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Green Tea epigallocatechin-3-gallate Protects Against Oxidative Stress-Induced Nuclear Translocation of p53 and Apoptosis in Retinal Pigment Epithelial Cells, ARPE-19

Peeradech Thichanpiang[1], Kornnika Khanobdee[2], Yindee Kitiyanant[1,3] & Kanokpan Wongprasert[1]

[1] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, Thailand

[2] Research Center for Neuroscience, Institute of Molecular Biosciences, Mahidol University, Nakhorn Pathom, Thailand

[3] Reproductive and Stem Cell Research Group, Institute of Molecular Bioscience, Mahidol University, Nakhorn Pathom, Thailand

Correspondence: Kanokpan Wongprasert, Department of Anatomy, Faculty of Science, Mahidol University, Bangkok, Thailand. Tel: 66-2-201-5447. E-mail: kanokpan.won@mahidol.ac.th

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Abstract

Epigallocatechin-3-gallate (EGCG), the most abundant polyphenolic flavonoids in green tea has been shown to possess strong antioxidant activities. Oxidative stress causes the defect of retinal pigment epithelial (RPE) cells that contribute to several retinal diseases. Several studies have shown that increasing the body's defenses against oxidative stress with specific antioxidants and mineral supplements could preserve the vision. Therefore, the purpose of this study was to determine the protective role of EGCG against exogenous reactive oxygen species hydrogen peroxide (H_2O_2)-induced cell death in ARPE-19 cells, human retinal pigment epithelial cell line. ARPE-19 cells were pretreated with EGCG in the presence/absence of H_2O_2. The protective effects of EGCG and the underlined mechanisms against H_2O_2 were evaluated. The present study demonstrated that 400 μM H_2O_2 significantly decreased cell viability, increased the accumulation of intracellular reactive oxygen species (ROS) and increased the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells and chromatin condensed nuclei. In addition, H_2O_2 induced p53 nuclear translocation, up-regulated Bax and down-regulated Bcl-2 expression thereby increased Bax/Bcl-2 ratio. These toxic effects of H_2O_2 were reversed by 100 μM EGCG pretreatment. These studies suggest that EGCG protects H_2O_2-induced cell death in ARPE-19 cells by its antioxidant property and attenuation of p53 nuclear translocation.

Keywords: epigallocatechin-3-gallate (EGCG), oxidative stress, p53, apoptosis, ARPE-19 cells

1. Introduction

Epigallocatechin-3-gallate (EGCG), the most abundant (40-60%) component of polyphenolic flavonoids in green tea, has been shown to possess a strong and active antioxidant, according to its two triphenolic groups in its molecular structure (Singh, Akhtar, & Haqqi, 2010; Weinreb, Mandel, Amit, & Youdim, 2004). EGCG has been shown to protect H_2O_2-induced oxidative stress in various types of cell such as PC12 cells (Koh et al., 2003), G93A motorneuron cells (Koh et al., 2004), auditory neurons (Xie, Liu, Zhu, Wu, & Ge, 2004) and INS-1 insulinoma cells (Kim et al., 2010) through different anti-apoptotic mechanisms.

It is well established that free hydroxyl radicals generated from excess H_2O_2 leading cell apoptosis since H_2O_2 is a potent DNA damage inducer (Jornot, Petersen, & Junod, 1998). For intrinsic apoptosis pathway, Bcl-2 family proteins are particularly important as they are potent regulators involving in the mitochondria-initiated intrinsic apoptosis (Brunelle & Letai, 2009; Sharpe, Arnoult, & Youle, 2004). This family consists of both pro- and anti-apoptotic members that perform the opposing effects on mitochondria, including the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax (Brunelle & Letai, 2009). Therefore, the ratio of Bax to Bcl-2 serves as a rheostat to determine the susceptibility of cells to apoptosis.

The p53 tumor suppressor protein mediates as a major role in a complex signaling pathway evolved in cellular stresses induced onset of apoptosis. Under certain stress conditions, p53 is phosphorylated and is shown to

translocate from cytosol and accumulate within the nucleus where it enhances the expression of target apoptotic genes, particularly Bax by binding on the promoter of gene (Samuels-Lev et al., 2001). Consequently, Bax forms a homodimer and releases cytochrome c from the mitochondria resulting in caspase-9 activation, which subsequently activates the effectors, caspase-3 and caspase-7, which are responsible for the dismantling of an apoptotic cell (Haupt, Berger, Goldberg, & Haupt, 2003).

Retinal pigment epithelium (RPE) is a single layer of cuboidal cells that forms the outer blood-retinal barrier. It is essential as nutritional or metabolic support for photoreceptor, phagocytosis and degradation of shed photoreceptor outer segments (Strauss, 2005). RPE is particularly susceptible to oxidative stress by reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, singlet oxygen, and H_2O_2, due to its locations and functions (Liang & Godley, 2003). Dysfunction of RPE contributes to retinal diseases such as retinitis pigmentosa (RP) (Strauss, 2005) and age-related macular degeneration (AMD) (Zarbin, 2004) leading to visual impairment. H_2O_2 has been widely used as the oxidative stress-induced apoptosis model in RPE (Godley, Jin, Guo, & Hurst, 2002; Jin, Hurst, & Godley, 2001), and has been shown to mediate apoptosis through p53 dependent pathway in several circumstances cells such as human fibroblasts (Youn et al., 2007), glioma cells (Datta, Babbar, Srivastava, Sinha, & Chattopadhyay, 2002) and RPE cells (Jin et al., 2001). The present study, therefore, aimed to investigate the protective effects of EGCG against H_2O_2-induced apoptosis in ARPE-19 cells focusing on the lineage of p53 dependent pathway. The results help enlighten the underlined protective mechanism of EGCG against oxidative stress-induced RPE apoptotic cell death.

