

Protection of Differentiating Neuronal Cells from Amyloid β Peptide-induced Injury by Alkaline Extract of Leaves of *Sasa senanensis* Rehder

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Abstract. *Background/Aim:* We have previously reported the protection of doxorubicin-induced keratinocyte toxicity by alkaline extract of the leaves of *Sasa senanensis* Rehder (SE). In order to extend the generality of the cell protective effect of SE, we investigated whether it also protects rat PC12 and human SH-SY5Y neuron model cells from amyloid β -peptide ($A\beta$)-induced injury. *Materials and Methods:* Viability of cells was determined by the MTT method. Cytotoxicity was evaluated by the concentration that reduces the cell viability by 50% (CC_{50}). Protection from $A\beta$ -induced cytotoxicity was evaluated by the concentration that reversed the $A\beta$ -induced reduction of viability by 50% (EC_{50}). The selectivity index (SI) of neuroprotective activity was defined as the ratio of EC_{50} to CC_{50} . $A\beta_{1-42}$ aggregation was assayed using $A\beta_{1-42}$ ammonium hydroxide. *Results:* SE showed hormetic growth stimulation at lower concentrations in both neuron precursors and differentiated cells. SE reproducibly inhibited $A\beta$ -induced cytotoxicity against both undifferentiated and differentiated neuron cells. Both the extent of differentiation induction and viability depended on the cell density, suggesting the release of growth and

differentiation stimulation substances into culture supernatant. Higher concentrations of SE partially reduced the $A\beta_{1-42}$ aggregation. *Conclusion:* Hormetic growth stimulation and inhibition of aggregation may be involved in the neuroprotective activity of SE.

We previously reported diverse biological activity of alkaline extract of the leaves of *Sasa senanensis* Rehder (SE), a group III over-the-counter drug (1). Among them, antiviral (2) and anti-UV activities (3) were prominent, possibly due to the presence of lignin carbohydrate complex in the extract (4, 5). We also manufactured a tooth paste that have anti-halitosis effects (6). Recently we found that SE alleviated the doxorubicin-induced cytotoxicity against primary human gingival epithelial cells (HGEP), by its growth promoting activity (7). However, it was not clear whether the SE's cell protective or growth promoting activities are a universal phenomenon or not, regardless of cell types.

Alzheimer's disease (AD) is the most common neurodegenerative disease and the leading cause of dementia in elderly people, and characterized by extracellular neuritic plaques made of aggregated forms of neurotoxic amyloid β -peptide ($A\beta$) and intracellular deposits of abnormally phosphorylated tau protein (8). In lipid rafts of cell membranes, amyloid precursor protein (APP) is enzymatically cleaved into $A\beta$ peptides. Especially the 42-amino acid fragment ($A\beta_{1-42}$) easily aggregates and self-assembles to form a heterogeneous mixture of oligomers and protofibrils, which ultimately deposit in senile plaques. Small soluble $A\beta$ oligomers are considered as the major neurotoxic species in AD (9). $A\beta$ s have been reported to induce apoptosis in neuronal cells by mitochondrial dysfunction and oxidative

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Key Words: Nerve cell, NGF, differentiation, amyloid β -peptide, protection, hormesis, bamboo leaf extract.

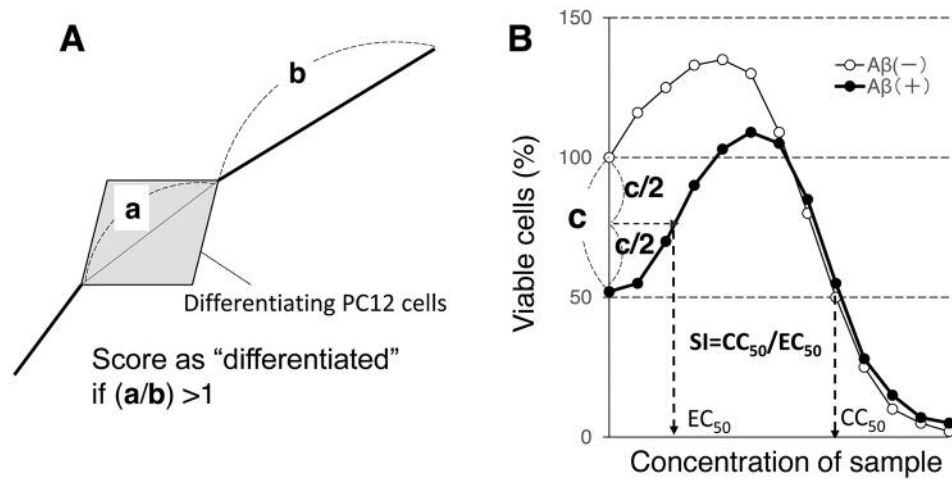


Figure 1. Criteria for differentiated cells (A) and selectivity index (SI) for quantifying the protective effects for A β -toxicity.

