Effects of resveratrol on hydrogen peroxide-induced oxidative stress in embryonic neural stem cells

Sibel Konyalioglu[1], Guliz Armagan[1], Ayfer Yalcin[1], Cigdem Atalayin[2], Taner Dagci[3, 4]

1 Department of Biochemistry, Faculty of Pharmacy, Ege University, Bornova-Izmir 35100, Turkey
2 Department of Restorative Dentistry and Endodontics, Faculty of Dentistry, Ege University, Bornova-Izmir 35100, Turkey
3 Department of Physiology, School of Medicine, Ege University, Bornova-Izmir 35100, Turkey
4 Center for Brain Research, Ege University, Bornova-Izmir 35100, Turkey

Abstract

Resveratrol, a natural phenolic compound, has been shown to prevent cardiovascular diseases and cancer and exhibit neuroprotective effects. In this study, we examined the neuroprotective and antioxidant effects of resveratrol against hydrogen peroxide in embryonic neural stem cells. Hydrogen peroxide treatment alone increased catalase and glutathione peroxidase activities but did not change superoxide dismutase levels compared with hydrogen peroxide + resveratrol treatment. Nitric oxide synthase activity and concomitant nitric oxide levels increased in response to hydrogen peroxide treatment. Conversely, resveratrol treatment decreased nitric oxide synthase activity and nitric oxide levels. Resveratrol also attenuated hydrogen peroxide-induced nuclear or mitochondrial DNA damage. We propose that resveratrol may be a promising agent for protecting embryonic neural stem cells because of its potential to decrease oxidative stress by inducing higher activity of antioxidant enzymes, decreasing nitric oxide production and nitric oxide synthase activity, and alleviating both nuclear and mitochondrial DNA damage.

Key Words

Research Highlights

(1) Resveratrol treatment can prevent hydrogen peroxide-induced oxidative stress due to the altered activities of catalase, glutathione peroxidase, nitric oxide synthase and nitric oxide levels.
(2) Resveratrol treatment can also provide a protection against hydrogen peroxide-induced nuclear DNA or mitochondrial DNA damage in embryonic neural stem cells.
(3) Resveratrol treatment has a neuroprotective potential against oxidative stress-induced damages in embryonic neural stem cells.

INTRODUCTION

Oxidative stress contributes to the pathogenesis of acute or chronic neurodegenerative processes[1-2]. The main cytotoxic reactive oxygen species are hydrogen peroxide and free radicals, such as superoxide anions and hydroxyl radicals. It has been suggested that overproduction of superoxide anions is involved in N-methyl-D-aspartate-induced neurotoxicity[1, 3], but the potential toxic effects of hydrogen peroxide and hydroxyl radicals in neurons still need to be elucidated. The overproduction of

hydrogen peroxide is associated with amyloid aggregation[4-5], dopamine oxidation[6], and brain ischemia/reperfusion[7]. Cellular conversion of hydrogen peroxide into hydroxyl radicals involves Fenton reactions and transition metals. The excessive production of free radicals damages cellular macromolecules such as lipids, proteins, and DNA[8-10], which results in mitochondrial and nuclear genome damage, calcium imbalance, and induction of apoptosis in neuronal cells[11].

Embryonic neural stem cells exist in various regions of the central nervous system throughout the mammalian lifespan, and they can be expanded and induced to differentiate into neurons and glia both *in vitro* and *in vivo*. Embryonic neural stem cells-based approaches have received much attention because of their potential in the treatment of neurodegenerative disorders[12-13]. In animal models of neurodegenerative diseases, transplantation of stem cells or their derivatives induces the regeneration of the lost neurons and glial cells and elicits remyelination, has trophic effects, and modulates inflammation[14-17]. However, little is known regarding how embryonic neural stem cells affect the cellular distributions or activities of antioxidant enzymes.

Food-derived antioxidants have received growing attention as chemopreventive agents to prevent oxidative damage. However, the brain penetrance of antioxidants is an important parameter in their neuroprotective capacities for acute or chronic neurological disorders. Therefore, flavonoids with lipophilic chemical structures and antioxidant properties are promising candidates for neurodegenerative interventions[18]. Resveratrol is a polyphenolic compound with strong antioxidant properties that is found abundantly in grapes, berries, nuts, and red wine[19]. Previous studies suggest chemopreventive[20], cardioprotective[21], and neuroprotective[22-26] effects of resveratrol.

The present study was designed to investigate the effects of resveratrol and trolox treatments on nuclear DNA and mitochondrial DNA damage, the activities of antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase), and both nitric oxide and nitric oxide synthase activity in embryonic neural stem cells that attenuate hydrogen peroxide-induced oxidative stress. In this study, hydrogen peroxide was used to elicit oxidative stress in embryonic neural stem cells to provide a better understanding of the effects of resveratrol in this experimental system. In addition, the neuroprotective effects of resveratrol on embryonic neural stem cells were compared with trolox, a reference compound for

specific antioxidants[27-30].

