S9 - 2

Antioxidant Properties and Neuroprotective Effects of *Shaengshik* Extracts

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INTRODUCTION

Reactive oxygen or nitrogen species (ROS or RNS) are generated by the ordinary metabolic processess as well as by the external environmental stimuli such as ultraviolet light, gamma radiation and some kinds of xenobiotics in the living system.[1,2] Oxidative stress can occur when the production of free radicals surpasses free radical scavenging or damaged macromolecule repairing capacities. A growing body of evidence suggests that oxidative stress be implicated as a major cause of cellular injuries in a vast variety of clinical abnormalities including neurodegenerative disorders.[3,4] As one of the most vulnerable structures, mitochondria are exposed to high concentrations of ROS and may therefore play a pivotal role in the cell death decision. A decrease in mitochondrial energy charge and redox states, loss of transmembrane potential (depolarization), mitochondrial respiratory chain impairment, and release of substances such as calcium and cytochrome c all contribute to apoptosis.[5-8] These kinds of mitochondrial abnormalities may constitute a part of the spectrum of chronic oxidative stress in Alzheimer's disease. Accumulation of amyloid beta (Aβ) in form of senile plaques is also thought to play a central role in the pathogenesis of Alzheimer's disease mediated by oxidative stress.[9]

One of the plausible ways to prevent the cellular injuries induced by oxidative stress is to augment or potentiate endogenous oxidative defense capacity through dietary or pharmacological intake of antioxidants. Many natural antioxidants such as vitamin E, vitamin C or many kinds of phytochemicals are recognized as antioxidant agents that prevent oxidation *in vivo* and as free radical scavengers in lipophilic and water-soluble sites *in vitro*.[10-12] The contribution of antioxidant capacity through consumption of natural ingredients may play a significant protection against oxidative stress. *Shaengshik*, an uncooked natural powder, consumed mostly as a meal substitute or dietary supplement to promote health, may serve as an important natural antioxidant source. Recently, it has been reported that Aβ produces ROS, and antioxidants such as vitamin E or melatonin exert neuroprotective effects against Aβ-induced cytotoxicity.[13,14] Moreover, intracellular levels of antioxidative defense enzymes, such as superoxide dismutase, catalase and glutathione peroxidase are also changed during the progression of AD.[15,16] Intracellular accumulation of free radicals results in damage to critical cellular macromolecules including DNA, lipid and proteins thereby causing functional as well as structural alterations in these biomolecules, which ultimately leads to cell death. Several studies have addressed implications of apoptotic cell death in Aβ-mediated neurotoxicity.[17,18] Apoptosis is a tightly regulated process, which involves changes in the expression of distinct sets of enzymes and their substrate proteins.[19] Apoptosis plays an important homeostatic role in several cellular processes as well as various pathological conditions such as neurodegenerative disorders such as Alzheimer disease and Parkinson disease, and cerebral ischemia. Brain tissue from Alzheimer patients contains not only deposits of oxidized β-amyloid, but also activated caspase-3, a cysteine protease that mediates mitochondrion-initiated apoptosis. Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive degeneration and

loss of neurons in the brain. The appearance of neurofibrillary tangles and accumulation of senile plaques are two distinct neuropathological hallmarks of AD.[20,21] Several lines of evidence suggest that enhanced oxidative stress induced by ROS or RNS is associated with pathogenesis and progression of AD.[22,23]

The goal of present study was to examine the protective mechanisms of the standardized extract of *Shaengshik* against Aβ-induced apoptosis in relation to oxidative status as well as measuring *in vitro* antioxidant activities. The importance of diet-derived antioxidants in the management of Alzheimer's disease will be also discussed.

