PHYTOTHERAPY RESEARCH
Phytother. Res. **24**: 76–84 (2010)
Published online 13 May 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2871

Neuroprotective Effects of *Triticum aestivum* L. against β-Amyloid-induced Cell Death and Memory Impairments

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β-Amyloid (Aβ) is a key component of senile plaques, neuropathological hallmarks of Alzheimer's disease (AD) and has been reported to induce cell death via oxidative stress. This study investigated the protective effects of *Triticum aestivum* L. (TAL) on Aβ-induced apoptosis in SH-SY5Y cells and cognitive dysfunctions in Sprague-Dawley (SD) rats. Cells treated with Aβ exhibited decreased viability and apoptotic features, such as DNA fragmentation, alterations in mitochondria and an increased Bax/Bcl-2 ratio, which were attenuated by TAL extract (TALE) pretreatment. To elucidate the neuroprotective mechanisms of TALE, the study examined Aβ-induced oxidative stress and cellular defense. TALE pretreatment suppressed Aβ-increased intracellular accumulation of reactive oxygen species (ROS) via up-regulation of glutathione, an essential endogenous antioxidant. To further verify the effect of TALE on memory impairments, Aβ or scopolamine was injected in SD rats and a water maze task conducted as a spatial memory test. Aβ or scopolamine treatment increased the time taken to find the platform during training trials, which was decreased by TALE pretreatment. Furthermore, one of the active components of TALE, total dietary fiber also effectively inhibited Aβ-induced cytotoxicity and scopolamine-caused memory deficits. These results suggest that TALE may have preventive and/or therapeutic potential in the management of AD. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: apoptosis; β-amyloid; memory; oxidative stress; scopolamine; *Triticum aestivum* L.

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disorder in the elderly population characterized by progressive degeneration and loss of neurons in the brain. The accumulation of senile plaques and neurofibrillary tangles in vulnerable brain regions form the two typical neuropathological markers for AD (Citron, 2002, 2004). The senile plaques are mainly composed of β-amyloid peptide (Aβ) generated from amyloid precursor protein (APP) by β- and γ-secretases. One of the commonly accepted hypotheses underlying the neuropathogenesis of AD is that abnormal proteolytic cleavage of APP leads to an excess extraneuronal accumulation of Aβ which has been shown to be toxic to neurons as well as glia. Experimental data from *in vitro* and *in vivo* studies indicate that Aβ affects a wide array of brain functions, thereby leading to neuronal cell death and cognitive impairments (Vila and Przedborski, 2003).

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Oxidative stress is involved in Aβ-induced neurotoxicity and neuronal cell death, thereby mediating the pathogenesis and/or progression of AD (Markesbery, 1997). Aβ has been shown to increase intracellular reactive oxygen species (ROS) levels and to induce oxidative damage to cellular macromolecules, such as DNA, protein and lipid (Markesbery, 1997). ROS have been reported to cause cell death via apoptosis in various cell types, and such proapoptotic effects can be blocked or delayed by a wide array of intra- and extracellular antioxidants (Carmody and Cotter, 2001). Overexpression of antioxidant enzymes and/or pretreatment with antioxidant compounds has also been shown to attenuate Aβ-induced apoptotic cell death (Aruoma *et al.*, 2003; Behl *et al.*, 1994).

Besides oxidative stress, accumulated evidence supports that continuous infusion of Aβ into the cerebral ventricle in animal models causes cognitive dysfunction via deterioration of the cholinergic system (Nitta *et al.*, 1994). Cholinergic deficits have been associated with memory loss and correlated with the severity of AD (Bierer *et al.*, 1995). In line with this notion, scopolamine, a muscarinic cholinergic receptor antagonist has widely been used to impair learning acquisition and short-term memory, and utilized as a model for

Received 02 January 2009
Revised 16 March 2009
Accepted 18 March 2009

screening antiamnesic drugs (Bartus *et al.*, 1982). Pharmacological intervention to increase cholinergic transmission is a potential candidate for the development of preventive and/or therapeutic agents against cognitive impairments in AD (Terry and Buccafusco, 2003). Some synthetic acetylcholine esterase inhibitors, such as tacrine (Cognex®) and donepezil (Aricept®), have been approved by the FDA and prescribed for the treatment of dementia.

One of the plausible ways to prevent neuronal damage induced by Aβ is to augment the endogenous defense capacity and to improve cholinergic activity through daily intake of phytochemicals. Nutraceuticals and functional food ingredients have been searched for the treatment of AD patients to improve their memory and cognitive function (Deschamps *et al.*, 2001; Florent-Béchard *et al.*, 2007). Natural products derived from diets are known to exhibit a variety of biological effects including antioxidant, antiinflammatory, anticarcinogenic, antimutagenic and antiaging activities. Epidemiological, clinical and laboratory studies demonstrating the association between nutrition and cognition highlight the importance of dietary intake in preventing or delaying AD (Youdim and Joseph, 2001; Sano *et al.*, 1996). Because of their relatively low side-effects and a long history of human use, edible phytochemicals may be good candidates for therapeutic and/or preventive application in AD. However, their effectiveness and underlying molecular mechanisms have not been scientifically verified.

Triticum aestivum L. (TAL), a common wheat has been a food crop since the beginning of agriculture, used whole or ground, mainly made into flour, and served as the basic materials of most baked foods, including bread. Wheat is composed of protein, fat, carbohydrate and fiber and has been regarded as a rich source of vitamins, minerals and natural antioxidants, including ferulic, caffeic, *p*-hydroxybenzoic and vanilic acids (Zhou *et al.*, 2004). Wheat grass has been clinically manipulated in the treatment of thalassaemia, distal ulcerative colitis and rectal bleeding (Marawaha *et al.*, 2004; Ben-Arye *et al.*, 2002). In addition, wheat extracts from sprout and germ have been reported to exhibit antimutagenic and anticarcinogenic activities (Peryt *et al.*, 1992; Boros *et al.*, 2005). Particularly, these beneficial effects of wheat have been ascribed to its potent antioxidant properties (Kulkarni *et al.*, 2006).

In the light of various physiological roles of TAL with its antioxidant potential, this compound is anticipated to exert neuroprotective effects via the regulation of cellular homeostasis and augmentation of self-defense to oxidative stress. In the previous study, we and others have shown that some antibiotics such as tetracyclines, aminoglcosides and quinolones were effective in reducing apoptotic cell death under ischemic conditions (Lee *et al.*, 2004; Park *et al.*, 2007) and confer protection against myocardial infarction, stroke and dementia (Lee *et al.*, 2004; Park *et al.*, 2007; Yrjanheikki *et al.*, 1999; Choi *et al.*, 2007). In a similar ischemic cell culture model, TAL extract (TALE) treatment also prevented neuronal cell death by inhibiting the apoptotic process (unpublished data), supporting the possible application of TALE for the management of ischemic diseases and vascular dementia. Recently, it was also reported that TALE and its starch fraction reduced the ischemic area and neutrophil- or microglia-related pro-inflammatory responses in a focal cerebral ischemic animal model caused by transient middle cerebral artery occlusion (MCAO) (Han *et al.*, 2008).

