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Full Length Research Paper

Protective effect of extracts of calyx from *Diospyros kaki* on H_2O_2-induced cytotoxicity in PC12 cells

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Oxidative stress is considered a major cause of cellular injuries, including various clinical abnormalities. Here, the neuroprotective effect of extracts from the calyx of *Diospyros kaki* (DCE), peel of *D. kaki* (DPE), and flesh of *D. kaki* (DFE) of *Diospyros kaki* on oxidative stress-induced apoptosis in PC12 cells was investigated by an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. The results showed that H_2O_2 significantly decreased cell viability (50.9%), and DCE exerted the highest neuroprotective effect on H_2O_2-induced cytotoxicity. We identified that treatment with DCE of H_2O_2-stressed PC12 cells caused dose-dependent suppression of H_2O_2-induced leakage of lactate dehydrogenase (LDH) as released amount (11.1 to 30.4%); we further verified these findings by observing morphological features. H_2O_2 also induced severe apoptosis of the PC12 cells, as determined by Hoechst 33342 staining and flow cytometric analysis. DCE (100, 500 µg/ml) exerted a significantly high protective effect on PC12 cells against H_2O_2-induced cell injury, and the percentage of cells in the sub-G1 phase decreased to 3.7 and 4.6%. These results suggest that DCE can protect cells against H_2O_2-induced apoptosis, and might be a potential therapeutic agent for treating or preventing neurodegenerative diseases that are related to oxidative stress.

Key words: Apoptosis, *Diospyros kaki*, neurodegenerative disease, neuroprotective effect, oxidative stress.

INTRODUCTION

Oxidative stress is believed to play important roles in neuronal cell death associated with many neurodegenerative disease, including stroke, Alzheimer's disease (AD), and Parkinson's disease (PD) (Yoon et al., 2007; Lee et al., 2008; Lee et al., 2008), the development of which is mediated by reactive oxygen species (ROS). The ROS hydrogen peroxide (H_2O_2) is generated during various processes, including the enzymatic or spontaneous dismutation of superoxide anions produced as by-products of mitochondrial respiration (Yin et al., 2008).

H_2O_2 readily penetrates cells and reacts with intracellular metal ions, such as iron or copper, to generate highly reactive hydroxyl radicals that successively attack cellular components, such as lipids, proteins, DNA, and cause a wide variety of oxidative insults (Saito et al., 2008). In addition, excessive levels of H_2O_2 are toxic to the brain (Zhang et al., 2007). Compared to other organs, the brain is particularly vulnerable to oxidative insult, on account of the high rate of oxygen utilization, a relative paucity of antioxidant levels, and the high content of polyunsaturated lipids (Lawrence et al., 2008; Emerit et al., 2004; Floyd 1999). Moreover, there are regionally high concentrations of redox-active transition metals capable of the catalytic generation of ROS (Desagher et al., 1997; Subir et al., 2007; Hwang et al., 2008). In this manner,

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oxidative stress has long been linked to neuronal cell death that is associated with certain neurodegenerative diseases (Jang et al., 2001). Currently, a certain cure for neurodegenerative diseases is not available. Many synthetic chemicals, such as phenolic compounds, have been proved to be strong radical scavengers, but they usually have some severe adverse effects (Heilmann et al., 1995).

Many natural products have pharmacological applications and potential for chemotherapeutic use (Keating et al., 2008). Plant products are used extensively in testing, owing to their low toxicity and considerable medicinal value (Hong et al., 2006). Therefore, further understanding of the natural products and regulation of compensatory responses to oxidative stress by these products may provide novel insights into pathogenesis and potential therapies for neurological diseases.

Thus, a number of researchers have attempted to prevent or diminish ROS-induced damage and use natural products for treatments that prevent ROS generation and reduce neuronal damage (Gouazé et al., 2004; Kodach et al., 2006).

