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Full Paper

Phloroglucinol Exerts Protective Effects Against Oxidative Stress–Induced Cell Damage in SH-SY5Y Cells

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Abstract. In this study, we explored whether phloroglucinol (1,3,5-trihydroxybenzene) exerts protective effects against oxidative stress–induced cytotoxicity in SH-SY5Y cells, a neuroblastoma cell line. The central nervous system is especially vulnerable to oxidative stress because the brain consumes large amounts of energy to perform its complicated functions, but contains a lower level of anti-oxidant enzymes compared with other peripheral tissues. In SH-SY5Y cultures, pretreatment with 10 μg/ml of phloroglucinol attenuates the cytotoxicity of hydrogen peroxide. In addition, when assessed with 2′,7′-dichlorofluorescein diacetate dye, the increase in reactive oxygen species (ROS) induced by hydrogen peroxide was significantly reduced by pretreatment with phloroglucinol. ROS is known to cause lipid peroxidation of the cell membranes, the carbonylation of intracellular proteins, and DNA damage, which can lead to various cellular dysfunctions and eventually cellular death. We found that pretreatment with phloroglucinol down-regulated the levels of 8-isoprostane, protein carbonylation, and 8-hydroxy deoxyguanine caused by hydrogen peroxide treatment in SH-SY5Y cells. These results indicate that phloroglucinol exerts protective effects against oxidative stress-induced cell damage in SH-SY5Y cells.

Keywords: brown algae, hydrogen peroxide, phloroglucinol, reactive oxygen species, SH-SY5Y cell

Introduction

Oxygen (O_2) is required by prokaryotic and eukaryotic cells for energy production (1 – 3). More than 90% of the oxygen that enters mammalian cells is used inside the mitochondria to produce 80% of the adenosine triphosphate needed by human cells. Some of the oxygen taken into cells forms partially reduced oxygen species such as reactive oxygen species (ROS). Some of these ROS contain an unpaired electron and are referred to as free radicals (4). ROS react quickly with other molecules (2). The generation of excessive ROS in normal cells can initiate tissue damage and cause the destruction of cellular components, including lipids, proteins, and DNA, which can induce a variety of cellular responses by generating secondary reactive species and ultimately lead to cell death via apoptosis and necrosis (5 – 7).

The brain is especially vulnerable to oxidative stress because it consumes large amounts of energy to perform its complicated functions, but contains lower levels of anti-oxidant enzyme, compared to other peripheral tissues. Oxidative stress has been implicated in the pathogenesis of several disease processes, including ischemia and reperfusion injury, Alzheimer's disease, Parkinson's disease, and diabetes mellitus (5). A primary goal of this study is to search for a molecule derived from natural

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products that can protect against oxidative stress in neuronal cells.

Phloroglucinol (1,3,5-trihydroxybenzene) is a member of the polyphenol group and one of the phlorotannin components abundantly present in *Ecklonia cava* (edible brown algae), which belongs to the *Laminariaceae* family (8). Seaweeds have commonly been eaten in East Asia for centuries, and their phytochemicals have been studied for their anti-oxidant and free radical properties (9 – 12). Treatment of cells with phloroglucinol has been shown to result in reduced cell damage caused by hydrogen peroxide in lung fibroblasts, as well as reduced γ ray-induced oxidative stress via the quenching of ROS and the activation of anti-oxidant systems (8, 9). Recently, we reported that phloroglucinol provided cytoprotection against UVB-irradiated human HaCaT keratinocytes (13). Apart from their oxidative damage defense properties, seaweeds, particularly brown alga species, have been suggested to be involved in protective biological events such as bactericidal activity, radical scavenging activity, antimutagenic activity, and inhibition of anti-plasmin activity (14).

In this study, we demonstrate that pretreatment with phloroglucinol protects SH-SY5Y cells from hydrogen peroxide–induced cytotoxicity. In addition, the increases in ROS, lipid peroxidation, protein carbonyl formation, and 8-hydroxy deoxyguanine (8-OhdG) induced by hydrogen peroxide treatment are ameliorated by phloroglucinol.

