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Strawberry and Its Anthocyanins Reduce Oxidative Stress-Induced Apoptosis in PC12 Cells

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Because strawberries are known to contain higher concentrations of phytochemicals and have higher antioxidant capacity among common fruits, their neuroprotective activity was tested in vitro on PC12 cells treated with H_2O_2. Their protective effect and antioxidant capacity were also compared with those of banana and orange, which are the fresh fruits consumed at highest levels in the United States. The cell viability test using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay showed that strawberry phenolics significantly reduced oxidative stress-induced neurotoxicity. Because oxidative stress is also known to increase neuronal cell membrane breakdown, lactate dehydrogenase (LDH) and trypan blue exclusion assays were also performed. Strawberry showed the highest cell protective effects among the samples. The overall relative neuronal cell protective activity of three fruits by three tests followed the decreasing order strawberry > banana > orange. The protective effects appeared to be due to the higher phenolic contents including anthocyanins, and anthocyanins in strawberries seemed to be the major contributors.

KEYWORDS: Alzheimer's disease; strawberry; phenolics; anthocyanins

INTRODUCTION

Neurodegenerative disorders such as Alzheimer's disease (AD) are chronic diseases characterized by loss of memory and cognition. Many studies indicate that the brain of an AD patient is subjected to increased oxidative stress resulting from free radical damage, and the resulting cellular dysfunctions are widely believed to be responsible for neuronal degeneration in AD (*1*). Oxidative neuronal cell damage has been implicated in neurodegenerative disorders such as AD (*2*). It is mediated by reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals, which are generated as byproducts of normal and irregular metabolic processes that utilize molecular oxygen. ROS are known to attack cellular biomolecules and are responsible for increased oxidation of protein, DNA, polyunsaturated fatty acids, and lipid. These oxidative stress-induced damages disrupt cellular function and membrane integrity, thereby leading to apoptosis (*3*). It has been demonstrated that ROS generate cell death through apoptosis (*4*). There are many types of physiological oxidative stress inducers, which are able to cause apoptotic cell death. For instance, H_2O_2 induces apoptosis in many different cell types (*4*), and this effect will be inhibited by the addition of antioxidants such as vitamin C (*5*). As the major component of ROS, H_2O_2 has been extensively used as an inducer of oxidative stress in many in vitro models (*6*).

Natural antioxidants have been reported to play a major role in blocking oxidative stress induced by free radicals. Recently, natural foods and food-derived components, such as antioxidative vitamins and phenolic phytochemicals, have received a great deal of attention because they are safe and not considered to be "medicine"; some of these are known to function as chemopreventive agents against oxidative damage. The contribution of vitamin C to the total antioxidant activity of fruits was determined to be generally <15% (*7*), whereas polyphenolic phytochemicals contribute significantly to the total antioxidant capacity of fruits (*8*). The phytochemicals in plant tissues responsible for the antioxidant capacity can largely be attributed to the phenolics, such as anthocyanins and other flavonoid compounds (*9*). Several studies have shown that the strawberry generally possessed a high level of antioxidant activity, which is linked to the levels of phenolic compounds in the fruit (*10*). Strawberry extracts have been found to have higher antioxidative effects than plum, red grape, tomato, banana, and orange (*7*). Anthocyanins are found in a variety of highly pigmented fruits and seem to play a role in preventing human diseases related to oxidative stress (*11*). Therefore, we investigated the effects of strawberries and anthocyanins on oxidative stress-induced apoptosis of neuronal cells and compared them with those of banana and orange, which are major fruits consumed in the United States (*12*). In addition, we also compared the effect of anthocyanin with those of catechin and naringenin, which are major antioxidative phenolics in banana and orange, respectively (*13*, *14*).

MATERIALS AND METHODS

Materials. Ham's F12K medium, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from ATCC (Manassas, VA). All other chemicals were the products of Sigma (St. Louis, MO). Fresh

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strawberry and banana used in this study were obtained from local supermarkets in Geneva, NY, in 2002. Immediately upon arrival in the laboratory, strawberry and banana were stored in a 2−5 °C cold room. They were carefully cut into slices, and the freeze-dried samples were ground to powder using a laboratory mill (Thomas-Willey) and then stored at −20 °C until analyzed. California navel orange was purchased from a local supermarket in Geneva, NY, in 2004. Cells were preincubated with fruit extracts (strawberry, banana, and orange) and three phenolics (cyanidin, catechin, and naringenin) for 10 min. After 10 min, 400 μM H_2O_2 was treated for 2 h. Cyanidin, catechin, and naringenin (10−100 μmol/L) were added from stock solutions (1 mM) in deionized distilled water. The fruit extracts (100−2000 μg/mL) were added from stock solutions (20 mg/mL) in deionized distilled water. Data are presented as mean ± SD for one representative triplicate determination.