Consequently, over the course of our screening program on the neuroprotective effect of natural products, we found that extracts from the calyx of *Diospyros kaki* (DCE) exerted a neuroprotective effect against oxidative stress-induced cell death in PC12 cells. *D. kaki*, a deciduous fruit cultivated in Japan, Korea, China, Brazil, and Italy, contains many medicinally bioactive compounds, such as polyphenols, flavonoids, terpenoids, steroids, dietary fiber, carotenoids, naphthoquinones, sugars, amino acids, and minerals (Yokozawa et al., 2007; Kawase et al., 2003; Mallavadhani et al., 1998). *D. kaki* is used both in Japanese folk medicine and in Chinese medicine for the treatment of hiccoughs (Matsuura et al., 1985). The main constituent of *D. kaki* can act as an antifeedant, insecticide, insect growth regulator, sterilant (Gujar et al., 1990), and antioxidant (Jang et al., 2010). The protective effect of DCE on H_2O_2-induced cell death had not been previously assessed. In this study therefore, we determined the neuroprotective effect of DCE against H_2O_2-induced cell death in PC12 cells.

MATERIALS AND METHODS

The calyx, peel, and flesh of *D. kaki* were kindly supplied by Jinyoung, South Korea. H_2O_2 solution and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). The lactate dehydrogenase (LDH) release assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). PC12 cells were obtained from the Korean Cell Line Bank (KCLB). Dulbeco's modified eagles medium (DMEM), fetal bovine serum (FBS), horse serum (HS), and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY, USA). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Preparation of extracts from *Diospyros kaki*

Five gram (5 g) of calyx, peel, and flesh of *D. kaki* was extracted with 100 ml of methanol for 3 days at room temperature and then passed through Whatman No. 1 filter paper (Advantec, Tokyo, Japan). The methanol solvent was removed by evaporation to obtain the dried methanol extract. Hereafter, for simplicity, methanol extracts from the calyx, peel, and flesh of *D. kaki* are denoted as DCE, DPE, and DFE, respectively. These samples were then dissolved in dimethyl sulfoxide (DMSO) to a concentration of 50 mg/ml for the experiments and added to the culture medium so that the final concentration of DMSO was less than 1%.

Cell culture and treatments

Rat pheochromocytoma PC12 cells line were maintained in DMEM supplemented with 10% FBS, 5% HS, 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 3.7 mg/ml of $NaHCO_3$. PC12 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. In all experiments, the cells were treated with DCE, DPE, and DFE before they were treated with H_2O_2 for the indicated times.

MTT reduction assay for cell viability

Cell viability was measured using blue formazan, which is metabolized from colorless MTT by the mitochondrial dehydrogenases, active only in live cells. PC12 cells were preincubated in 96-well plates at a density of 1×10^5 cells/ml for 24 h. The cells, exposed to various concentrations (50, 100, 500 µg/ml) of DCE, DPE, and DFE, were pretreated with 0.5 mM H_2O_2 for 2 h. After the incubation, an MTT reagent (5 mg/ml) was added to each of the wells, and the plate was incubated for additional 2 h more at 37°C. The intracellular formazan product was dissolved in 100 µl of DMSO. The absorbance of each well was then measured at 540 nm with an ELISA reader (model 680, BioRad, USA), and the percentage viability was calculated.

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was determined by measuring the release of LDH. PC12 cells, treated with various concentrations of DCE, were incubated with H_2O_2 for 2 h, and the supernatant was used to assay the LDH activity. The reaction was initiated by mixing 50 µl of the cell-free supernatant with a potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate to a final volume of 100 µl in a 96-well plate. The absorbance of the sample was read at 540 nm. Data were normalized to the activity of LDH released from vehicle-treated cells and are expressed as a percentage of the control value.

Observation of morphologic changes

PC12 cells were seeded at 2×10^5 cells/well in a 6-well plate and incubated overnight in a humidified atmosphere at 37°C in the presence of 5% CO_2. The cells were pretreated with various concentrations of DCE. After they were incubated for 30 min, the cells were treated with 0.5 mM of H_2O_2 for 2 h. The cellular morphology was observed using a phase-contrast microscope (Nikon, Japan).

