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Poria cocos water extract (PCW) protects PC12 neuronal cells from beta-amyloid-induced cell death through antioxidant and antiapoptotic functions

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Received April 22, 2009, accepted May 29, 2009

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Pharmazie 64: 760–764 (2009) *doi: 10.1691/ph.2009.9614*

Beta-amyloid (Abeta)-induced neurotoxicity is considered to be mediated through the formation of reactive oxygen species (ROS). In this study, the protective effects of *Poria cocos* water extract (PCW) against Abeta1-42-induced cell death were investigated using rat pheochromocytoma (PC12) cells. Exposure of PC12 cells to the Abeta1-42 (20 μM) for 48 h resulted in neuronal cell death, whereas pretreatment with PCW at the concentration range of 5-125 μg/ml reduced Abeta1-42-induced cell death. In addition, PC12 cells treated with Abeta1-42 exhibited increased accumulation of intracellular oxidative damages and underwent apoptotic death as determined by characteristic morphological alterations and positive *in situ* terminal end-labeling (TUNEL staining). However, PCW attenuated Abeta1-42-induced cytotoxicity, apoptotic features, and accumulation of intracellular oxidative damage. Moreover, PCW (5 to 125 μg/ml) decreased expression of apoptotic protein Bax and activity of caspase-3, but enhanced expression of anti-apoptotic protein Bcl-2. These results suggest that PCW may protect cells through suppressing the oxidative stress and the apoptosis induced by Abeta1-42, implying that PCW may be potential natural agents for Alzheimer's diseases.

1. Introduction

Beta-amyloid (Abeta) is the major component of amyloid deposits found in the plaques characterizing Alzheimer's disease (AD). The Abeta peptides having 40–42 amino acids are formed through the processing of amyloid precursor peptide (APP) by α-, β-, and γ-secretase (Selkoe 1994; Checler 1995). Abeta appears to promote the generation of oxidative stress (Keller et al. 1997). Abeta1-40, Abeta1-42 or Abeta25-35, the truncated Abeta fragments exhibit toxicity to neurons *in vitro* and *in vivo* (Varadarajan et al. 2000). Intracellular accumulation of Abeta-induced H_2O_2 may lead to the peroxidation of membrane lipids and finally to cell death (Lyras et al. 1997). It is believed that the generated reactive oxygen species (ROS) and oxidative damage may be involved in the pathogenesis of neurodegenerative disorders. *In vitro* and *in vivo*, several antioxidants and free radical scavengers, have been shown to be neuroprotective against Abeta-induced toxicity (Heo et al. 2004; Wang et al. 2001). Since antioxidative activity may play an important role in inhibiting the progression of Alzheimer's disease, less toxic and more effective antioxidant substances among natural products had been screened in this study. *Poria cocos* has been used for its sedative, diuretic and tonic effects in traditional medicine for several hundred years (Tai et al. 1995). Recently, *Poria cocos* has been reported to exhibit a removable scavenging activity of free radicals (Schinella et al. 2002; Lin et al. 2008). Considering the several activities of *Poria cocos,* we investigated whether PCW could exhibit inhibitory effects on the Abeta-induced neuronal cell death through its antioxidant property. We have evaluated the inhibitory effect of PCW on Abeta-induced oxidative damage in PC12 cell lines derived from a pheochromocytoma of the rat adrenal medulla. We then examined the inhibitory effect of PCW on neuronal cell death and changes of apoptosis machinery such as Bax, Bcl-2 and caspase-3.

2. Investigations, results and discussion

2.1. PCW attenuated Abeta1-42-induced cell death

Changes in cell morphology were assessed by light microscopic examination. PC12 cells treated for 48 h with Abeta1-42 alone at 20 μM, exhibited morphological alterations such as cell shrinkage and membrane blebbings that are nor-

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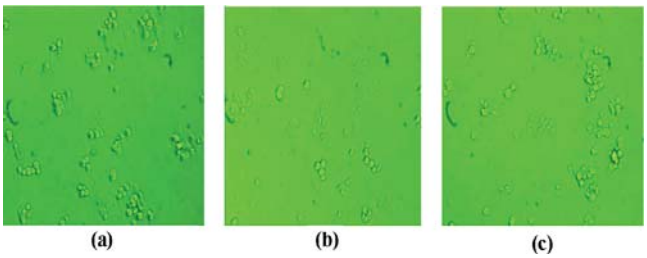