Extraction of Phenolics. The phenolics in fruits were extracted from 10 g of dried sample using 80% aqueous methanol according to the ultrasound-assisted method (*15*). 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 no. 2 filter paper (Whatman International Limited, Kent, U.K.) using a chilled Büchner funnel and rinsing with 50 mL of absolute methanol. Extraction of the residue was repeated using the same conditions. The two filtrates were combined and transferred into a 1 L evaporating flask with an additional 50 mL of 80% aqueous methanol. The solvent was removed using a rotary evaporator at 40 °C. The remaining phenolic concentrate was first dissolved in 50 mL of absolute methanol and diluted to a final volume of 100 mL using deionized distilled water (ddH_2O). The mixture was centrifuged at refrigerated temperatures at 12000*g* for 20 min and stored at −20 °C until analyses.

Determination of Total Phenolic Content. The total phenolic phytochemical concentration was measured using the Folin−Ciocalteu method (*15*). The absorbance was measured at 750 nm using a spectrophotometer. The total phenolic contents of the samples are expressed in milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight. All samples were prepared in three replications.

Determination of Anthocyanin Content. The total anthocyanin content of the methanolic extract was determined using a modified pH differential method described previously (*16*). A Hewlett-Packard 8452A diode array spectrophotometer was used to measure the absorbance at 510 and 700 nm in buffers at pH 1.0 and 4.5. The absorbance reading was converted to total milligrams of cyanidin 3-glucoside per 100 g of fresh weight using the molar extinction coefficient of 26900 and absorbance of $A = [(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}]$. All samples were prepared in six replications.

Vitamin C Equivalent Antioxidant Capacity (VCEAC) Assay. Blue-green ABTS radicals were used to evaluate the antioxidant capacity of samples (*17*). A radical initiator, 1.0 mM AAPH, was added to 2.5 mM ABTS in phosphate-buffered saline (PBS; pH 7.4; 0.1 M K_2HPO_4/KH_2PO_4 buffer; 150 mM NaCl). The mixed solution was heated in a water bath at 68 °C. The resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm with additional PBS. Twenty microliters of sample was added to 980 μL of the ABTS radical solution. The mixture was incubated in a 37 °C water bath under restricted light for 10 min and measured at 734 nm. The ABTS radical loses color when its unpaired electron is paired by the electron from antioxidants in samples. The antioxidant capacity of samples was expressed on a fresh weight basis as milligrams per 100 g of vitamin C equivalents (VCEAC).

Cell Culture. PC-12 cells (ATCC) were cultured in Ham's F12K medium containing 15% horse serum, 2.5% fetal bovine serum, 50 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 5% CO_2. The PC12 cell line was derived from a transplantable rat pheochromocytoma. The cells respond reversibly to nerve growth factor (NGF) by induction of the neuronal phenotype.

Determination of Cell Viability. PC12 cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μL of Ham's F12K, and the cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (*18*). The cells were incubated with 0.25 mg of 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 the absorbance using a microplate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Table 1. Levels of Total Phenolics, Anthocyanins, and Vitamin C Equivalent Antioxidant Capacity (VCEAC) from Samples[a]

fruit	total phenolics (mg of GAE/100 g of fresh wt)	total anthocyanins (mg of cyanidin 3-glucoside/100 g of fresh wt)	VCEAC (mg of VCE/100 g of fresh wt)
strawberry	155 ± 3.4	19.430 ± 1.11	363 ± 8.5
banana	100 ± 2.7	0.005 ± 0.06	188 ± 5.7
orange	91 ± 5.6	0.010 ± 0.09	169 ± 9.4

[a] The data are presented as mean ± SD of three replications. GAE, gallic acid equivalent; VCE, vitamin C equivalent.

Measurement of Cell Membrane Toxicity. PC12 cells were precipitated by centrifugation at 2000*g* for 2 min at room temperature, 50 μL of the supernatants was transferred into new wells, and LDH was determined using the in vitro toxicology assay kit (Sigma, St. Louis, MO). 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. Because viable PC12 cells maintained membrane integrity, the cells did not allow trypan blue dye to pass through the cell membrane. Cells with damaged membrane appeared blue due to their accumulation of dye and were counted as dead. The dye of 0.4% trypan blue was added to PC12 cells, and after 5 min, cells were loaded into a hematocytometer and counted for the dye uptake. The number of viable cells was calculated as percent of the total cell population (*19*).