Based on these results, present study investigated the possible protective effects of TALE on Aβ-induced apoptosis in cultured SH-SY5Y neuroblastoma cells and the cholinergic memory deficits caused by Aβ or scopolamine in Sprague-Dawley (SD) rats.

MATERIALS AND METHODS

Materials. β-Amyloid ($A\beta_{25\text{-}35}$) was supplied from Bachem Inc. (Torrance, CA, USA). $A\beta_{25\text{-}35}$, which is the most toxic peptide fragment derived from APP, was dissolved in deionized distilled water at a concentration of 1 mM and stored at –20°C. The stock solution was diluted to desired concentrations immediately before use. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were provided from Gibco BRL (Grand Island, NY, USA). Dichlorofluorescein diacetate (DCF-DA) and tetramethylrhodamine ethyl ester (TMRE) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Scopolamine hydrochloride and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Preparation of TALE. The grains of TAL were ground in a mill (KF-20, KoreaMedi, Korea) using a 2 mm screen, and 1 kg of the product was extracted in 10 L of water at 70–90°C for 1 h (extractor, NO-300, Kyung Seo Machine, Korea). The whole extract was centrifuged at 14000 × **g** for 30 min and the supernatant was concentrated (vacuum dryer, N-1000, EyelA Co., Japan) and freeze-dried into powder (freezer dryer, 79340–00, Labconco Co., MO, USA). TALE was analysed to be composed of 4.9% water, 5.9% ash, 7.9% protein, 0.2% fat and 81.1% carbohydrate, including 15.4% total dietary fiber (TDF). TAL was collected at Gurye, Jeonnam Province, Korea, in 2006. Kumkang, TAL used in this study, is deposited at Korea Seed & Variety Service, with Plant Variety Protection Registration Number 153.

Preparation of TDF. TDF with ash was prepared by following previously developed (Lee *et al.*, 1992) and recently modified methods (Total Dietary Fiber Assay, cat. no. TDFR 06/10, Megazyme, Ireland). Ten grams of TALE was completely dispersed with stirring in 400 mL of MES/TRIS buffer (0.05 M of each, pH 9.0). After addition of α-amylase (Novozymes, Denmark), the mixture was incubated at 80°C for 30 min with continuous stirring at low speed. The solution was cooled down to 60°C and treated with protease (Megazyme) at 60°C for 30 min. Following incubation with amyloglucosidase (Megazyme) at 60°C for another 30 min, 1.6 L of 95% ethanol preheated to 65°C was added, and the mixture was kept at room temperature for 60 min without stirring. Finally, 1.5–2.0 g of TDF with ash was obtained by centrifugation and drying.

Cell culture. SH-SY5Y cells were grown routinely in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin–fungizone mixture (Cambrex, East Rutherford, NJ,

DOI: 10.1002/ptr

USA) at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. The medium was changed every other day and the cells were plated at an appropriate density before treatments. In all experiments, the cells were preincubated with indicated concentrations of TALE or TDF for 1 h, and then $A\beta_{25\text{-}35}$ was added to the medium.

Measurement of cell viability. SH-SY5Y cells were plated at a density of 4×10^4 cells/300 μL in 48-well plates and the cell viability was determined by the MTT reduction assay as described previously (Jang *et al.*, 2004). After incubation of SH-SY5Y cells with $A\beta_{25\text{-}35}$ in the presence or absence of TALE for 22 h, the cells were treated with the MTT solution (final concentration, 1 mg/mL) for another 2 h. The dark blue formazan crystals formed in viable cells were solubilized with dimethylsulfoxide (DMSO), and the absorbance was measured at 570 nm with a microplate reader (TECAN GmbH, Salzhurg, Austria). The results were expressed as a percentage (%) of MTT reduction with the absorbance of vehicle-treated control cells being 100%.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL staining). To detect DNA fragmentation, the commercially available *in situ* cell death detection kit (Roche Diagnostic GmbH, Mannheim, Germany) was utilized. SH-SY5Y cells (8×10^4 cells/600 μL in a 4-well chamber slide) were fixed in 10% neutral-buffered formalin solution at room temperature for 30 min. Endogenous peroxidase was inactivated by the treatment with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and the cells were further incubated in a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4°C. Then, the TUNEL reaction mixture was added for 60 min at 37°C, followed by labeling with peroxidase-conjugated anti-goat antibody for an additional 30 min. After being stained with diaminobenzidine for 10 min, the cells were rinsed with phosphate-buffered saline (PBS), mounted with 50% glycerol, and examined under a microscope (LABO America Inc., CA, USA).

Measurement of the mitochondrial membrane potential ($\Delta\Psi m$). To measure the mitochondrial transmembrane potential, TMRE was utilized. TMRE rapidly equilibrates between cellular compartments due to potential differences in membrane. After incubation of cells with $A\beta_{25\text{-}35}$ in the presence or absence of TALE, the cells were loaded with TMRE (150 nM) for 30 min and fluorescence was monitored using a fluorometer (SpectraMax Gemini XS, Molecular Device) with excitation at 544 nm and emission at 590 nm.

Western blot analysis. After incubation with $A\beta_{25\text{-}35}$ in the presence or absence of TALE, the cells (2×10^6 cells/2 mL in 60 mm dish) were harvested and cell lysis was carried out at 4°C in RIPA buffer (Novagen, Gibbtown, NJ, USA) for 15 min. The lysates were centrifuged at $12000 \times \mathbf{g}$ for 15 min and the supernatant was collected. The protein concentration was quantified by the bicinchrinic acid (BCA) method provided from Pierce (Rockford, IL, USA). Protein samples mixed with loading buffer were resolved on a 12.5% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 2 h. The membrane was blocked with 5% non-fat dried milk in 0.1% Tween-20 and PBS (PBST) for 1 h at room temperature and further incubated with primary anti-Bcl-2 (1:1000) or anti-Bax (1:1000) antibody solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) made in 3% non-fat dry milk (in PBS) for 3 h at room temperature. Following three washes with PBST, the membrane was treated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies in 3% non-fat dry milk (in PBS) for 1 h at room temperature. After being washed again three times in PBST buffer, the membrane was reacted with ECL substrate solution (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions and visualized using chemiluminescent immunoblotting imaging (97-UV94–11, UVP, Upland, CA, USA).