Fig. 1: Microscopic analysis of PCW on Abeta1-42-induced cell death. Representative phase-contrast photographs of PC12 cells after Abeta1-42 treatment in the absence or presence of PCW: (a) no treatment; (b) PC12 cells exposed to 20 μM Abeta1-42 for 48 h; and, (c) PC12 cells pretreated with 5 μg/ml PCW for 2 h followed by exposure to 20 μM Abeta1-42 for an additional 48 h

mally associated with the occurrence of apoptotic cell death (Wyllie et al. 1980). Compared to the normal PC12 cells (Fig. 1a), cells treated with Abeta1-42 alone treatment became round up, detached from the bottom, and aggregated as assessed by phase-contrast microscopy (Fig. 1b). However, PCW pretreatment mitigated such morphological features of damaged cells (Fig. 1c).

2.2. PCW protected PC12 cells against Abeta1-42-induced cytotoxicity

The Ab1-42 cytoxicity was evaluated by determining the percentage of WST-1 reduction after incubation of PC12 cells for 48 h with increasing concentrations of Ab1-42 (data not shown). EC_{50} of Abeta1-42 was 20 μM in PC12 cells. Cytotoxic effects of Ab1-42 were attenuated dose-dependently and significantly in the presence of 5 to 125 μg/ml of PCW (Fig. 2). PCW alone at each of these concentrations did not cause any apparent cytotoxicity (data not shown).

2.3. Protection from Ab1-42 toxicity by PCW

When exposed to 20 μM Ab1-42 alone for 48 h, PC12 cells increased LDH release, indicative of neuronal toxicity to 168 ± 0.5% over the vehicle-treated controls. Treatments with PCW at 5 to 125 μg/ml concentrations reduced in a dose-dependent manner LDH release against Ab1-42-induced toxicity (156 ± 4.3%, 137 ± 6.8% and 140 ± 7.5%, respectively) (Fig. 3). Protective effects of PCW were proportional to its concentrations at low levels.

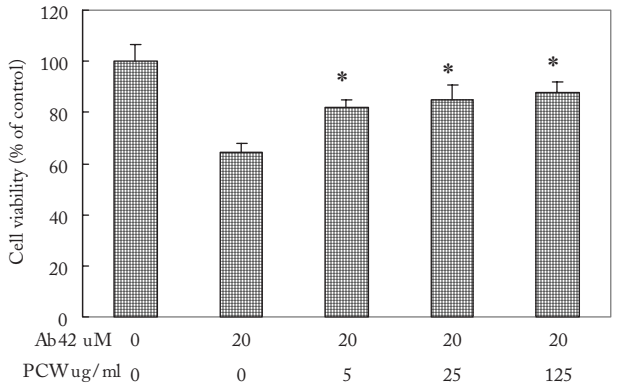

Fig. 2: Protective effect of PCW on Ab1-42-induced cytotoxicity in PC12 cells. PC12 cells were treated with indicated concentrations of Ab1-42 in the absence or presence of 5 to 125 μg/ml of PCW for 48 h at 37 °C. Viable cells were determined using the WST-1 reduction assay. PCW was added to the media 2 h prior to the Ab1-42 treatment. Values are means ± SD ($n = 3$). *Significantly different from the viability of cells treated with Ab1-42 alone ($p < 0.05$)

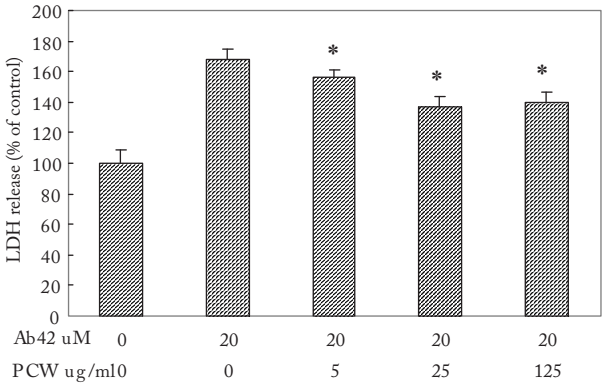

Fig. 3: Protective effect of PCW in PC12 cells against neuronal damage induced by Ab1-42. PC12 cells in culture were treated with 20 uM Ab1-42 in the absence or presence of various concentrations of PCW for 48 h. The absorbance of non-treated cells (control) was regarded as 100%. Results are expressed as means ± SD (n = 3). * indicates significant difference compared with 20 μM Ab1-42 alone treated group, *$P < 0.05$

