Effect and mechanism of epigallocatechin-3-gallate (EGCG). against the hydrogen peroxide-induced oxidative damage in human dermal fibroblasts

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Synopsis

This study was conducted to investigate the protective effects of epigallocatechin-3-gallate (EGCG) on hydrogen peroxide (H_2O_2)-induced oxidative stress injury in human dermal fibroblasts. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and the use of Hoechst staining and terminal deoxynucleotidyl transferase dUTP nick end labeling for apoptosis detection indicated that the administration of H_2O_2 to human dermal fibroblasts caused cell damage and apoptosis. The incubation of human dermal fibroblasts with EGCG markedly inhibited the human dermal fibroblast injury induced by H_2O_2. The assay for 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity indicated that EGCG had a direct, concentration-dependent antioxidant activity. Treatment of human dermal fibroblasts with EGCG significantly reversed the H_2O_2-induced decrease of superoxide dismutase (SOD) and glutathione peroxidase (GSH-px), and the inhibition of malondialdehyde (MDA) levels. These results showed that EGCG possessed antioxidant activity and was effective against H_2O_2-induced human dermal fibroblast injury by enhancing the activity of SOD and GSH-px, and by decreasing the MDA level. Our results suggested that EGCG should have the potential to be used further in cosmetics and in the prevention of aging-related skin injuries.

INTRODUCTION

Human dermal fibroblasts are the predominant cell type in the dermis and play an important role in the aging process. Apoptosis of human dermal fibroblasts can decrease cell number and cell activity, leading to reduced synthesis of collagen and other extracellular matrix and finally to change in morphology and function of dermal layer (1–3). The clinical symptoms include many skin aging changes such as dry rough skin, loose skin, reduced elasticity, and increased wrinkles. Therefore, the study of human dermal fibroblasts apoptosis is crucial for the research and development of personal antiaging protective products.

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Green tea contains polyphenolic compounds, mainly catechin, which is further composed of several monomers including epicatechin, epicatechin gallate, epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). Among all the monomers, EGCG has the highest proportion, accounting for about 50% of catechins (4,5). It has a strong antioxidant activity and can effectively remove the active free radicals within human body, inhibit lipid peroxidation (LPO), and reduce oxygen free radical (OFR) and LPO-induced damage to cells, DNA, or other biological macromolecules (6–8). Through its strong antioxidant effects, EGCG can also reduce the intracellular reactive oxygen species activity, block the ultraviolet (UV) radiation, inhibit the matrix metalloproteinase activation and collagen damage on human dermal fibroblasts, and increase the extracellular matrix (9–11). Exogenous sources for the production of cellular hydrogen peroxide (H_2O_2), especially in the skin, are UVA and UVB irradiation (12). In the study of skin photoaging, the most common skin lesions are caused by chronic UV irradiation, which involves the demise, aging, and apoptosis of human dermal fibroblasts. Therefore, the establishment of an in vitro model of H_2O_2-induced human dermal fibroblasts apoptosis is crucial for future studies on the oxidative damage of human skin, screening of antioxidants, and its application in the cosmetics. Although many studies have reported that EGCG has strong antioxidant activity (13,14), little is known about its role in the model of H_2O_2-induced human dermal fibroblast apoptosis (15). In this study, for the first time, we address the effect of EGCG on oxidative damage and apoptosis induced by H_2O_2 in human dermal fibroblasts and explore the mechanism of its protective role, which provides a theoretical basis for the application of EGCG in cosmetics.

In this study, we used H_2O_2-induced oxidative damage in human dermal fibroblasts as a model to study the effects of EGCG on H_2O_2-induced oxidative damage and apoptosis and examined its underlying mechanism.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco Life Technologies (Grand Island, NY). H_2O_2 (3%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Propidium Iodide (PI), and EGCG (purity 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis detection kit was purchased from Roche (Basel, Switzerland). Superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-px) detection kit were purchased from Jiancheng Biological Engineering Academy (Nanjing, China).