Measurement of intracellular ROS accumulation. To monitor the net intracellular accumulation of ROS, DCF-DA was used. After incubation with $A\beta_{25\text{-}35}$ for 6 h in the presence or absence of TALE, the cells (4×10^4 cells/300 μL in 48-well plates) were treated with DCF-DA (15 μM) for 15 min at 37°C. Then, the medium was removed and DMSO was added for cell lysis. DCF fluorescence intensity was measured by a microplate reader (TECAN GmbH) with excitation at 485 mm and emission at 535 nm.

Assessment of intracellular reduced glutathione (GSH) levels. The intracellular GSH levels were assessed by an ApoGSH™ glutathione colorimetric detection kit (Biovision Research, CA, USA). The cells were harvested and lysed in an ice-cold glutathione buffer. Cell lysates were incubated on ice for 10 min and 5% sulfosalicylic acid was added. After centrifugation, the reaction solution (NADPH generating mix, glutathione reductase and glutathione reductase reaction buffer) was mixed with supernatant, gently vortexed and incubated for 10 min at room temperature. After addition of substrate solution, the mixtures were kept at room temperature for another 10 min and the absorbance of the clear supernatant was read at 405 nm using a microplate reader (TECAN GmbH).

Animals. Male SD rats, aged 7 weeks and weighing 200–250 g (Hyochang Science, Korea), were housed in a temperature- and light-regulated environment (21 ± 2°C, 12 h light/12 h dark cycle: light period starting at 6 a.m.) with free access to food and water. All experiments were conducted according to the institutional guidelines for the care and use of laboratory animals.

$A\beta_{25\text{-}35}$-induced cognitive dysfunction. The rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the infusion cannulae were inserted into the lateral ventricle (AP, –0.8 mm; ML, 1.5 mm; DV, 3.6 mm). The rats were randomly assigned into three groups: the sham group, the $A\beta_{25\text{-}35}$-treated (15 nmol/day, i.c.v. for 14 days) group, and the co-treatment group with $A\beta_{25\text{-}35}$ and TALE (200 mg/kg/day, p.o. for 14 days after finishing 14-day infusion of $A\beta_{25\text{-}35}$). The rats in the sham group were infused with vehicle control. $A\beta_{25\text{-}35}$ was administered for 14 days, and then TALE was given orally for the subsequent 14 days. As a behavior test for spatial memory, the Morris water maze was performed after the last treatment with TALE for 5 consecutive days.

DOI: 10.1002/ptr

Scopolamine-induced memory impairment. The rats were randomly assigned into three groups: the sham group, the scopolamine-treated (0.5 mg/kg, i.p.) group, and the co-treatment group with TALE (or TDF) and scopolamine. The rats in the sham group were injected with vehicle control. TALE or TDF was dissolved in 0.9% physiological saline and orally administered for 5 days prior to the start of training trials and 30 min before scopolamine injection during training periods. Rats, other than those from the sham group, received scopolamine (0.5 mg/kg, i.p.) 30 min before the training trial of the Morris water maze test to induce memory deficit.

Morris water maze task. The water maze test was conducted in a circular tank (diameter: 180 cm and height: 60 cm) filled with water which was maintained at 21 ± 2°C. The tank was divided into four quadrants with a hidden escape platform (diameter: 12 cm and height: 38 cm). The platform was submerged in the center of one quadrant, 2 cm below the surface of the water. The rats were trained to find the hidden platform based on several cues external to the maze. The position of the cues remained unchanged throughout the experiments. During the training period, three trials were conducted on each day for 5 consecutive days. Each rat was given 90 s to find the hidden platform and then allowed to remain on it for another 30 s. The mean time spent to escape onto the platform during training trials was calculated. If a rat failed to escape from the water within 90 s, it was guided to the platform and stayed there for another 30 s. Immediately after the last training trial, the platform was removed and rats were tested on probe trial for 30 s. The time spent in the target quadrant, where the platform had been located during training trials, was measured (probe test).

Statistical analysis. Values were expressed as mean ± SD or mean ± SEM. Statistical analysis for single comparison was performed by Student's *t*-test. When necessary, multiple comparisons were performed using one-way ANOVA followed by post-hoc Tukey analysis using SPSS software (SPSS 12.0 KO for Windows). The statistical significance was considered to be $p < 0.05$.

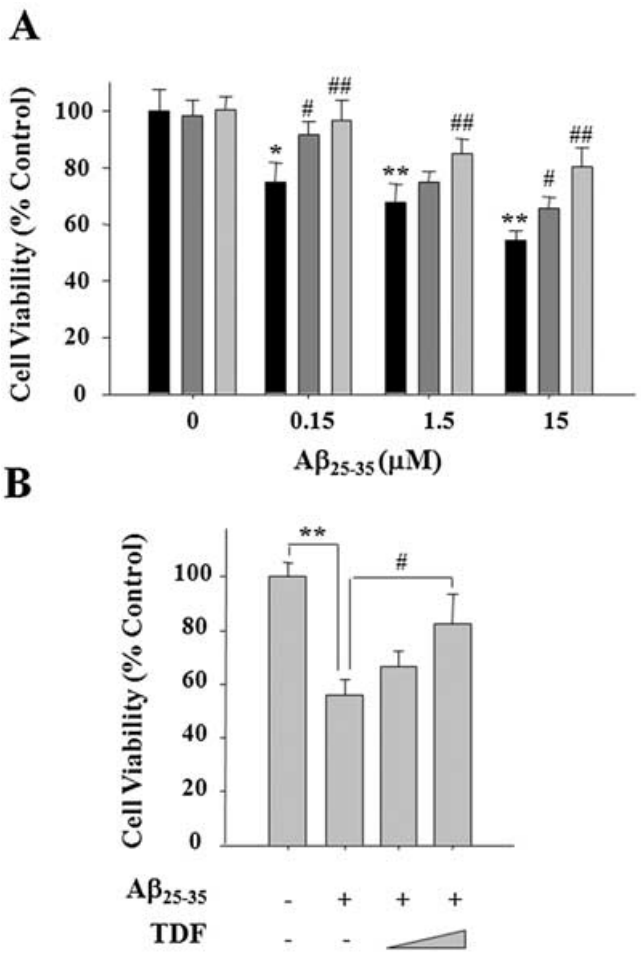

Figure 1. Protective effect of TALE and its active constituent TDF on $A\beta_{25\text{-}35}$-induced cytotoxicity in SH-SY5Y cells. (A) SH-SY5Y cells were treated with indicated concentrations of $A\beta_{25\text{-}35}$ in the absence (■) or presence of 0.5 mg/mL (▩) and 1 mg/mL (□) TALE for 24 h at 37°C. (B) SH-SY5Y cells were treated with $A\beta_{25\text{-}35}$ (10 μM) in the absence or presence of TDF (0.5, 1 mg/mL) for 24 h at 37°C. Cell viability was determined using the MTT reduction assay. TALE or TDF was added to the media 1 h prior to the $A\beta_{25\text{-}35}$ treatment. Data represent the mean ± SD. *$p < 0.05$ and **$p < 0.01$ vs vehicle-treated control group; #$p < 0.05$ and ##$p < 0.01$ compared with the $A\beta_{25\text{-}35}$-treated group.