Statistical Analysis. The results were expressed as means ± SD. Differences among different experimental groups were tested for significance using one-way analysis of variance (ANOVA; SPSS Inc., Chicago, IL), taking $P < 0.05$ as significant. Data were compared with control group by one-way ANOVA test.

RESULTS AND DISCUSSION

The contents of total phenolics and anthocyanins and the antioxidative capacity of these fruits are presented in **Table 1**. Strawberry contained much higher levels of the average concentration of total phenolics (155 mg of GAE/100 g of fresh weight) and total anthocyanins (19.4 mg of cyanidin 3-glucoside/100 g of fresh weight). Although banana and orange showed phenolic contents of ∼91−100 mg of GAE/100 g of fresh weight, they have very little anthocyanin. Several previous studies showed the total phenolics (150−280 mg of GAE/100 g of fresh weight) and the anthocyanins (15−45 mg of cyanidin 3-glucoside/100 g of fresh weight) of strawberry (*20*, *21*). The total phenolics of banana and orange were 85 and 90 mg of GAE/100 g of fresh weight, respectively (*22*). The relative total antioxidant capacities of the samples were as follows in decreasing order: strawberry > banana > orange. The differences in VCEAC between strawberry and others were ∼2-fold, and the differences of antioxidative capacity may be due to the contents of phenolics including anthocyanins. Phytochemicals from fruits and vegetables have long been recognized to possess many properties including antioxidant activity (*23*). Oxidative damage has been found in all classes of organic molecules that are critical for maintaining neuronal structure and function. Excessive lipid, protein, and DNA peroxidation have all been studied in neurodegenerative disorders (*24*). In this respect, diets rich in fruits may reduce the risk of oxidative stress-induced chronic disease. Our results suggested that strawberries with

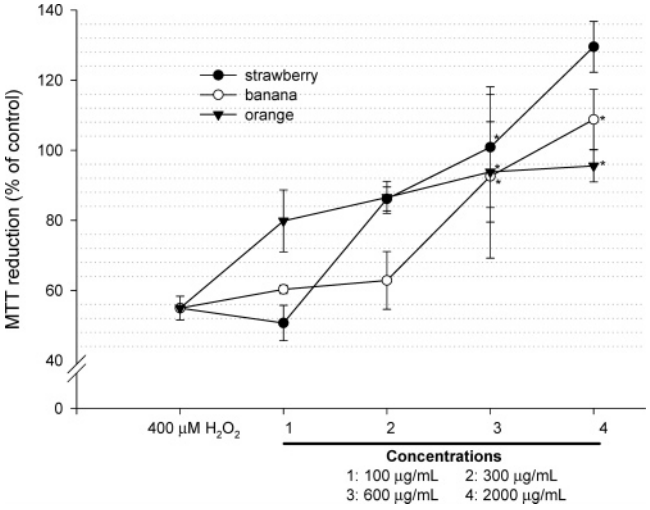

Figure 1. Cell viability effect of fruit phenolics on H_2O_2-induced cytotoxicity in PC12 cell system. PC12 cells were pretreated for 10 min with various concentrations. The cells were then treated with 400 μM H_2O_2 for 2 h. Levels of cell viability were measured using the MTT assay as described under Materials and Methods. Results shown are means ± SD ($n = 3$). Significant difference ($P < 0.05$) was observed on the H_2O_2-induced apoptosis

high anthocyanin content are a more important source of antioxidant phytochemicals than others.