RESULTS AND DISCUSSION

On the basis of numerous *in vitro* and *in vivo* studies, it has been suggested that natural antioxidants play important role in the prevention of damage occurred as a result of oxidative stress induced by reactive oxygen species in degenerative diseases such as Alzheimer's disease. The investigation of *in vi*tro antioxidant properties and neuroprotective effects of *Shaengshik* extract was performed to identify the beneficial effect of *Shaengshik.* The antioxidant activity of the *Saengshik* extract was assessed by means of two different *in vitro* tests. The Trolox equivalent antioxidant activities were determined using the stable DPPH*. The Trolox equivalent values of *Saengshik* extracts were evaluated and methanol extract of *Saengshik* showed highest value of 386.16 mg or 1.542 mM. The antioxidant activities was in the order of MeOH extract > EtOH extract > water extract. In fact, water extract did not show very much activity at the concentration used. The SOD like activities of *Saengshik* EtOH and MeOH extract showed that 10.74% and 16.83% inhibition of oxidation process respectively, whereas water extract did not show any activity. The Trolox equivalent values were calculated as the concentration of Trolox with the same antioxidant activity of the *Saengshik* extracts in DPPH inhibition test.

The possible cytotoxicity of *Shaengshik* extracts in PC12 cells was evaluated based on their effects on cell growth (MTT assay). At concentrations ranging from 0.0625 to 1 mg/mL, water extract of *Shaengshik* slightly inhibited cell growth concentration dependently. MeOH extracts showed almost negligible cell cytotoxicity in all concentrations. In constrast, considerable cell cytotoxicity of EtOH extracts specially at the higher concentrations. Among the three kinds of extracts tested that of MeOH significantly protected PC-12 cells from the toxic effect of H_2O_2. About 58% of PC12 cells survived after 24 h treatment of the highest concentration of H_2O_2 (400 μM).

Shaengshik extract prevented $A\beta_{25-35}$-induced viability loss of PC12 cells. Aβ toxicity was evaluated by the trypan blue dye exclusion assay upon incubation of PC12 cells for 36 h. MeOH extract increased the cell survival rate with a concentration-dependent manner and 0.05 mg/mL showed the highest protection rate among the three concentrations tested (0.0125, 0.025 and 0.05 mg/mL). *Shaengshik* extract inhibited the $A\beta_{25-35}$-induced intracellular ROS accumulation accompanied with the decrease in MDA and increase in GSH levels. To examine the possible inhibition of *Shaengshik* extract of ROS in $A\beta_{25-35}$-induced PC12 cells, the accumulation of intracellular ROS using the DCF-DA was measured pretreated with MeOH extracts. DCF-DA is a nonfluorescent dye highly permeable to cells. Once inside cells, the compound is hydrolyzed by the esterase activity to DCF that is trapped intracellularly. DCF then interacts with peroxides and forms the fluorescent product can be readily detected by confocal microscopy. PC12 cells treated with 25 μM $A\beta_{25-35}$ for 6 h displayed intense fluorescence after staining with DCF dye and intracellular ROS accumulation resulting from $A\beta_{25-35}$ treatment was significantly reduced when *Shaengshik* extract was treated to the media. The GSH content in the control PC 12 cells was significantly reduced by the treatment with $A\beta_{25-35}$, and this depletion of the GSH contents was recovered almost up to the level of the control at the 0.05 mg/mL of MeOH extract. *Shaengshik* MeOH extract was found to effectively scavenge free radicals generated by $A\beta_{25-35}$ treatment as measured by malondialdehyde (MDA) production.

Oxidative damage has been involved in Aβ-induced cell death PC 12 cells treated with $A\beta_{25-35}$ underwent peroxidation of their lipid bilayer, leading to increased levels of MDA derived from lipid peroxides.