2.4. PCW suppressed Ab1-42-induced intracellular oxidative damage

Polyunsaturated fatty acids in membrane lipids are prone to be attacked by reactive oxygen species (ROS), and the resulting lipid peroxides can cause injury and cell death. Generation of cellular lipid peroxides produced by Abeta1-42 treatment was assessed by measurement of fluorescence of malondialdehyde-modified protein adducts. Malondialdehyde, one of lipid peroxides, can react and modify intracellular proteins. To determine whether PCW may attenuate cell death through reducing the effect on the oxidative damage, the fluorescence of intracellular malondialdehyde-modified protein adducts was measured. Treatment of PC12 cells with 20 μM Ab1-42 alone increased intracellular malondialdehyde-modified protein adducts but PCW significantly reduced generation of Ab1-42-induced intracellular malondialdehyde-modified protein adducts (190 ± 11.9 % vs. 104.8 ± 4.5 % (PCW alone) and 114.3 ± 7.7 %) (Fig. 4).

2.5. PCW reduced Abeta1-42-induced apoptotic cell death

The presence of apoptotic cells was further confirmed by terminal end labeling (TUNEL staining), which is widely used in detecting DNA fragmentation *in situ*. In this histochemical technique, the appearance of intensely stained nucleus is indicative of terminal incorporation of labeled dUTP into the 3′-end of fragmented DNA derived from apoptotic nuclei. Treatment

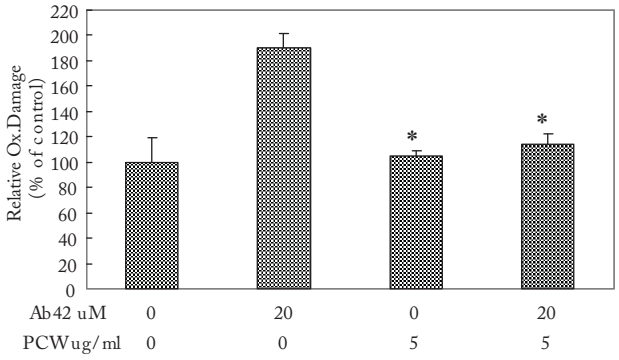

Fig. 4: Protective effect of PCW on Ab1-42-induced intracellular oxidative damage. Intracellular oxidative damage levels were determined by the measurement of fluorescence of malondialdehyde-modified protein adducts. PC12 cells were cotreated by 5 μg/ml PCW with 20 μM Ab1-42 for 48 h. Values are means ± SD from three experiments with duplicates. (*) Significantly different from Ab1-42 alone treated group ($p < 0.05$)

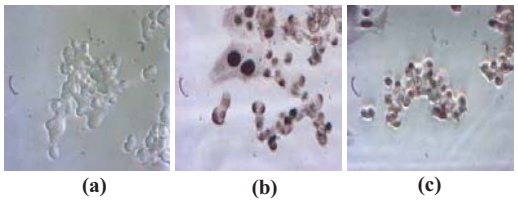

Fig. 5: Microscopic analysis of protective effect of PCW on Abeta1-42-induced cell death. DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (lower panels): (a) no treatment; (b) PC12 cells exposed to 20 μM Abeta1-42 for 48 h; and, (c) PC12 cells pretreated with 5 μg/ml PCW for 2 h followed by exposure to 20 μM Abeta1-42 for an additional 48 h

with 20 μM Abeta1-42 alone greatly increased the proportion of TUNEL-positive cells, which was reduced by 5 μg/ml PCW treatment (Fig. 5).

2.6. PCW inhibited Abeta1-42-induced caspase-3 activation of PC12 cells

The activation of caspases is now well accepted to be responsible for apoptotic cell death (Imawati et al. 1999). In addition, caspase-3 activation due to Abeta-induced cell injury in neurons has been reported (Famer et al. 2004). In particular, caspase-3 is a major activator of apoptotic signals. We therefore evaluated the Abeta1-42 toxicity with a caspase-3 inhibitor of Ac-DEVD-AMC. Caspase-3 activities at 48 h after PCW administration were similar to those in the control (data not shown). Caspase-3 activity in the treatment of 20 μM Abeta1-42 alone significantly increased compared with that of the control (544.6 ± 35.0 % vs. 100.0 ± 21.7 %). However, in case of pretreatment of 5 μg/ml PCW, caspase-3 activity significantly decreased compared with that in Abeta1-42 alone treatment (446.0 ± 29.9 % vs. 544.6 ± 35.0 %) (Fig. 6).