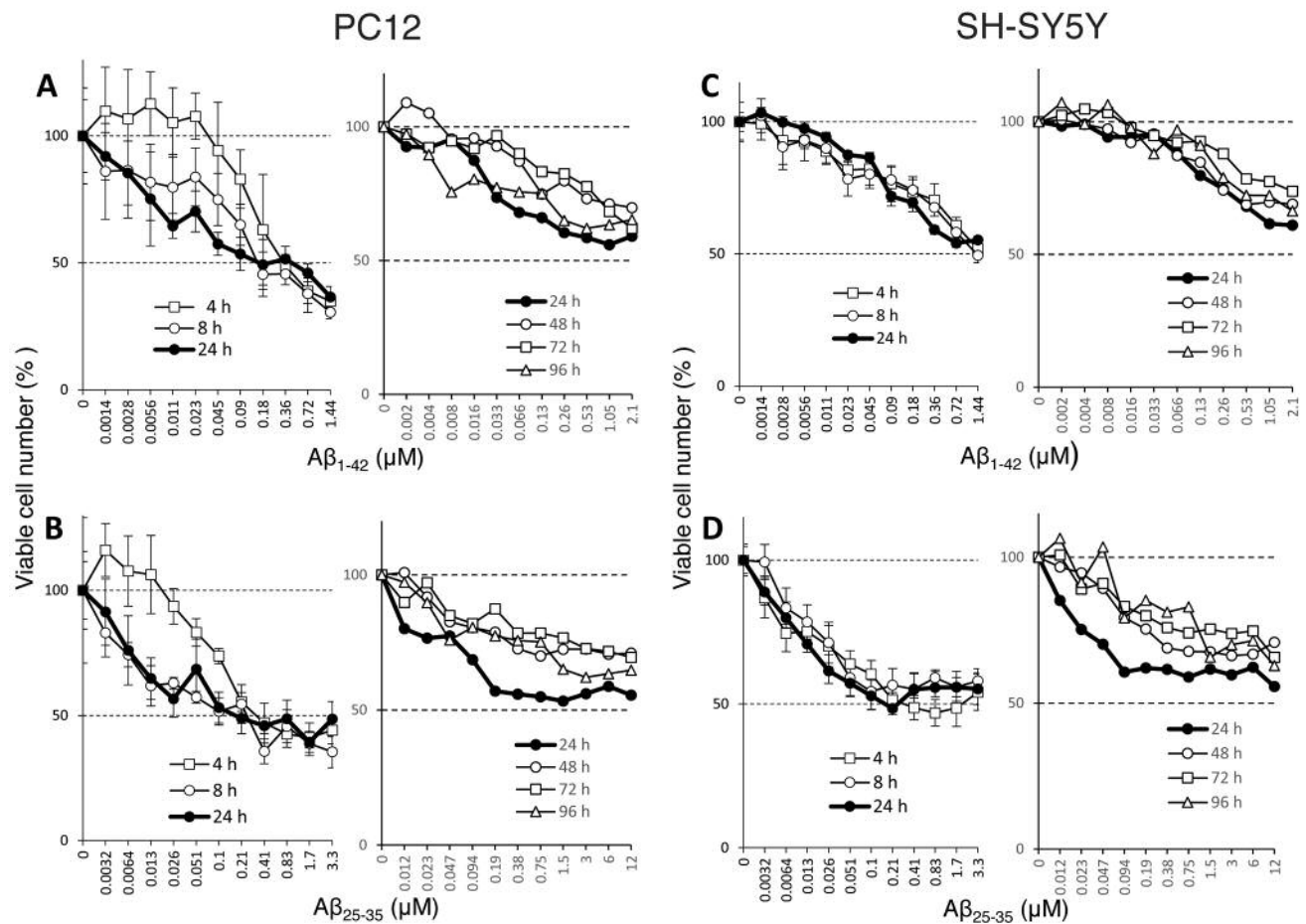


Figure 2. Determination of optimal treatment time with A β peptides to express maximum toxicity. PC12 and SH-SY5Y cells were inoculated at 3 and $2 \times 10^4/\text{cm}^2$, and incubated to achieve complete cell attachment. Cells were treated for 4, 8, 12, 48, 72 or 96 h without (control) or with the indicated concentrations of A β_{1-42} (upper column) or A β_{25-35} (lower column), and then viable cell number was determined by MTT method, and expressed as % of control. Each value represents mean \pm S.D. ($n=4$).