Nuclear staining for apoptosis

Chromosomal condensation and morphological changes in the

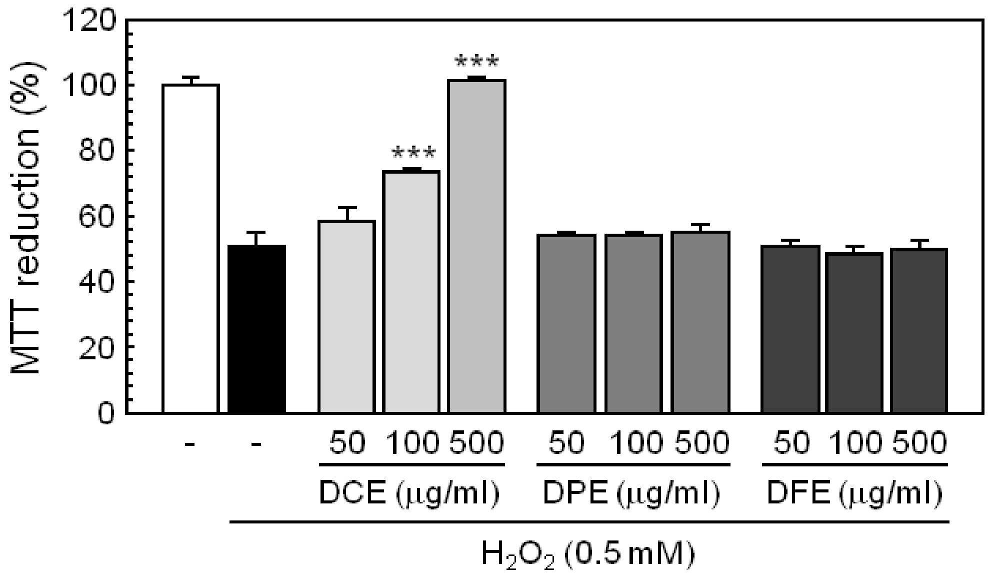

Figure 1. The protective effect of extracts from various parts of *Diospyros kaki* on H_2O_2-stressed PC12 cells (DCE; calyx of *Diospyros kaki*, DPE; peel of *Diospyros kaki*, DFE; flesh of *Diospyros kaki*). After pretreatment of PC12 cells for 30 min with 50, 100 and 500 µg/ml of various parts of *D. kaki*, the cells were then treated with 0.5 mM of H_2O_2 for 2 h. After a MTT reduction assay, the MTT reduction rate (mean ± SD of triplicate determinations) was calculated by setting each of control survival rates. ***significant versus H_2O_2-treated control group ($p < 0.001$).

nucleus were observed using the chromatin dye, Hoechst 33342 (Sigma, MO, USA). PC12 cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 4 h at room temperature. After the cells were rinsed twice with PBS, the cells were stained with Hoechst 33342 for 30 min at room temperature. The cells were then washed twice with PBS, and the Hoechst-stained nuclei were visualized under a fluorescence microscope (Nikon, Japan).

Flow cytometric detection of apoptotic cells

Flow cytometric analysis of cellular DNA content was performed as described previously (Suzuki et al., 2001). Briefly, PC12 cells were harvested and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 µg/ml of propidium iodide (PI) at room temperature in the dark for 30 min. Suspensions of treated PC12 cells were analyzed by cytometry (Beckman Coulter, Fullerton, CA, USA) with a laser-excitation wavelength of 488 nm and an emission wavelength of 620 nm (FL3) to quantify the red PI fluorescence. The percentages of cells in various phases of the cell cycle, namely sub-G_1, G_1, S, and G_2/M, were assessed using WINCYCLE 32 software (Beckman Coulter). Every measurement usually counted at least 10,000 events. Apoptotic rates were determined by the percentage of hypoploid nuclei accumulated at the peak of the sub-G_1 phase (Erba et al., 1999).

Statistical analysis

All data are expressed as the means of three determinations and the data were analyzed using the Statistical Package for Social Sciences (SPSS) package for Windows (Version 11.5; Chicago, IL, USA). The data were evaluated by one-way analysis of variance (ANOVA) followed by Scheffe's test.