2.7. PCW down-regulates the expression of Bax with a concomitant up-regulation of Bcl-2 in PC12 cells in vitro

The Bcl-2 protein family plays a pivotal role in the regulation of apoptosis, and their major site of action is on the regulation of Cyt c release from mitochondria into the cytosol. The present results showed that the level of pro-apoptotic protein Bax in PC12 cells was significantly down-regulated by treatment with PCW at 5 μg/ml compared with the Abeta1-42 alone

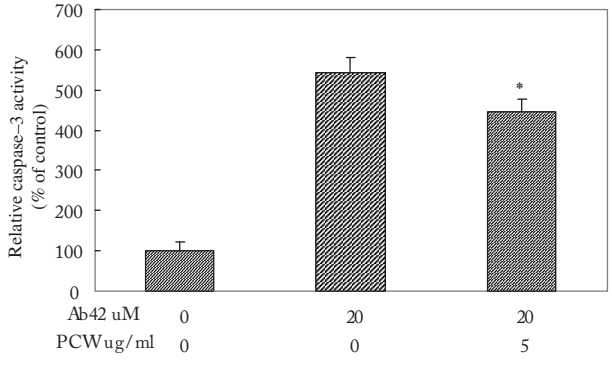

Fig. 6: Prevention of Abeta1-42-induced caspase-3 activation by PCW in PC12 cells. PC12 Cells were preincubated with PCW for 2 h and additionally treated with Abeta1-42 for 48 h. PC12 Cells were lysed with lysis buffer and 30 μg of protein for each sample was incubated with the fluorogenic substarate, Ac-DEVE-MCA (20 μM). Cleavage of the substrate emitted a fluorescent signal that was measured using a fluorometer (Excitation at 380 nm, Emission at 460 nm). Values represent the mean ± SD ($n = 3$) of caspase-3 activity as % of control. * $p < 0.05$ compared with Abeta1-42 alone treated group