Materials and Methods

Chemicals and reagents

Phloroglucinol, hydrogen peroxide, and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was obtained from Invitrogen (Carlsbad, CA, USA).

Cell culture

SH-SY5Y cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 units/ml).

Cell viability

Cell viability was determined by MTT assay, which is based on the cleavage of tetrazolium salt by mitochondrial dehydrogenase in viable cells (15). Cells were seeded in a 96-well plate at 1.5×10^4 cells/well. To evaluate the protective effects of phloroglucinol against hydrogen peroxide, cells were pre-incubated with different concentrations of phloroglucinol for 1 h and then treated with 0.8 mM hydrogen peroxide (IC_{50}) for an additional 12 h. Subsequently, 50 μl of MTT stock solution (2 mg/ml) was added to each well for a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800 × *g* for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethyl sulfoxide (DMSO), and the absorbance (540 nm) was measured by a scanning multi-well spectrophotometer.

Intracellular ROS measurements

The DCF-DA method was used to detect the level of intracellular ROS (16). Cells were seeded in a 96-well plate at 1.5×10^4 cells/well and treated with 10 μg/ml phloroglucinol. After 1 h, 0.8 mM hydrogen peroxide was added to the plates. The cells were incubated for an additional 6 h at 37°C. After the addition of 25 μM DCF-DA solution for 10 min, the DCF fluorescence was detected using a Perkin Elmer LS-5B spectrofluorometer and flow cytometry (Becton Dickinson, Mountain View, CA, USA), as previously described (8). In addition, microscopic images were collected using a Laser Scanning Microscope 5 PASCAL (Carl Zeiss, Jena, Germany).

Lipid peroxidation assay

Cells were pretreated with 10 μg/ml phloroglucinol for 1 h and exposed to hydrogen peroxide, followed by incubation for an additional 12 h at 37°C. Lipid peroxidation was assayed by determining the 8-isoprostane levels in the culture medium (17). A commercial enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) was employed according to the manufacturer's instructions. The detection limit of 8-isoprostane in this assay was 5 pg/ml. The intra-assay and inter-assay coefficients of variation were 7.2% and 18.5%, respectively. Lipid peroxidation was also estimated using diphenyl-1-pyrenylphosphine (DPPP), a fluorescent probe (18). After the cells were incubated with 5 mM DPPP for 15 min in the dark, they were treated with eckol. The DPPP fluorescence images were analyzed using a Zeiss Axiovert 200 inverted microscope at an excitation wavelength of 351 nm and an emission wavelength of 380 nm.

Protein carbonylation

The cells were pretreated with 10 μg/ml phloroglucinol for 1 h. Then, 0.8 mM hydrogen peroxide was added to the plate, and the mixture was incubated for 12 h. The amount of carbonyl formation in protein was determined using an Oxiselect^{TM} protein carbonyl enzyme-linked

immunosorbent assay (ELISA) kit purchased from Cell Biolabs (San Diego, CA, USA), according to the manufacturer's instructions.

8-OhdG

The 8-OhdG content in DNA was determined using a Bioxytech 8-OHdG-ELISA kit purchased from OXIS Health Products (Portland, OR, USA) and performed according to the manufacturer's instructions. Cellular DNA was isolated using the DNAzol reagent (Life Technologies, Grand Island, NY, USA) and quantified using a spectrophotometer.

Single-cell gel electrophoresis (Comet assay)

Cells were pretreated with phloroglucinol for 1 h and exposed to hydrogen peroxide, followed by incubation for an additional 12 h at 37°C. The degree of oxidative DNA damage was determined via Comet assay. The cell suspension was mixed with 75 μl of 0.5% low melting agarose (LMA) at 39°C, and the mixture was then spread onto a fully frosted microscopic slide that was pre-coated with 200 μl of 1% normal melting agarose (NMA). After the agarose solidified, the slide was covered with another 75 μl of 0.5% LMA and then immersed in a lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4°C. The slides were then placed in a gel-electrophoresis apparatus containing 300 mM NaOH and 10 mM Na-EDTA (pH 13) for 40 min to allow sufficient time for DNA unwinding and the expression of the alkali-labile DNA damage. An electrical field was then applied (300 mA, 25 V) for 20 min at 4°C to induce migration of the negatively charged DNA towards the anode. The slides were washed three times for 5 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5), then stained with 75 μl propidium iodide (20 μg/ml), and observed using a fluorescence microscope equipped with an image analyzer (Komet 5.5; Kinetic Imaging, Nottingham, UK). The percentage of total fluorescence in the DNA tails and the tail lengths for 50 cells per slide were recorded.