2. Materials and Methods

2.1 Chemicals

The mouse monoclonal anti-α-tubulin FITC-conjugated secondary antibody and TO-PRO-3 were obtained from Zymed Laboratories Invitrogen (South San Francisco, CA). The rabbit polyclonal anti-Bcl-2, the rabbit polyclonal anti-Bax and the mouse monoclonal anti-p53 and rabbit polyclonal anti-phospho-p53 (at serine 15) were purchased from Santa Cruz Biotech (Santa Cruz, CA). HRP-conjugated anti-rabbit, anti-mouse antibodies and ECL Plus Western Blotting reagent were purchased from Amersham Biosciences (Piscantaway, NJ). Ac-DEVD-CHO [caspase-3 inhibitor (aldehyde); Ac-Asp-Glu-Val-Asp-CHO] was purchased from Alexis Biochemicals (San Diego, CA, USA). All other chemicals used in this study were obtained from Sigma (St. Louis, MO).

2.2 Cell Culture and Treatment with EGCG, H_2O_2, and Caspase 3 Inhibitor

ARPE-19 cells (ATCC, Manassas, VA) have been well characterized and possess differentiated properties similar to primary human RPE (Dunn, Aotaki-Keen, Putkey, & Hjelmeland, 1996). Cells were grown in DMEM/F12 (Gibco, Grand Island, NY) containing 10% Fetal Bovine Serum, 2% L-glutamine and 100 U/mL penicillin, and 100 μg/mL streptomycin at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were divided into normal control, EGCG, H_2O_2, pretreated with EGCG prior H_2O_2 exposure and Ac-DEVD-CHO caspase-3 inhibitor groups. Cells in the control group were incubated under the normal growth conditions. ARPE-19 cells were incubated with H_2O_2 (100, 200, 300, 400 and 500 μM) for dose selection then cells were exposed to the selected dose with different periods (0.5, 1, 2, 4 and 6 h). For the EGCG groups, the cells were pre-incubated for 2 h with different final concentrations of EGCG (1, 10, 25, 50 and 100 μM) followed by a 6 h incubation with 400 μM H_2O_2. For the Ac-DEVD-CHO caspase-3 inhibitor group, cells were pre-incubated for 1 h with 250 μM Ac-DEVD-CHO, and then incubated for an additional 6 h with 400 μM H_2O_2.

2.3 Cell Viability Measurement by MTT Assay

ARPE-19 cells (1×10^5 cells/well) were seeded and grown in 96-well plates for 24 h. At the designated times, cell viability was determined using methylthiazolium bromide (MTT) assay. Briefly, 100 μl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h at 37ºC in the dark. After incubation, 100 μl of dimethylsulfoxide (DMSO) was added to each well to lyse the cells. The absorbance of the sample was measured at 490 nm on a Versamax microplate reader using SoftMax Pro 4.8 analysis Molecular Devices software (Sunnyvale, CA).

2.4 Intracellular Reactive Oxygen Species (ROS) Measurement

The levels of the intracellular ROS was measured by the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method. Cells were grown on a 96 well-plate (1×10^5 cells/well) for 24 h and then incubated with 10 μM DCFH-DA for 1 h at 37°C in the dark. After incubation, DCFH-DA was removed and washed. Cells were then performed experiments. At different time periods (0.5, 1, 2, 4, 6 h), the fluorescence of 2′,7′-dichlorofluorescein (DCF) was detected at 485 nm excitation and 535 nm emission by a fluorescent plate reader (Wallac 1420 Victor,

Perkin-Elmer, Foster City, CA).

2.5 *Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay*

Nuclear DNA fragmentation of apoptotic cells was determined using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) following the manufacture's instruction. ARPE-19 cells were grown on Lab-Tek chamber slides (Nunc, Thermo Fisher Scientific, NY) until 95-100% confluence and then were performed the experiments. Cells were fixed with 4% methanol-free paraformaldehyde for 25 min at 4°C, washed with PBS, permeabilized with 0.2% Triton X-100 for 5 min and then washed. Fixed monolayer cell cultures were then covered with 100 µl of equilibration buffer for 10 min, followed with 50 µl of TUNEL reaction mixture containing equilibration buffer, nucleotide mix and TdT enzyme for 60 min at 37°C. The reaction was terminated by in 2X saline sodium citrate for 15 min at RT and then washed. The cells were then counterstained with Hoechst 33342 for 10 min in the dark, washed in de-ionized water and mounted with anti-fade solution. TUNEL-positive cells (green fluorescence) were observed by a fluorescent microscope (Nikon E600). Percentage of TUNEL-positive cells was averaged from a total of 500 cells from 3 independent slides.

2.6 Western Blot Analysis

Cells were grown in 6 well plates until 95-100% confluence then were performed experiments. Cells were lysed in lysis buffer [3 mM $MgCl_2$, 1 mM EGTA, 10 mM Na Orthovanadate, 10 mM Na Pyrophosphate, 50 mM NaF, 1 mM protease inhibitor (Roche)] for 30 min. The lysate was collected in to a 1.5 ml microfuge tube, centrifuged at 12000 g for 15 min. The supernatant was then kept at -80°C until used. Protein concentration was determined using BCA™ Protein Assay Kit (Pierce, Rockford, IL). Twenty micrograms of protein was mixed with loading buffer and run in a 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 100 V. The proteins were then transferred onto a nitrocellulose membrane and blocked with 5% nonfat dry milk in 1X Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.1% Tween 20) for 2 h. Membranes were incubated with primary then secondary antibodies and developed using the Amersham Biosciences chemiluminescent ECL kit and Hyperfilm ECL (Piscataway, NJ). Primary antibodies used in this study were rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-phospho-p53 (at serine 15) and mouse anti α-tubulin. HRP conjugated anti-rabbit and HRP conjugated anti-mouse IgG were used as secondary antibodies. The band density was normalized by α-tubulin.

2.7 Immunocytochemistry

To investigate the nuclear translocation of p53, immunocytochemistry was performed using anti-p53 antibody as a primary antibody. Cells were grown to 95-100% confluence on round coverslips then performed experiments. Cells were fixed in 4% paraformaldehyde for 5 min, blocked with 1% normal goat serum in 0.05% Triton X-100 for 30 min, then incubated overnight at 4°C with the diluted primary antibody: anti-p53 (1:500). After washed with PBS-Tween, cells were incubated with FITC-conjugated secondary antibody for 1 h at 37°C. Nuclei of cells were counterstained with TO-PRO-3. Immunofluorescence was visualized under a confocal laser-scanning microscope (FV 1000, Olympus, Tokyo, Japan). Negative controls were cells omitted anti-p53 antibody.