RESULTS

Kinetic characteristics of hydrogen peroxide-induced neurotoxicity

To determine the working concentration of hydrogen peroxide, we performed a series of dose-response assays using the lactate dehydrogenase assay[31-35]. Treatment with increasing concentrations of hydrogen peroxide engendered a dose-dependent loss of cell viability. Because 100 µM of hydrogen peroxide produced approximately a 50% reduction in viability, we used this concentration in subsequent experiments (Figure 1A).
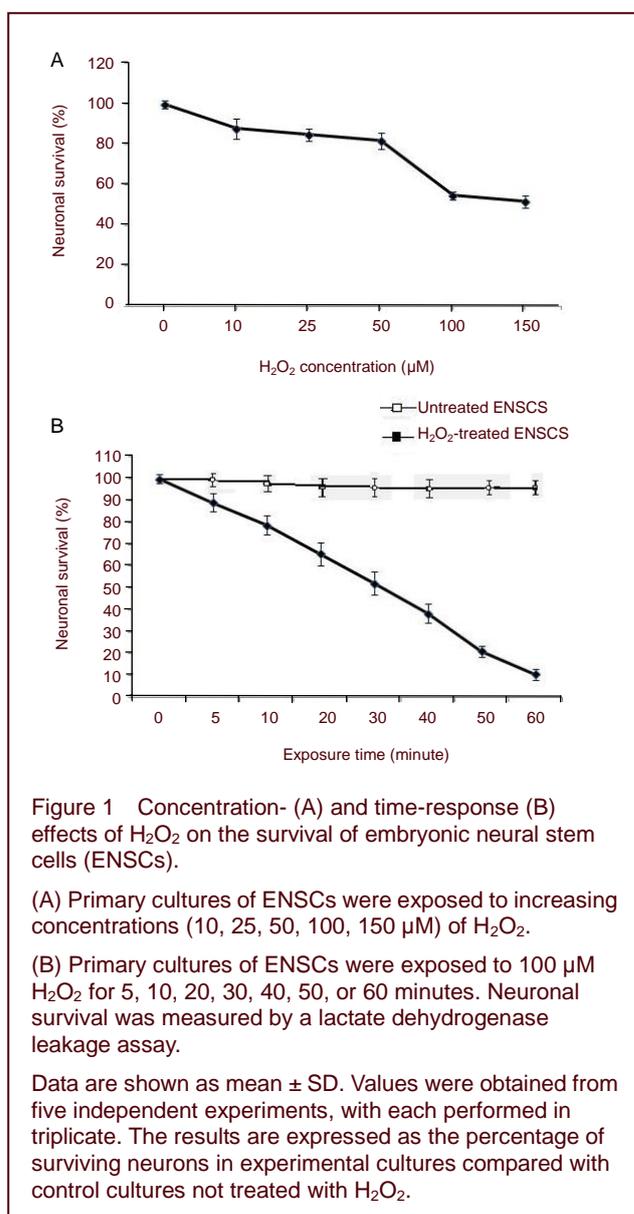
Figure 1 Concentration- (A) and time-response (B) effects of H_2O_2 on the survival of embryonic neural stem cells (ENSCs).

(A) Primary cultures of ENSCs were exposed to increasing concentrations (10, 25, 50, 100, 150 µM) of H_2O_2.

(B) Primary cultures of ENSCs were exposed to 100 µM H_2O_2 for 5, 10, 20, 30, 40, 50, or 60 minutes. Neuronal survival was measured by a lactate dehydrogenase leakage assay.

Data are shown as mean ± SD. Values were obtained from five independent experiments, with each performed in triplicate. The results are expressed as the percentage of surviving neurons in experimental cultures compared with control cultures not treated with H_2O_2.

Next, we determined the appropriate exposure time to 100 µM hydrogen peroxide for neuronal survival.

Exposure to hydrogen peroxide at 100 μM decreased cell viability time dependently. As after 30 minutes of exposure to 100 μM hydrogen peroxide, neuronal survival was reduced by 55%, we used this exposure time in subsequent experiments (Figure 1B).

The effective concentration of resveratrol to attenuate hydrogen peroxide-induced toxicity was determined in primary embryonic neural stem cell cultures using the lactate dehydrogenase leakage assay. The cultures were exposed to increasing concentrations of resveratrol (5–250 μM) combined with 100 μM hydrogen peroxide for 30 minutes. Because resveratrol (100 μM) significantly increased cell viability in hydrogen peroxide-treated embryonic neural stem cells, we used this concentration in subsequent examinations of hydrogen peroxide-induced toxicity (Figure 2).
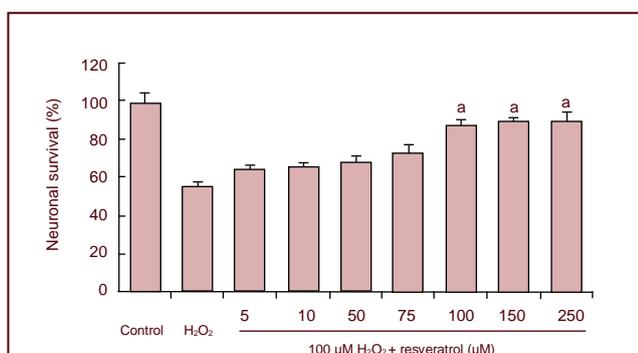
Figure 2　Effects of resveratrol on the survival of embryonic neural stem cells treated with hydrogen peroxide (H_2O_2).