CELL CULTURE

Primary cultures of skin fibroblasts from a healthy boy's foreskin left over from surgery were grown on plastic flasks under standard conditions: DMEM supplemented with 10% CS at 37°C in a humidified atmosphere of 5% CO_2. According to the different purposes of experiments, cells were quantitatively planted onto the cell culture bottles or 30-mm petri dishes for future experiments. Third- to sixth-generation cells were used for
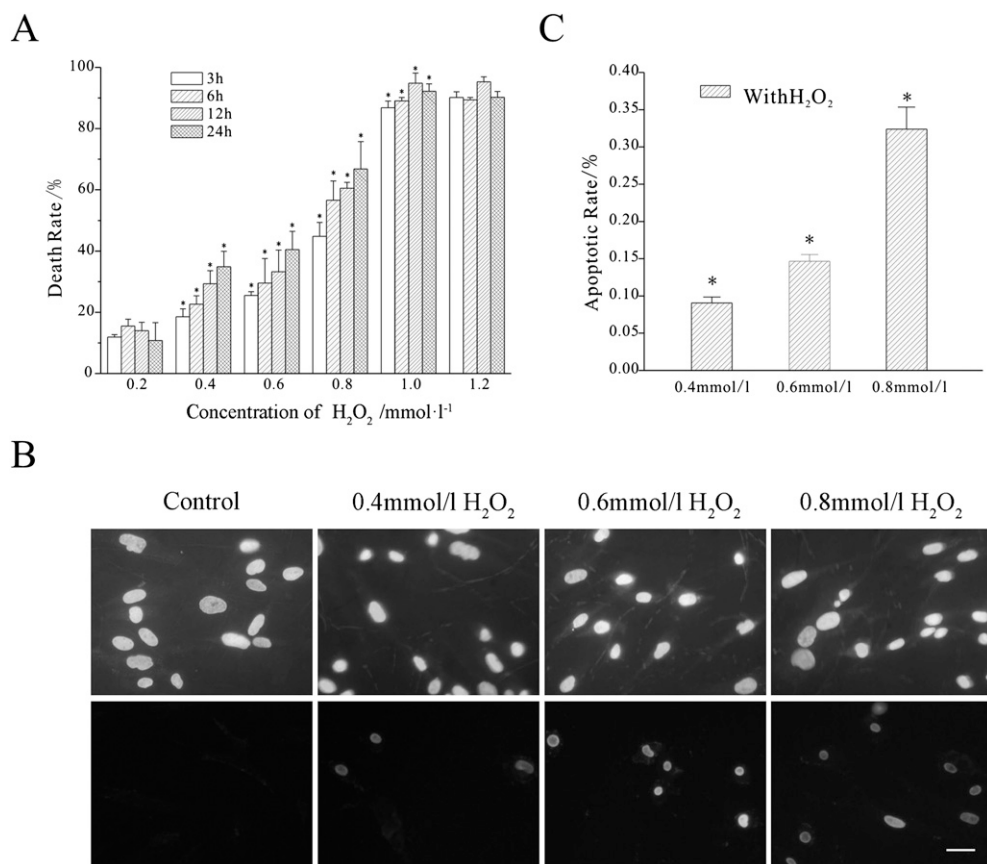
Figure 1. Oxidation damage to human dermal fibroblasts induced by different concentrations of H$_2$O$_2$. (A) Statistical analysis of cell death rate measured by an MTT colorimetric assay with different concentrations of H$_2$O$_2$ and treatment times. *$p < 0.05$ according to one-way ANOVA. Data are obtained from five independent experiments. (B) Cell viability and damaged cell nuclei detected by Hoechst staining and TUNEL assay after exposure of human dermal fibroblasts to different concentrations of H$_2$O$_2$. The scale bar represents 100 µm. (C) Statistical analysis of TUNEL-positive cells resulting from treatments with different concentrations of H$_2$O$_2$. *$p < 0.05$ according to one-way ANOVA. Data are obtained from five independent experiments.

experiments. For each experiment, we used a seeding density of 5×10^4 cells/well plated onto 30-mm petri dishes. The cells were grown to 70% confluency over 24 h.