The oxidative stress-induced neurotoxicity was examined by determining the percentage of MTT reduction after incubation of PC12 cells for 2 h with H_2O_2. Hydrogen peroxide (400 μM) caused a significant decrease in cell viability (45 ± 3.2%), but pretreatment of PC12 cell with fruit phenolics blocked oxidative stress-induced cytoxicity in a dose-dependent manner (**Figure 1**). The protective effects of fruit phenolics were ~97−127%. The effect of strawberry was much higher than others at the highest concentration (2000 μg/mL) and showed a concentration-dependent manner. These data showed the same pattern of the results on their antioxidant capacity (**Table 1**). This result clearly demonstrated that PC12 cell apoptosis by oxidative stress was suppressed by pretreatment with the fruit phenolics. Especially, strawberry phenolics with high anthocyanin content showed the most effective protection. MTT dye reduction assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria (*5*). Mitochondria may be one of the most sensitive primary targets of oxidative injury in neuronal cells (*25*). This may be due to the fact that mitochondrial DNA (mtDNA) does not encode for any repair enzymes and, unlike nuclear DNA, it is not shielded by protective histones. In addition, mtDNA is close to the site where free radicals are generated during oxidative phosphorylation (*26*). Indeed, an increased frequency of mutations in mtDNA has been found in autopsy samples of AD brains (*27*), and many studies have suggested mitochondrial defects in the pathogenesis of AD patients (*26*). Therefore, these results suggest that PC12 cell protection by strawberry is partially due to the mitochondrial protection mechanisms.

It has been proposed that amyloid β (Aβ) proteins produced from amyloid precursor protein (APP) play an important role in AD (*28*). The Aβ protein has been identified as a possible source of oxidative stress in the AD brain because it can acquire a free radical state contributing to its toxic effects (*29*). In another study, experiments on a transgenic mouse model of AD sustain that Aβ-induced neurotoxicity is mediated by oxidative stress. For example, it has been reported (*29*) that Cu/Zn superoxide dismutase (SOD) and hemoxygenase-1 (HO-1), biomarkers of oxidative stress, were promoted in aged transgenic mice. This study was further verified in the PC12 cell system. Both SOD and HO-1 levels were increased in PC12 cells following treatment with Aβ or H_2O_2. Hence, these studies strongly suggest that free radicals are involved in Aβ-induced neurotoxicity. The brain is considered to be abnormally sensitive to oxidative damage (*30*), and in fact early studies on the peroxidation of brain membranes supported this conception (*31*). Because the brain is enriched in the more easily peroxidizable fatty acids [arachidonic acid and docosahexaenoic acid (DHA)], the cell membrane of the brain is considered to be abnormally sensitive to oxidative damage (*30*). To examine the probability of oxidative stress-induced membrane damage, we have assessed the protective effect of strawberry phenolics on H_2O_2-induced cytotoxicity using the LDH assay, measuring the activity of this stable enzyme released into the medium from apoptotic PC12 cells. A quantitative analysis of LDH activity can determine what percentage of cells is dead. Treatment with H_2O_2 caused an increase in LDH release into the medium and a decrease in the number of viable cells (81 ± 2.3%) (**Table 2**). Pretreatment with the fruit extracts exhibited efficient inhibitory activity (40−68% at 2000 μg/mL) of LDH release in PC12 cell system, and strawberry extracts with high anthocyanin content showed the highest inhibitory effect (~68%) on LDH release.

Table 2. Inhibition of LDH Release of Fruit Phenolics on H_2O_2-Induced Membrane Damage in PC12 Cells (Cell Viability: Percent of Control)[a]

concn (μg/mL)	strawberry	banana	orange
100	20.9 ± 5.3*	18.1 ± 0.6*	2.4 ± 0.1
300	41.8 ± 4.7	27.3 ± 1.2	21.2 ± 1.3*
600	50.0 ± 9.3	34.5 ± 1.8	30.3 ± 1.2
2000	67.8 ± 3.9	43.2 ± 2.3	39.9 ± 4.5

[a] PC12 cells were pretreated for 10 min with various concentrations. The cells were treated with H_2O_2 (400 μM) for 2 h. LDH activity in culture supernatants was measured with a colorimetric LDH assay kit. All data are represented as the means ± SD ($n = 3$) and values obtained from three separate cultures. The cell viability of 400 μM H_2O_2 was 18.9 ± 2.4. Statistical analysis indicated that the influence of the compounds used had a significant effect on H_2O_2-induced membrane toxicity (LDH release) ($P < 0.05$); *, indicates no significantdifferentce from control.

To confirm if strawberry phenolics block the H_2O_2-induced membrane damage, the trypan blue exclusion assay, which directly measures the viable cells maintaining the capability of excluding the dye and may reflect more precisely the integrity of viable cell membrane, was also used. H_2O_2-induced oxidative stress increased plasma membrane damage and the fruit phenolics protected the PC12 cells from membrane toxicity (**Figure 2**). These data showed the same pattern as the LDH assay; the protective activity of strawberry extracts was much more effective than others (106% at 2000 μg/mL).

tert-Butyl hydroperoxide (TBHP) is a short-chain analogue of lipid hydroperoxides that mimics the toxic effect of peroxidized fatty acids. Lipid hydroperoxides are formed in living cells by lipid peroxidation via formation of oxygen-derived free radicals from multiple sources such as oxidative stresses (*32*). It has also been reported that TBHP leads to cell death by inducing changes in mitochondrial permeability in hepatocytes accompanied by a depolarization of the mitochondrial potential (*33*). Lazze et al. (*34*) found that anthocyanins inhibit the TBHP-induced cytotoxicity like lipid peroxidation and DNA damage. Therefore, these results indicate double protective effects of the strawberry with the much higher antioxidative capacity and anthocyanin content on mitochondrial disruption and oxidative stress-induced membrane damage.