Shaengshik extract attenuated $A\beta_{25-35}$-induced PC12 apoptotic ce death. Apopototic cells were detected by TUNEL staining, which is a widely used immunostaining method in detecting DNA fragmentation *in situ*. In this histochemical technique, the appearance of intensely stained nuclei is indicative of terminal incorporation of labeled dUTP into fragmented DNA derived from apoptotic nucleus. Treatment with $A\beta_{25-35}$ significantly increased the proportion of TUNEL-positive cells and this was reduced by *Shaengshik* extract. *Shaengshik* extract also showed a protective effect on $A\beta_{25-35}$ induced dissipation of the mitochondrial membrane potential. When PC12 cells were exposed to $A\beta_{25-35}$, the $\Delta\Psi m$ rapidly depolarized, as shown by the decrease in voltage-sensitive dye, TMRE compared to the controland pretreatment with *Shaengshik* extract reduced the changes of $\Delta\Psi m$ as indicated by restoration of TMRE dye. $A\beta_{25-35}$-induced dissipation of the $\Delta\Psi m$ was blocked by the pretreatment with *Shaengshik* extract in a concentration dependent manner. It was found that *Shaengshik* decreased caspase-3 activity. There was approximately 3-fold induction of caspase-3 activity in PC12 cells treated with $A\beta_{25-35}$, which was suppressed by *Shaengshik* extract. By treating 0.05 μM MeOH extract caspase-3 activity returned to the control level.

The purpose of present study was to examine the antioxidant activity and the neuroprotective effects of *Saengshik* which is consisted of whole grain, essential vegetables, seaweeds, mushrooms and other minor ingredients. The one meal portion of *Saengshik* provides one-third of nutrients such as iron, folic acid, zinc, vitamin A, vitamin B_1, vitamin B_2, niacin, vitamin C, vitamin B_6 and vitamin D with the limited content of energy (170 kcal). *In vitro* antioxidative activity of *Saengshik* extract was measured using the two commonly used methods-DPPH radical scavenging and SOD activity. The findings from these experiments demonstrate that the MeOH and EtOH fractions possess relatively strong antioxidant/free radical scavenging properties. However, with the present limitations in the methodology evaluating total antioxidant activities in complex mixtures as stated by Liesuy et al.,[24] further investigations are required to assess the more accurate antioxidative capacity of complex food such as *Saengshik*. Also, the nature of compounds responsible for the antioxidant activity has to be elucidated in further study.

In this study *Shaengshik* extract has shown to protect cells from β-amyloid toxicity, and it was also demonstrated Aβ-induced apoptotic death via oxidative stress in PC12 cells was suppressed by pretreatment with *Shaengshik* extract. Beta-amyloid caused a decrease in cell viability by trypan blue dye exclusion assay, which was restored in the presence of *Shaengshik* extract. In this experiment, trypan blue assay is used instead of MTT dye reduction assay, since the MTT assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria, it may not precisely reflect the cell death that is mediated by mechanisms that do not include disruptions in mitochondrial function.

Alzheimer's disease is characterized by a pronounced loss of neurons in susceptible regions of the brain. Evidence suggests that this neuronal loss occur through apoptosis, a type of cell death with distinct morphological and biochemical characteristics. The principle component of senile plaques in AD brain is β-amyloid, a 39～43 amino acid peptide derived from amyloid precursor protein.[25] Aβ has been shown to be neurotoxic *in vivo* and *in vitro*, and is generally believed to contribute to the etiology of AD. Oxidative stress caused by increased accumulation of ROS in cells has been implicated in pathophysiology of several neurodegenerative diseases including AD.[26,27] In line with this notion, Aβ which is associated with senile plaques formation in the brains of patients with AD was found to induce apoptosis in cultured neurons through generation of ROS.[28,29] There has

been a growing body of data implicating free radical toxicity, radical-induced mutations and oxidative enzyme impairment, mitochondrial dysfunctions, and excitotoxic mechanisms in the pathogenesis of neurodegeneration.[30,31] The brain may be particularly vulnerable to oxidative stress in that it consumes a large amount of the body's oxygen and has relatively poor antioxidant protection as seen by low levels of the antioxidant enzyme glutathione perxodiase as well as antioxidants, such as glutathione and vitamin E.[32]