Embryonic neural stem cells were treated with different concentrations (5, 10, 50, 75, 100, 150, 250 μM) of resveratrol and 100 μM H_2O_2 for 30 minutes. Neuronal survival was measured by a lactate dehydrogenase leakage assay. The data are shown as mean ± SD.

All values were obtained from five independent experiments, with each performed in triplicate. The results are expressed as the percentage of surviving neurons in experimental cultures compared with control cultures not treated with H_2O_2. [a]$P < 0.05$, *vs.* H_2O_2-treated cells (one-way analysis of variance followed by Tukey's *post hoc* test).

Antioxidant enzyme activities, nitric oxide synthase activity, and nitric oxide levels during the modulation of hydrogen peroxide-induced neurotoxicity by resveratrol

To evaluate the status of the antioxidant enzymes, we used standard spectrophotometric assays in cells treated with only hydrogen peroxide, hydrogen peroxide and resveratrol, or hydrogen peroxide and trolox. Catalase and glutathione peroxidase enzyme activities in the hydrogen peroxide group were significantly higher than those in the control group ($P < 0.05$) (30.7% for catalase and 38.1% for glutathione peroxidase, respectively). Resveratrol

treatment to attenuate the effects of hydrogen peroxide significantly decreased catalase and glutathione peroxidase activities compared to the hydrogen peroxide group, by 15.3% and 34.5%, respectively ($P < 0.05$). Trolox treatment had no significant effects on catalase activity compared to the hydrogen peroxide group, but it significantly decreased glutathione peroxidase activity by 45.5% ($P < 0.05$). For superoxide dismutase, only a slight increase in enzyme activity was observed in embryonic neural stem cells treated with only hydrogen peroxide, hydrogen peroxide and resveratrol, or hydrogen peroxide and trolox ($P > 0.05$; Figure 3).
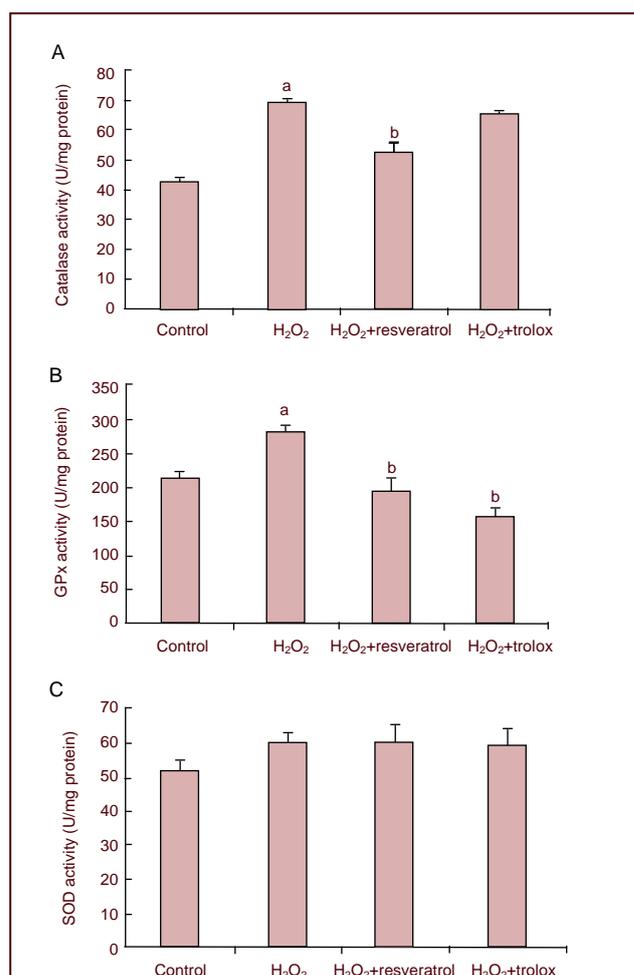
Figure 3　Effects of hydrogen peroxide (H_2O_2) alone, H_2O_2 and resveratrol, or H_2O_2 and trolox treatment on catalase (A), glutathione peroxidase (GPx; B) and superoxide dismutase (SOD; C) enzyme activities in embryonic neural stem cells.

Antioxidant enzyme activities in embryonic neural stem cells were measured following treatment with H_2O_2 (100 μM) alone, H_2O_2 (100 μM) and resveratrol (100 μM), or H_2O_2 (100 μM) and trolox (100 μM) for 30 minutes. The data are shown as mean ± SD. All values were obtained from five independent experiments, with each performed in triplicate. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* H_2O_2 group (one-way analysis of variance followed by Tukey's *post hoc* test).