2.8 Statistical Analysis

Data was presented as means ± SEM from three or more independent experiments. Significance was accessed by one way analysis of variance (ANOVA) followed by a Tukey multiple comparison test in the GraphPad Prism program version 5 (GraphPad software, San Diego, CA). Differences with *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1 Effect of H_2O_2, EGCG, and Caspase 3 Inhibitor on ARPE-19 Cell Viability

The viability of ARPE-19 cells treated with 100, 200, 300, 400 and 500 µM H_2O_2 at 6 h were 80.4 ± 3.0%, 65.7 ± 8.0%, 59.8 ± 3.8%, 25.5 ± 4.4% and 20.1 ± 4.1% of the control value, respectively (Figure 1A). Moreover, cells treated with 400 µM H_2O_2 at 0.5, 2, 4, and 6 h showed time-dependent decrease of cell viability (Figure 1B). Cell viability was dramatically decreased to 25.5% (< 50%) of the control after treating the cells with 400 µM H_2O_2 at 6 h. Therefore, the 400 µM H_2O_2 for 6 h incubation was a suitable condition as oxidant-induced oxidative stress in subsequent experiments.

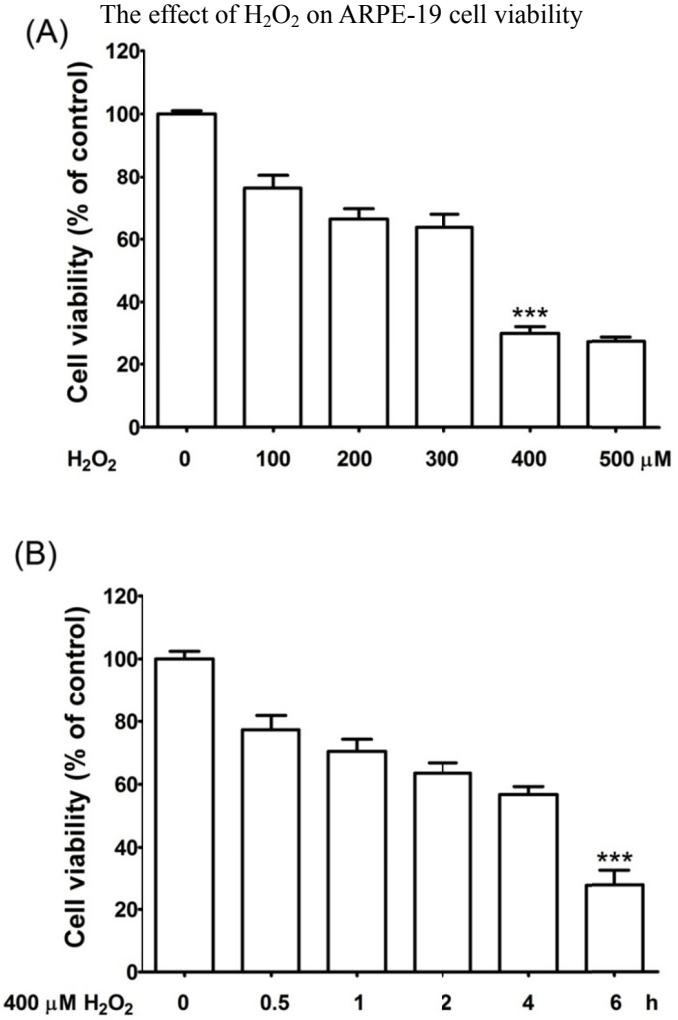

Figure 1. The effect of H_2O_2 on ARPE-19 cell viability determined by MTT assay. (A), Cells were incubated with different H_2O_2 concentrations (100, 200, 300, 400 and 500 μM) for 6 h. (B), Cells were treated with 400 μM with various times (0.5-6 h). The results were expressed as mean ± SEM of three independent experiments. The ANOVA was performed for statistical analysis, ***$p < 0.001$ compared with the control group

Pretreatment the cells with 100 μM EGCG for 2 h prior to exposure with 400 μM H_2O_2 for additional 6 h significantly increased cell viability to 52.4 ± 1.9% of the control value (Figure 2A). The viability of cells incubated with 250 μM caspase-3 inhibitor, Ac-DEVD-CHO, for 2 h prior to treatment with H_2O_2 also increased cell viability to 55.9 ± 1.5% of the control value (Figure 2 A). EGCG at 1, 10, 25, 50 and 100 μM did not affect the cell viability whereas 200 μM EGCG slightly decreased cell viability (91.8 ± 1.3%) (Figure 2B). These results suggested that 400 μM H_2O_2 caused ARPE-19 cells death whereas cells pretreatment with 100 μM EGCG or Ac-DEVD-CHO could protect the cells from H_2O_2-induced cell death.