MTT ASSAY

Cell suspensions were made in fibroblast growth medium containing different drug concentrations with a density of 5×10^4 cells/ml. Cells were plated at a density of 5×10^3 cells/well in 96-well plates (200 µl/well, equivalent to 1×10^4 cells/well). Each different concentration of drugs occupied six holes, repeated twice, and incubated at 37°C with 5% CO$_2$. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (20 µl/well) was added to each hole 4 h before the experiment ended. The supernatant was discarded 4 h later. Finally, 150 µl dimethyl sulfoxide was added and shaken for another 15 min until the crystals were completely dissolved. The absorbance was measured at a wavelength of 570 nm (A570) by a microplate reader.
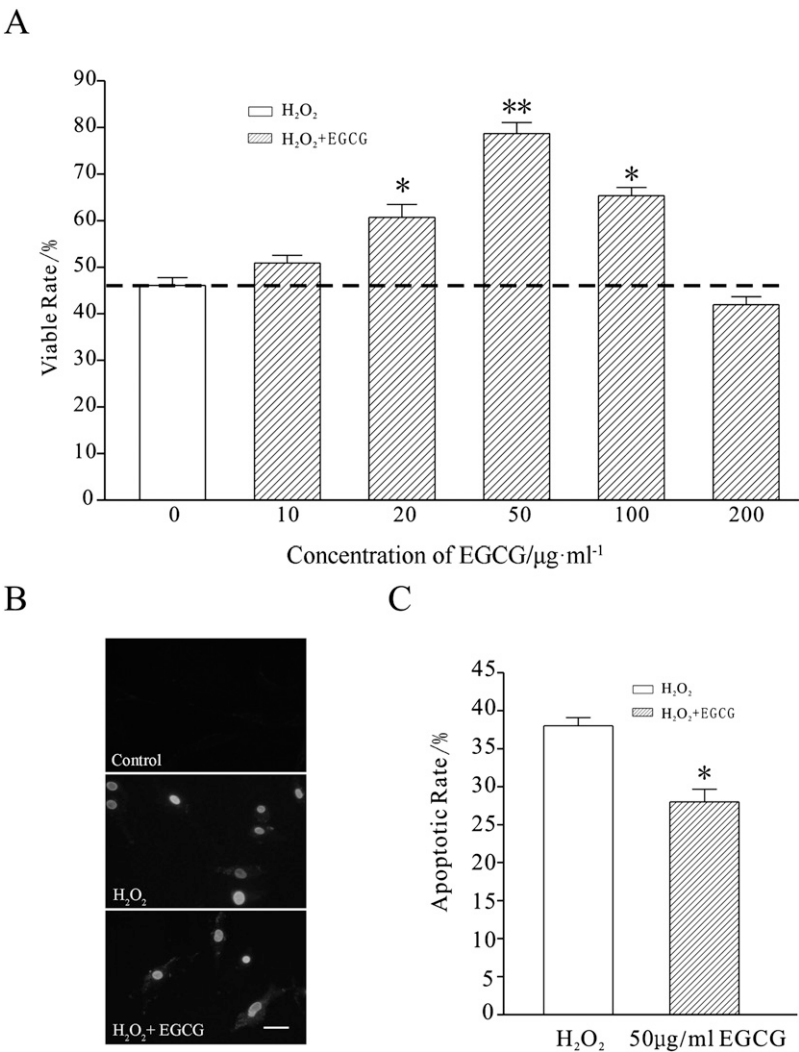
Figure 2. Effect of EGCG on H_2O_2-induced dermal fibroblast injury. (A) Statistical analyses of the effect of EGCG on viability rates of human dermal fibroblasts undergoing concentration of 0–200 μg/ml with H_2O_2 injury. The viability rates were measured with an MTT colorimetric assay. *$p < 0.05$; **$p < 0.01$ according to the Student's t-test compared with H_2O_2 alone. Data are obtained from five independent experiments. (B) Effect of EGCG on H_2O_2-induced apoptosis detected by the TUNEL assay. (C) Statistical analysis of the effect of EGCG on the apoptotic rate induced by H_2O_2. *$p < 0.05$ by Student's t-test, compared with H_2O_2 alone.