The exposure of embryonic neural stem cells to 100 µM hydrogen peroxide led to approximately a three-fold increase in nitric oxide synthase activity and a two-fold increase in nitric oxide levels compared with the control group ($P < 0.05$). Treatment with resveratrol to attenuate the effects of hydrogen peroxide significantly decreased nitric oxide synthase activity and nitric oxide levels compared with hydrogen peroxide-treated cells ($P < 0.05$; Figure 4). Similar to resveratrol, trolox treatment significantly decreased the activity of nitric oxide synthase and the levels of nitric oxide in hydrogen peroxide-treated embryonic neural stem cells.
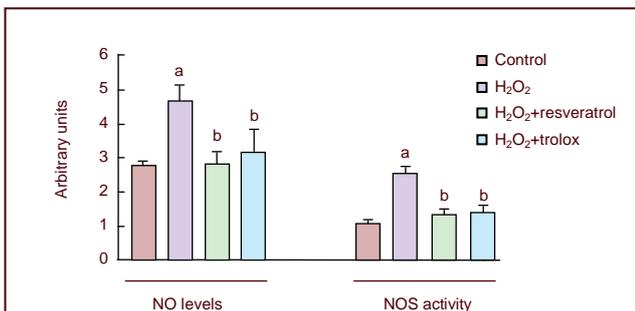
Figure 4 Effects of treatment with hydrogen peroxide (H_2O_2) alone, H_2O_2 and resveratrol, or H_2O_2 and trolox on nitric oxide synthase (NOS) enzyme activity and nitric oxide (NO) levels in embryonic neural stem cells.

NOS enzyme activity and NO levels in embryonic neural stem cells were measured following treatment with H_2O_2 (100 µM) alone, H_2O_2 (100 µM) and resveratrol (100 µM), or H_2O_2 (100 µM) and trolox (100 µM) for 30 minutes.

The data are shown as mean ± SD. All values were obtained from five independent experiments, with each performed in triplicate. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* H_2O_2 group (one-way analysis of variance followed by Tukey's *post hoc* test).

Influence of resveratrol on the genotoxic effects of hydrogen peroxide

The comet assay is a technique used to investigate primary genotoxic effects in target organs following acute and chronic oxidative stress. In particular, the determination of DNA in the tail is linearly related to the frequency of DNA strand breaks. Comet assay parameters (including percentage of tail DNA, tail length and tail moment) in control and treated cells are shown in Figure 5. Resveratrol attenuated hydrogen peroxide-induced nuclear DNA damage.

Effects of resveratrol on mitochondrial DNA

The measurements of mitochondrial DNA damage by agarose gels were determined using the ratios of the peak areas between the upper and lower bands. The data in Figure 6 show the density of mitochondrial DNA shearing in control and treated-cells. Similar to the

findings of the comet assay, mitochondrial DNA damage in the hydrogen peroxide group was significantly higher than that in the control group ($P < 0.05$). The density of mitochondrial DNA shearing was 2.4-fold higher in the hydrogen peroxide group than the control group. Resveratrol treatment to attenuate the effects of hydrogen peroxide significantly decreased mitochondrial DNA shearing in embryonic neural stem cells compared to hydrogen peroxide group. Specifically, 100 µM of hydrogen peroxide increased whereas resveratrol decreased the shearing of mitochondrial DNA in embryonic neural stem cells ($P < 0.05$).
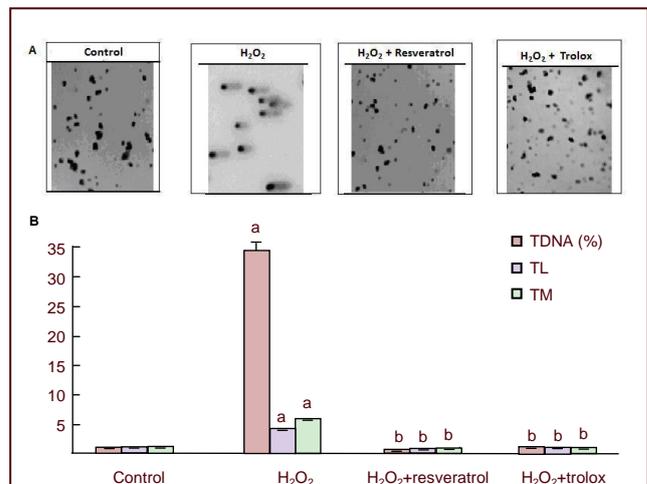
Figure 5 Qualitative (A) and quantitative (B) determination of nuclear DNA damage in embryonic neural stem cell cultures treated with H_2O_2 (100 µM) alone, H_2O_2 (100 µM) and resveratrol (100 µM), or H_2O_2 (100 µM) and trolox (100 µM) for 30 minutes.

The comet assay software program (version 1.2.2) was used to determine the extent of DNA damage after electrophoretic migration of DNA fragments in the agarose gel.

The data are shown as mean ± SD. All values were obtained from five independent experiments, with each performed in triplicate. Microscope magnification × 20. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* H_2O_2 group (one-way analysis of variance followed by Tukey's *post hoc* test).

TDNA: Tail DNA; TM: tail moment; TL: tail length; H_2O_2: hydrogen peroxide.

DISCUSSION

In this study, we determined the activities of antioxidant enzymes in embryonic neural stem cells exposed to hydrogen peroxide with or without resveratrol or trolox. The protective effects of resveratrol or trolox on embryonic neural stem cells were determined by changes in nitric oxide synthase activity, nitric oxide levels, and damage to nuclear and mitochondrial DNA. Although its antioxidant

effects have been reported in other cell types, the present results provide, to the best of our knowledge, the first evidence of a neuroprotective role of resveratrol against hydrogen peroxide-induced toxicity in embryonic neural stem cells[35-38].