RESULTS AND DISCUSSION

The neurotoxic activity of Aβ is attributable to the presence of amino acids located in positions 25–35 of the full-length Aβ. After incubation of SH-SY5Y cells with increasing concentrations of $A\beta_{25\text{-}35}$ (0.15, 1.5 and 15 μM) for 24 h in the presence or absence of TALE, $A\beta_{25\text{–}35}$-induced cytoxicity was evaluated by calculating the percentage (%) of MTT reduction (Fig. 1A). $A\beta_{25\text{-}35}$ treatment decreased the cell viability in a concentration-dependent manner, which was attenuated by pretreatment with TALE at concentrations of 0.5 and 1 mg/mL (Fig. 1A). TALE at each of these concentrations alone did not cause any apparent self-cytotoxicity to SH-SY5Y cells.

Epidemiological data indicate that increased consumption of whole grains, including wheat, may reduce the incidence of chronic disease such as cardiovascular disease and certain types of cancer (Smith *et al.*, 2003). Such beneficial effects have been ascribed to the presence of dietary fiber and other phytochemicals, including phenolic acids. In another experiment, in order to verify the neuroprotective constituents of TALE, the effect of total dietary fiber (TDF), one of the ingredients of TALE, against $A\beta_{25\text{-}35}$-induced cytotoxicity was examined. SH-SY5Y cells were exposed to $A\beta_{25\text{-}35}$ (10 μM) in the absence or presence of TDF (0.5, 1 mg/mL), and the cell viability was measured 24 h after incubation. TDF pretreatment effectively inhibited the $A\beta_{25\text{-}35}$-induced decrease in MTT reduction (Fig. 1B).

To further estimate the effect of TALE on $A\beta_{25\text{-}35}$-induced apoptotic cell death, TUNEL staining was conducted which is widely used in detecting DNA fragmentation *in situ*. This histochemical analysis relies on the specific labeling of the 3′-OH end of DNA breaks with modified nucleotides by terminal deoxynucleotidyl transferase. The intensely stained nucleus serves as a marker for fragmented DNA derived from apoptotic cells. Treatment of SH-SY5Y cells with $A\beta_{25\text{-}35}$ significantly increased the number of TUNEL-positively stained cells, which was effectively reduced by 1 mg/mL TALE pretreatment (Fig. 2A). TALE at this concentra-

DOI: 10.1002/ptr

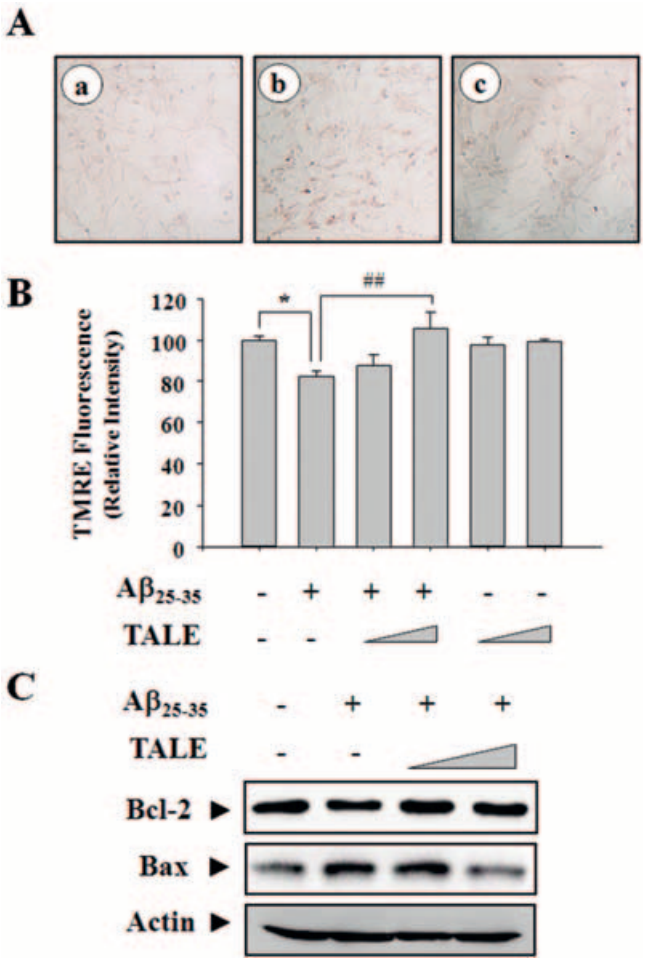

Figure 2. Protective effect of TALE on $A\beta_{25\text{-}35}$-induced apoptotic cell death in SH-SY5Y cells. (A) DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL staining). a, vehicle-treated control; b, SH-SY5Y cells exposed to 15 μM $A\beta_{25\text{-}35}$ alone for 24 h; c, SH-SY5Y cells pretreated with 1 mg/mL TALE for 1 h followed by exposure to $A\beta_{25\text{-}35}$ for additional 24 h. (B) ΔΨm was assessed based on the TMRE fluorescence as described in Materials and Methods. Quantitative analysis of the TMRE fluorescence intensity was conducted after treatment with $A\beta_{25\text{-}35}$ (15 μM) for 24 h in the absence or presence of TALE (0.5, 1 mg/mL). Data represent the mean ± SD. $^{*}p < 0.05$ vs vehicle-treated control group; $^{\#\#}p < 0.01$ compared with $A\beta_{25\text{-}35}$-treated group. (C) SH-SY5Y cells were treated with $A\beta_{25\text{-}35}$ (15 μM) for 24 h after 1 h preincubation with TALE (0.5, 1 mg/mL). Protein samples from cell lysates were resolved by SDS-PAGE and subjected to western blot analysis with Bcl-2 or Bax antibody. Actin levels were measured for the confirmation of equal loading of protein samples.

tion alone did not cause any morphological alterations related to apoptosis (data not shown).