HOECHST 33342 STAINING

According to the instructions of the Hoechst 33342 staining kit, fibroblasts were plated onto the glass coverslips at a density of 1×10^4 cells/well, and left overnight. The adherent cells were pretreated with 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/l H_2O_2 to prepare the H_2O_2-induced cell injury model. After 3-, 6-, 12-, and 24-h treatment, the culture medium was discarded and fixed with 4% paraformaldehyde for 20 min. The fixative solution was discarded and rinsed 3 times every 5 min with phosphate-buffered saline (PBS).
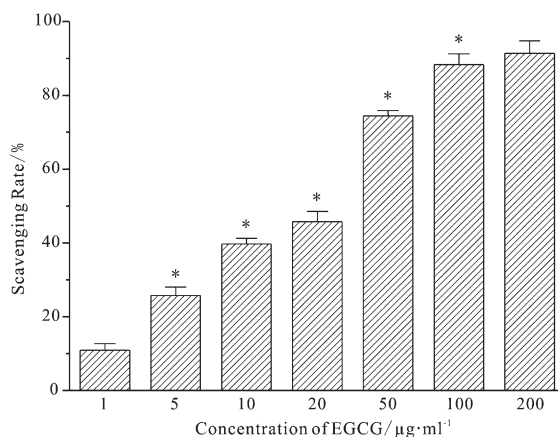
Figure 3. Antioxidant activity of EGCG detected by a scavenging DPPH radical assay. EGCG significantly increased the scavenged DPPH radicals in a concentration-dependent manner. *$p < 0.05$ according to one-way ANOVA. Data are presented as ± standard error of the mean (SEM), obtained from three independent experiments.

Finally, 1 ml of Hoechst 33342 was added per well, stained for 20 min, and rinsed 3 times with PBS. Coverslips on slide containing antiquench ingredients were sealed and protected from light. The nucleus can be visualized with a fluorescence microscope.

TUNEL STAINING

The TUNEL was used to detect the apoptosis of human dermal fibroblasts. Cells were plated onto 35-mm petri dishes, fixed in 4% paraformaldehyde for 25 min at 4°C, and permeabilized with 0.1% Triton-X-100/PBS. Fluorescein isothiocyanate (FITC)-labeled fluorescein nucleotide and terminal deoxynucleotidyl transferase (TDT) mixture were then added for 60 min at 37°C. When visualized under a fluorescence microscope, the FITC-stained nucleus was scored as a positive result. Negative control group was used without TDT solution. Counting was based on five randomly selected areas across horizon, and the apoptotic rate is equal to the number of green fluorescent cells divided by the total number of living cells and the green fluorescent cells.

ASSAY FOR DPPH RADICAL SCAVENGING ACTIVITY

Samples (1 ml) at different concentrations of EGCG and control were added to 1 ml of DPPH solution (12 mg/100 ml). Ultrapure water was added at a final volume of 4 ml, shaked vigorously for 30 s, and left in a dark room for 5 min at room temperature. Its absorbance was recorded at 517 nm. The effect of DPPH radical scavenging of the sample was calculated according to the following formula: DPPH scavenging effect (%) = $\{1 - (T - T_0)/(C - C_0)\} \times 100\%$, where T_0 is the absorbance of the contrast sample, T is the absorbance of the sample, C is the absorbance of the positive control, and C_0 is the absorbance of the negative control.