Statistical analysis

All data are presented as the mean ± S.E.M. Statistical significance was tested using a one-way ANOVA followed by Tukey *post-hoc* tests using PASW statistics 18 software (SPSS, Inc., Chicago, IL, USA). The results were considered to be statistically significant at a threshold of $P < 0.05$.

Results

Phloroglucinol protects SH-SY5Y cells against hydrogen peroxide

We first performed an MTT assay to confirm the cytotoxic effects of hydrogen peroxide in SH-SY5Y cells, a neuroblastoma cell line. Treatment with hydrogen peroxide at 0.8 mM for 12 h was found to induce 50% cytotoxicity in the cells (Fig. 1A). On the basis of these results, we used the concentration of 0.8 mM hydrogen peroxide for further experiments.

Next, we pretreated the cells with various concentrations (5, 10, 15, 20, and 40 μg/ml) of phloroglucinol for 1 h and determined cell viability after hydrogen peroxide treatment for 12 h. We found that 5, 10, 15, 20, and 40 μg/ml phloroglucinol pretreatment resulted in 53%, 77%, 74%, 71%, and 64% cell viability, respectively, whereas 49% cell viability was observed in cells treated with hydrogen peroxide only (Fig. 1B). Based on the results that 10 μg/ml phloroglucinol showed maximal protective effects against hydrogen peroxide, we evaluated whether phloroglucinol was toxic to SH-SY5Y cells. Among the doses of 5, 10, 20, and 40 μg/ml, 20 and 40 μg/ml phloroglucinol treatment for 12 h significantly reduced cell viability by 8% and 21%, respectively, whereas 5 and 10 μg/ml phloroglucinol did not induce any changes in cell viability, compared to the vehicle-treated control (Fig. 1C). For our subsequent experiments, we used 10 μg/ml phloroglucinol.

Phloroglucinol reduces intracellular ROS caused by hydrogen peroxide treatment

We evaluated whether phloroglucinol exerts effects on ROS formed by hydrogen peroxide treatment. The radical-scavenging effect of phloroglucinol was determined by measuring ROS levels generated by 6 h of hydrogen peroxide treatment. As shown in Fig. 2A, the increase in ROS following hydrogen peroxide treatment was attenuated by phloroglucinol-pretreatment for 1 h from 227% to 108%, as determined spectrofluorometrically. These results were also confirmed by flow cytometry, which showed a 38% reduction in the fluorescence (arbitrary units 66 vs. 106) in cells treated with phloroglucinol pretreatment plus hydrogen peroxide compared with cells given vehicle pretreatment plus hydrogen peroxide treatment (Fig. 2B). Furthermore, red fluorescence intensity, an indicator of ROS observed using confocal microscopy, was enhanced in hydrogen peroxide–treated cells, whereas phloroglucinol pretreatment reduced the fluorescence intensity of the hydrogen peroxide–treated cells (Fig. 2C).

