

Apple Phenolics Protect in Vitro Oxidative Stress-induced Neuronal Cell Death

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ABSTRACT: Oxidative stress induced by reactive oxygen species may be linked to neurodegenerative diseases. Fresh Red Delicious apples, having 232.9 mg/100 g vitamin C equivalent antioxidant capacity, protected the rat pheochromocytoma neuronal (PC-12) cells from H₂O₂-induced oxidative toxicity in vitro in a dose-dependent manner. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay showed significant increase in cell viability when PC-12 cells were treated with apple extracts. This indicates that the apple phenolics protected oxidative stress-induced neurotoxicity. Because oxidative stress is also known to increase neuronal cell membrane breakdown, we further investigated by lactate dehydrogenase and trypan blue exclusion assays. Apple phenolics inhibited oxidative stress-induced membrane damage in neuronal cells. Therefore, these results may suggest that naturally occurring antioxidants, such as phenolic phytochemicals in fresh apples, may reduce the risk of neurodegenerative disorders.

Keywords: apple, oxidative stress, neurodegeneration, phenolic phytochemicals, reactive oxygen species

Introduction

Neuronal damage of the brain resulting from oxidative stress is believed to be responsible for the development of neurodegenerative disorders such as Alzheimer's disease (AD) (Smith and others 2000). It is mediated by reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), superoxide radical anion, and hydroxyl radical. ROS are generated as byproducts of normal and irregular metabolic processes that utilize molecular oxygen in biological system. ROS can attack cellular biomolecules. H₂O₂, produced by the human body, is an important risk factor to oxidative stress in neurodegeneration because it can pass easily through biological membranes (Varadarajan and others 2000).

Recently, there has been a growing interest in pharmacological approaches to slow the rate of both cognitive and functional declines associated with neurodegeneration. Studies show that chronic disease prevention is closely linked to the effects of environmental factors such as nutrition and exposure to toxic substances (Moskaug and others 2004). Toxic substances induce the components capable of damaging cellular lipid, protein, carbohydrates, and DNA, whereas nutrition may serve to improve cellular defense systems against overwhelming oxidative stress. Cellular damage of exposure to many toxicants seems to be related to their ability to generate ROS (Moskaug and others 2004). Many agents have been proposed to slow neurodegenerative progression. Some agents include naturally occurring antioxidants and antioxidant-rich foods, which are known to play a major role in preventing oxidative damage affected by ROS. Phytochemicals in fruits and vegetables have been considered to possess many beneficial properties, including antioxidant, antiproliferative, and anticarcinogenic effects (Eastwood 1999; Hollman and Katan 1999). Continued research is being undertaken to further understand the biological actions of fruits and vegetables, and the beneficial effects of phytochemicals

on neurological dysfunctions are only beginning to receive attention.

The biofunctional value of fruits depends not only on phytochemical concentration but also on the amount of such foods consumed daily. Apples are one of the major fruits frequently consumed by Americans (Putnam and Allshouse 1994). We previously showed the antioxidative and antiproliferative activities of apples (Eberhardt and others 2000). In the present study, we examined the possible protective effects of phenolic phytochemicals in apple against oxidative cell death in cultured PC-12 cells.

Materials and Methods

Materials

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, N.Y., U.S.A.). All other chemicals were the products of Sigma (St. Louis, Mo., U.S.A.). Fresh Red Delicious apples were picked at commercial maturity during the 2002 harvest season at the New York State Agricultural Experiment Station orchard in Geneva, N.Y., U.S.A. Immediately on arrival in the laboratory after harvest, apples were stored in a 2 °C to 5 °C cold room. Apples were carefully cut into slices, and the pits were removed. Apple slices were frozen. The freeze-dried samples were ground to powder using a laboratory mill (Thomas Scientific, Swedesboro, N.J., U.S.A.) and then stored at -20 °C until analyzed.

Extraction of apple phenolics

The phenolics in fruits were extracted from 10 g of dried sample using 80% aqueous methanol by the ultrasound-assisted method (Kim and Lee 2002). The mixture was sonicated for 20 min with a continual stream of nitrogen gas purging to prevent possible oxidative degradation of phenolics. The mixture was filtered through Whatman nr 2 filter paper (Whatman Intl., Kent, U.K.). Extraction of the residue was repeated using the same conditions. The 2 filtrates were combined, and the solvent was removed using a rotary

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evaporator at 40 °C. The remaining phenolic concentrate was stored at -20 °C until analyses.