DETERMINATION OF SOD, GSH-PX, AND MDA LEVELS

The concentration of SOD and the activities of MDA were determined using commercially available kits purchased from the Jiancheng Biological Engineering Academy
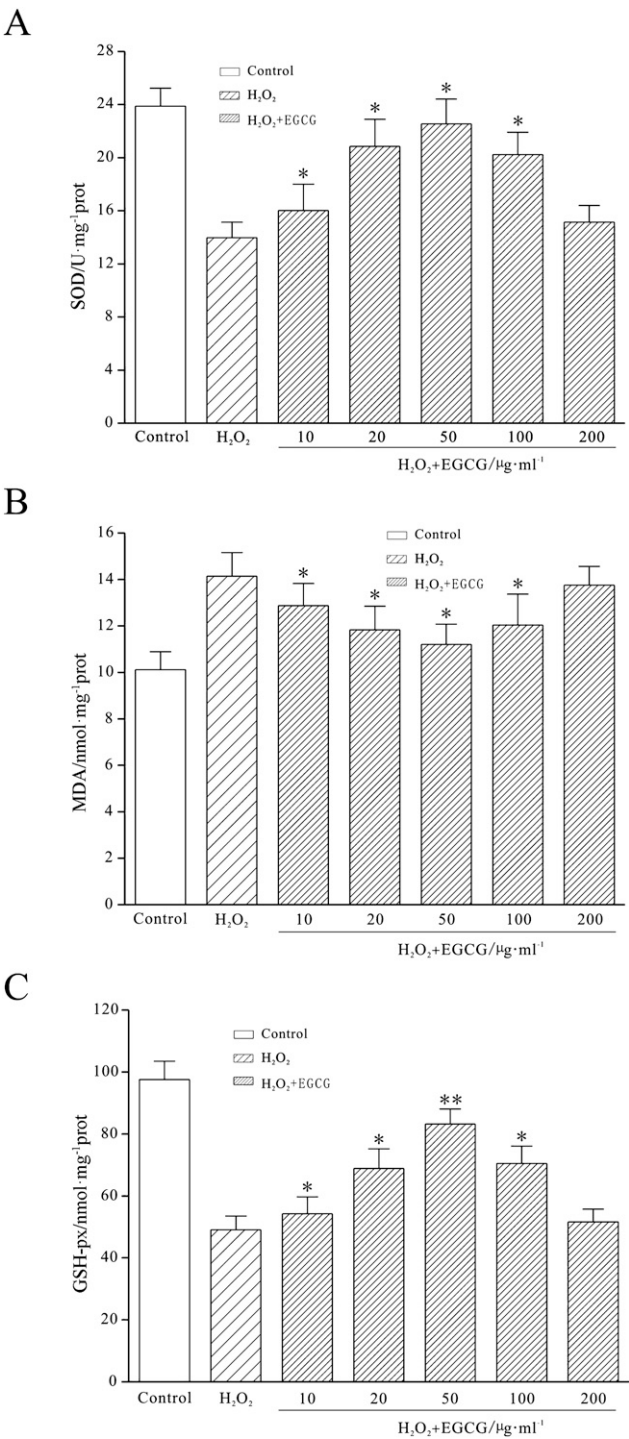
Figure 4. Effect of EGCG on antioxidant enzymes SOD and GSH-px and the production of LPO MDA. Data for this statistical analysis were obtained from four independent experiments. *$p < 0.05$; **$p < 0.01$ by Student's t-test, compared with H_2O_2 alone.

(Nanjing, China). Fibroblasts were plated onto 30-mm petri dishes with a density of 1×10^4 cells/dish, and left overnight. After the cells were completely adherent, they were incubated with H$_2$O$_2$ and H$_2$O$_2$ and EGCG for 6 h, respectively, and the cell protein was extracted. Finally, the contents of SOD, LPO product MDA and GSH-px were determined.

DATA ANALYSIS

SAS 6.0 was used for statistics analysis. Results were expressed as mean ± standard deviation. The analysis of variance (ANOVA) test was used for comparison among groups. The mean values between groups were compared using Student's t-test. A p value< 0.05 indicated a statistical significance.