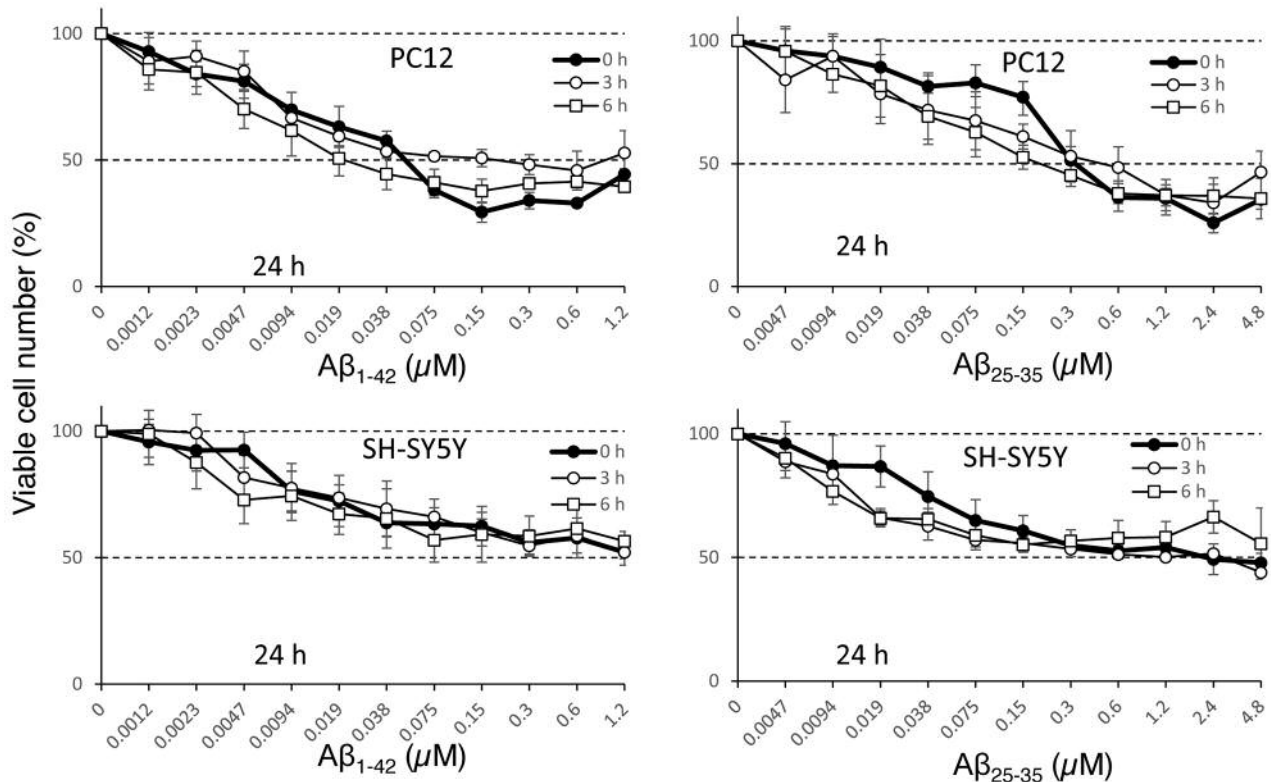


Figure 3. Effect of preincubation of $A\beta$ peptides on the cytotoxicity induction. $A\beta_{1-42}$ and $A\beta_{25-35}$ solution were preincubated at 37°C for 0 (control), 3 or 6 h. PC12 (upper column) and SH-SY5Y cells (lower column) were then exposed to the indicated concentrations of $A\beta_{1-42}$ or $A\beta_{25-35}$, and then viable cell number was determined by MTT method, and expressed as % of control. Each value represents mean \pm S.D. ($n=4$).

stress (10), alteration of metal ion influx (11), endoplasmic reticulum stress (12) and impaired autophagy (13). In order to test the generality of cell protective activity of SE, we investigated here whether SE protects the neuronal cell death induced by amyloid β -peptides ($A\beta_{1-42}$ and $A\beta_{25-35}$).

We have used two different assay systems using undifferentiated PC12 pheochromocytoma cells (14) and human SH-SY5Y neuroblastoma cells (15, 16), grown in DMEM medium supplemented with 10% FBS in two uncoated culture plates, and (ii) differentiating PC12 cells, induced by 50 ng/ml nerve growth factor (NGF) in DMEM supplemented with 1%FBS in five different 96-microwell plates (two uncoated, one poly-L-lysine coated, two collagen-coated plates).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), human recombinant NGF, dimethyl sulfoxide (DMSO) (Wako Pure Chemical Ind., Ltd., Osaka, Japan); fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) (Sigma-Aldrich Inc., St. Louis, MO, USA); $A\beta_{1-42}$ and $A\beta_{25-35}$ (Cosmo Bio Co., Ltd., Tokyo, Japan). NGF (dissolved in water at 0.5 mg/ml), $A\beta_{1-42}$ (dissolved in DMSO at 1 mM) and $A\beta_{25-35}$ (dissolved in water at 0.1 mg/ml) were frozen at $-20^{\circ}C$. The following 96-microwell plates were purchased from the indicated companies: TrueLine (Nippon Genetics Co., Ltd., Tokyo, Japan), TPP (Techno Plastic Products AG, Trasadingen, Switzerland), BioCoat™ Collagen I 96-well plate, BioCoat™ Collagen I 96-well plate, BioCoat™ Poly-L-Lysine 96-well plate (Corning Inc., Corning, Canton, NY, USA).

Cell culture. PC12, a cell line derived from a pheochromocytoma of the rat adrenal medulla (14), and SH-SY5Y, cloned from a human bone marrow biopsy derived line called SK-N-SH (15, 16) were purchased from Riken Cell Bank (Tsukuba, Japan). These cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml, penicillin G and 100 μg /ml streptomycin under a humidified 5% CO_2 atmosphere. The superiority of DMEM over mixed culture medium (DMEM+Ham12) has been reported in our previous paper (17).

Determination of viable cell number. The culture medium was removed, and replaced with fresh medium containing 0.2 mg/ml MTT in the presence (for differentiated cells) or absence (for non-differentiated cells) of 50 ng/ml NGF. The cells were incubated for 1 h, and the formazan precipitate was dissolved in DMSO to