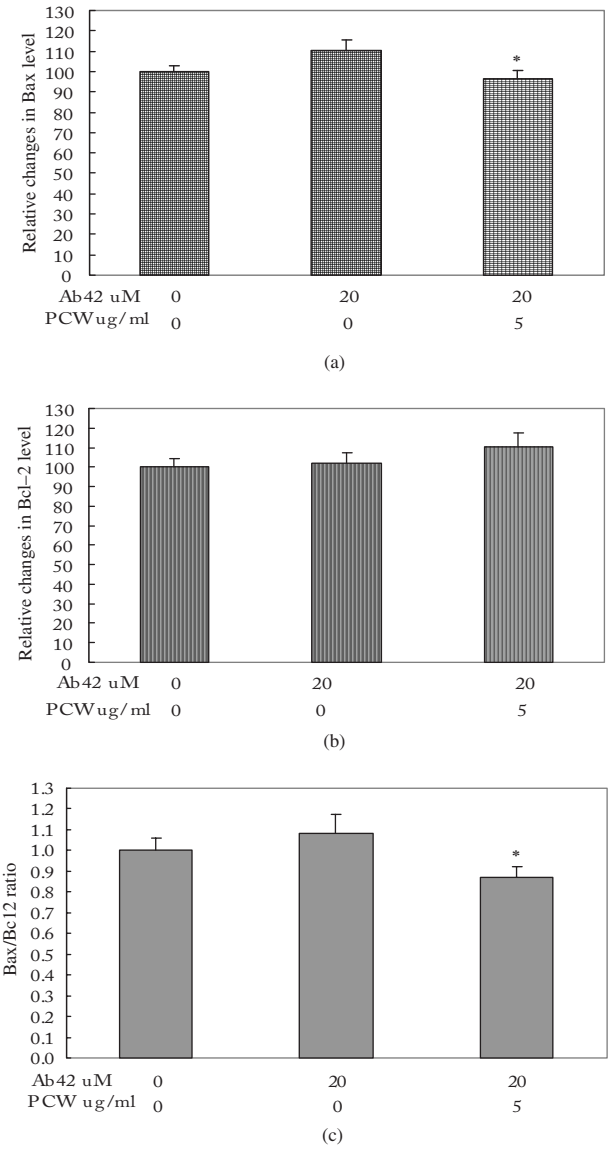

Fig. 7: Effects of the PCW on expression of (a) Bax and (b) Bcl-2 protein, and (c) Bax/Bcl-2 ratio in PC12 cells. Cells were incubated for 48 h with culture medium alone, 20 μM Abeta1-42, and 20 μM Abeta1-42 at 2 h after 5 μg/ml PCW pretreatment in culture medium. The levels of Bax and Bcl-2 protein were measured by Bax ELISA and Bcl-2 ELISA kits, respectively. Results were expressed as the mean ± SD in duplicate experiments. *$p < 0.05$ compared with Abeta1-42 alone treated group

treatment at 20 μM (96.2 ± 4.3 % vs. 110.2 ± 5.2 %) (Fig. 7a). In contrast, the level of anti-apoptotic protein Bcl-2 was not increased significantly by the PCW treatment compared with the Abeta1-42 alone treatment (101.8 ± 5.2 % vs. 110.3 ± 6.9 %) (Fig. 7b). It was notable that the ratios of Bax/Bcl-2 protein level were amplified with both the down-regulation of Bax protein and the up-regulation of Bcl-2 protein. The ratio of Bax/Bcl-2 protein level was reduced significantly by the PCW treatment compared with the Abeta1-42 alone treatment (0.87 ± 0.05 vs. 1.08 ± 0.09) (Fig. 7c). Therefore, PCW treatment at 5 μg/ml influenced on the suppression of the apoptosis pathway of Abeta1-42-induced cell death.

3. Discussion

Many studies investigated the relationship between oxidative stress and apoptosis in AD. Accumulation of Abeta-induced ROS (reactive oxygen species) causes damage to neuronal membrane lipids, proteins and nucleic acids, which ultimately

leads to apoptosis (Rupniewska and Bojarska-Junak 2004). For this reason, oxidative damages are believed to play a critical role in cell loss during progression of AD (Varadarajan et al. 2000; Domenico 2005). Hence, for prevention or attenuation of AD development or progression, numerous antioxidant materials have been demonstrated high efficacy (Grundman et al. 2002; Patel et al. 2008). Recently, several studies reported that antioxidant properties of *Poria cocos* play a most important role for scavenging free radicals (Schinella et al. 2002; Nobuyasu et al. 2003). This study has estimated whether PCW prevented Abeta1-42-induced cell death of PC12 cells accompanied by the suppression of Abeta1-42-induced oxidative damage, inhibition of pro-apoptotic related signaling proteins and increment of anti-apoptotic processes. Abeta1-42 treatment changed the cells to apoptotic morphologies such as shrinkage, rounding up, and detachment from the culture dish, which were ameliolated by the treatment with PCW (Fig. 1). Moreover, PCW significantly recovered viability of PC12 cells from Abeta1-42-induced cytotoxicity and apoptosis ($p < 0.05$) (Fig. 2). In order to determine a protective effect of PCW, activity of lactate dehydrogenase (LDH) released from the cells was assessed. LDH release increased significantly after cells were treated with Abeta1-42 but pretreatment with PCW significantly attenuated increased LDH leakage in PC12 cells (Fig. 3). The results show that PCW has a protective effect against Abeta1-42-induced cytotoxicity. In order to investigate the functions of PCW, the protective effects of PCW against Abeta-induced neurotoxicity were evaluated on the aspects of oxidative stress. It is reported that Abeta-induced neurotoxicity is mediated by free radicals *in vitro* and in a transgenic mouse model of AD (Bruce et al. 1996; Pappolla et al. 1998). As shown in Fig. 4, consistent with the above result of increasing cell viability, PCW significantly decreased Abeta1-42-induced intracellular oxidative damage, suggesting that antioxidant properties of PCW may play an important role in cell viability (Fig. 2). Meanwhile, Zhou et al. reported that none of nine lanostane-type triterpenes isolated after ethanolic extraction of *Poria cocos* wolf, shows promising antioxidant activity (Liang et al. 2008). Therefore, antioxidant activity of PCW isolated from an aqueous extract of *Poria cocos* wolf, may be related to the other components which are contained in the plant. In order to confirm the effect of PCW on the apoptosis process of Abeta1-42-induced neuronal cell death, we examined the apoptosis images of PC12 cells by TUNEL assay. In agreement with the previous results, exposure of PC12 cells to Abeta1-42 resulted in an apoptotic cleavage of DNA and then the appearance of nuclear fragmentations and intensely stained nucleus in PC12 cells as shown in the TUNEL staining (Fig. 5). This study also showed that PCW changed the biochemical alternations participated in the process of Abeta1-42 induced apoptotic cell death (Figs. 6 and 7). That is, Abeta1-42 increased the activity of pro-apoptotic caspase-3 (Famer et al. 2004), which was suppressed by PCW. Meanwhile, caspase-3, a family of cysteine proteases, can be activated by ROS (Masahiro et al. 1998). Hence, the suppressive effect of PCW on the activity of caspase-3 may contribute to the inhibitory effect of PCW on apoptosis through its antioxidant property. Meanwhile, PCW decreased significantly expression of pro-apoptotic protein Bax but increased a few expression of anti-apoptotic protein Bcl-2. As a consequence, a significant down-regulation of the ratio of Bax/Bcl-2 was found after the PC12 cells were treated with PCW, suggesting that PCW suppresses the process of Abeta1-42-induced apoptosis (Fig. 7). In summary, PCW significantly inhibited Abeta1-42-induced cell death as well as intracellular oxidative damage, and decreased the activity of pro-apoptotic caspase-3 and expression of pro-apoptotic protein Bax but increased expression of anti-apoptotic protein Bcl-2.