Figure 6 Qualitative (A) and quantitative (B) determination of mitochondrial DNA (mtDNA) damage in embryonic neural stem cell cultures treated with hydrogen peroxide (H_2O_2; 100 μM), H_2O_2 (100 μM) and resveratrol (100 μM), or H_2O_2 (100 μM) and trolox (100 μM) for 30 minutes.

The mtDNA (2 μg/μL) was loaded onto a 0.5% agarose gel and electrophoresed for 50 minutes at 80 V. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) and photographed. Densitometric analysis was performed using Biocapt (Vilber-Lourmat, France) software. M: Marker (100 bp).

The data are shown as mean ± SD. All values were obtained from five independent experiments, with each performed in triplicate. The results are expressed as the ratio of mtDNA damage in experimental cultures compared with control cultures not treated with H_2O_2. [a]$P < 0.05$, *vs.* control group; [b]$P < 0.05$, *vs.* H_2O_2 group (one-way analysis of variance followed by Tukey's *post hoc* test).

Hydrogen peroxide originates from the enzymatic or spontaneous dismutation of superoxide anions, which are the byproducts of a wide and ubiquitous variety of oxidases. Because of its high membrane permeability, intracellularly formed hydrogen peroxide induces deleterious intracellular effects and in neighboring cells. Although hydrogen peroxide-induced toxicity has been observed in different cell types[32, 39], neurons are particularly vulnerable to hydrogen peroxide-induced toxicity[40-42].

In the present study, treatments with hydrogen peroxide alone, starting at a concentration of 10 μM, decreased embryonic neural stem cell survival in primary cultures, and up to 45% of the cells were killed by 100 μM of hydrogen peroxide. Resveratrol partially protected embryonic neural stem cells from hydrogen peroxide-induced toxicity. According to our results, treatment with resveratrol (100, 150, and 250 μM) decreased hydrogen peroxide-induced cell death by 35%. Therefore, we suggest that resveratrol provides cytoprotection by inhibiting nitric oxide production and glutathione depletion, and by reducing intracellular reactive oxygen species accumulation. We make this suggestion because resveratrol protects hippocampal neurons from nitric oxide-induced neurotoxicity[43], inhibits MPP^+-induced glutathione depletion[39], and reduces the accumulation of reactive oxygen species in cellular systems[40]. Antioxidant enzyme regulation in cells is an important process that balances the generation and the elimination of reactive oxygen species. Among the antioxidant enzymes, catalase is more crucial and eliminates hydrogen peroxide directly. Superoxide dismutase and glutathione peroxidase are essential in the attenuation of oxidative stress as well. For this reason, we determined possible changes in catalase, superoxide dismutase, and glutathione peroxidase activities following hydrogen peroxide treatment with or without resveratrol and trolox. According to our results, catalase activity was higher (1.5-fold) in the hydrogen peroxide group compared to the control group. Also, glutathione peroxidase activity was significantly higher in hydrogen peroxide-treated embryonic neural stem cells. Our results seem to be consistent with the study of Spanier *et al* [43]. However, co-treatment with resveratrol resulted in a significant decrease in catalase and glutathione peroxidase activities, indicating a reduction in reactive oxygen species production. In contrast, superoxide dismutase activity did not significantly vary in cells treated with hydrogen peroxide and resveratrol or trolox compared to those only treated with hydrogen peroxide. Therefore, we suggest that superoxide dismutase levels did not change because of low levels of superoxide radical production.

Nitric oxide, is a highly diffusible free radical with a short half-life. In the nervous system, it is involved in neurotransmitter release, long-term potentiation and depression, neuronal differentiation, synaptogenesis, cell death, and neurodegeneration[11, 44]. Nitric oxide synthase activity is primarily responsible for the production of nitric oxide radicals[11], and the role of overproduction of nitric oxide by inducible nitric oxide synthase in neurological

disorders has been reported[45-48]. Nitric oxide radicals promote oxidative cell damage by reacting with superoxide anions to form peroxynitrite, a highly toxic molecule that modifies biomacromolecules, and by induction of apoptotic and necrotic pathways[49]. Our results show that hydrogen peroxide treatment significantly increased nitric oxide synthase activity with concomitant nitric oxide production. In addition, resveratrol or trolox treatment both decreased nitric oxide synthase activity and nitric oxide levels. Therefore, we suggest that hydrogen peroxide treatment triggers nitric oxide production by altering nitric oxide synthase activity, and we suggest that the increase in nitric oxide levels may facilitate their transformation into peroxynitrite radicals, leading to neurotoxicity in embryonic neural stem cells. Furthermore, the increased activities of antioxidant enzymes, catalase and glutathione peroxidase, and the increased levels of nitric oxide by hydrogen peroxide treatment support the idea that hydrogen peroxide causes oxidative stress. It has been suggested that nitric oxide synthase inhibition is a therapeutic target in diseases associated with pathophysiological overproduction of nitric oxide[49]. Accordingly, resveratrol may be a potential agent to provide neuroprotection by inhibiting nitric oxide synthase activity.