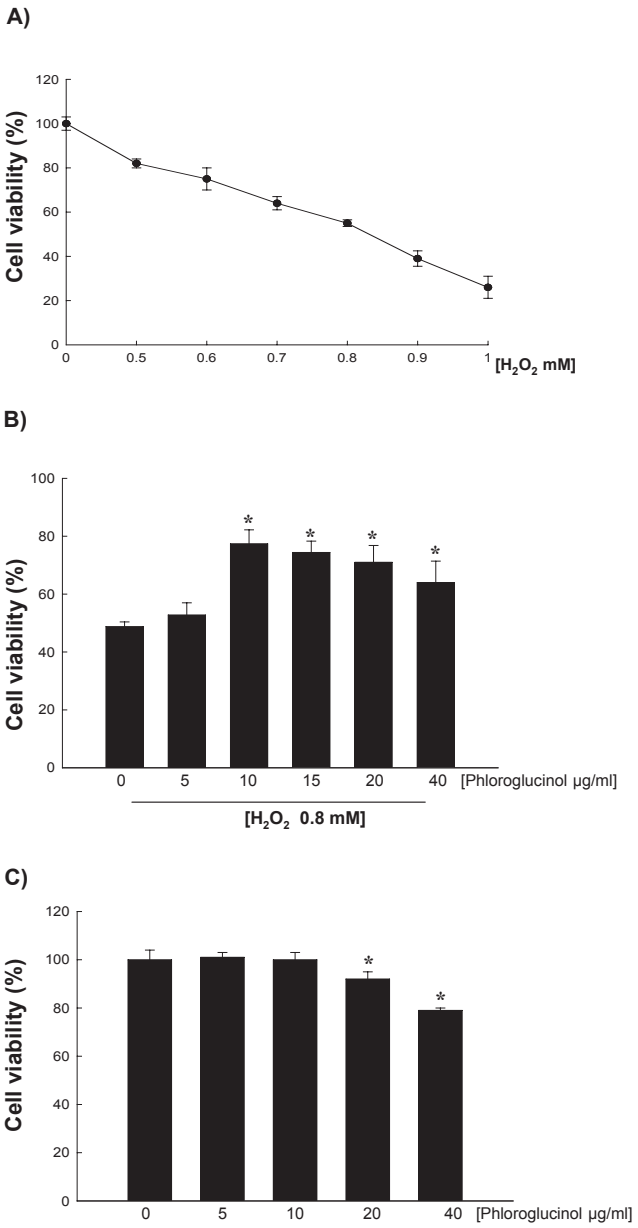


Fig. 1. Phloroglucinol protects SH-SY5Y cells against hydrogen peroxide. Cell viability (%) was measured by MTT assay. A: SH-SY5Y cells were treated with different ranges of hydrogen peroxide concentrations (0, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 mM). IC_{50} was determined at 0.8 mM hydrogen peroxide. B: Phloroglucinol in a range of different concentrations (5, 10, 15, 20, and 40 μg/ml) was used to pretreat SH-SY5Y cells for 1 h, and hydrogen peroxide was then used to treat the cells for an additional 12 h. The cell viability percentage was evaluated. C: The toxic effects of phloroglucinol on cell viability were examined with a range of different concentrations (5, 10, 20, and 40 μg/ml). The cells were treated with phloroglucinol for 13 h and the cell viability percentage was evaluated. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$).

Phloroglucinol diminishes the upregulated lipid peroxidation induced by hydrogen peroxide treatment

Increased ROS levels are known to induce lipid peroxidation, protein carbonylation, and DNA oxidation. In this study, we assessed whether pretreatment with phloroglucinol affected the marker levels for lipid peroxidation by examining 8-isoprostane levels. The 8-isoprostane levels were reduced from 1,061 ± 21 to 968 ± 26 ng/ml after phloroglucinol pretreatment for 1 h (Fig. 3A). Lipid peroxidation was also monitored using the DPPP reagent, which reacts with lipid hydroperoxides to produce the highly fluorescent product DPPP oxide (18). The fluorescence intensity of DPPP oxide was enhanced in hydrogen peroxide–treated cells and greatly reduced in cells treated with phloroglucinol (Fig. 3B).

Phloroglucinol down-regulates the increase in protein carbonylation induced by hydrogen peroxide treatment

Proteins are known to be carbonylated by intracellular ROS. We measured the level of protein carbonylation with a protein carbonyl ELISA kit. Protein carbonyl concentration was significantly down-regulated from 12.2 ± 1 to 8.9 ± 1 nM after pretreatment with phloroglucinol for 1 h prior to hydrogen peroxide treatment for 12 h (Fig. 4).