RESULTS AND DISCUSSION

Protective effect of DCE on H_2O_2-induced PC12 cells

Cell damage by oxidative stress has been implicated in the physiological process of aging as well as in a variety of neurodegenerative disorders (Olanow et al., 1993). It is mediated by ROSs which are generated as by-products of normal and irregular metabolic processes that utilize molecular oxygen (Halliwell et al., 1992). The excessive production of ROS in living systems is responsible for extensive damage to membrane lipids, proteins, and other biomolecules (Gardner et al., 1997). Thus, the removal of excess ROS, or suppression of their generation by antioxidants, may be effective in preventing oxidative cell death. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly related to the living cell number. We examined the effects of DCE, DPE, and DFE on H_2O_2-stressed PC12 cells by performing an MTT reduction assay. As shown in Figure 1, PC12 cells treated for 2 h with 0.5 mM H_2O_2 showed a 50.9% reduction in cell viability, compared to the control cells. On the other hand, after treatment with various concentrations (50, 100, and 500 µg/ml) of DCE for 30 min and then exposure to 0.5 mM H_2O_2 for 2 h, the viability of the H_2O_2-stressed PC12 cells was 58.5, 73.4, and 101.5%, respectively, compared to the viability of the control cells. DPE and DFE did not exert a neuroprotective effect on H_2O_2-stressed PC12 cells.

Table 1. Protective effect of DCE on H_2O_2-stressed PC12 cells.

Treatment	LDH release (%)
Control	23.5±0.26
H_2O_2	57.4±1.14
DCE (50 µg/ml)+H_2O_2	46.3±0.68***
DCE (100 µg/ml)+H_2O_2	44.8±2.10***
DCE (500 µg/ml)+H_2O_2	27.0±0.46***

Cell viability was measured using LDH release assay in cells those were exposed to DCE under H_2O_2-stressed conditions. PC12 cells were pretreated for 30 min with indicated concentrations (50, 100 and 500 µg/ml) of DCE. The cells were then treated with 0.5 mM H_2O_2 for 2 h. The results from the LDH release assay were normalized to the activity of LDH released from the H_2O_2-treated cells (100%) which had been obtained by separately plating. Data (mean ± SD of triplicate determinations) are representative of at least three independent experiments. ***significant versus H_2O_2-treated control group ($p < 0.001$).

To further investigate the neuroprotective effect of DCE, we performed an assay to determine LDH release as another indicator of cytotoxicity. LDH is a stable cytoplasmic enzyme present in all cells, and it is rapidly released into the cell culture supernatant upon damage to the plasma membrane. An increase in the number of dead or plasma membrane-damaged cells results in an increase in LDH activity in the culture supernatant. PC12 cells were treated with various concentrations (50, 100, and 500 µg/ml) of DCE for 30 min and then exposed to 0.5 mM H_2O_2 for 2 h. As expected, DCE reduced cell damage in a dose-dependent manner, as evident by an 11.1 to 30.4% decrease in the amount of LDH released from the H_2O_2-stressed PC12 cells (Table 1). DCE offered almost complete protection against H_2O_2-induced cell death, and the viability was restored nearly to the control level. Morphology of the cultured cells was assessed by transmitted phase-contrast microscopy. As shown in Figure 2, the control group had rounded cell bodies with clear edges and a fine dendritic network. However, after 2 h of exposure to 0.5 mM H_2O_2, most of the cell bodies showed cytoplasmic shrinkage and aggregation and were either detached from each other or floating in the medium. In contrast, DCE significantly alleviated the morphological manifestations of cell damage, indicating that DCE offered protection to the H_2O_2-stressed PC12 cells.

From the results, we identified that among the extracts from *D. kaki*, DCE had the highest neuroprotective effect on H_2O_2-stressed PC12 cells. DCE offered almost complete protection against H_2O_2-induced cell death, and the cell viability was reinstated nearly to the control level. Taken together, these results allow us to conclude that DCE is effective for the protection and viability of PC12 cells.