RESULTS AND DISCUSSION

H$_2$O$_2$-INDUCED HUMAN DERMAL FIBROBLASTS INJURY AND APOPTOSIS

We first used MTT assay to examine the survival rate of H$_2$O$_2$-induced human dermal fibroblasts and established the cell oxidative injury model. The H$_2$O$_2$ treatment time was 3, 6, 12, and 24 h with the concentration of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2mmol/l. The results showed that the death rate was highly correlated with the concentration of the treatment, and was less correlated with the treatment time. At the same concentration, there is no significant difference among the groups with different treatment time (Figure 1A). Based on the MTT assay results, we chose 6 h and 0.4–0.8mmol/l as the optimal induction conditions for the next series of experiments. Because the MTT assay could not show the kind of cell damage H$_2$O$_2$ treatment caused, we used Hoechst staining to determine whether H$_2$O$_2$-induced fibroblast damage was associated with apoptosis. Hoechst 33342 is a specific fluorescent dye that binds to the minor groove of DNA bases and can detect changes in the nuclear morphology of the apoptotic cells. Under the fluorescence microscope, the control group showed uniformly stained larger nuclei and evenly dispersed blue fluorescence, whereas the H$_2$O$_2$-treated cells showed dense nuclei with brighter fluorescence in blue and white than normal cells. In the nuclei of the apoptotic cells, dense granular fluorescence was also observed. They exhibited a typical nuclear condensation and fragmentation, especially under H$_2$O$_2$ concentration of 0.8 mmol/l (Figure 1B, upper panel). We also used the TUNEL method to check the cell apoptosis under a fluorescence microscope and semiquantitatively determined the rate of cell apoptosis. The control group showed almost no green fluorescence, whereas the H$_2$O$_2$-treated group showed positive green fluorescent cells. The nuclei of the H$_2$O$_2$-treated group showed a typical form of apoptosis with nuclear condensation and fragmentation, and the number of apoptotic cells increased with the H$_2$O$_2$ concentration (Figure 1B, lower panel). In 100× high magnification lens, the number of apoptotic cells randomly selected from five discrete horizons in each group also increased with the H$_2$O$_2$ concentration (Figure 1C). It further confirmed that H$_2$O$_2$-induced oxidative damage led to apoptosis. H$_2$O$_2$ is a reactive oxygen species with a high reactivity that can promote the generation of free radicals, causing membrane LPO, and can freely diffuse in the membrane, resulting in cell necrosis and apoptosis. The result of the current study further consolidated this conclusion.

EGCG PROTECTS CELLS AGAINST H_2O_2-INDUCED CELL INJURY AND APOPTOSIS

We next studied whether the EGCG-treated human dermal fibroblasts had the ability to fight against the oxidative stress by using the H_2O_2-induced human dermal fibroblasts apoptotic model. We first added EGCG with concentrations ranged from 10 to 200 µg/ml and tested the survival rate of the cells after H_2O_2 treatment with MTT assay. The results suggested that EGC effectively improved the survival rate of H_2O_2-treated cells. In particular, they showed significant differences in 20, 50, and 100 µg/ml groups ($p < 0.05$), and the 50 µg/ml group showed the most prominent effect. The statistics from three repeated experiments are shown in Figure 2A. The results from TUNEL assay experiments showed that by adding EGCG (50 µg/ml) the number of positive green fluorescence cells is significantly less than that of the cells treated with H_2O_2 only (Figure 2B). In 100× high magnification lens, the number of apoptotic cells in the group treated with both H_2O_2 and EGCG was significantly less than that in the group treated with H_2O_2 alone ($p < 0.05$, Figure 1C). The above results indicated that EGCG had a protective effect against the H_2O_2-induced oxidative stress injury in human dermal fibroblasts.