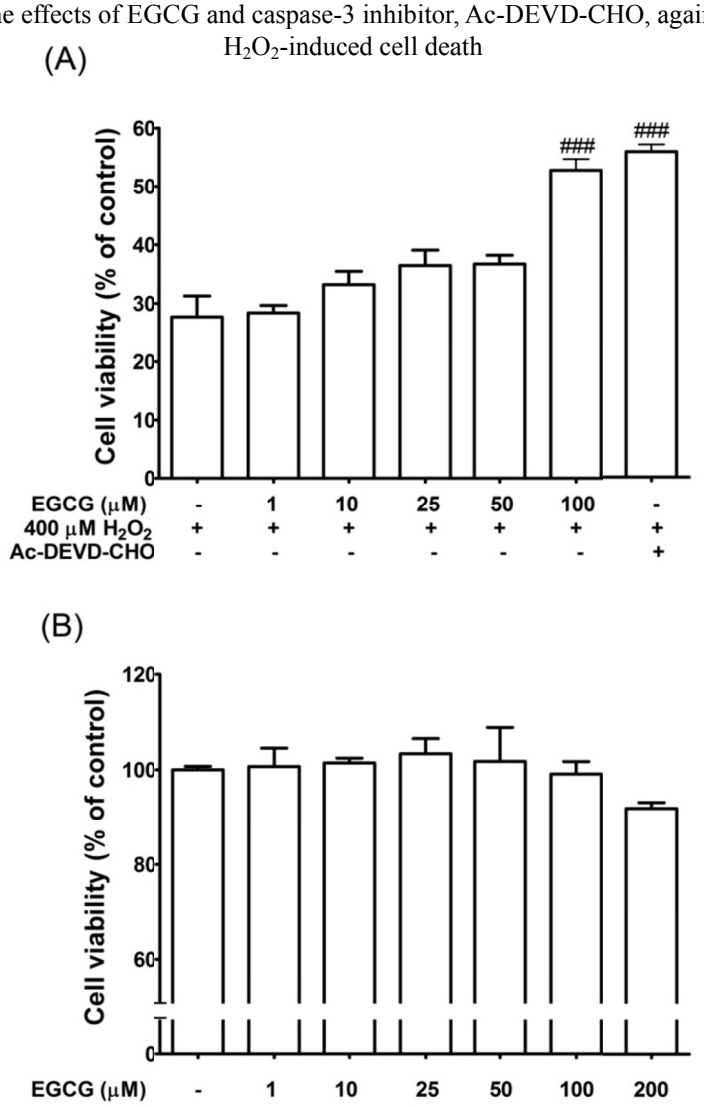

Figure 2. The effect of EGCG and caspase-3 inhibitor, Ac-DEVD-CHO, against H_2O_2-induced cell death in ARPE-19 cell. Cell viability was determined using MTT assay. (A), Cells were pretreated with EGCG (0, 1, 10, 25, 50, 100 μM) or 250 μM Ac-DEVD-CHO for 2 h prior to exposure with 400 μM H_2O_2 for 6 h. (B) Cells were treated with various concentrations of EGCG (1, 10, 25, 50, 100 and 200 μM) for 6 h. The results were expressed as mean ± SEM of three independent experiments. The ANOVA was performed for statistical analysis, ###$p < 0.001$ compared with H_2O_2-treated alone

3.2 Intracellular ROS Scavenging Ability of EGCG

After exposure with 400 μM H_2O_2 for 0.5, 1, 2, 4 and 6 h, the level of intracellular ROS generation in ARPE-19 cells was dramatically increased to 2.8 ± 0.2, 2.9 ± 1.2, 3.1 ± 0.2, 3.6 ± 0.1 and 3.5 ± 0.4 folds of control, respectively (Figure 3A). Pretreatment cells with 100 μM EGCG for 2 h prior to exposure with 400 μM H_2O_2 for 6 h significantly decreased intracellular ROS level to 1.1 ± 0.1 folds of control in ARPE-19 cells, compared with H_2O_2 treated cells without EGCG pretreatment (Figure 3B). Cells treated with 100 μM EGCG alone did not increase ROS level (Figure 3B). These findings indicated that pretreatment with 100 μM EGCG could effectively prevent H_2O_2-induced ROS production.

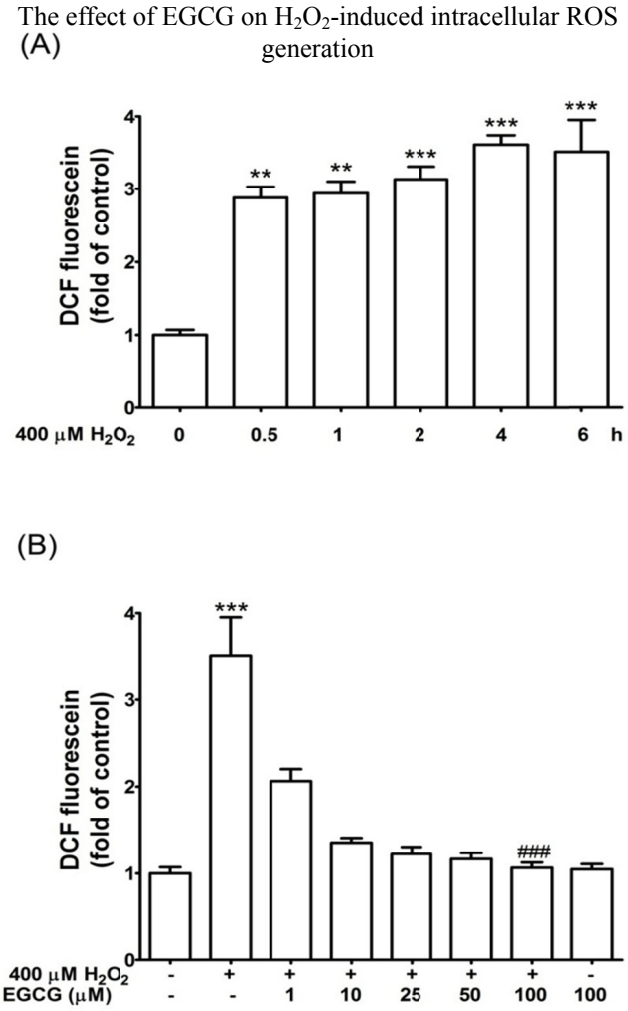

Figure 3. The effect of EGCG on H_2O_2-induced intracellular ROS generation. Before the experiment, all cell groups were incubated with 10 μM of DCFH-DA at 37°C in the dark for 1 h. (A), Cells were incubated with 400 μM H_2O_2 for 0.5, 1, 2, 4 and 6 h. (B), Cells were pretreated with 1, 10, 25, 50 and 100 μM EGCG or culture media for 2 h prior to incubation with 400 μM H_2O_2 for 6 h. Intracellular ROS level was expressed as fold of control (mean ± SEM of three independent experiments). The ANOVA was performed for statistical analysis, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control, and $^{###}p < 0.001$ compared with the H_2O_2-treated alone

