HCAECs were exposed to 750 µM H_2O_2 for 1 h. The effects observed in 10 and 50 µM concentrations at all preincubation periods were not observed in 100 µM resveratrol. Preincubation with 10 and 50 µM resveratrol for 1, 12, and 24 h significantly decreased H_2O_2-induced cell death in HCAECs as shown in Figure 3 (P = 0.020).

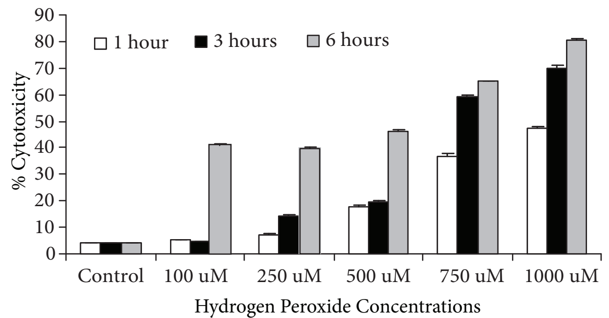

Figure 2. The effects of H_2O_2 on the LDH rele ase. Cells were treated with various concentrations of H_2O_2 (100, 250, 500, 750, and 1000 µM for 1, 3, and 6 h) in HCAEC cultures. LDH release was increased in all concentrations of H_2O_2 as compared to control in HCAECs (P = 0.020). Results are expressed as mean ± SD. The experiments were repeated 3 times with similar results.

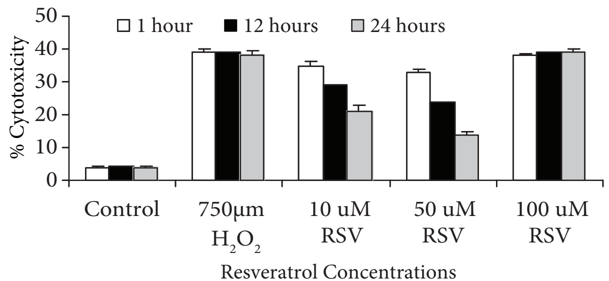

Figure 3. Protective effects of resveratrol on H_2O_2-induced LDH release in HCAEC. Cells were pretreated with different concentrations of resveratrol and for different time periods (10, 50, and 100 µM; 1, 12, and 24 h). After preincubation the medium was changed to a medium without resveratrol, and 750 µM H_2O_2 was added. All results are expressed as mean ± SD. For 1, 12, and 24 h and 10 and 50 µM resveratrol concentrations P = 0.020, as compared with the H_2O_2 group.

The inhibitory effect of resveratrol on ROS injury in HCAECs

Increased fluorescence intensity was determined when cells were treated with various concentrations of H_2O_2. ROS levels were markedly increased after 15 min of treatment with various concentrations of H_2O_2 and reached a peak which gradually decreased in a 6

h lapse of time (P = 0.020) (Figure 4). Figure 5 shows the effect of resveratrol on HCAEC cultures subjected to ROS injury. All concentrations of resveratrol (10, 50, and 100 µM) significantly decreased H_2O_2-induced ROS injury at all preincubation time periods (P = 0.020).

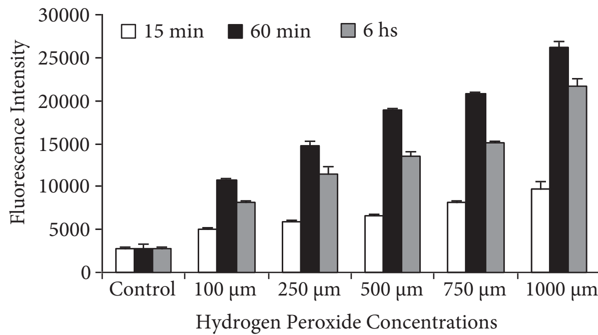

Figure 4. Effect of H_2O_2 on ROS formation. ROS formation was increased significantly in all concentrations of H_2O_2 as compared to control HCAEC (P = 0.021). Results are expressed as mean ± SD.

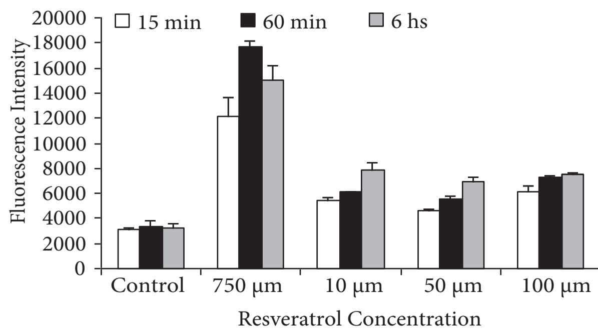

Figure 5. Effect of resveratrol on H_2O_2-induced ROS formation. HCAEC were pre-treated with various concentrations of resveratrol for 24 h. After pre-treatment, the cells were exposed to 750 µM H_2O_2 for 15 min, 60 min, and 6 h. Resveratrol decreased H_2O_2 induced ROS formation in HCAEC. Statistically significant differences were found for all concentrations of resveratrol at all time periods compared with cells treated only with H_2O_2 (P = 0.021). Results are expressed as mean ± SD.