The production of hydroxyl radicals radicals from hydrogen peroxide through the Fenton reaction is dependent on transient metal ions, such as iron (Fe^{2+}/Fe^{3+}). These hydroxyl radicals damage DNA[50]. Through site-specific reactions, metal ions tightly bind to chromatin, and an affinity site arises on DNA for hydroxyl radicals derived from the Fenton reaction. The oxidant agent used in this study, hydrogen peroxide, probably triggers hydroxyl radicals production *via* Fenton reactions in embryonic neural stem cells. Several studies have provided inconsistent results regarding the influence of resveratrol on genome integrity, which could be related to the use of different methodologies and cell types[50-51]. In general, genomic DNA damage is detected through DNA breaks, micronuclei, sister chromatid exchanges, or chromosome aberrations. DNA breaks scored using the comet assay may reflect incomplete base excision repair, nucleotide excision repair, or double-strand break repair[51]. This method has many applications in the estimation of oxidative damage and DNA crosslinks, in apoptosis, and in genotoxicity induced by chemical compounds[25, 52]. Quincozes-Santos *et al*[25] showed that pretreatment of resveratrol has genoprotective effects against hydrogen peroxide-induced DNA damage in C6 glioma cells. Consistently, our comet-assay results

demonstrate hydrogen peroxide-triggered nuclear DNA damage in embryonic neural stem cells. Meanwhile, we observed a significant decrease in the DNA damage in embryonic neural stem cells treated with resveratrol and hydrogen peroxide or trolox and hydrogen peroxide.

Mitochondria play important roles in cell proliferation, energy production, and apoptosis. These organelles contain their own maternally inherited genetic material, called mitochondrial DNA. Mitochondrial DNA is smaller than nuclear DNA. It plays a crucial role in ageing and carcinogenesis because mitochondria represent the major site for the generation of cellular oxidative stress and mediate mitochondria-dependent apoptotic processes. The deficiency in mitochondrial antioxidant status causes mitochondrial overproduction of reactive oxygen species. The generation of reactive oxygen species by mitochondria leads to irreversible damage of mitochondrial DNA and membrane lipids and proteins, resulting in mitochondrial dysfunction and ultimately cell death[52]. Damage to mitochondrial DNA therefore has an important role in ageing, cancer, and neurodegenerative diseases[53]. It has been shown that resveratrol prevents mitochondrial damage induced by hydrogen peroxide[54-55]. Okawara *et al*[56] showed that resveratrol protects brain mitochondrial functions after hypoxia-reoxygenation by reducing reactive oxygen species generation in rat brain mitochondria. Resveratrol counteracts the effects of uncouplers on mitochondrial respiration and oxidative phosphorylation, both *in vivo* and *in vitro*[55-56]. Zini *et al*[57] suggested that resveratrol can preserve mitochondrial functions through its antioxidant properties, by acting on complex III, and by stabilizing mitochondrial membranes. Also, resveratrol reversed neuronal dysfunction induced by polyglutamine tracts in the Huntington protein in *C. elegans*[58] and significantly delayed age-dependent impairment of locomotor activity and cognitive performance[43]. Therefore, we suggest that resveratrol preconditioning can promote cerebral mitochondrial biogenesis, including increasing the number and size of the mitochondria and the content of mitochondrial DNA. Mitochondrial biogenesis can also exert neuroprotective actions against cerebral injuries induced by oxidative stress. Our study demonstrated that mitochondrial DNA damage was significantly increased by hydrogen peroxide treatment, and it was significantly decreased by resveratrol treatment. The observed mitochondrial DNA damage can be explained by higher concentrations of reactive oxygen species and their deleterious effects on DNA following hydrogen peroxide treatment. Therefore, we suggest that resveratrol may be a potential protective agent to inhibit

oxidative mitochondrial DNA damage caused by hydrogen peroxide in embryonic neural stem cells. In addition, these data suggest that the protective effects of resveratrol are mediated by its radical scavenging ability.

To conclude, in the present study, it is clear that the beneficial effects of resveratrol are mediated, at least in part, by inhibition of oxidative stress. This inhibition lead to increased antioxidant enzyme activity, decreased nitric oxide production and nitric oxide synthase activity, and alleviated nuclear and mitochondrial DNA damage. In the light of these findings, further studies on its molecular mechanisms are needed to clarify the protective role of resveratrol against neurodegenerative processes that involved oxidative or nitrosative stress.

MATERIALS AND METHODS

Design
A randomized, controlled, *in vitro* cell culture experiment.

Time and setting
Experiments were performed at the Brain Research Center, Ege University and Biochemistry Department, Faculty of Pharmacy, Ege University, Turkey from September 2009 to January 2010.

Materials
Neuronal restricted precursors and glial restricted precursors from embryonic neural stem cells were isolated from embryonic day 13.5 Sprague-Dawley rats ($n = 5$). The Appropriate Animal Care Committee of Ege University approved the protocol for the experiment. All efforts were made to minimize the number of animals used and their suffering.