Protective effect of DCE against H_2O_2-induced apoptosis in PC12 cells

Oxidative stress can cause cell death via apoptosis in many cell types, and such an effect can be blocked or delayed by a wide variety of antioxidants (Kehrer et al., 1944). Markesbery et al. (1999) reported that oxidative stress played a key role in the neuronal apoptosis induced by reactive oxygen species. To determine whether cell protection by DCE was due to the inhibition of apoptosis, we treated the PC12 cells with H_2O_2 and various concentrations of DCE. Apoptotic cells show chromatin condensation, which can be visualized using the DNA-binding fluorescent dye Hoechst 33342. Nuclei of control cells appeared round to oval, with a separate pattern of blue fluorescence. After treatment with H_2O_2 at a concentration of 0.5 mM for 2 h, cell nuclei became increasingly bright. The nuclei decreased in size and fragmented into apoptotic bodies. In contrast, pre-treatment with 100 and 500 µg/ml of DCE prevented these H_2O_2-induced morphological alterations of nuclei, and the number of cells with nuclear condensation and fragmentation was remarkably decreased in the DCE-treated group compared to the H_2O_2 control group (Figure 3).

In order to analyze the protective effects of DCE against H_2O_2-induced cell injury, we investigated nuclear changes. PC12 cells were stained with PI to analyze the percentage of apoptotic cells by using flow cytometry. The apoptotic cells were distributed according to the cell cycle phase by showing a sub-G1 DNA content. As shown in Figure 4, in PC12 cells that were exposed to 0.5 mM H_2O_2 for 2 h to induce apoptosis, approximately 20.4% of apoptotic cells were in the sub-G1 phase. On the other hand, pretreatment of cells with either 100 or 500 µg/ml of DCE was very effective for attenuating H_2O_2-induced apoptotic cell death, and the percentage of cells in the sub-G1 phase decreased to 3.7 and 4.6%, respectively. When the cells were exposed to H_2O_2, there was a distinct increase in the percentage of cells with a sub-G1 DNA content, which is representative of programmed cell death. However, DCE significantly reduced the number of cells having characteristics of apoptosis.

Through the results, we clarified that DCE have inhibitory effect against neuronal cell apoptosis. Exposure of PC12 cells to H_2O_2 triggered DNA damage as assessed by a DNA-sensitive dye, showed a significant sub-G1 peaks, indicating the presence of an apoptotic component in H_2O_2-induced cell injury. We suggest that DCE has neuroprotective effects, indicated by the reduction in cell death and apoptosis in DCE-treated PC12 cells. Also, various parts of *D. kaki* have well-known neuroprotective effects, and it has been reported that *D. kaki* stem and peel can protect neuronal cells from oxidative stress (Yoo et al., 2009; Lee et al., 2008). However, in this study, we first provided evidence that pretreated cells with DCE resulted in mitigation to H_2O_2