Cell culture

PC-12 cells (ATCC, Manassas, Va., U.S.A.) were cultured in Ham's F12K medium containing 15% horse serum, 2.5% fetal bovine serum, and 50 units/mL penicillin and 100 µg/mL streptomycin in a humidified incubator at 5% CO₂.

Measurement of oxidative stress

Levels of cellular oxidative stress were measured using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) (Heo and others 2004). PC-12 cells were then pretreated for 10 min at the levels of various concentrations of apple phenolics. The cells were then treated with or without 400 µM H₂O₂ for 2 h. At the end of the treatment, cells were incubated in the presence of 50 µM DCF-DA in phosphate-buffered saline. Fluorescence was finally quantified using Tecan SER-NR 94572 fluorometer (San Jose, Calif., U.S.A.) equipped with 485-nm excitation and 530-nm emission filters. The results were expressed in percent relative to the oxidative stress of the control cells set to 100%.

Determination of cell viability

PC-12 cells were plated at a density of 10⁴ cells/well on 96-well plates in 100 µL RPMI. Cell viability was evaluated using 3 different assays (MTT reduction, lactate dehydrogenase [LDH] release, and trypan blue exclusion assays). The cell viability was determined by the conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay (Heo and others 2001). The cells were incubated with 0.25 mg MTT/mL (final concentration) for 2 h at 37 °C, and the reaction was stopped by adding solution containing 50% dimethylformide and 20% sodium dodecyl sulfate (pH 4.8). The amount of MTT formazan product was determined by measuring absorbance using a micro-plate reader (Bio-Rad, Hercules, Calif., U.S.A.) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

For LDH assay, cells were precipitated by centrifugation at 2000 × g for 2 min at room temperature, 50 µL of the supernatants was transferred into new wells. The in vitro toxicology assay kit (Sigma) was used for LDH assay. Total cellular LDH activity was determined by solubilizing the cell with 0.2% Triton X-100. Damage of the plasma membrane was evaluated by measuring the amount of the intracellular enzyme LDH released into the medium.

The trypan blue exclusion assay was based on the capability of viable cells to exclude the dye. Cells with damaged membrane appeared blue due to their accumulation of dye. The dye of 0.4% trypan blue was added to PC-12 cells. After 5 min, cells were loaded into a hemacytometer and counted for the dye uptake. The number of viable cells was calculated as a percentage of the total cell population.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed by Student *t* test. Statistical comparisons within the same group were performed for paired observations. *P* ≤ 0.05 was considered significant.

Results and Discussion

Apple phenolics and their effects on reactive oxygen intermediates accumulation

The contents of total phenolics, total flavonoids, and antioxidant capacity of apple extracts were measured according to a previous

Table 1—Total phenolics, total flavonoids, and vitamin C equivalent antioxidant capacity (VCEAC) of fresh apple cv. Red Delicious^a

	Concentration
Total phenolics (mg GAE ^b /100 g)	155.6 ± 6.1
Total flavonoids (mg CE ^c /100 g)	99.0 ± 1.0
VCEAC (mg vitamin C/100 g)	232.9 ± 10.5

^aThe method of Kim and others (2003) was used. Each value is presented as mean ± standard deviation (*n* = 6).

^bGAE = gallic acid equivalent.

^cCE = catechin equivalent.

method (Kim and others 2003) and presented in Table 1. The average concentration of total phenolics and flavonoids were 155.6 mg and 99.0 mg per 100 g fresh apples, respectively. Vitamin C equivalent antioxidant capacity (VCEAC) calculated on a weight basis (mg/100 g) shows the total antioxidant capacity of foods (Kim and others 2002). The level of VCEAC in apples was calculated using vitamin C standard curves and it was 232.9 mg/100 g of fresh apples. Our previous study showed that the vitamin C content of apples ranged between 4 to 15 mg/100 g, and its contribution to the total antioxidative activity of apple is less than 11% (Lee and others 2003). Therefore, phenolics in apples are the major contributors to the total antioxidant capacity of apples (approximately 90%).