Methods
Preparation of embryonic neural stem cells
The preparation of the neuronal restricted precursors and glial restricted precursors has been described previously[59-60]. Briefly, embryos were isolated in Dulbecco's Modified Eagle Medium/Nutrient F12 Ham's (DMEM/F12; Sigma, St. Louis, MO, USA). Trunk segments (approximately 0.96 mm) were incubated in a solution of collagenase type I, dispase II, and Hanks balanced salt for 8 minutes at room temperature to remove the meninges from the brain and to remove all of the spinal cord. Cords were dissociated using a 0.05% trypsin and ethylenediamine tetraacetic acid solution for 20 minutes at 37°C. Cells were plated in the complete medium (DMEM/F12, bovine serum albumin, B27, basic

fibroblast growth factor, penicillin-streptomycin, N2, and neurotrophin-3) on poly-L-lysine and laminin-coated dishes. After dissection, neuronal restricted precursors and glial restricted precursors were co-cultured for 5–10 days in the complete medium to generate a mixed population for grafting. Previous studies[60-61] verified that these cultures contained only the precursors that were devoid of multipotent stem cells and mature cell types. The purity of the culture in lineage-restricted precursors was verified by staining the immature neural marker nestin. The neuronal restricted precursor/glial restricted precursor ratio was determined using embryonic neural cell adhesion molecule and A2B5. Embryonic neural stem cells were cultured at 37°C in 5% CO_2 95% humidified atmosphere. The cells were cultured in DMEM containing: 25 mM glucose, 1 mM pyruvate, 4.02 mM l-alanyl-glutamine and 10% fetal calf serum (Sigma). The cells were cultured 1 day prior to the treatments. Confluent cells were detached with 0.15% trypsin for 5 minutes. Afterwards, the cells were mixed with 2 mL of complete medium and centrifuged at 1 000 r/min (180 × *g*) for 5 minutes. Cells were transferred to 96-well microtitre plates (Corning, Acton, MA, USA) with a cell number of 1×10^4 cells per well (at the concentration of 1×10^6/mL).

Cell treatment
Cells were exposed to different concentrations (10–150 μM) of hydrogen peroxide or 100 μM of hydrogen peroxide for 60 minutes to determine the appropriate concentration and exposure time for decreased neuronal survival. Cells were exposed to hydrogen peroxide (100 μM) and resveratrol (5–250 μM) for 30 minutes at 37°C to determine whether resveratrol has cytoprotective effects that attenuate the effects of hydrogen peroxide. After these experiments, the cells were divided into four groups as follows: (1) control group, cells treated with PBS; (2) hydrogen peroxide group, cells treated with 100 μM hydrogen peroxide for 30 minutes only; (3) hydrogen peroxide + resveratrol group, cells treated with hydrogen peroxide (100 μM) and resveratrol (100 μM) for 30 minutes; (4) hydrogen peroxide + trolox group, cells treated with hydrogen peroxide (100 μM) and trolox (100 μM) for 30 minutes. Resveratrol (5–250 μM) and trolox (100 μM) were dissolved in PBS. Trolox, a well-known antioxidant and oxygen free radical scavenger, was used to calibrate the effects of resveratrol in embryonic neural stem cells. The concentration used for trolox (100 μM) was chosen according to a previous study in which trolox produced significant protection against oxidative stress in cultured hippocampal neurons[43].

Lactate dehydrogenase assay

Viability of the cultures following hydrogen peroxide alone or hydrogen peroxide and resveratrol treatment was assessed using a lactate dehydrogenase efflux assay[62-63]. Culture medium (90 µL) was assayed by adding 100 µL PBS containing pyruvate (1.54 mM) and nicotinamide adenine dinucleotid (0.5 mM). The conversion of nicotinamide adenine dinucleotid to oxidized nicotinamide adenine dinucleotide (NAD^+) was monitored at 340 nm on a microplate reader (Molecular Devices Versamax BN 03252, Sunnyvale, CA, USA). Absorbance was recorded every minute for 5 minutes and the change in absorbance (ΔA_{340nm}) was determined. In the absence of media or lysate samples, ΔA_{340nm}/min was negligible over 5 minutes. Total lactate dehydrogenase activity was determined by adding ΔA_{340nm}/min in lysate and media samples. Lactate dehydrogenase release was determined by dividing ΔA_{340nm}/min in the media sample by total ΔA_{340nm}/min. Viability was determined by dividing total ΔA_{340nm}/min in treated samples by total ΔA_{340nm}/min for control samples, and expressed as a percentage. The neuronal survival following hydrogen peroxide alone or hydrogen peroxide and resveratrol treatment is represented as a percentage of control.

Catalase activity in embryonic neural stem cell cultures

The activity of catalase was determined spectrophotometrically[64]. The reaction mixture consisted of 1 mL of PBS (50 mM, pH 7.0) and 2 mL of diluted cell homogenate. The mixture was incubated at 25°C for 3 minutes. The reaction was started by the addition of 1 mL of 30 mM hydrogen peroxide. The decomposition of hydrogen peroxide was followed directly by a decrease in absorbance at 240 nm at 25°C, as measured using a temperature-controlled Shimadzu UV-1601 spectrophotometer (Kyoto, Japan). The results are expressed as U/mg protein. One unit of activity is equal to 1 mM of hydrogen peroxide degraded per minute and is expressed as units per mg of protein.