Discussion

Resveratrol shows its antioxidant effect with 2 different cellular protective mechanisms. Resveratrol directly catches the free radicals (hydroxyl radical and superoxide anion radical). It also has an antioxidant capacity due to the balance of hydroxyl phenolic groups and delocalization of the electrons (27). Ungvari et al. (14) showed in a cell-free assay the direct free radical capturing effect of 1, 10, and 100 µM resveratrol, which decreased the H_2O_2-induced increases in dichlorofluorescein fluorescence as concentration increased. The other mechanism involved in resveratrol's antioxidant effect is an increase in antioxidant enzyme activity. In this study the protective effect of resveratrol against H_2O_2 damage in HCAECs was evaluated with LDH and ROS. Rubiolo et al.'s study (17) was the first to show the protective effect of resveratrol in primary rat hepatocytes with ROS and LDH. Our study is the first in the literature showing the protective effect of resveratrol with ROS and LDH tests.

Enzymatic sources of ROS are the mitochondrial electron transport chain, lipooxygenase, cyclooxygenase, cytochrome P450s, xanthine oxidase, NAD(P)H oxidase, uncoupled eNOS, and other hemoproteins. These systems produce a 1-electron reduction of molecular oxygen to form a superoxide anion. Superoxide anion converts to H_2O_2 either spontaneously or by SOD enzyme pathway. Subsequently H_2O_2 converts to water and oxygen through catalase, glutathione peroxidase, and thiols. H_2O_2 has 3 fates: it reacts with nitric oxide to form nitrogen dioxide anion; it is catalyzed by enzymes such as glutathione peroxidase and catalase to form water and oxygen; or, in the presence of heavy metals, it undergoes a Fenton reaction to form a hydroxyl radical (29). Increased H_2O_2 in the cells leads to oxidative stress that may contribute to endothelial dysfunction (9).

Resveratrol affects the intracellular redox state. Resveratrol and its antioxidant mechanisms inhibit lipid peroxidation in carcinogenic compounds, depending on the concentration and the cell type, which leads to oxidative breakage of cellular DNA. Recently it has been proposed that such pro-oxidant action could be an important action mechanism of its anti-cancer and apoptotic-inducing properties. It has been shown that there is an interesting correlation among the antioxidant and pro-oxidant activities and cytotoxicity of dietary polyphenols. Every antioxidant is in fact a redox (reduction-oxidation) agent and thus might become a pro-oxidant, accelerating lipid peroxidation and/or inducing DNA damage

under special conditions. Studies have revealed the pro-oxidant effects of antioxidant vitamins and several classes of plant-derived polyphenols such as flavonoids, tannins, and curcumin. Resveratrol probably mobilizes endogenous copper ions, possibly chromatin bound copper, as a cytotoxic mechanism. First, resveratrol undergoes oxidation in the presence of Cu(II). The oxidative product of resveratrol is a dimer, possibly formed by dimerization of the resveratrol phenoxyl radicals as a result of the reductive activation of molecular oxygen. Indeed, this initial electron transfer generates the reduction of Cu(II) to Cu(I). Interestingly, DNA strand scission occurred at a neutral pH, indicating that resveratrol can induce DNA cleavage without the oxygenation of the benzene nuclei to the catechol moiety. However, the structural feature of the copper-peroxide complex as the reactive species responsible for the DNA cleavage is still unknown. Secondly, the Cu(II)-peroxide complex is capable of binding DNA and forms a DNA-resveratrol-Cu(II) ternary complex. The high binding affinity of a 4-hydroxy group at the 4-position with both Cu(II) and DNA makes this possible and cleaves DNA efficiently (30).

In this study we used H_2O_2 as an oxidative injury model. However, we optimized the conditions at 750 µM H_2O_2 concentration and 1 h of incubation, using the data obtained from our optimization work (Figure 2). Other investigators have used different H_2O_2 injury conditions in their studies. Wang et al. (31) have shown that icariin protects in vitro human umbilical vein endothelial cells from H_2O_2-induced oxidative injury (H_2O_2 concentration of 750 µM and an incubation period of 18 h). Cortese et al. (32) have shown that zinc supplementation protects rat aorta endothelial cells from H_2O_2-induced injury (H_2O_2 concentration of 1 µM and an incubation period of 24 h).