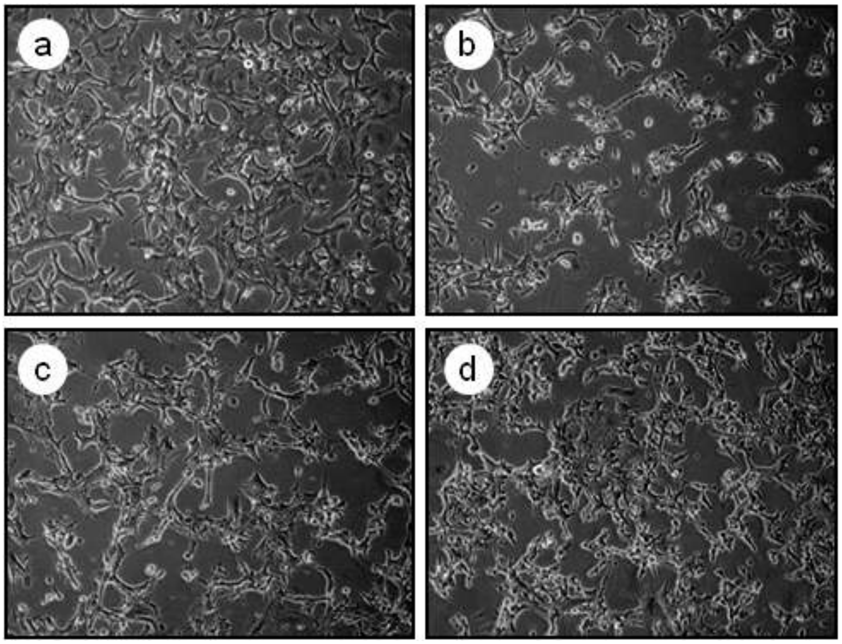

Figure 2. The protective effect of DCE on H_2O_2-induced morphological changes in PC12 cells. (a) Control; (b) PC12 cells exposed to 0.5 mM H_2O_2 for 2 h; (c) PC12 cells treated with 100 µg/ml of DCE for 30 min before exposure to 0.5 mM H_2O_2 for 2 h, (d) PC12 cells treated with 500 µg/ml of DCE for 30 min before exposure to 0.5 mM of H_2O_2 for 2 h. Photographs were taken with a phase-contrast microscope at 100× magnification and showed that H_2O_2 treatment decreased the number of viable cells and induced shrinkage and aggregation of cell bodies, whereas DCE pretreatment attenuated the effects of H_2O_2 treatment.

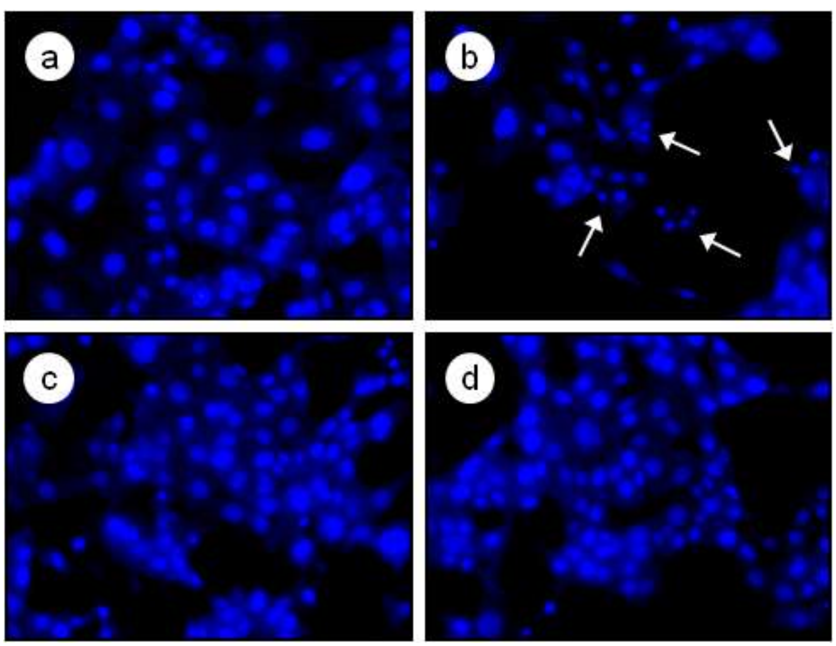

Figure 3. Inhibition of H_2O_2-induced apoptosis in PC12 cells by DCE. The cells were preincubated with DCE at the indicated concentrations for 30 min. The cells were then stimulated with 0.5 mM H_2O_2 for 2 h. Fixed cells were stained with Hoechst 33342 (10 µM) and examined by fluorescence microscope (Magnification ×400). (a), control; (b), 0.5 mM H_2O; (c), 0.5 mM H_2O_2 + DCE 100 µg/ml; (d), 0.5 mM H_2O_2 + DCE 500 µg/ml. The arrows indicate apoptotic cells.