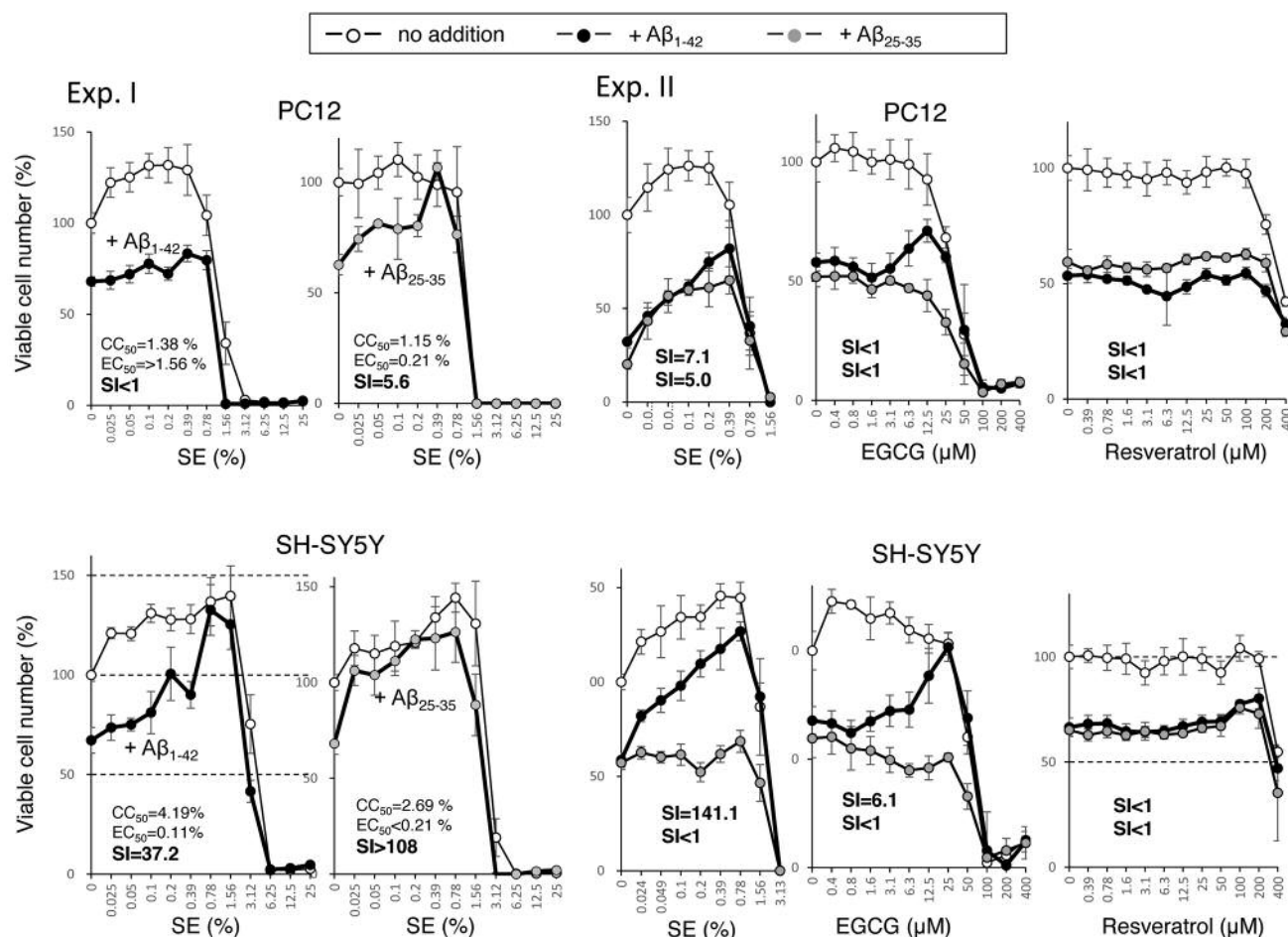


Figure 4. Protective activity of SE and other antioxidants against the $A\beta$ -induced cytotoxicity in PC12 (upper column) and SH-SY5Y cells (lower column). Cells were incubated for 24 h with the indicated concentrations of SE, EGCG or resveratrol in the presence of 400 nM $A\beta_{1-42}$ or 1,600 nM $A\beta_{25-35}$. Each value represents mean \pm S.D. (n=4).

measure the absorbance at 560 nm with a plate reader (Infinite F 50 R, TECAN, Kawasaki, Japan).

Induction of differentiation toward neurons. For the induction of differentiation, PC12 cells were incubated for the indicated times with differentiating inducing medium (DMEM supplemented with 1%FBS, 50 ng/ml NGF and antibiotics). Differentiated cells were defined as the cells in which the extended neuritis exceeds the longest diameter of each cell, assessed under the light microscope (EVOSfl, ThermoFisher Scientific, Waltham, Mass, USA) (Figure 1A).

Neuroprotection assay. Undifferentiated (PC12, SH-SY5Y) and differentiated PC12 cells were treated with $A\beta_{1-42}$ and $A\beta_{25-35}$ for the 24~48 hours in the presence of increasing concentrations of test samples. The viable cell number was determined as described above. The 50% cytotoxic concentration (CC₅₀) was determined from the amyloid peptide-free culture, and the 50% effective concentration (EC₅₀) that reduced the amyloid peptide-induced cytotoxicity by 50% were calculated (Figure 1B). The protective

effect was quantified by the selectivity index (SI), using the following formula: $SI = CC_{50}/EC_{50}$ (Figure 1B).

Detection of $A\beta$ fibril formation. $A\beta_{1-42}$ aggregation was measured using $A\beta_{1-42}$ ammonium hydroxide (rPeptide, Georgia, USA). To measure the $A\beta_{1-42}$ fibril formation in 96-well black microplates, 25 μ l of 400 μ M Thioflavin (ThT) and 10 μ l of each concentration of SE were added into each well and then mixed with 23 μ l of 221 μ M $A\beta$ solution. Final $A\beta_{1-42}$ peptide concentration were ~50 μ M, and final SE concentrations were 0.5, 0.1, 0.05 and 0.01%. ThT fluorescence signal was monitored at intervals of 10 min for 3 h at 37°C with an excitation wavelength (λ_{ex}) of 440 nm and an emission wavelength (λ_{em}) of 485 nm, and 15 sec shaking between reads to facilitate aggregation using a SpectraMax i3 (Molecular Devices, Sunnyvale, CA, USA). This gave 6 readings for each well sample.