With the criteria that we used in the selection of the optimal injury conditions we were able to observe enough injury, but were unable to attain the maximum cell death, as observed in the above-mentioned studies. Finally, our main concern in the selection of a H_2O_2 injury model was to attain a better standardization than other in vitro oxidative stress injury models.

In this study we showed that resveratrol has a protective effect on HCAECs facing oxidative-stress-induced cell injury. This protection was observed in 1, 12, and 24 h preincubation periods. Similarly, Rubiolo et al. (17) have shown in primary rat hepatocytes that resveratrol decreases cell death for up to 24 h, but this inhibition was not observed at 48 h. The authors explained this difference with the hypothesis that resveratrol reacts with the oxidative-stress-inducing agent (tert-butyl hydroperoxide), and the remaining tert-butyl hydroperoxide damages the cell and increases cell death at 48 h. Brito et al. (25) have shown that the antioxidant effect of resveratrol occurs via an increase in the intracellular GSH within in vitro bovine aortic endothelial cells. Their results show that a long pre-incubation (14 h) with resveratrol at 50 µM protects in vitro bovine aortic endothelial cells from peroxynitrite-mediated cell death.

In our study we also tested the effect of resveratrol itself on HCAEC. We used 100 µM resveratrol as the highest concentration, and we found that this concentration caused 15% liberation of LDH under in vitro conditions for human coronary artery endothelial cells. Rubiolo et al. (18) have also shown that resveratrol (75 mM) could be cytotoxic for their cells (at a 5% LDH liberation) when the primary rat hepatocytes were preincubated with this concentration of resveratrol. Even at this concentration resveratrol-incubated cells have shown less injury than cells incubated only with tert-butyl hydroperoxide. The difference between our results and those of Rubiolo et al. (18) may be due to the difference in the cell lines as well as the different agents used to create oxidative stress.

Increased formation of ROS enhances oxidative stress. ROS-induced oxidative stress has played a significant role in various pathological conditions including cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion injury, and ageing (12). Our data showed that 10 and 50 µM resveratrol decreased ROS generation, suggesting that the ROS- scavenging capacity of resveratrol may be related to decreased cell death. Rubiolo et al. (17) have similarly shown that 50 µM resveratrol decreased the fluorescence intensity in cells treated with only tert-butyl hydroperoxide.

Other investigations have shown that pretreatment with 25-100 µM resveratrol for 72 h decreased intracellular accumulation of ROS in the in vitro rat

aortic smooth muscle cells (13). Kode et al. (33) have shown that treatment of human primary small airway epithelial and human alveolar epithelial (A549) cells with a cigarette smoke extract (CSE) enhanced generation of ROS. Preincubation of the cells with 10 µM resveratrol for 24 h decreased CSE-induced ROS production and this case may be related to the free radical scavenging ability of resveratrol. Vieira de Almeida et al. (6) have shown that 50 µM resveratrol protected primary cortical astrocyte cultures from H_2O_2 -induced oxidative stress (100 µM H_2O_2 for 30 min) by inhibiting intracellular ROS production. Hou et al. (34) have shown that human umbilical vein endothelial cells pretreated with *Ligusticum chuanxiong* and *Angelica sinensis* were protected against H_2O_2 (2 µM for 5 h) damage, and ROS and production was inhibited. It has been reported that chitosan oligosaccharides protected human umbilical vein endothelial cells from H_2O_2-induced oxidative stress. Viability loss was observed for the human umbilical vein endothelial cells exposed to 300 µM H_2O_2 for 12 h. This effect was associated with a decrease in intracellular ROS (10).

In the literature the cardioprotective effects of resveratrol are shown in various in vivo studies. Buluc et al. (35,36) showed that resveratrol depresses cardiac muscle contraction in rats and protects the heart. Dernek et al. (37) showed that resveratrol protects rat hearts from ischemia-reperfusion injury. Tatlidede et al. (38) reported that resveratrol can be used to prevent oxidative stress due to doxorubicin toxicity.

In conclusion, this study has clearly demonstrated the protective effect of resveratrol on cell death in HCAECs as well as its effect in decreasing the formation of ROS by these cells under the in vitro conditions used. These effects were found both in 10 and 50 µM concentrations of resveratrol and for 1, 12, and 24 h preincubation periods. These data on HCAECs are presented for the first time in scientific literature and provide interesting supportive evidence for the study of pathophysiological phenomena related to HCAECs. These findings may also suggest that decreased cell death can be related to the ROS-scavenging capacity of resveratrol. However, in addition to the direct antioxidant capacity resveratrol's protective effect could also be effectuated by resveratrol-induced intracellular antioxidant enzyme activities. Further studies need to be carried out in order to elucidate these mechanisms in HCAECs.

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