During the apoptotic process, mitochondrial transmembrane potential (ΔΨm) decreases and this change facilitates the release of signaling molecules from mitochondria, which manifest the apoptotic cell death. The dissipation of ΔΨm and permeability changes were observed in cells exposed to $A\beta_{25\text{-}35}$ alone (15 μM) as shown by the statistically significant decrease in TMRE fluoresence (Fig. 2B). The $A\beta_{25\text{-}35}$-induced reduction in ΔΨm was blocked by the pretreatment with 1 mg/mL TALE (Fig. 2B). As another representative marker for apoptotic cell death, the levels of Bcl-2 family proteins were compared. Among the Bcl-2 family proteins, the relative ratio of proapoptotic Bax and the antiapoptotic Bcl-2 plays an important role in determining cell survival and death. As shown in Fig. 2C, $A\beta_{25\text{-}35}$ (15 μM) treatment increased the expression of proapoptotic Bax with a simultaneous decrease in the level of antiapoptotic Bcl-2 (Fig. 2C). However, TALE (1 mg/mL) pretreatment substantially reduced the elevated ratio of Bax to Bcl-2 (Fig. 2C).

Among a wide array of mediators explaining neuronal cell death, oxidative stress caused by increased intracellular accumulation of ROS has been implicated in the pathophysiology of diverse neurodegenerative disorders, such as AD (Markesbery, 1997). Consistent with this notion, Aβ associated with senile plaques formed in the brains of patients with AD, was found to induce apoptosis in cultured neurons and glia via the generation of ROS (Vila and Przedborski, 2003; Davis, 1996; Qin *et al.*, 2002). Aβ-produced free radical mediated neurotoxic effects and a number of antioxidants have been shown to protect cells from Aβ-induced toxicity (Xiao *et al.*, 2002; Jang and Surh, 2003).

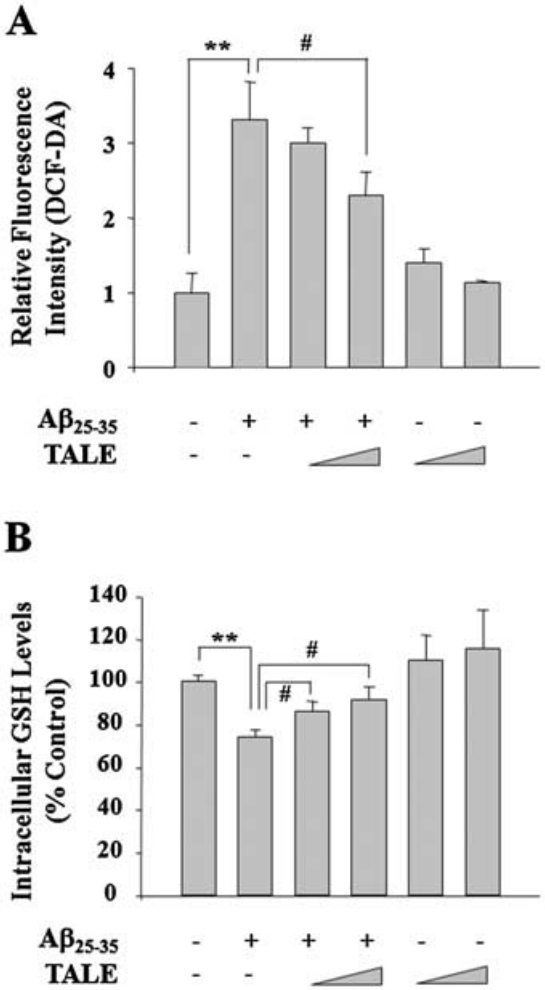

Figure 3. Protective effect of TALE on $A\beta_{25\text{-}35}$-induced oxidative damage. SH-SY5Y cells were exposed to $A\beta_{25\text{-}35}$ (15 μM) in the absence or presence of TALE (0.5, 1 mg/mL). TALE was added to the media 1 h prior to the $A\beta_{25\text{-}35}$ treatment. (A) Intracellular peroxide levels were determined based on the DCF fluorescence as described in Materials and Methods. Data are expressed as relative fluorescence intensity compared with the vehicle-treated control group. (B) Intracellular GSH levels were determined using a ApoGSH™ glutathione colorimetric detection kit as described in Materials and Method. Data are expressed as the % decrease in GSH levels compared with the vehicle-treated control group. Data represent the mean ± SD. $^{**}p < 0.01$ vs vehicle-treated control group; $^{\#}p < 0.05$ vs $A\beta_{25\text{-}35}$-treated group.

DOI: 10.1002/ptr

In the present study, to elucidate the possible neuroprotective mechanisms of TALE, intracellular accumulation of ROS was monitored by DCF-DA staining. DCF-DA is a non-fluorescent dye permeable to the cell membrane. However, the compound is hydrolysed by cellular esterase to DCF which can interact with peroxides forming fluorescent 2′,7′-dichlorofluorescein. SH-SY5Y cells treated with 15 μM $A\beta_{25\text{-}35}$ displayed intense fluorescence after staining with DCF-DA dye (Fig. 3A). Intracellular ROS accumulation resulting from $A\beta_{25\text{-}35}$ treatment was significantly decreased by TALE (1 mg/mL) pretreatment (Fig. 3A). This concentration alone did not cause any change in the fluorescence intensity compared with vehicle-treated control cells.

Wheat, including TALE, is a rich source of vitamins, antioxidants and minerals. It has been reported to exhibit an antimutagenic property against benzo[*a*] pyrene-induced mutagenicity and antioxidant activity by scavenging superoxide anion (Peryt *et al.*, 1992). The antioxidant potential of wheatgrass was verified at different levels through FRAP (ferric reducing antioxidant power), ABTS (2,2′-azobis-3-ethylbenzthiazoline-6-sulfonic acid) and DPPH (1,1'-diphenyl-2-picrylhydrazyl) assays (Kulkarni *et al.*, 2006). TALE was reported to significantly inhibit ascorbate-Fe^{2+}-induced lipid peroxidation, a representative marker for oxidative stress, in rat liver mitochondria (Kulkarni *et al.*, 2006). Significant radical scavenging and metal chelating activities detected in the three hard winter wheat varieties are correlated with high contents of phenolic acids, tocopherols and carotenoids (Yu *et al.*, 2002). The antioxidative potential of hard red winter wheat bran was also explained on the basis of the total phoenolic content, total antioxidant activity and free radicals scavenging and metal chelating capacity (Zhou *et al.*, 2005).