EFFECT OF EGCG ON DPPH RADICAL SCAVENGING

We further studied whether EGCG has the ability to scavenge the free radicals using the DPPH radical spectrophotometric analysis. The effects of different concentrations of EGCG on free radical scavenging are shown in Figure 3. The results showed that with a concentration range of 1–200 µg/ml, EGCG had the ability to scavenge the free radicals and its effect increased as concentrations increased. With 200 µg/ml EGCG, its effect on radical scavenging reached up to 91.42%. Thus, EGCG has significant effects on free radical scavenging.

EFFECT OF EGCG ON SOD, GSH-PX ACTIVITY, AND MDA LEVEL OF HUMAN DERMAL FIBROBLASTS

Lipid peroxide-mediated cell damage is one of the major causes of cell injury. MDA is the main product of LPO, and its level indirectly reflects the degree of oxidative damage to cells. MDA level is also widely used as a biomarker for oxidative stress (16). In addition, antioxidant enzymes such as SOD and GSH-px are also considered to be effective in enhancing the cellular antioxidant defense system (17). Therefore, we further explored the effects of EGCG on its protection against H_2O_2-induced cell oxidative stress and apoptosis by measuring SOD, GSH-px activity, and MDA levels. The results showed that H_2O_2 dramatically reduced the SOD level in the human dermal fibroblasts compared to the control group. After adding EGCG, the SOD level could be significantly recovered. Moreover, within the concentration range of 10–50 µg/ml, the effect of EGCG on recovery of SOD level was found to be concentration-dependent (Figure 4A). When the EGCG concentrations exceeded 50 µg/ml, the ability of EGCG on the recovery of SOD reduced and showed no significant difference compared to the group treated with H_2O_2 alone (Figure 4A). This may result from the high concentration of EGCG-induced cell toxicity.

EGCG also has effect on increasing the activity of GSH-px, which is another antioxidant enzyme. As shown in Figure 4B, H_2O_2 decreased the GSH-px activity of human dermal fibroblasts, and after adding EGCG, the GSH-px activity can be significantly recovered. Similarly, the recovery of GSH-px activity showed a concentration-dependent effect when the EGCG concentration was in the range of 10–50 µg/ml. When the EGCG concentration

exceeded 50 µg/ml, the ability of EGCG on the recovery of GSH-px activity also decreased, showing no significant difference compared to the group treated with H$_2$O$_2$ alone (Figure 4B). This result further supported the cell toxicity caused by the high concentration of EGCG.

Similar conclusions were also obtained from the experiments of MDA level measurements. As shown in Figure 4C, H$_2$O$_2$ increased the MDA level of human dermal fibroblasts and after adding EGCG, the increased MDA level was significantly reduced. However, this effect disappeared under high concentrations of EGCG.

In summary, our results showed that EGCG can increase endogenous antioxidant enzymes activity (SOD and GSH-px) and can enhance the OFR scavenging capacity of human body's own antioxidant defense system while reducing the MDA level.

CONCLUSIONS

In this study, for the first time we have established the H$_2$O$_2$-induced human dermal fibroblasts as an oxidative injury model. By using different methods such as MTT assay, Hoechst staining, and the in situ TUNEL assay, we confirmed that H$_2$O$_2$ could inhibit the viability of human dermal fibroblasts and induced the apoptosis at a certain concentration. EGCG showed a good protective antioxidative effect and significantly inhibited the oxidation-induced apoptosis in the TUNEL experiments. We also tried to detect the apoptosis by using flow cytometer. However, the result is not as convincing as the TUNEL experiments.

This study also confirmed that EGCG increases SOD and GSH-px activity in human dermal fibroblasts and lowers the MDA level, significantly inhibits the LPO in the human dermal fibroblasts, and shows a clear protective effect against H$_2$O$_2$ damage. Therefore, this method provides a theoretical basis for the application of EGCG in cosmetics and is a simple, fast way to screen the cosmetics with antioxidant effect. This is also worthy of further research and be a useful application in terms of screening cosmetics raw materials.

ACKNOWLEDGMENT

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