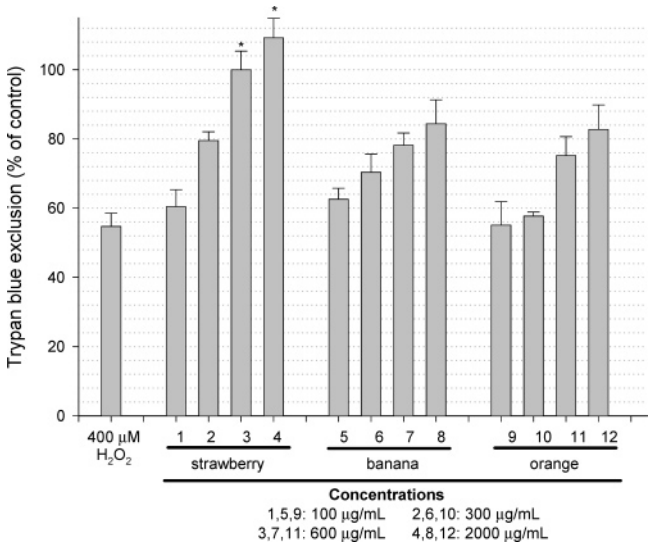

Figure 2. Preventive effects of fruit phenolics on H_2O_2-induced membrane damage in PC12 cells. PC12 cells were plated at low density in a 24-well plate (4×10^5/mL). Cells were incubated with the phenolics for 10 min before the addition of 400 μM H_2O_2. Cultures were observed after an additional 2 h, and trypan blue exclusion staining was performed. Data are presented as mean ± SD for one representative triplicate determination and are expressed as the percent survival compared to the corresponding controls ($P < 0.05$).