Based on the oxidative hypothesis in AD, it is important to maintain a balance between antioxidants and oxidants in living organisms, and increased intake of dietary antioxidants may help in maintaining an adequate antioxidant status. GSH, a sulphydryl compound, has detoxifying and antioxidative effects against oxidative stress and plays an important role in maintaining the intracellular redox status. In support of this notion, intracellular GSH was substantially depleted at 24 h after $A\beta_{25\text{-}35}$ treatment (15 μM), which was significantly restored after the pretreatment with TALE (0.5 and 1 mg/mL) compared with the basal GSH levels in the vehicle-treated control cells (Fig. 3B). GSH has been shown to inhibit or retard apoptosis triggered by many different stimuli including oxidants (Bains and Shaw, 1997). Aβ treatment significantly decreased GSH in both neurons and astrocytes, while NAC pretreatment has been shown to exhibit protective effects against Aβ-induced oxidative cell death (Olivieri *et al.*, 2001).

In another experiment, to further investigate the memory enhancing effects of TALE, $A\beta_{25\text{-}35}$ was intracerebroventrically injected into the lateral ventricle of SD rats as an *in vivo* model system for AD. The Morris water maze test was performed to monitor and compare the spatial memory. The $A\beta_{25\text{-}35}$-infused group exhibited significantly impaired learning and memory and took a longer time to find the hidden platform during 5 training days (Fig. 4A). However, oral administration of TALE

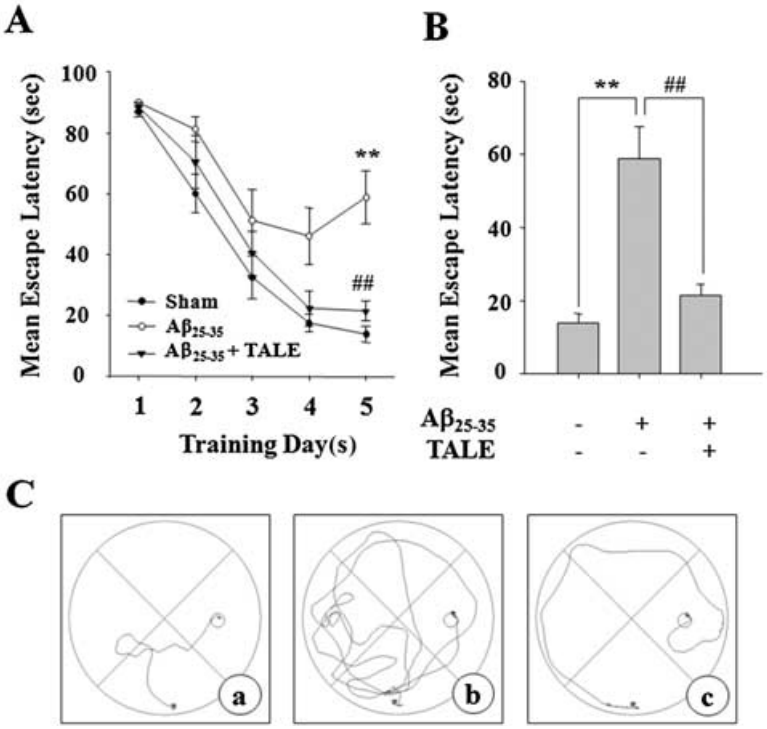

Figure 4. Effect of TALE on the learning and memory impairments induced by continuous i.c.v. infusion of $A\beta_{25\text{-}35}$ in SD rats. (A) Mean escape latencies during training trials of the Morris water maze task are represented. The rats were randomly assigned into three groups ($n = 6$): the sham control group, the $A\beta_{25\text{-}35}$-treated (15 nmol/day, i.c.v.) group, and the co-treatment group with TALE (200 mg/kg/day, p.o.) and $A\beta_{25\text{-}35}$ (15 nmol/day, i.c.v.). The rats in sham group were infused with vehicle control. $A\beta_{25\text{-}35}$ was administrated for 14 days, and then TALE was given for subsequent 14 days. (B) Mean escape latencies of each group to find the hidden platform on training day 5 are represented. Data represent the mean ± SEM. $^{**}p < 0.01$ vs sham group; $^{\#\#}p < 0.01$ compared with $A\beta_{25\text{-}35}$-injected group. (C) The typical swimming-tracking paths of each group in Morris water maze task on training day 5. a, vehicle-treated control; b, $A\beta_{25\text{-}35}$ treatment alone (15 nmol/day, i.c.v.); c, $A\beta_{25\text{-}35}$ (15 nmol/day, i.c.v.) + TALE (200 mg/kg/day, p.o.).

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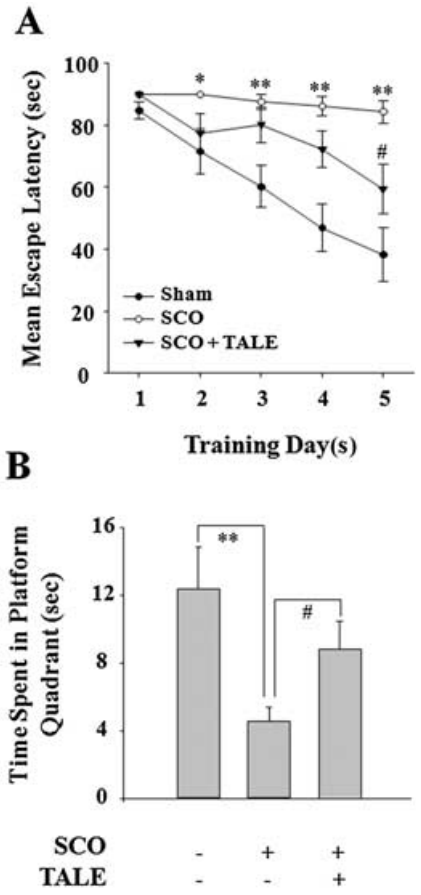

Figure 5. Effect of TALE on the learning and memory deficits caused by scopolamine in SD rats. Mean escape latencies during training trials (A) and time spent in the platform quadrant during probe trial (B) of the Morris water maze task are represented. The rats were randomly assigned into three groups (n = 9): the sham control group, the scopolamine-treated (SCO : 0.5 mg/kg, i.p.) group, and the co-treatment group (SCO + TALE) with TALE (200 mg/kg, p.o.) and scopolamine (0.5 mg/kg, i.p.). Data represent the mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs sham group; $^{\#}p < 0.05$ compared with scopolamine-injected group.

(200 mg/kg/day, for 14 days) effectively reduced the mean escape latency, thereby ameliorating the learning and memory deficits caused by continuous infusion of $A\beta_{25\text{-}35}$ (Fig. 4A). The mean escape latencies and representative swimming-tracking paths of each group on day 5 are shown in Fig. 4B and Fig. 4C, respectively.