3.3 Determination of Apoptosis Morphology by TUNEL and Nuclear Chromatin Condensation

DNA fragmentation and nuclear chromatin condensation which were the hallmark of apoptosis were determined by TUNEL method and Hoechst 33342 staining, respectively. The results demonstrated that treatment ARPE-19 cells with ARPE-19 cells with 400 μM H_2O_2 for 12 h induced nuclear chromatin condensation (Figure 4E) and TUNEL-positive cells (59.8 ± 2.0%) (Figure 4F) in ARPE-19 cells. Cells pretreatment with 100 μM EGCG showed a reduction in number of cells with nuclear chromatin condensation (Figure 4G) and number of TUNEL-positive cells (33.8 ± 2.4%) (Figure 4H). Figure 4I shows the percentage of TUNEL-positive cells which significantly decreased in cells pretreated with EGCG prior to H_2O_2 insult. No TUNEL-positive cell was observed in control cells (Figure 4B). Cells which were treated with DNase I, as positive control, are shown in Figure 4C and D. The results suggested that EGCG could protect ARPE-19 cells from apoptotic cell death.

The effect of EGCG on H_2O_2-induced nuclear chromatin condensation

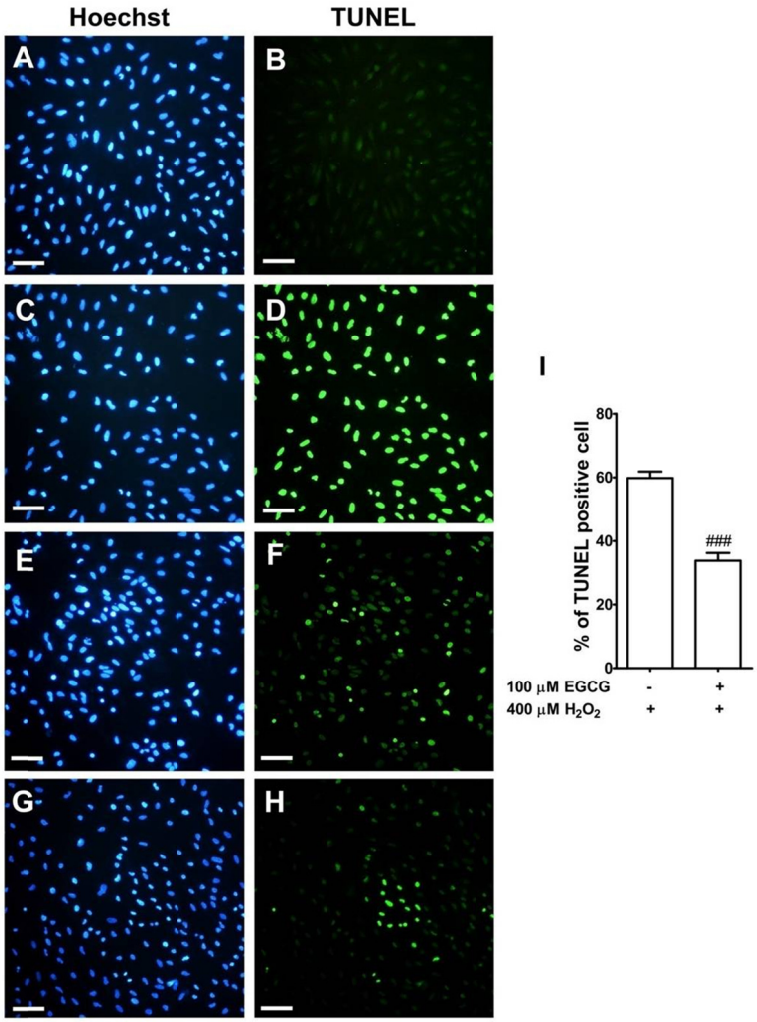

Figure 4. Fluorescent micrographs showing effects of EGCG on H_2O_2-induced nuclear chromatin condensation in ARPE-19 cells determined by TUNEL/Hoechst 33342 staining. (A) and (B), Control. (C) and (D), Positive control (DNase I). (E) and (F), Cells treated with 400 μM H_2O_2-treated cells for 12 h. (G) and (H), Cells pretreated with 100 μM EGCG for 2 h before 400 μM H_2O_2 for 12 h. (I), Percentages of TUNEL-positive cells. The results were expressed as mean ± SEM of three independent experiments. The ANOVA was performed for statistical analysis, ###$p < 0.001$ compared with the H_2O_2-treated alone. Scale bar = 50 μm

3.4 Determination of the Apoptosis Signaling Pathway by Measuring the Bax/Bcl-2 Ratio

Expressions of apoptosis related Bcl-2 family proteins, Bax and Bcl-2 were determined by Western blot analysis. The results demonstrated that the expression level of a pro-apoptotic protein, Bax, significantly increased in cells treated with 400 μM H_2O_2 for 2 h whereas the expression level of Bcl-2, an anti-apoptotic protein, significantly decreased (Figure 5A). Pretreatment cells with 100 μM EGCG for 2 h prior to exposure with 400 μM H_2O_2 for additional 2 h caused the decrease of Bax and increase of Bcl-2 expressions, compared to H_2O_2 treated alone (Figure 5A). The results suggested that H_2O_2 induced up-regulation of Bax and down-regulation of Bcl-2 expressions, whereas EGCG protected the H_2O_2 induced ARPE-19 cell death by inhibiting Bax up-regulation and Bcl-2 down-regulation induced by H_2O_2; reversed the expressions of Bax and Bcl-2 to as control. Therefore, the ratio of the expression of Bax/Bcl-2 significantly increased in H_2O_2 treated alone (1.8 ± 0.1) compared with control while EGCG pretreated group significantly decreased the ratio (1.1 ± 0.2) compared with H_2O_2 treated alone (Figure 5B).