Taken together, this study suggests that PCW may have a protective effect on Abeta1-42-induced neuronal cell death due to its anti-oxidant properties and in an anti-apoptotic functions, and so may be a promising candidate in novel neuroprotective therapy strategies for AD.

4. Experimental

4.1. Materials

Beta-amyloid (Abeta1-42) was purchased from rPeptide Co. (GA, USA) and WST-1 cell proliferation reagent was purchased from Roche Diagnostic (Penzberg, Germany). The cell damage was measured by a colorimetric lactate dehydrogenase (LDH) assay (TOX-7; Sigma-Aldrich Co., MO, USA). TUNEL was performed with an In Situ Cell Death Detection Kit-AP (Roche Diagnostics, Mannheim, Germany). Caspase-3 activity was measured with the Caspase-3/CPP32 Fluorometric Assay Kit (K105) (Biovision, Inc., Calif., USA). Bax and Bcl-2 protein amount were determined using Express ^{TM} ELISA commercial kit (Genscript Corporation, NJ, USA) Ab1-42 was prepared as a 1 mM solution in 0.1% ammonium hydroxide. The stock solutions were diluted to the desired concentrations immediately before use and added to the distilled water. RPMI 1640 medium, FBS and other supplements were obtained from Gibco BRL (NY, USA).

4.2. Preparation of PCW extract

Poria cocos, purchased from a Kyung-Dong chinese medicine store (Seoul, Korea) was identified by the Center of Herbal Authentication of college of oriental medicine, Wonkwang University and stored at $-20\,^{\circ}C$. After slicing, the air-dried materials from *Poria cocos* (1 kg) were extracted with distilled water by refluxing for 5 h (three times × 2L) on a water bath at 100 °C. The extract was filtered through a Buchner funnel using Whatman No. 1 filter paper, concentrated by rotary evaporator, and freeze dried. The dried extract was stored at $-70\,^{\circ}C$ and was used in all experiments.

4.3. Cell culture

PC12 cells were cultured routinely in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO^2/95% air. The medium was changed every 24 h, and cells was plated at an appropriate density (6.25×10^4 cells/cm^2). Cells were incubated with Abeta1-42 in the absence or presence of PCW for 48 h, and then determined cell viability, cell damage, morphology of apoptotic cell death and expression of apoptotic signal proteins. Oxidative damage was determined by measuring the fluorescence intensity of malondialdehyde-modified biomolecules.