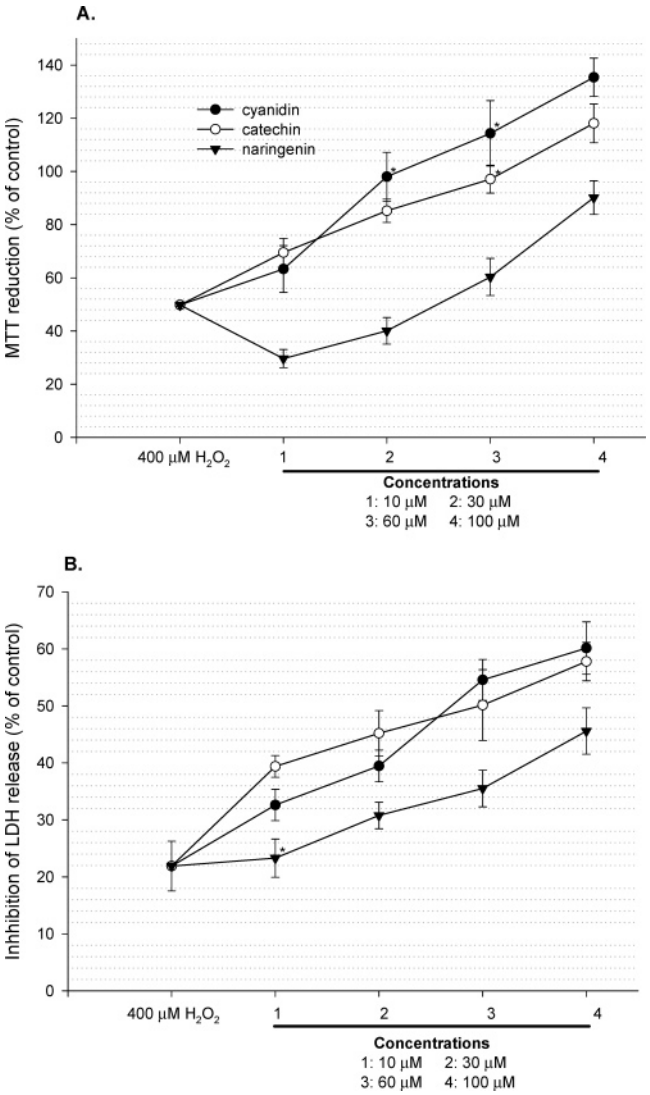

Figure 3. Cell viability effect of cyanidin, catechin, and naringenin on H_2O_2-induced cytotoxicity in PC12 cell system (**A**, MTT reduction assay; **B**, LDH assay). PC12 cells were pretreated for 10 min with various concentrations. The cells were then treated with 400 μM H_2O_2 for 2 h. Results shown are means ± SD ($n = 3$). Significant difference ($P < 0.05$) was observed on the H_2O_2-induced apoptosis.

Several studies showed that the antioxidant capacity of commercial banana was attributed to its catechins (*13*) and that of orange to naringenin (*14*). To determine whether the highest neuronal cell protection of strawberry is due to the anthocyanin, we compared three phenolics (cyanidin, catechin, and naringenin) in cell viability assays. In **Figure 3**, the cell protection of cyanidin was the most effective at 100 μM concentration. The relative protective effects of three phenolics were as follows in decreasing order: cyanidin > catechin > naringenin. These results accorded with the total antioxidative effect (VCEAC) (**Table 1**) and cell protective effect of the three samples (**Table 2** and **Figure 2**). These data thus suggest that the neuronal cell protective capacities of strawberry might be due to total phenolic contents and antioxidative activity (VCEAC). Especially, the fact that strawberry has the much higher anthocyanin content among the three samples may play a pivotal role in cell viability.

The inflammatory reaction hypothesis of AD provoked interest when it was demonstrated that the products of inflammatory reaction, such as cytokines (*35*) and free radicals (*36*), were neurotoxic in experimental neuron models. These products of inflammatory reactions may represent extracellular signals, which initiate and promote neuronal degeneration in AD. Mitogen-activated protein (MAP) kinase (e.g., p38 protein families) initiates the induction of genes such as inducible nitric oxide synthase (iNOS) and cycloxygenase-II (COX-II) as well as several cytokines, and these genes make cytotoxic inflammatory reaction in neuronal cells (*37*). Therefore, anti-inflammatory agents may play an important role in neurodegenerative disorders such as AD. Several studies have indicated that phenolics from fruits and vegetables inhibited the production of inflammatory reaction-induced nitric oxide (NO, inflammatory mediator) through the blocking of the expression level of iNOS, COX-II gene in a concentration-dependent manner (*5*, *38*). In addition, Lietti et al. (*39*) showed significant anti-inflammatory activity of *Vaccinium myrtillus* anthocyanosides in rats and rabbits. Several studies suggested that strawberries improved measures of cognitive function in animal studies, and they had variable health benefits including an anticarcinogenic effect (*40*, *41*). Therefore, the consumption of strawberries with high anthocyanin content will supply beneficial antioxidative phytochemicals, and these bioavailable phytochemicals may also play an important role in reducing the oxidative stress-induced risk of neurodegenerative disorders such as AD.

In summary, chemoprevention has been suggested as a useful strategy for the management of neurodegenerative disorders such as AD. Many natural substances present in the human diet have been known as potential chemopreventive agents (*38*). Our study suggested that strawberries protect the oxidative stress-induced neuronal damage, and the protection was due to the antioxidant properties against neurodegeneration. Consistent with the antioxidant capacity, the protective effects on neuronal cell viability were as followed in decreasing order: strawberry > banana > orange. The degrees of neuronal cell protection from MTT, LDH, and Trypan blue assays were closely correlated with their phenolic contents ($r^2 = 0.935$, 0.999, and 0.996, respectively) and their antioxidative capacities ($r^2 = 0.913$, 0.999, and 0.999, respectively). Anthocyanins in strawberries

appeared to be the major contributors. Therefore, additional consumption of strawberries may be beneficial to increase chemopreventive effects in neurodegenerative diseases such as AD.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-aminopropane)dihydrochloride; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); AD, Alzheimer's disease; Aβ, amyloid β; APP, amyloid precursor protein; COX-II, cycloxygenase-II; GAE, gallic acid equivalent; HO-1, hemoxygenase-1; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; MAP kinase, mitogen-activated protein kinase; mtDNA, mitochondrial DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase; TBHP, *tert*-butyl hydroperoxide; VCEAC, vitamin C equivalent antioxidant capacity.

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