Oxidative stress caused by increased accumulation of ROS has been implicated in neurodegenerative diseases such as AD (Smith and others 2000). Intracellular ROS accumulation resulting from H₂O₂ treatment was significantly reduced when PC-12 cells were treated with the apple extracts compared with those treated with H₂O₂ only (Figure 1). Exposure of PC-12 cells to H₂O₂ for 2 h resulted in a 5 times increase in oxidative stress levels compared with that

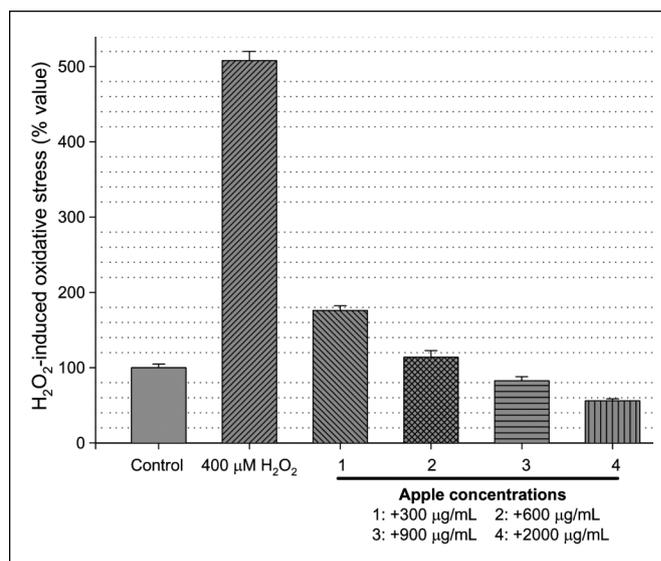


Figure 1—Reduction of H₂O₂-induced oxidative stress by apple phenolics in neuronal PC-12 cells. PC-12 cells were pretreated with apple extracts (100 µg extract = 500 mg fresh apple) for 10 min. The cells were then treated with 400 µM H₂O₂ for 2 h. The inhibition effect of apples on H₂O₂-induced oxidative stress was determined by 2,7-dichlorofluorescein diacetate assay. The data are displayed with mean ± standard deviation (bars) of 3 replications. Statistical analysis indicated that the influence of the compounds used had significant effect on the H₂O₂-induced oxidative stress (*P* < 0.05).

of the control, whereas cells pretreated with apple extracts (300 to 2000 $\mu\text{g}/\text{mL}$) before H_2O_2 exposure showed significant reduction (70% to 90%) of oxidative stress (Figure 1). This result suggests that apple phenolics with a strong antioxidant activity may play an important role to reduce the oxidative stress-induced risk of AD.

Effect of apple phenolics on H_2O_2 -induced neurocytotoxicity

The cytotoxicity of apple extracts (100 μg extract = 500 mg fresh apple) was measured by various methods using PC-12 cells. The MTT assay is a simple colorimetric assay to measure cell cytotoxicity. Hydrogen peroxide caused a significant decrease in cell viability (about 30%) when measured by MTT reduction assay, but apples inhibited H_2O_2 -induced oxidative injury (Figure 2). The cellular reactions involved in MTT assay are not completely understood today; however, the involvement of active mitochondria, namely the mitochondrial succinate dehydrogenase system, has been indicated (Carmichael and others 1987). Besides, mitochondria may be one of the most sensitive primary targets of oxidative injury in neuronal cells (Wallace 1992). Therefore, these results suggest that PC-12 cell protection by apple phenolics is partially because of the mitochondrial protection mechanisms.

The neuronal cells of brain is especially vulnerable to ROS damage as a result of the brain's high oxygen consumption rate, its abundant lipid content, high levels of membrane unsaturated fatty acid that are easily oxidizable by free radicals, and the relative paucity of antioxidant enzymes compared with other organs (Coyle and Puttfarcken 1993; Varadarajan and others 2000). Loss of membrane phospholipids may be because of decreased biosynthesis, increased degradation, and increased lipid peroxidation (Markes-

bery 1997). Lipid peroxidation produces many aldehydes, especially the 4-hydroxyalkenals, which are produced by the oxidation of polyunsaturated fatty acid (PUFA). One of the major products of PUFA (arachidonic and docosahexenoic acids) peroxidation is 4-hydroxynonenal (4-HNE). The 4-HNE is an α , β -aldehyde, which is highly reactive and responsible for cytotoxic effects induced by oxidative stress. The 4-HNE is capable of inhibiting DNA, RNA, and protein synthesis, glycolysis, and degradation of physiological enzymes. Studies of non-neuronal cells indicate that 4-HNE contributes to membrane damage and cell death because of a variety of oxidative insults (Esterbauer and others 1991). In this respect, we investigated the membrane protective effect of apple extracts using LDH release assay. LDH assay is a means of measuring either the number of cells via total cytoplasmic LDH or membrane integrity as a function of amount of cytoplasmic LDH released into the medium. In our work, H_2O_2 -induced oxidative stress increased plasma membrane damage, whereas apple phenolics protected the PC-12 cells from neurotoxicity in a dose-dependent manner (Figure 2). In addition, to confirm the cytoprotection of apple on the H_2O_2 -induced membrane damage, the trypan blue exclusion assay was also used. These data also showed the membrane protection effect of apple phenolics. As shown in Figure 2, H_2O_2 -induced oxidative stress caused significant damage of plasma membrane (about 50%). However, the damage was significantly reduced after exposure to apple extract at above 300 $\mu\text{g}/\text{mL}$: at 2000 $\mu\text{g}/\text{mL}$, protection was over 130%. These results indicate the membrane protection effects of apple phenolics with antioxidative activity on oxidative stress-induced cytotoxicity.