4.4. WST-1

Cell viability was evaluated by the WST-1 assay. WST-1 assay measures the activity of intramitochondrial and extramitochondrial dehydrogenases. Briefly, tetrazolium salts are cleaved by dehydrogenases of viable cells to produce formazan and the change of absorbance is detected spectrophotometrically. Dehydrogenase activity was assayed in collagen-coated 96 well-plates (density of 6.25×10^4cells/well). The cells were exposed with Ab1-42 at 20 μM concentration with/without various concentrations of PCW (5-125 μg/ml) for 48 h. At required time, the medium was removed and the WST-1 reagent (diluted 1:10 in medium) was added. The cells were incubated in a gassed atmosphere (5% CO^2) for 2 h. The changes in absorbance were measured using a Biotek Synergy 2 Multi-Detection Microplate Reader (Vermont, USA) at wavelength 450 nm. Results were expressed as the percentage of WST-1 reduction, assuming that the absorbance of control cells was 100%.

4.5. Lactate dehydrogenase (LDH) assay

The cell membrane integrity (cell damage) of the PC12 cell cultures was measured by a colorimetric lactate dehydrogenase (LDH) assay according to the recommendations from the supplier. This assay measures membrane integrity as function of the amount of cytoplasmic LDH released into the culture mediums. Briefly, assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor and dye solutions. For all cultures, assay mixture was added to the culture mediums. After incubation for 30 min at room temperature in the dark, the colour reaction was stopped by 1 M HCl. Absorbance was determined at 490 nm using a Biotek Synergy 2 Multi-Detection Microplate Reader (Vermont, USA). Background correction was performed at 650 nm.

4.6. Preparation of subcellular fractions and Lipid peroxidation assays

A fluorescence assay procedure was used to measure lipid peroxidation according to slightly modified published procedures (Biasi et al. 2002). After scraped and collected into an Eppendorff tube, the cells were washed twice with cold PBS. There after 0.5 ml of mixed buffer of 50 mM potassium phosphate and 100 mM glycine, pH 7.4, was added, the suspensions were sonicated (sonicator Virsonic 300, Virtis, Danbury, CT, USA) for four 5-s intervals for a total of 20 s. The sonicated suspensions were centrifuged at 2,000 × *g* for 10 min, and the precipitates were discarded. The fluorescence intensities of malondialdehyde-protein adducts were measured in the supernatants by a PerkinElmer LS 55 Fluorescence Spectrometer (Waltham, Massachusetts, USA) as fluorescence exhibited by interaction between protein amino functions and malondialdehyde (MDA) at wavelengths of 390/460 nm (excitation/emission) and filter bandwidth: 2.5 nm, respectively.

4.7. Detection of DNA Fragmentation by Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Biotin Nick End Labeling (TUNEL)

TUNEL was performed with an In Situ Cell Death Detection Kit-AP according to the manufacturer's instructions. Briefly, after the cultivation, the PC12 cells were fixed with 3.7% formaldehyde and washed with PBS, and then permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min on ice. After permeabilization, the cells were incubated in a TUNEL reaction mixture comprising TdT for 60 min at 37 °C, washed with PBS, and incubated in anti-fluorescein antibody conjugated with alkaline phosphatase (AP). After a reaction using nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl-phosphate and 4-toluidine salt (BCIP) as substrates for AP, the stained cells were analyzed under a light microscope.

4.8. Caspase-3 activity

Caspase-3 activity was measured with the Caspase-3/CPP32 Fluorometric Assay Kit (K105), in accordance with the protocol supplied by the manufacturer. For each assay, 50 μg of tissue cell lysate was used. Samples were read in a PerkinElmer LS 55 Fluorescence Spectrometer (Waltham, Massachusetts, USA) with a 400-nm excitation and a 505-nm emission filter. The relative fluorescence units were normalized to total protein content of sample determined by the bicinchoninic acid assay (Pierce, IL,USA). The values of specific activity (S.A., unit = pmol/min/μg) were calculated. Results were expressed as the percentages of control.

4.9. Expression of bax and bcl-2

Bax and Bcl-2 protein amount were determined in PC12 cell lysate using Express ™ ELISA commercial kit purchased from Genscript Corporation following the manufacturer's instructions. Standards were performed in duplicate.

4.10. Statistical analysis

The means and standard deviations (SD) were calculated for all experiments. The data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test to determine whether means were significantly different from the control. In all cases, * a p value of <0.05 was used to determine the significance.

Acknowledgement: This study was supported by a grant of the Oriental Medicine R&D Project, Ministry for Health & Welfare & Family Affairs, Republic of Korea (B070030).

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