Statistical treatment. Experimental values are expressed as the mean \pm standard deviation (SD) of triplicate or quadruplicate samples.

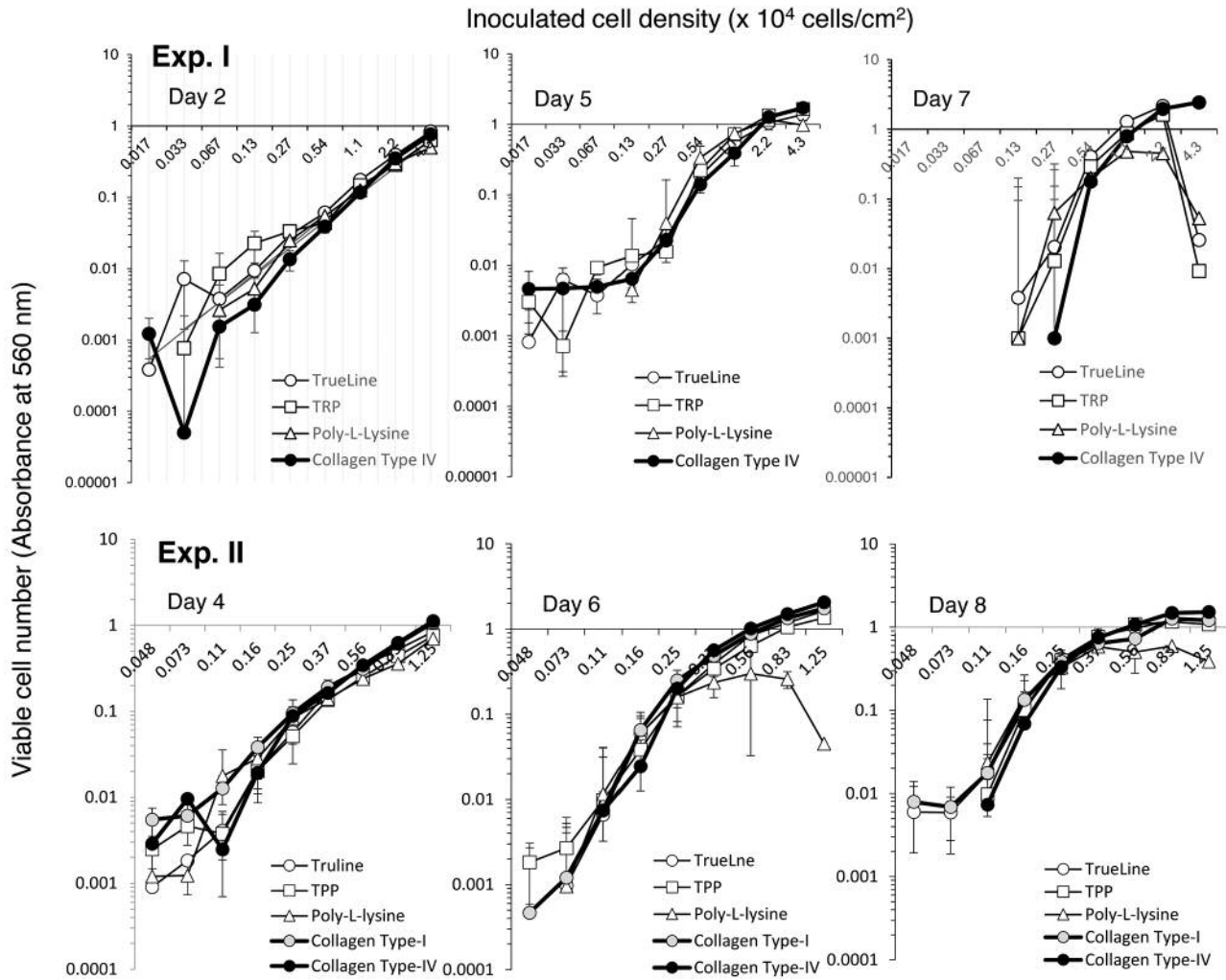


Figure 5. Comparison of cell growth on five different 96-microwell plates. PC12 cells were inoculated on four (Exp. I) or five different plates (Exp. II) at the indicated cell density, and incubated for 2 days to complete cell attachment. Cells were cultured for 2–8 days in fresh differentiation medium containing 50 ng/ml NGF and 1%FBS, and viable cell number was determined. Each value represents mean \pm S.D. (n=6).

Statistical analysis was performed by using Student's *t*-test and Bonferroni *post hoc* test. A *p*-value <0.05 was considered significant.

Results

Optimal condition for cytotoxicity induction by amyloid peptides. We first investigated the optimal treatment time with amyloid β -peptides. When PC12 cells were incubated for 4, 8, 24, 48, 72 or 96 h with increasing concentrations of $A\beta_{1-42}$ (A) and $A\beta_{25-35}$ (B) (Figure 2), their cytotoxicity became maximum after 24 h. The optimal concentration of $A\beta_{1-42}$ and $A\beta_{25-35}$ was 0.08–2.1 and 0.1–12 μ M, respectively. When SH-SY5Y cells were similarly incubated with $A\beta_{1-42}$ (C) and $A\beta_{25-35}$ (D) (Figure 2), strongest

cytotoxicity emerged 24 h after treatment. The optimal concentration of $A\beta_{1-42}$ and $A\beta_{25-35}$ was 0.36–2.1 and 0.1–12 μ M, respectively. Their viability declined to nearly half of the control, consistent with previous reports (18–21).