Our results clearly demonstrated that apple phenolics protect PC-12 cells from oxidative H_2O_2 toxicity in vitro. Quercetin, one of the major flavonoids in apples, appeared to be the main agent responsible for this beneficial effect (data not shown). Because the possibility that nutritional scarcities may play an important role in cognitive defects has been recognized recently (Youdim and Joseph 2001), the identification of potentially protective factors for memory deficits or dementia is becoming a gradually important subject. In this respect, the consumption of more fresh fruits and vegetables high in phenolics is strongly recommended to provide sufficient antioxidants to the human body. Among fresh fruits consumed in the United States, apples (8.76 kg/person/y = about 1/4 of an apple/person/d) ranked 2nd to bananas (12.7 kg/person/y). However, the antioxidant activity of apple is much higher than that of bananas or oranges (date not shown). Therefore, as we have already shown, apples have strong anticancer activities in vitro (Eberhardt and others 2000), additional apple consumption (1 per day in our diet) may supply major bioactive compounds, which may also play an important role in reducing the risk of neurodegenerative disorders such as AD.

Conclusions

ROS play an important role in the progression in neurodegenerative disorders such as AD. Natural foods possessing antioxidative components (vitamins and phenolics) have received a great deal of attention because they are safe and not considered as medicine; some of these are known to function as chemopreventive agents against oxidative damage. Hence, natural food-derived multiple antioxidant supplements would be more useful than individual agents alone. Apple, having vitamin and antioxidative phenolics, has shown a strong anti-neurodegenerative activity in vitro study. Because apple is one of the rich sources of antioxidant among fruits and has many important bioactive compounds, daily consumption of an apple may provide the beneficial effects that may reduce a risk of chronic diseases including AD. Therefore, it

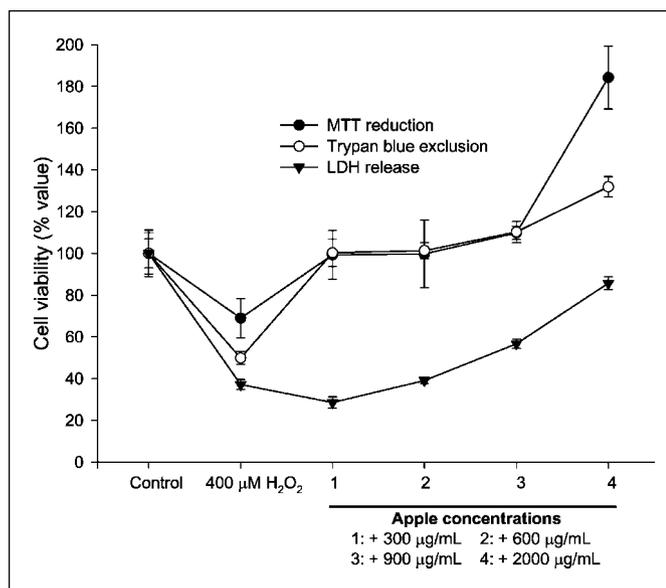


Figure 2—Effects of the apple phenolics on the viability of PC-12 cells with exposure of H_2O_2 . The cell viability was evaluated using 3 different methods (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction, lactate dehydrogenase release, and trypan blue exclusion assays). PC-12 cells were pre-incubated for 10 min with various concentrations of apple extracts (100 μg extract = 500 mg fresh apple), and then the cells were treated with H_2O_2 (400 μM) for 2 h. The control group received treatment with neither H_2O_2 nor apple extracts. Results are shown as mean \pm standard deviation ($n = 3$). Significant difference ($P < 0.05$) was observed on the H_2O_2 -induced cell death.

would be proposed that natural antioxidants from apple phenolics could reduce the risk of neurodegenerative disease such as AD.

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