As the learning process involves activation by neurotransmitters such as acetylcholine (Winkler *et al.*, 1995; Decker and McGaugh, 1991), the cholinergic system has been reported to play an important role in learning and memory. Cholinomimetic drugs have been reported to enhance memory, whereas centrally acting cholinergic antagonists are reported to impair memory. Therefore, *in vivo* experimental animal models with decreased cholinergic function have been used widely to study the antiamnestic potential of new drugs (AchCuadra *et al.*, 1994). A muscarinic antagonist, scopolamine, blocks the post-synaptic muscarinic M1 transmission and this leads to disruption of the hippocampal functions and produces deficits in the processes of learning acquisition and short-term memory which are considered as characteristics of AD (AchCuadra *et al.*, 1994). To investigate the effect of TALE treatment on scopolamine-induced learning and memory impairments, the Morris water maze task was conducted and the mean escape latency to find the hidden platform during consecutive training days was calculated in each group. The rats in the sham control group rapidly learned the location of the submerged platform which was reached within 40 s by day 5 of the training period (Fig. 5A). In contrast, the performance in the scopolamine-injected group was significantly impaired compared with that in the vehicle-injected control group. Repeated daily administration of TALE at dose of 200 mg/kg/day reduced the mean escape latencies through the training period (Fig. 5A). To further verify the spatial memory of rats, the time spent in the platform quadrant during the probe trial was measured after the last training trial on day 5. The time spent in the target quadrant to search for the removed platform reflects retention of learning and memory. The sham and TALE-treated groups spent more time in the platform quadrant than the scopolamine alone-treated rats (Fig. 5B).

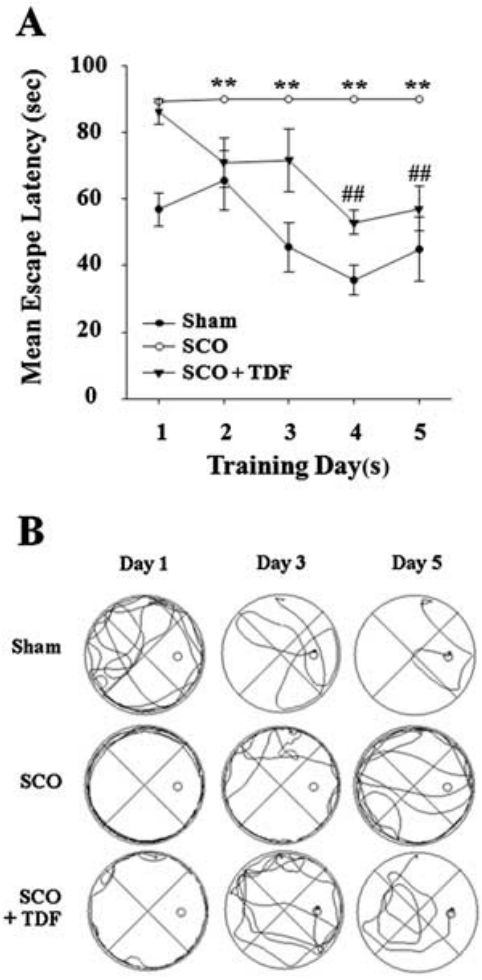

Figure 6. Effect of TDF on the cognitive dysfunction induced by scopolamine in SD rats. The rats were randomly assigned into three groups: the sham group, the scopolamine-treated (SCO : 0.5 mg/kg, i.p.) group, and the co-treatment group (SCO + TDF) with TDF (50 mg/kg, p.o.) and scopolamine (0.5 mg/kg, i.p.). (A) Mean escape latencies during training trials of the Morris water maze task are represented. Data represent the mean ± SEM. $^{**}p < 0.01$ vs sham group; $^{\#\#}p < 0.01$ compared with scopolamine-injected group. (B) The typical swimming-tracking paths of each group in Morris water maze task on training days 1, 3 and 5 are represented.

DOI: 10.1002/ptr

In another experiment, oral administration of TDF, an active component of TALE, significantly ameliorated scopolamine-decreased spatial memory as assessed by the water maze task. The changes in the mean escape latency to find the submerged platform during training trials of the Morris water maze task are shown in Fig. 6A. The performance in the scopolamine-injected group was significantly deteriorated compared with that in the vehicle control group (Fig. 6A). However, the scopolamine-prolonged mean escape latency, an indicative of deteriorated memory, was effectively reduced by repeated daily administration of TDF at a dosage of 50 mg/kg/day (Fig. 6A). The representative swimming-tracking paths of each group on days 1, 3, 5 of training trials are shown in Fig. 6B.

In summary, $A\beta_{25\text{-}35}$ caused cytotoxicity and apoptosis in SH-SY5Y cells through intracellular accumulation of ROS and induction of oxidative stress, which could be protected by TALE pretreatment. In addition, TALE markedly improved cognitive dysfunction caused by Aβ or scopolamine injection. These results suggest that TALE may have preventive and/or therapeutic potential in the management of AD. The elucidation of intracellular signaling cascades mediating Aβ-induced neurological damage and their regulation by TALE or its active constituents may provide the molecular basis for neuroprotection of numerous phytochemicals.

Acknowledgements

This work was supported by a grant (PF06221–00) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government (J. W. Lee, J. H. Jang and H. S. Han) and a grant from the Regional Innovation Center Program of the Ministry of Commerce, Industry and Energy through the Research Center for Biomedical Resources of Oriental Medicine at Daegu Haany University (J. W. Lee, J. H. Jang and C. H. Yang).

REFERENCES

AchCuadra G, Summers K, Giacobini E. 1994. Cholinesterase inhibitor effects on neurotransmitters in rat cortex *in vivo*. *J Pharmacol Exp Ther* **270**: 277–284.

Aruoma OI, Bahorun T, Jen LS. 2003. Neuroprotection by bioactive components in medicinal and food plant extracts. *Mutat Res* **544**: 203–215.

Bains JS, Shaw CA. 1997. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Rev* **25**: 335–358.

Bartus RT, Dean RL 3rd, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**: 408–414.

Behl C, Davis JB, Lesley R, Schubert D. 1994. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* **77**: 817–827.

Ben-Arye E, Goldin E, Wengrower D, Stamper A, Kohn R, Berry E. 2002. Wheat grass juice in the treatment of active distal ulcerative colitis: a randomized double-blind placebo-controlled trial. *Scand J Gastroenterol* **37**: 444–449.