Phloroglucinol down-regulates the DNA damage induced by hydrogen peroxide treatment

Together with the markers for lipid peroxidation and protein carbonylation, the contents of 8-OhdG representing DNA oxidation are well-correlated with oxidative stress (19). We found that phloroglucinol pretreatment for 1 h significantly attenuated the increase in 8-OhdG induced by hydrogen peroxide treatment for 12 h from 1,262 to 635 pg/ml (Fig. 5A). Cellular DNA damage induced by hydrogen peroxide was also detected via Comet assay. Exposure of cells to hydrogen peroxide increased the percentage of DNA in the tails of the cells by 72% compared with the control value. The percentage of DNA in the tails was reduced to 30% in cells treated with phloroglucinol (Fig. 5B).

Discussion

Phloroglucinol is a compound derived from natural products such as edible brown algae, including *Ecklonia cava* (8). It is generally accepted that the development of a protective or therapeutic agent from an edible natural product is safer than that from chemical synthesis.

As life expectancy increases, populations worldwide show an increased prevalence of various neurodegenerative diseases. Although each neurodegenerative disease has a distinct etiological process and affects different

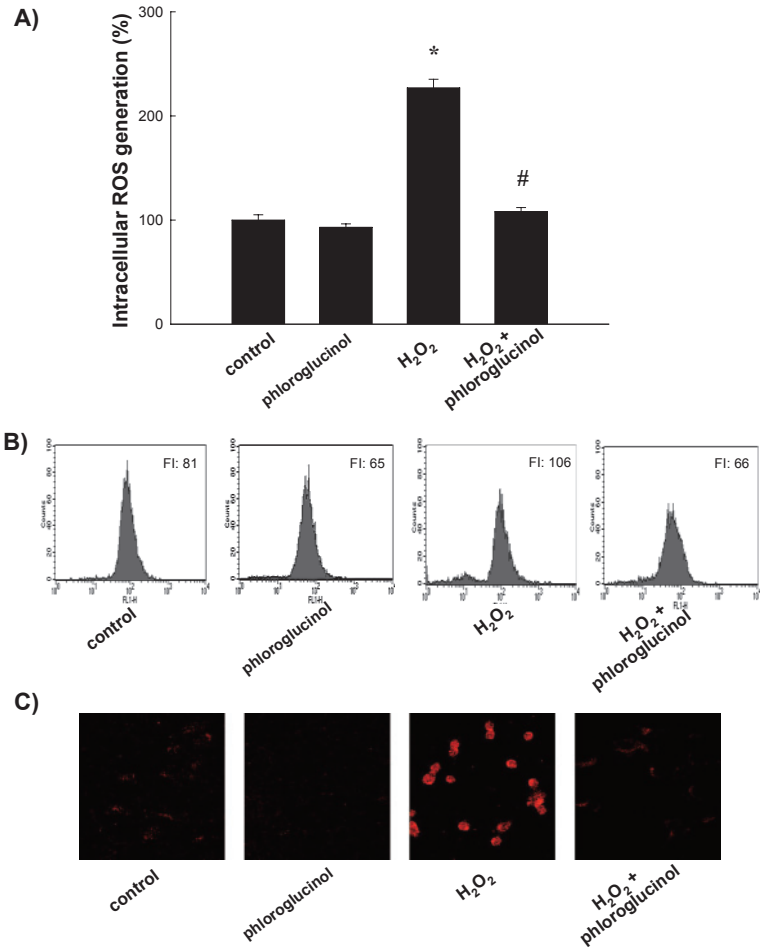


Fig. 2. Phloroglucinol reduces intracellular ROS levels caused by hydrogen peroxide treatment. A: Cells were seeded in a 96-well plate at 1.5×10^4 cells/well. At 16 h after plating, the cells were pretreated with 10 μg/ml phloroglucinol for 1 h and 0.8 mM hydrogen peroxide was added to the plates. The cells were incubated for an additional 6 h at 37°C. After the addition of 25 μM DCF-DA solution for 10 min, the DCF fluorescence was detected using a Perkin Elmer LS-5B spectrofluorometer. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$) and #significantly different from hydrogen peroxide–treated cells ($P < 0.05$). B: The intracellular ROS level was evaluated using flow cytometry. C: Microscopic images were collected using the laser scanning confocal microscope 5 PASCAL.