Superoxide dismutase activity in embryonic neural stem cell cultures

We used xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitropheno)-5-phenyltetrazolium-chloride (INT) to form red formazan dye[65]. Superoxide dismutase activity was measured based on inhibition of this reaction and expressed as U/mg of protein. One unit of superoxide dismutase was defined by a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Glutathione peroxidase activity in embryonic neural stem cell cultures

Glutathione reductase and nicotinamide-adenine dinucleotide phosphate convert oxidized glutathione to its reduced form through oxidation of nicotinamide-adenine dinucleotide phosphate to $NADP^{+}$[66]. The decrease in absorbance at 340 nm was measured spectrophotometrically and expressed as U/mg of protein. One unit of glutathione peroxidase induces formation of 1 µmol $NADP^+$ from nicotinamide-adenine dinucleotide phosphate per minute at pH 8.0 and 25°C.

Nitric oxide synthase activity in embryonic neural stem cell cultures

Nitric oxide synthase activity was measured using the nitric oxide synthase activity kit (Bioxytech, Oxis Inc., Dublin, USA), which determines total nitrite as an indicator of nitric oxide synthase activity. Nitrite production was measured during the timed reaction (60 minutes) and compared with heat-inactivated control samples. Nitrate reductase was used for the enzymatic reduction of nitrate to nitrite. Spectrophotometric quantitation of nitrite was performed using Griess reagents[57]. In acidic solution, nitrite converts into HNO_2 (diazotied sulfanilamide). Sulfanilamide-diazonium salt reaction with N-(1-naphthyl)-ethylenediamine leads to chromophore production which was measured at 540 nm and expressed as nmol/mg protein/min[57].

Measurement of nitric oxide levels in embryonic neural stem cells cultures

Chemical reduction of nitrate to nitrite was performed with the kit (Assay Designs, New York, NY, USA) following the manufacturer's protocol. Afterwards, nitrite was measured with Griess reagent. In acidic solutions, nitrite converts to HNO_2. We used a sulfanilamide-diazonium salt reaction with N-(1-naphthyl)-ethylenediamine to produce the chromophore, which was measured at 540 nm and expressed as nmol/mg of protein[57].

Single-cell gel electrophoresis (the comet assay)

Single-cell gel (comet) assay was carried out following the protocol described by Tice et al [41]. Slides were prepared in duplicate for each treatment. Thus, a volume of 10 µL of treated or control cells (~1 × 10^4 cells) were added to 120 µL of 0.5% low melting point agarose at 37°C, layered onto a precoated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in a refrigerator, the coverslip was removed and slides immersed in lysis solution (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid; 10 mM

Tris-HCl buffer pH 10; 1% sodium sarcosinate; with 1% Triton X-100 and 10% dimethyl sulfoxide) for approximately 1 hour. Prior to the electrophoresis, the slides were left in alkaline buffer (0.3 mM NaOH and 1 mM ethylenediaminetetraacetic acid, pH > 13) for 20 minutes and electrophoresed for another 20 minutes at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized with 0.4 M Tris-HCl buffer (pH 7.4) and stained using a silver-based method[38]. The dried microscope slides were covered with a cover-glass prior to analysis with a light microscope (Olympus, Japan). The microscope was connected to a charge-coupled device camera and a computer with the Comet Assay Software Program version 1.2.2 installed to determine the extent of DNA damage after electrophoretic migration of DNA fragments in the agarose gel.

The three standard parameters of the assay, percentage of tail DNA, tail length (in μm) and the tail moment, were scored. Tail length is the measurement from the center of the head area (nucleus) towards the end of the tail, percentage of tail DNA is the percentage of DNA in the tail, and tail moment is a parameter calculated using the formula: Tail moment = tail length × percentage of tail DNA[67]. The results are expressed as a percentage of tail DNA, tail moment, and tail length. All of the steps described above were conducted in the dark to prevent additional DNA damage. Throughout this study, several diluted and treated aliquots were tested for viability by trypan blue exclusion[68-69].

Mitochondrial DNA isolation and agarose gel electrophoresis

Mitochondrial DNA was isolated from control and treated cells with a BioVision mitochondrial DNA isolation kit (BioVision, Mountain View, CA, USA) following the manufacturer's protocol[70]. The concentration of mitochondrial DNA was determined by measuring absorbance at A_{260nm}. The purity of the mitochondrial DNA was determined by the ratio of absorbance at $A_{260nm/280nm}$. The mitochondrial DNA was separated by agarose gel electrophoresis as described previously[70], with some modifications. Briefly, mitochondrial DNA (2 μg/μL) was dissolved in 5 μL of 6xgel loading buffer and then loaded into a well of a 0.5% agarose gel in a tris, boric acid, and ethylenediaminetetraacetic acid buffer (89 mM Tris boric acid, 2 mM ethylenediaminetetraacetic acid, pH 8.0). Electrophoresis was performed for 50 minutes at 80 V. After electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL in a tris, boric acid, and ethylenediaminetetraacetic acid buffer) and

photographed. Densitometric analysis was performed using Biocapt software (Vilber-Lourmat, France).

Statistical analysis

All results are expressed as mean ± SD from five independent experiments for all indices. Comparisons of means between groups were performed by one-way analysis of variance followed by Tukey's *post hoc* test. A *P* value less than 0.05 was considered statistically significant. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Version 13.0 for Windows (SPSS Inc, Chicago, IL, USA).

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