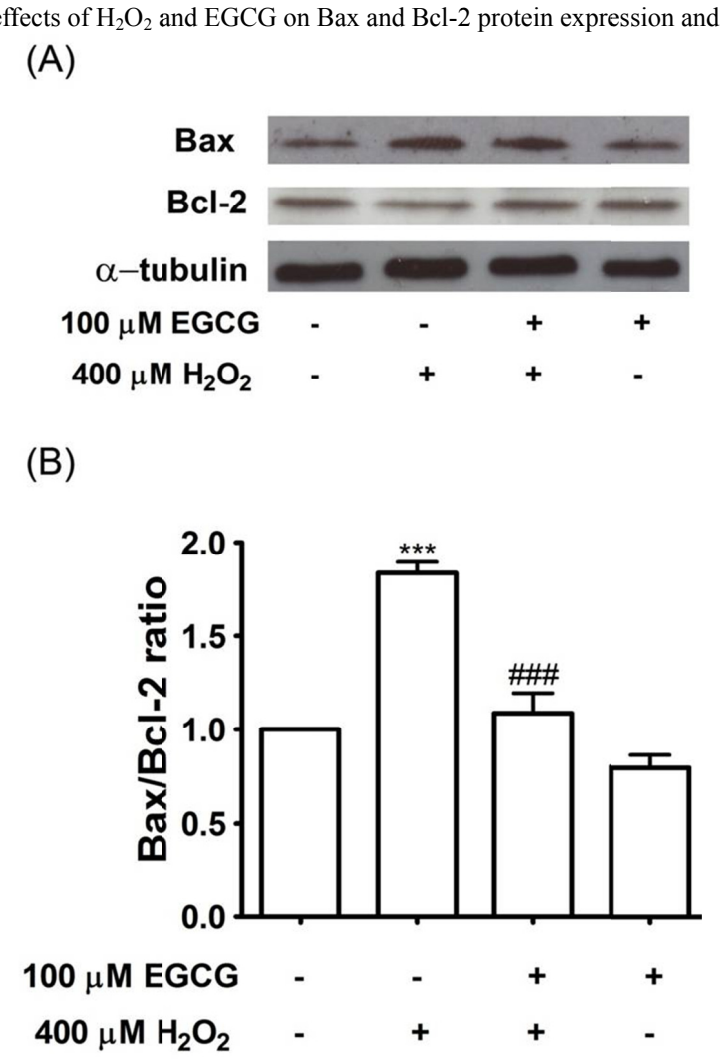

Figure 5. The effect of H_2O_2 and EGCG on Bax and Bcl-2 protein expressions and the Bax/Bcl-2 ratio in ARPE-19 cells. Cells were pretreated with either 100 μM EGCG or culture media for 2 h prior to exposure with 400 μM H_2O_2 for 2 h. (A), The alterations of Bax and Bcl-2 expressions determined using Western blot analysis. Expression of α-tubulin represented the equal protein loading. Bands were representative of three independent experiments. (B), Bax/Bcl-2 ratio. The ANOVA was performed for statistical analysis, ***$p < 0.001$ compared with the control and ###$p < 0.001$ compared with the H_2O_2-treated alone

3.5 Nuclear Translocation of p53 and phospho-p53 Expression

The immunocytochemistry of p53 in ARPE-19 cells is shown in Figure 6. The immuno-reactivity of p53 is shown in green whereas nuclei of cells are shown in red. The result showed that p53 was immuno-localized densely in nuclei of cells treated with H_2O_2 for 2 h (Figure 6C), compared to control (Figure 6A). Whereas, cells pretreated for 2 h with 100 μM EGCG prior to exposure with H_2O_2 for additional 2 h showed much less p53 immuno-reactivity in nuclei when compared to H_2O_2 treated alone (Figure 6D). Negative control cells are shown in Figure 6B. These findings suggested that ARPE-19 cells pretreatment with EGCG could reduce H_2O_2-induced nuclear translocation of p53.

To further investigate the effect of EGCG on p53, the phosphorylation/activation state of p53 was determined. The ARPE-19 cells treated with 400 μM H_2O_2 showed the higher level of phospho-p53 expression (3.9 ± 0.4) while pretreatment with 100 μM EGCG decreased the level of phospho-p53 to 2.4 ± 0.4 (Figure 6E). These results suggested that EGCG attenuated H_2O_2-induced the level of phospho-p53 expression.

The effects of H_2O_2 and EGCG on p53 translocation and the expression of phospho-p53