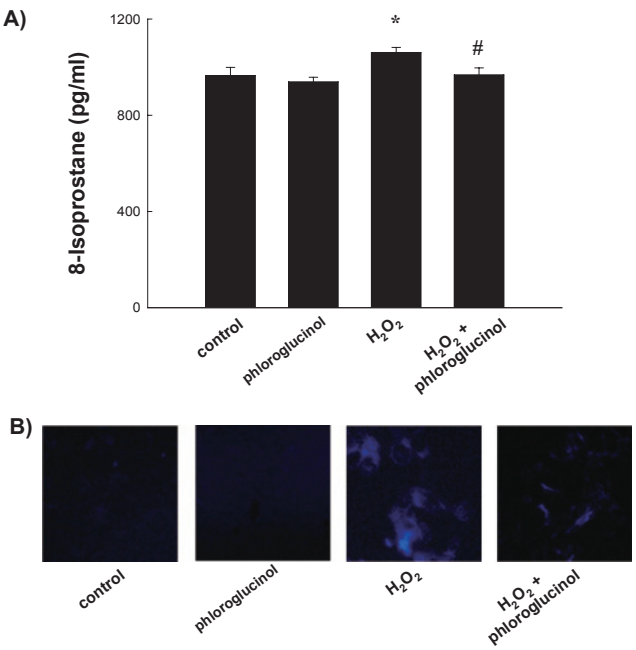


Fig. 3. Phloroglucinol diminishes the upregulated lipid peroxidation induced by hydrogen peroxide treatment. A: Lipid peroxidation was assayed by determination of 8-isoprostane levels. 8-Isoprostane levels were determined in the culture medium using a commercial enzyme immunoassay. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$) and #significantly different from hydrogen peroxide–treated cells ($P < 0.05$). B: Microscopic images were collected using the laser scanning confocal microscope 5 PASCAL.

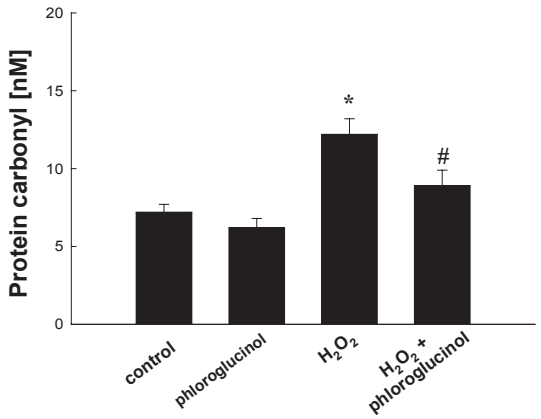


Fig. 4. Phloroglucinol down-regulates the increase in protein carbonylation induced by hydrogen peroxide treatment. The cells were pretreated with 10 μg/ml phloroglucinol for 1 h and 0.8 mM hydrogen peroxide was added to the plate, and the mixture was incubated for 12 h. The amount of carbonyl formation in protein was determined using an Oxiselect TM protein carbonyl ELISA kit. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$) and #significantly different from the hydrogen peroxide–treated cells ($P < 0.05$).