Bierer LM, Haroutunian V, Gabriel S *et al*. 1995. Neurochemical correlates of dementia severity in Alzheimer's disease: relative importance of the cholinergic deficits. *J Neurochem* **64**: 749–760.

Boros LG, Nichelatti M, Shoenfeld Y. 2005. Fermented wheat germ extract (Avemar) in the treatment of cancer and autoimmune diseases. *Ann N Y Acad Sci* **1051**: 529–542.

Carmody RJ, Cotter TG. 2001. Signalling apoptosis: a radical approach. *Redox Rep* **6**: 77–90.

Citron M. 2002. Alzheimer's disease: treatments in discovery and development. *Nat Neurosci* **5**: 1055–1057.

Citron M. 2004. Strategies for disease modification in Alzheimer's disease. *Nat Rev Neurosci* **5**: 677–685.

Choi Y, Kim HS, Shin KY *et al*. 2007. Minocycline attenuates neuronal cell death and improves cognitive impairment in Alzheimer's disease models. *Neuropsychopharmacology* **32**: 2393–2404.

Davis JB. 1996. Oxidative mechanisms in beta-amyloid cytotoxicity. *Neurodegeneration* **5**: 441–444.

Decker MW, McGaugh JL. 1991. The role of interactions between the cholinergic system and other neuromodulatory systems in learning and memory. *Synapse* **7**: 151–168.

Deschamps V, Barberger-Gateau P, Peuchant E, Orgogozo JM. 2001. Nutritional factors in cerebral aging and dementia: epidemiological arguments for a role of oxidative stress. *Neuroepidemiology* **20**: 7–15.

Florent-Béchard S, Malaplate-Armand C, Koziel V *et al*. 2007. Towards a nutritional approach for prevention of Alzheimer's disease: biochemical and cellular aspects. *J Neurol Sci* **262**: 27–36.

Han HS, Choi JS, Kim YJ *et al*. 2008. Protective effect of *Triticum aestivum* L. and its component, starch, in rat focal cerebral ischemia. *Curr Top Neutraceut Res* **6**: 47–54.

Jang JH, Aruoma OI, Jen LS, Chung HY, Surh YJ. 2004. Ergothioneine rescues PC12 cells from beta-amyloid-induced apoptotic death. *Free Radic Biol Med* **36**: 288–299.

Jang JH, Surh YJ. 2003. Protective effect of resveratrol on β-amyloid-induced oxidative PC12 cell death. *Free Radic Biol Med* **34**: 1100–1110.

Kulkarni SD, Tilak JC, Acharya R, Rajurkar NS, Devasagayam TP, Reddy AV. 2006. Evaluation of the antioxidant activity of wheatgrass (*Triticum aestivum* L.) as a function of growth under different conditions. *Phytother Res* **20**: 218–227.

Lee J, Kim KW, Lee JK, Lee SJ. 2004. Therapeutic agent for ischemia which inhibits apoptosis under ischemic condition. *US Patent*, No. 6716822.

Lee SC, Prosky L, DeVries JW. 1992. Determination of total, soluble, and insoluble, dietary fiber in foods – enzymatic-gravimetric method, MES-TRIS buffer: Collaborative study. *J Assoc Off Anal Chem* **75**: 395–416.

Marawaha RK, Bansal D, Kaur S, Trehan A. 2004. Wheat grass juice reduces transfusion requirement in patients with thalassemia major: a pilot study. *Indian Pediatr* **41**: 716–720.

Markesbery WR. 1997. Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med* **23**: 134–147.

Nitta A, Itoh A, Hasegawa T, Nabeshima T. 1994. beta-Amyloid protein-induced Alzheimer's disease animal model. *Neurosci Lett* **170**: 63–66.

Olivieri G, Baysang G, Meier F *et al*. 2001. *N*-acetyl-L-cysteine protects SHSY5Y neuroblastoma cells from oxidative stress and cell cytotoxicity: effects on beta-amyloid secretion and tau phosphorylation. *J Neurochem* **76**: 224–233.

Park CH, Lee J, Jung HY *et al*. 2007. Identification, biological activity, and mechanism of the anti-ischemic quinolone analog. *Bioorgan Med Chem* **15**: 6517–6526.

Peryt B, Szymczyk T, Lesca P. 1992. Mechanism of antimutagenicity of wheat sprout extracts. *Mutat Res* **269**: 201–215.

Qin L, Liu Y, Cooper C, Liu B, Wilson B, Hong JS. 2002. Microglia enhance beta-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. *J Neurochem* **83**: 973–983.

Sano M, Ernesto C, Klauber MR *et al*. 1996. Rationale and design of a multicenter study of selegiline and alpha-tocopherol in the treatment of Alzheimer disease using novel clinical outcomes. *Alzheimer Dis Assoc Disord* **10**: 132–140.

Smith AT, Kuznesof S, Richardson DP, Seal CJ. 2003. Behavioural, attitudinal and dietary responses to the consumption of wholegrain foods. *Proc Nutr Soc* **62**: 455–467.

Terry AV Jr, Buccafusco JJ. 2003. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. *J Pharmacol Exp Ther* **306**: 821–827.

Vila M, Przedborski S. 2003. Targeting programmed cell death in neurodegenerative diseases. *Nat Rev Neurosci* **4**: 365–375.

Winkler J, Suhr ST, Gage FH, Thal LJ, Fisher LJ. 1995. Essential role of neocortical acetylcholine in spatial memory. *Nature* **375**: 484–487.

Xiao XQ, Zhang HY, Tang XC. 2002. Huperzine A attenuates amyloid beta-peptide fragment 25–35-induced apoptosis in rat cortical neurons via inhibiting reactive oxygen species formation and caspase-3 activation. *J Neurosci Res* **67**: 30–36.

Youdim KA, Joseph JA. 2001. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. *Free Radic Biol Med* **30**: 583–594.

Yrjanheikki J, Tikka T, Keinanen R, Goldstein G, Chan PH, Koistinaho J. 1999. A tetracycline derivative, minocycline, reduces inflammation and protects against focal cerebral ischemia with a wide therapeutic window. *Proc Natl Acad Sci USA* **96**: 13496–13500.

Yu L, Haley S, Perret J, Harris M, Wilson J, Qian M. 2002. Free radical scavenging properties of wheat extracts. *J Agric Food Chem* **50**: 1619–1624.

Zhou K, Laux JJ, Yu L. 2004. Comparison of Swiss red wheat grain and fractions. *J Agric Food Chem* **52**: 1118–1123.

Zhou K, Yin JJ, Yu LL. 2005. Phenolic acid, tocopherol and carotenoid compositions, and antioxidant functions of hard red winter wheat bran. *J Agric Food Chem* **53**: 3916–3922.