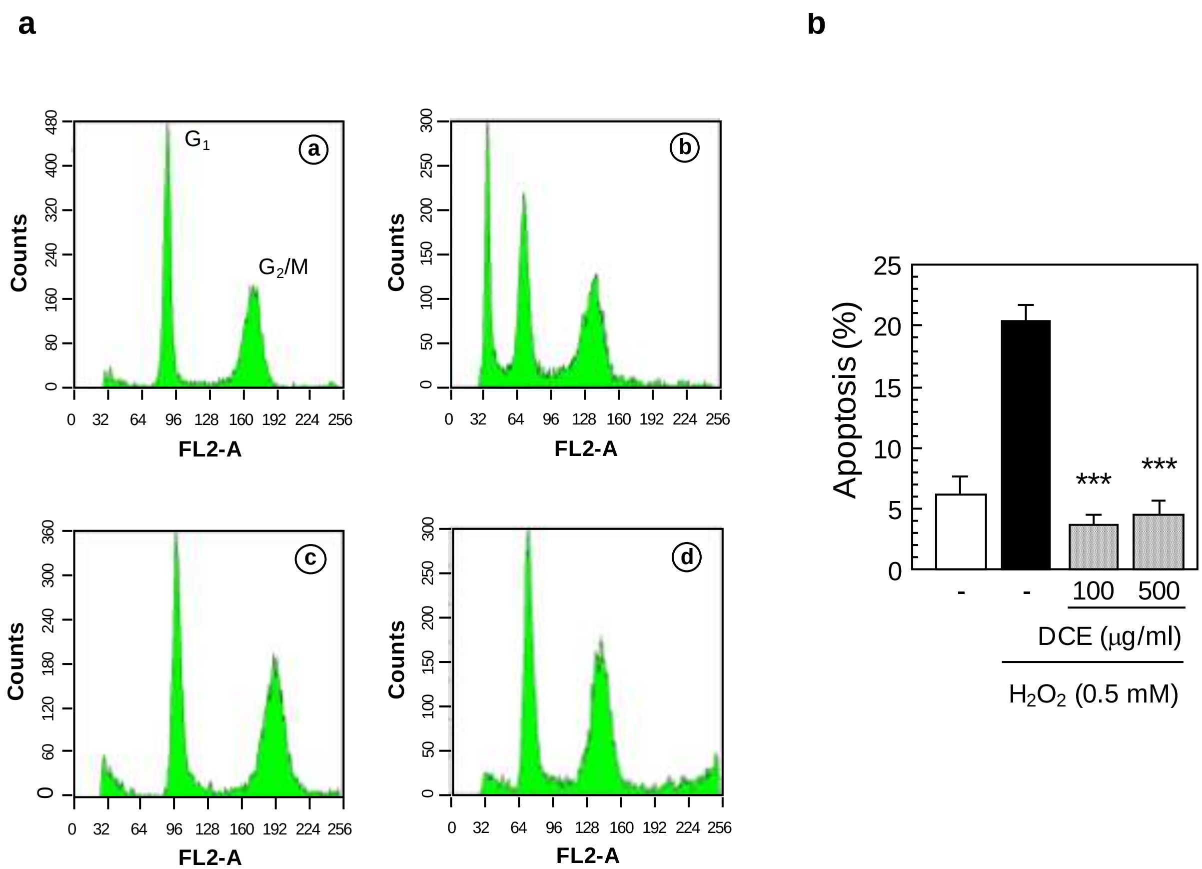

Figure 4. Effect of DCE for apoptotic repression on H_2O_2-stressd PC12 cells. Flow cytometric analysis of H_2O_2-induced apoptotic PC12 cells at the indicated concentrations (100 and 500 µg/ml) of DCE (a, control; b, 0.5 mM H_2O_2; c, 0.5 mM H_2O_2 + DCE 100 µg/ml; d, 0.5 mM H_2O_2 + DCE 500 µg/ml) was conducted after the cells had been incubated 2 h. The cell cycle distributions were determined by flow cytometric analysis of DNA content after staining with propidium iodide. The experimental details are described under "Material and methods". Data (means ± SD of triplicate determinations) are representative of at least three independent experiments. ***significant versus H_2O_2-treated control group ($p <$ 0.001).

toxicity in PC12 cells. The results indicate that DCE could ameliorate significantly, H_2O_2-induced neurotoxicity through the inhibition of oxidative stresses. The protective effects of DCE were related to inhibition of neuronal cell death from H_2O_2-induced apoptosis, further study on the detailed mechanisms of DCE is now in progress. Therefore, our results imply that DCE is a potential candidate for novel therapeutic agents for the treatment of neuronal diseases associated with oxidative stress.

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