It has been generally accepted that the oligomerization of $A\beta$ enhances its cytotoxicity (22). We investigated whether preincubation of amyloid peptides at 37°C, a condition that enhances oligomerization, may augment their cytotoxicity. In contrast to our expectation, cytotoxicity of both $A\beta_{1-42}$ and $A\beta_{25-35}$ did not change apparently without or with preincubation for 3 or 6 h. Comparable cytotoxicity was induced at the concentration range of 0.075–1.2 and 0.3–4.8 μ M, respectively (upper panel) (Figure 3). Similar superimposable dose-response curves were observed

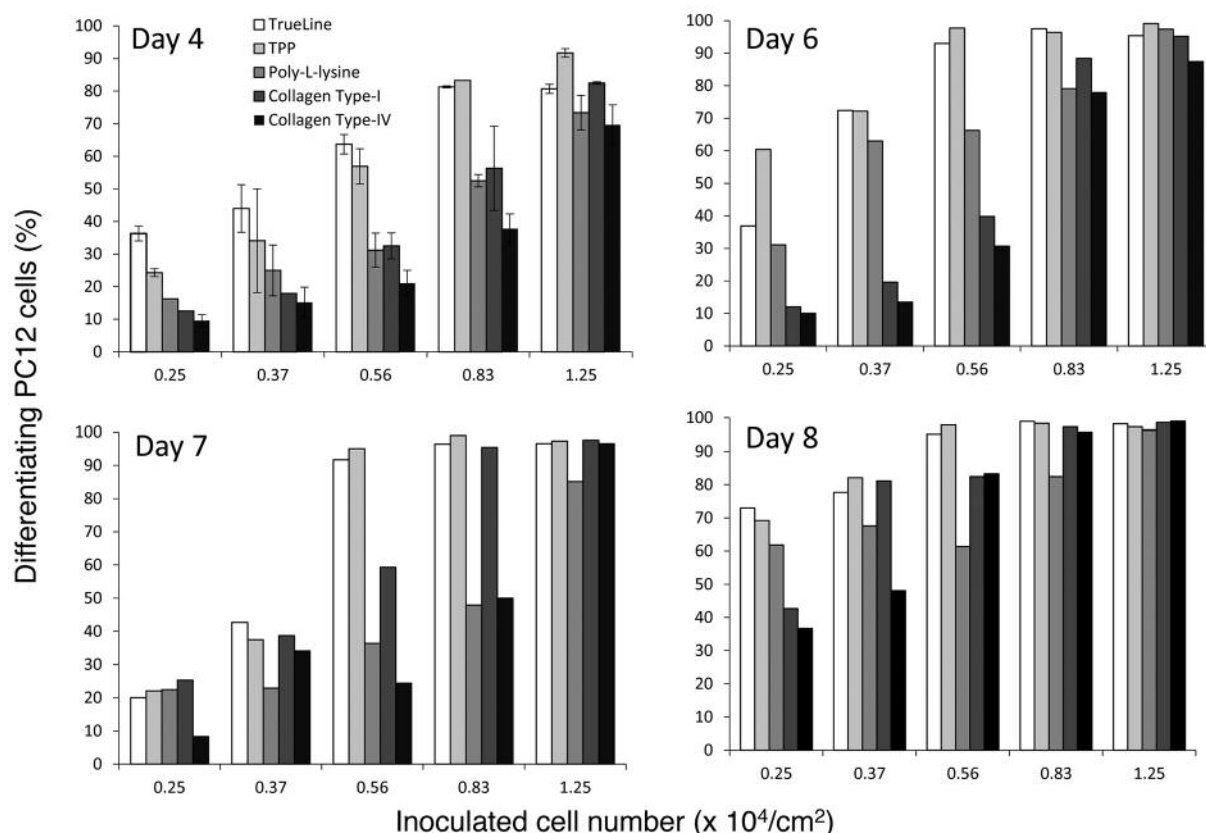


Figure 6. Effect of different types of plates on the incidence of nerve cell differentiation. PC12 cells were grown as in Exp. II of Figure 5, and approximately 100 cells on each well were checked for their morphology under light microscope to calculate the percent of differentiated cells, as described in Figure 1A.

regardless of preincubation in SH-SY5Y cells (lower panel) (Figure 3). Based on these experiments, we did not preincubate A β peptides for the following experiments. It is possible that A β gradually becomes aggregated during the 24-h incubation time, regardless of preincubation.

SE protects PC12 and SH-SY5Y cells from A β -induced toxicity. For the quantification of neuroprotective activity, we have to first calculate the CC₅₀ (for the cytotoxicity index) and EC₅₀ (for the efficacy index). The selection index (SI), that reflects both cytotoxicity and efficacy, was calculated by the following equation: SI=CC₅₀/EC₅₀ (Figure 1B).

SE potently protected undifferentiated PC12 cells (SI=<1; 5.6) and SH-SY5Y cells (SI=37.2; >108) from A β ₁₋₄₂ and A β ₂₅₋₃₅-induced toxicity, respectively (Exp. I, Figure 4). This indicates that SE can be used as a positive control.