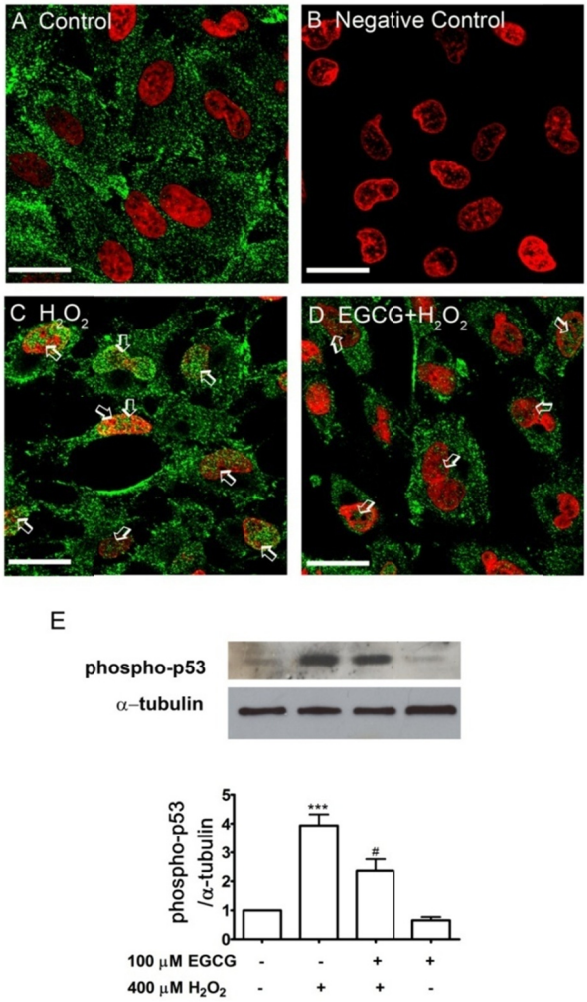

Figure 6. Immunocytochemistry showing p53 nuclear translocation, and Western blot analysis showing the expression of phospho-p53 after H_2O_2 exposure and protection by EGCG. (A), Control cells cultured with normal media. (B), Negative control. (C), ARPE-19 cells exposed to 400 μM H_2O_2 for 2 h. (D), Cells pretreated with 100 μM EGCG for 2 h before H_2O_2-exposure for another 2 h. Immunocytochemistry was performed using anti-p53 as a primary antibody while negative control was omitted the primary antibody. The secondary antibody was a FITC conjugated antibody (green), and nuclei were counterstained with TO-PRO-3 (red). (E), Relative expression of phospho-p53. .Expression of α-tubulin represented the equal protein loading. Bands are representative of three independent experiments, ***$p < 0.001$ compared with the control and #$p < 0.05$ compared with the H_2O_2-treated alone. Scale bar = 20 μm

4. Discussion

Oxidative stress is one of the key factors in the pathogenesis of aging associated diseases, including retinal degenerative disease such as AMD (Zarbin, 2004). It has been shown that chronic oxidative stress caused molecular and cellular damages in susceptible RPE cells, which in turn lead to visual dysfunction (Beatty, Koh, Phil, Henson, & Boulton, 2000; Zarbin, 2004). H_2O_2 has been widely used and well characterized in studies of oxidative stress on RPE cells, which have been validated for the rapid screening of potential protective compounds (Kook et al., 2008; Lu, Hackett, Mincey, Lai, & Campochiaro, 2006; Maher & Hanneken, 2005), therefore it was used as an oxidative insult in this study. The results from this study showed the cytotoxic effect of 400 μM H_2O_2 on ARPE-19 cells, which decreased in cell viability and the ability of EGCG to reverse the toxic effect of H_2O_2. Pretreatment the cells with 100 μM EGCG prior to exposure with 400 μM H_2O_2 significantly increased cell viability when compared with cells treated with H_2O_2 alone. This study suggested that EGCG might play a protective role against H_2O_2-induced ARPE-19 cell death. Cells pretreated with EGCG maintained a significantly higher viability compared to the H_2O_2-treated cells which indicated that EGCG provided the protection against

H_2O_2-induced ARPE cell death. These findings might support the previous studies about cytoprotective effect of EGCG against H_2O_2-induced oxidative stress in PC12 cells (Koh et al., 2003), G93A motorneuron cells (Koh et al., 2004), auditory neurons (Xie et al., 2004) and INS-1 insulinoma cells (Kim et al., 2010). *In vivo*, EGCG had highly potent activities in preventing striatal dopamine depletion and substantia nigra dopaminergic neuron loss caused by N-methyl-4- phenyl-1,2,3,6 tetrahydropyridine (MPTP) in mice (Levites, Weinreb, Maor, Youdim, & Mandel, 2001). Furthermore, EGCG exerted the protective effects against amyloid beta (Aβ) toxicity in PC12, SH-SHY5Y and mice hippocampus (Levites, Amit, Mandel, & Youdim, 2003). However, the previous study reported that EGCG did not protect against H_2O_2-induced oxidative stress in ARPE-19 cells (Hanneken, Lin, Johnson, & Maher, 2006), a finding which was not consistent with our study. This disparity may be due to differences in experimental procedure; in their studies ARPE-19 cells were exposed to H_2O_2 for a period of time before adding the EGCG, cell density and concentrations of EGCG used in their experiments were lower.

ROS is known as a second messenger that can activate the transduction pathways leading to apoptosis (Son et al., 2011). EGCG is regarded as a potent scavenger of free radicals including singlet oxygen, superoxide anions, hydroxyl radicals, and peroxy radicals based on stereochemistry, a trihydroxyl group on the B ring and the gallate moiety at the 3′ position in the C ring (Nanjo et al., 1996). In this current study, EGCG pretreatment decreased an intracellular accumulation of ROS in ARPE-19 cells injured by oxidant stress H_2O_2 thus the internalized EGCG may act as a scavenger of intracellular ROS.

We also demonstrated that ARPE-19 cells treated with H_2O_2 underwent apoptotic cell death and the caspase 3 inhibitor, Ac-DEVD-CHO, attenuated H_2O_2-induced the decrease of cell viability. These findings were consistent with the previous studies that H_2O_2-induced apoptosis in rat lens epithelial cell (Yao et al., 2003), chicken osteocytes (Kikuyama et al., 2002) and in human RPE cells (Cai, Wu, Nelson, Sternberg, & Jones, 1999; Jin et al., 2001). Pretreating the ARPE-19 cells with EGCG prior to H_2O_2 showed the significant reduction of the number of cells containing condensed nuclei and TUNEL positive cells. These findings demonstrated that EGCG showed a protective role in H_2O_2-induced apoptosis in ARPE-19 cells. Previous studies were reported that EGCG also has protective effects against apoptosis induced by the pro-parkinsonian neurotoxin 6-hydroxydopamine (6-OHDA) in SH-SY5Y cells (Guo, Bezard, & Zhao, 2005). It has also been reported that certain flavanoids including EGCG protected RPE cells from oxidative stress induce cell death (Hanneken et al., 2006) by inducing the expression of transcription factor Nrf2 (Nuclear factor-erythroid 2-related factor-2) and phase 2 enzyme heme-oxygenase, suggesting that EGCG afforded protection via its antioxidant properties. However, a direct action of EGCG on the apoptotic pathway in ARPE-19 cells has not been demonstrated. Therefore, our study determined the anti-apoptotic property of EGCG against H_2O_2-induced ARPE-19 cell death. Previous study reported that H_2O_2 caused mitochondrial DNA damage and promoted apoptosis through p53 dependent pathway with increased caspase-3 activity, decreased expression of Bcl-2 and increased expression of p53 protein in RPE (Jin et al., 2001). In the present study, we showed that treatment of ARPE-19 cells with H_2O_2 induced the p53 tumor suppressor protein migration into the cell nuclei, then followed by cell apoptosis, which supported the previous evidence that p53 may play a pivotal role in H_2O_2 induced apoptosis of ARPE-19. Moreover, pretreatment cells with EGCG prior to exposure with H_2O_2 could prevent p53 nuclear translocation. This finding suggested that EGCG may protect the ARPE-19 cell from apoptosis by interfering p53 pathway. Recently, EGCG also protected H9c2 cardiomyoblasts via the inhibition of p53 (Sheng, Gu, Xie, Zhou, & Guo, 2010). Moreover, oxidized LDL-induced apoptosis in endothelial cells by increasing p53 activity, the increase of which was strikingly downregulated by EGCG (Choi et al., 2008). EGCG treatment also decreased UVB- induced keratinocyte cytotoxicity and apoptosis via inhibition the mRNA expressions of apoptosis-regulatory gene p53 (Luo et al., 2006).