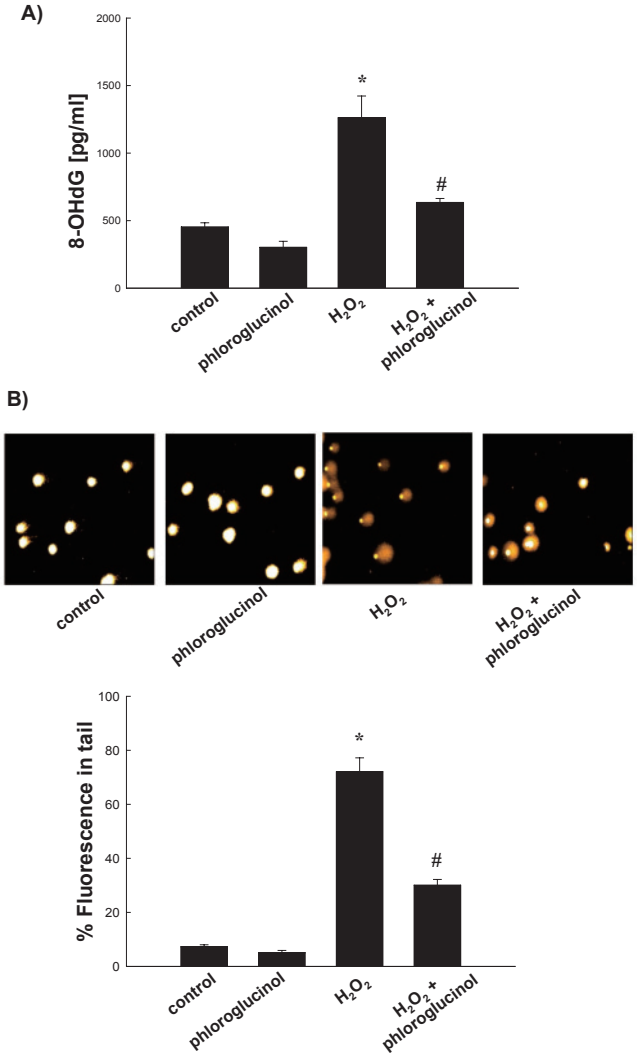


Fig. 5. Phloroglucinol down-regulates the DNA damage induced by hydrogen peroxide treatment. A: The 8-OhdG content in the DNA was determined using a Bioxytech 8-OhdG-ELISA kit. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$) and #significantly different from the hydrogen peroxide–treated cells ($P < 0.05$). B: Representative images and the percentage of cellular DNA damage were detected using a Comet assay. The statistical analysis was performed by a one-way ANOVA with Tukey's *post-hoc* test; the data are represented by means ± S.E.M., *significantly different from the control ($P < 0.05$) and #significantly different from the hydrogen peroxide–treated cells ($P < 0.05$).

brain regions, all neurodegenerative disorders share similar mitochondrial changes in varying degrees, suggesting a connection between disease pathology and the oxidative stress (3, 20). In the case of Alzheimer's and Parkinson's diseases, neuronal degeneration is associated with oxidative damage to all biomacromolecule types (3, 21): i) DNA and RNA oxidation is marked by increased levels of 8-OhdG and increased DNA oxidation and decreased repair in cerebrospinal fluid (22, 23); ii) Protein oxidation is marked by elevated levels of protein carbonyls and nitrotyrosine (24). Extensive literature supports a role for oxidative damage in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease and amyotrophic lateral sclerosis (3, 25).

We have previously reported that phloroglucinol treatment prevented cell damage induced by hydrogen peroxide in lung fibroblasts and reduced γ ray-induced oxidative stress via the quenching of ROS and the activation of anti-oxidant systems (8, 9).

Here, we investigated whether phloroglucinol has protective effects against hydrogen peroxide–induced cytotoxicity in SH-SY5Y cells, a neuroblastoma cell line. Phloroglucinol was found to protect SH-SY5Y cells against hydrogen peroxide–induced cell death, assessed by MTT assay (Fig. 1B). In addition, pretreatment with phloroglucinol reduced intracellular ROS levels (Fig. 2: A, B, and C) and oxidation markers in the cells (8-isoprotpane, protein carbonylation, and 8-OhdG) caused by hydrogen peroxide treatment (Figs. 3 – 5). In general, the criteria for measuring enhanced anti-oxidant activity reflect chemical structures like substitution patterns within hydroxyl groups and expanded conjugation systems after hydrogen or electron donation to radicals. The presence and availability of phenolic hydrogen and

the stabilization resulting from electron delocalization enhances anti-oxidant activity (26). In terms of structure and anti-oxidant activity, phloroglucinol is double-bond conjugated with a hydroxyl group and its structure provides anti-oxidant activity.

Collectively, our results provide evidence of a neuroprotective effect of phloroglucinol against oxidative stress–induced cell damage via the reduction of ROS and suggest that phloroglucinol may be a candidate compound for preventing or treating neurodegenerative diseases with additional research using experimental in vivo disease models. In the near future, we will be performing in vivo studies with phloroglucinol in a neurodegenerative disease animal model. In this future study, pharmacokinetic studies should be performed to examine whether phloroglucinol can pass through the blood–brain barrier.

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