We next investigated various antioxidants for their ability to protect PC12 and SH-SY5Y cells from A β -induced toxicity (Exp. II, Figure 4). SE, used as a positive control, reproducibly showed potent neuroprotective activity [SI=7.1

(A β ₁₋₄₂), SI=5.0 (A β ₂₅₋₃₅) in PC12 cells; SI=141.1 (A β ₁₋₄₂), SI=<1 (A β ₂₅₋₃₅) in SH-SY5Y cells]. On the other hand, epigallocatechin gallate (EGCG), a main component of green tea, showed much weaker protective activity [SI=<1 (A β ₁₋₄₂), SI=<1 (A β ₂₅₋₃₅) in PC12; SI=6.1 (A β ₁₋₄₂), SI=<1 (A β ₂₅₋₃₅) in SH-SY5Y]. Resveratrol, a popular stilbenoid contained in wine, showed no protective effect (SI=<1) (right panel).

SE protects the differentiated neuron cells from A β -induced toxicity. We first established which types of 96-microwell plates are the best for maintaining the growth and differentiation in the differentiation medium (Figure 5). We used the following four different plates (TrueLine, TPP, poly-L-lysine coated, collagen type-I-coated) (Exp. 1) and five different plates (TrueLine, TPP, poly-L-lysine coated, collagen type-I-coated, collagen type-IV-coated) (Exp. II). When the inoculated cell density was set to below 0.13×10⁴/cm² in either cases, cell viability declined dramatically, due to the induction of apoptosis (characterized by cell shrinkage) (observed under light microscope, data not shown). This

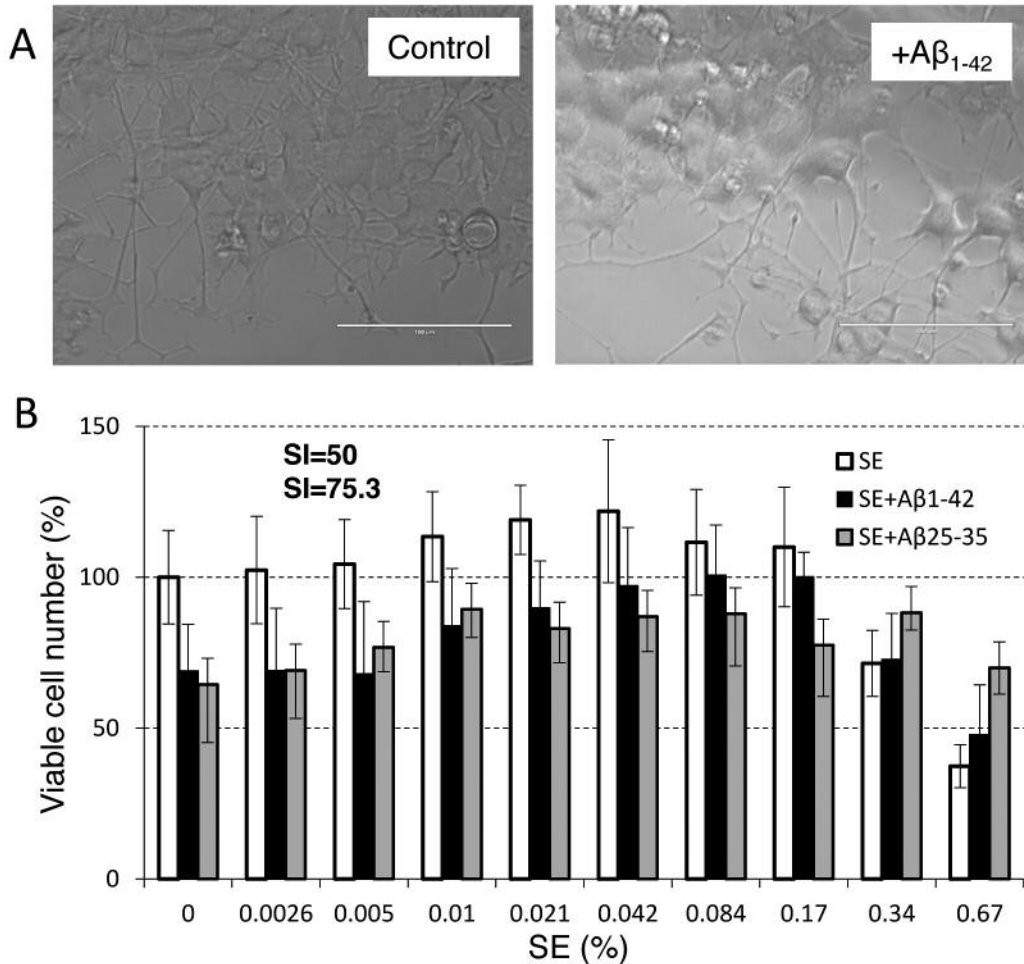


Figure 7. Protective effect of SE on A β -induced cytotoxicity in differentiating PC12 cells. PC12 cells were induced to differentiate with 50 nM NGF in differentiation medium, and then exposed to vehicle (control) or with 20 nM A β_{1-42} or 833 nM A β_{25-35} in the presence of increasing concentrations of SE. Viable cell number was then determined by MTT method, and expressed as % of control. Each value represents mean \pm S.D. (n=6).

suggests that a certain number of cells are required to maintain the growth and nerve cell differentiation. Cells inoculated on collagen type-IV coated plates, were still attached, even at extremely higher cell density (Exp. I). Repeated experiments confirmed that both collagen type-I and -IV plates maintained the highest cell density at day 8, without cell detachment, whereas significant cell detachment was observed in poly-lysine-coated plate even at an earlier stage (day 4) (Exp. II, Figure 5).