The tumor suppressor protein p53 is considered to be a major player in the apoptotic response to genotoxins. Therefore, we were interested in elucidating the role of EGCG on p53-dependent pathway for the induction of apoptosis. The phosphorylation of p-53 plays a critical role in the activation and up-regulation of p53 during cellular stress (Haupt et al., 2003). During cell stress, p53 translocates to the nucleus and induces pro-apoptotic gene expression and blocks anti-apoptotic gene expression (Miyashita & Reed, 1995). This leads to the disruption of the balance between pro- and anti-apoptotic proteins. Members of the Bcl-2 family are the mediators of cell survival and apoptosis (Brunelle & Letai, 2009). The interaction between the pro-apoptotic and anti-apoptotic Bcl-2 family members can alter the permeability of mitochondrial membrane and release cytochrome c then activate caspase cascade (Brunelle & Letai, 2009). To further investigate the downstream involvement of apoptotic proteins in p53 signaling pathway, the present study also investigated the expression of Bcl-2 family proteins; one was a pro-apoptotic protein, Bax and the other was anti-apoptotic protein, Bcl-2. Our study showed that H_2O_2 up-regulated Bax and down-regulated Bcl-2 expression thereby increase in Bax: Bcl-2 ratio which would lead to the caspase 3 activation (Jin et al., 2001). These results agreed with the previous studies that H_2O_2 induced

apoptosis via Bax and Bcl-2 pathway in PC12 (Jiang, Liu, Bao, & An, 2004) and human gastric carcinoma MGC803 cell line (Mao et al., 2006) by up-regulation of Bax and down-regulation of Bcl-2. The present study revealed that EGCG could protect H_2O_2-induced ARPE-19 cell death by reduction of p53 nuclear translocation and phospho-p53 expression, and then diminished the binding of activated p53 with the promoter of the Bax expression, decreased Bax: Bcl-2 ratio thereby inhibited caspase 3 cascade and finally attenuated apoptosis.

5. Conclusion

In summary (Figure 7), exogenous EGCG could exert a beneficial cytoprotective action in retinal pigment epithelial cells. Antioxidant EGCG inhibited activation and translocation of p53 transcription factor following DNA damage and apoptosis by H_2O_2 injury, and modulated the expression of its downstream genes of pro-apoptotic proteins, Bax, and perhaps other components of apoptotic pathway, Bcl-2. Schematic diagram shows the involvement of p53 and its apoptotic downstream Bax and Bcl-2 in the oxidant-induced retinal pigment epithelial cells apoptosis and the effects of EGCG on modulation of these genes. As depicted, H_2O_2-derived oxidants induced the increased expression of p53, and its downstream Bax in which expression ratio of Bax and Bcl-2 was increased. EGCG inhibited this direct apoptotic signaling and prevented against apoptosis cascades. Our findings clearly concluded that retinal pigment epithelial cell viability under oxidative stress might be a function of the expression ratio of Bax and Bcl-2 proteins, and that antioxidant EGCG could enhance the intrinsic cytosolic and mitochondrial tolerance against apoptotic triggers. These advantages make EGCG, an abundant polyphenolic flavonoids in green tea, attractive for clinical use, in particular, as agent that could help to protect cells from oxidative stress, and to provide potentially long-term protection of the eye.

Summary diagram showing survival signaling of EGCG in H_2O_2-induced RPE apoptosis

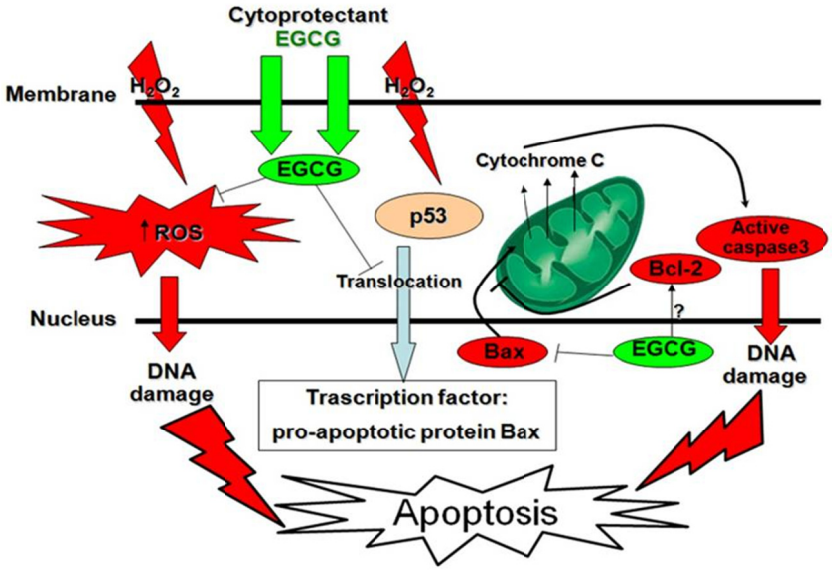

Figure 7. Schematic diagram showing survival signaling of EGCG in H_2O_2-induced RPE apoptosis
As depicted, ⟶ indicates activation or induction, and ⟞ indicates inhibition or blockade

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