The neurotoxic activity of Aβ has been attributed to amino acids present in positions 25~35 of the full-length beta-amyloid. In the present study, we have demonstrated that $A\beta_{25-35}$ induces apoptotic death via oxidative stress in PC12 cells. Thus, $A\beta_{25-35}$ induced intracellular accumulation of ROS at the concentrations that caused apoptosis. Transition metal ions such as iron, copper, aluminum and zinc have been proposed to play a role in Aβ-induced ROS generation.[33-36] The aggregation process of Aβ is accelerated by transition metal that catalyzes oxidation of Aβ[33,34] which aggravates Aβ-induced neurotoxicity by generating extremely reactive hydroxyl radial via the Fenton reaction.[35,36] It has also been reported that the concentration of transition metal ion is elevated in the brains of AD.[37,38] Antioxidants and antioxidant enzymes have been demonstrated to protect against Aβ-induced cytotoxicity. In this study, $A\beta_{25-35}$-induced intracellular accumulation of ROS was attenuated by *Shaengshik* extract as revealed by reduced distribution of DCF-fluorescence in PC12 cells pretreated with *Shaengshik*. The involvement of ROS in $A\beta_{25-35}$-induced cell death was further supported by increased accumulation of peroxides and the reduction of GSH in the treated cells. *Shaengshik* extract not only suppressed $A\beta_{25-35}$-induced cytotoxicity, but also decreased ROS accumulation accompanied with the elevation of GSH and reduction of MDA in pretreated cells. It has been shown that reduction of GSH levels increases the sensitivity of neurons to the toxic effect of neurotoxins,[39] and is associated with mitochondrial dysfunction.[40] ROS contribute to cell death, in part, through modulation of various cellular signaling cascades.

PC12 cells exposed to $A\beta_{25-35}$ showed characteristic morphological features of apoptosis, such as cell shrinkage and membrane blebbings and positive TUNEL staining. $A\beta_{25-35}$ treatment also induced cleavage of PARP, which is the substrate of active caspase mediating apoptosis. ROS can cause cell death via apoptosis in many cell types. $A\beta_{25-35}$ treatment caused impairment of the mitochondria in previous studies.[41,42] The present study revealed that $A\beta_{25-35}$-treated PC12 cells undergo apoptosis as determined by positive TUNEL staining. It has been shown that Aβ cause apoptosis in PC12 cells through induction of oxidative stress, which appears to be mediated by activation of signal cascades. It appeared that the increased apoptosis was protected by pretreatment with *Shaengshik*. Further elucidation of intracellular signaling cascades involved in Aβ-induced cell death and their modulation may provide insights into the molecular basis for neuroprotective effects of naturally occuring phytochemical compounds found in food.

CONCLUSIONS

Recently, considerable attention has been focused on dietary manipulation of oxidative and/or nitrosative damage. The various antioxidants have found to have the ability to scavenge free radicals. In this study, the antioxidative properties and neuroprotective effects of *Shaengshik* extracts were investigated. The *in vitro* antioxidant activities of *Saengshik* extracts were characterized by the DPPH method and Superoxide Dismutase (SOD) like activity. The ethanol and methanol extracts showed the considerable antioxidant activities determined by the both methods. In cultures of rat pheochromocytoma (PC12), the treatment with Aβ underwent apoptotic death as determined by positive *in situ* terminal end-labling (TUNEL stsining), decreased itochondrial transmembrane potential, elevated caspase-3 activity occurring with enhanced MDA accumulation and the reduction of GSH levels. *Shaengshik*

pretreatment attenuated Aβ-induced apoptosis in PC12 cells possibly by exerting antioxidant properties. The potential of *Shaengshik* as one of the neuroprotective agent has been suggested through this study, and the combination with other dietary antioxidants possessing ROS or RNS scavenging activities could provide better therpapeutic advantage for the management of Alzheimer diseases.

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