The percent of differentiated cells was declined with decreasing inoculation number of cells (Figure 6). At the later stage (day 7 or day 8), cells attached on four plates other than poly-L-lysine-coated plates became fully differentiated (Figure 6).

When PC cells were treated for 7 days with 50 ng/ml NGF in collagen type-I-coated plates, they extended well neurites,

characteristic to neurons (A) (Figure 7). Addition of 0.21 μ M A β_{1-42} for the last 2 days (day 5~day 7) resulted in some interruption of neurite outgrowth (A) and reduction of cell viability to 69% of control (B). Addition of SE dose-dependently reversed the A β_{1-42} -induced cell toxicity, yielding the SI value of 50 (B). Similarly, addition of 0.83 μ M A β_{25-35} reduced the cell viability to 65% of control, and addition of SE dose-dependently reversed the A β_{25-35} -induced cell toxicity, yielding the SI value of 75.3 (B). Cytotoxicity of higher concentration of SE was slightly inhibited by amyloid peptides, suggesting the interaction between these molecules.

Inhibition of A β_{1-42} aggregation by SE. SE at relatively higher concentrations (0.5~5%), but not lower concentrations (0.01~0.05%), significantly inhibited A β_{1-42} aggregation ($p<0.05$) (Figure 8).

Discussion

The present study demonstrated that SE inhibited the A β -induced toxicity against both undifferentiated PC12 and SH-SY5Y cells, and differentiated PC12 cells. We also found that lower concentrations (0.025~1.56%) of SE stimulated the growth of both PC12 and SH-SY5Y cells (Figure 4). Growth stimulation observed at lower doses of toxicants and radiations is known as hormesis (23). Maximum hermetic stimulation of growth was 32, 10 and 26% (mean=23%) in PC12 cells, and 40, 44 and 46% (mean=43%) in SH-SY5Y cells, respectively (Figure 4). EGCG, that also showed slightly lower protective effects, stimulated their growth by 6 and 23% (mean 15%). On the other hand, resveratrol, that showed no apparent protective activity, stimulated their growth only by 0~4% (Exp. II in Figure 4). These data suggest that the protective activity of SE may partially be due to its growth promoting activity. We have previously reported that SE also stimulated the growth of HGEP (7). It remained to be investigated whether hormetic response by SE is a universal phenomenon, or observed only in cells that express some specific receptors. Since the inhibition of A β ₁₋₄₂ aggregation was found only above 0.5% of SE, the action point of SE might not be A β ₁₋₄₂ aggregation.

Many antioxidants including resveratrol have been reported to inhibit the cytotoxicity of A β peptides (24-26). These findings are contradictory to our negative results of resveratrol (Figure 4). This discrepancy may be due to the difference in culturing conditions, or the quantitation methods. Most of the previous studies have used undifferentiated PC12 cells that have not extended neurites. We are now re-evaluating their neuroprotective activity using fully differentiated PC12 cells.

The present study also demonstrated that cells, inoculated at extremely low density, stopped their growth and differentiation (Figures 5 and 6), even if supplied by enough amounts of NGF. This suggests that neurite growth and maturation need the presence of a high density of differentiating cells, that may be a source of growth and differentiation stimulators other than NGF.

Since it is unlikely that higher-molecular weight substances present in SE pass through the blood brain barrier, fractionation and investigation of protective activity of lower molecular components present in SE are underway.

Conflicts of Interest

The first Author (HS) was supported by Daiwa Biological Research Institute Co., Ltd., Kanagawa, Japan. The Authors wish to confirm that such financial support has not influenced the outcome or the experimental data.

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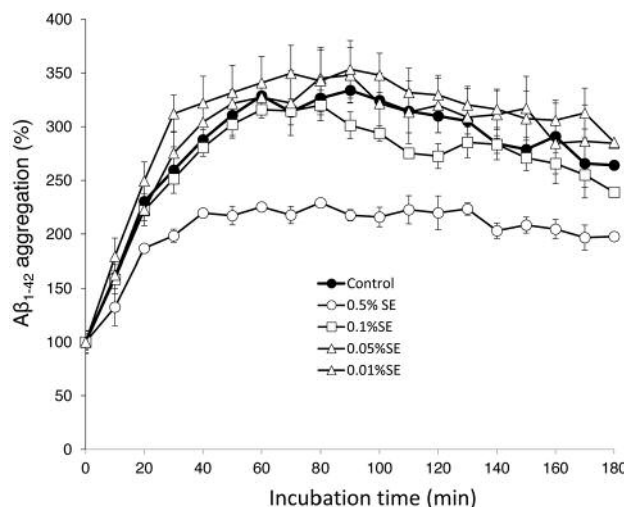


Figure 8. Dose-dependent inhibition of A β ₁₋₄₂ aggregation. A β ₁₋₄₂ aggregation was measured every 10 min at 37°C using Thioflavin, at Excitation 440 nm, Emission 485nm. Each value represents mean \pm S.D. (n=6). We found significant reduction of A β ₁₋₄₂ aggregation by 0.5% SE (0~150 min) ($p<0.005$).

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