The Study of Protective Effects of Low-Level Light and Donepezil Against β-Amyloid-Induced Cytotoxicity in SH-SY5Y Cells

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***Abstract*—The main purpose of this study was to evaluate whether donepezil and Low-Level Light Therapy (LLLT) shown to play neuroprotective effects by stimulating mitochondrial activity in the amyloid-beta 1-42 ($A\beta_{1-42}$) - induced neuronal toxicity model of Alzheimer's Disease (AD). This result indicates that the $A\beta_{1-42}$ accumulation in neuronal cells is related to the mitochondrial dysfunction and induced-neuronal cell death. While donepezil and Low-Level Light Therapy (LLLT; 660 nm, 5mW/cm^2, 3J/ cm^2) can reverse this situation. Donepezil is therapeutic acetylcholinesterase inhibitor currently being used for the treatment of AD. SH-SY5Y cells were pre-treated by donepezil at a concentration of 1 μM showed a maximum of neuronal viability compared to control cells. However, at higher concentrations, the neuronal viability was diminished. LLLT is a noninvasive therapy which showed significant increasing of neuronal viability and afforded protection against $A\beta_{1-42}$-induced toxicity. In addition, the combination treatment between 1 μM of donepezil pre-treatment and LLLT in SH-SY5Y cells induced by $A\beta_{1-42}$ toxicity had increased cell viability. In aggregate, these results demonstrate that LLLT has probably contributed to alternative treatment in neurodegenerative disease.**

***Index Terms*—Alzheimer's disease, Beta-Amyloid, low-level light therapy, donepezil hydrochloride**

I. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the presence of two kinds of abnormal protein deposits, extracellular deposits of β-amyloid peptide (Aβ) and intracellular neurofibrillary tangle (NFTs) in specific areas of the brain as playing a seminal role in the pathogenesis of AD. Aβ is a 39- to 42-amino acid peptide derived from the processing of proteolysis in integral membrane protein known as Aβ precursor protein (APP) [1], [2]. The Aβ hypothesis support from various studies showing that Aβ is toxic to neurons; such as, there is an increase in Aβ-released and apoptotic cell death involve overexpression of Aβ precursor protein (APP) [3]. Recent studies have also demonstrated that low concentration of soluble Aβ oligomers can induce synaptic dysfunction leading to the cognitive impairment and memory loss associated with AD [4].

Many compounds have been found to alleviate the progression or symptom of AD and reduction of Aβ aggregation or neurotoxicity in vitro. The role of cholinergic neurotransmission in-memory processing and cognitive function, regarding the progression of acetylcholinesterase (AChE) activity increasing in AD brains in parallel with memory deterioration. Donepezil is an AChE-inhibitor and is currently being used to treat AD-patient. These drug compounds act to inhibit the activity of the enzyme AChE, so increasing the levels of the neurotransmitter acetylcholine (ACh) at cholinergic synapses are involved in memory, thus enhancement of cholinergic function may stabilize or improve cognitive function and may affect behavior and daily function [5]. Moreover, donepezil pre-treatment showed the dose-dependent increase in the metabolism within the neuronal cell [6] and protect neuronal cultures from cytotoxicity [7].

Phototherapy has gained considerable interest in recent years as a therapy for the treatment of a variety of diseases. involving regenerative medicine and to promote wound healing as a relatively noninvasive technique [8], [9]. The ability of multiple light-emitting diodes in planar arrays were suitable for clinical applications because of large areas of tissue can be irradiated in one hands-off session. These arrays deliver almost laser-like wavelength specificity and clinically useful penetration depths and intensities. According to the previous study, LED-based systems have been successfully applied in an increasingly large number of fields and found the three-major wavelengths which a good photobiological basis and proven clinical utility: blue (around 415 nm), red (around 633 nm), near-infrared (around 830). Each has its own specific cellular targets and biological action spectrum

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and reaction [10]. The recent study demonstrated that the efficacy of LLLT in diseases related to the nervous system. Some of the results shown in the LLLT can activate the neurons growth and nerve regeneration both rat spinal cord [11], [12] and improvement of neurological functions in a mouse model of AD [13].

In the classic *in vitro* AD model, there were reported about the anti-Aβ-toxicity effect of donepezil. In this study, we sought to evaluate whether LLLT may protect Aβ-induced-toxicity in SH-SY5Y cells and whether the combination of LLLT and donepezil may have an additive effect.

II. Materials and Methods

A. Materials

Dulbecco's modified Eagle's Medium (DMEM; Carlsbad, CA, USA) Ham's F-12 nutrient mix (Carlsbad, CA, USA) and Fetal Bovine serums (FBS; Carlsbad, CA, USA), Trypan Blue stain (GIBCO, USA), 0.25% Trypsin/EDTA (Carlsbad, CA, USA), and cell viability reagent were purchased from GIBCO (Invitrogen Corporation, Grand Island, NY, USA). $A\beta_{1-42}$ was obtained from Sigma-Aldrich (Carlsbad, CA, USA). Donepezil hydrochloride was purchased from Chemical Express Co., Ltd (TCI/JAPAN). InGaAlP light-emitting diode (LEDs) at 660 nm.

B. Culture of SH-SY5Y Cells

The neuroblastoma cell line SH-SY5Y was cultured at 37ºC in a 5% CO_2, humidified air. SH-SY5Y cells were grown in DMEM and HAM's F-12 supplemented with 10% FBS and 1% antibiotic. For the assay, SH-SY5Y cells were sub-cultured in 96-well plates at a seeding density of 1 x 10^4 cells per well or in 6-well plates at a seeding density of 4 x 10^4 cells per well. Cells were treated with the 1µM donepezil hydrochloride before confluence in DMEM phenol red free. One group of cells maintained without any drug or light treatment served as the control and the remaining groups of cells were exposed to various concentrations of drug and light treatments.

C. $A\beta_{1-42}$ Oligomer Preparation

$A\beta_{1-42}$ Oligomer (1mg) was prepared according to the protocol outlined from Invitrogen (Carlsbad, CA, USA). Synthetic $A\beta_{1-42}$ was first dissolved in hexafluoro-2-propanol (HFIP, Sigma-Aldrich, MO, USA), distributed in aliquots, dried (HFIP film). The dried peptide was stored at -20ºC. Before each experiment, the dried peptide was resuspended in dimethyl sulfoxide (Sigma-Aldrich, MO, USA) to a final concentration of 5mM, vortexed thoroughly, and sonicated for 10 min. The peptide was further diluted in ice-cooled phenol red-free DMEM/F12 medium. Then, the peptide was placed at 4ºC overnight to form oligomers.

D. Fluorescent Microscopy

SH-SY5Y cells were seeded at a concentration of 4 x 10^4 cells/well (1 ml/well) in 6-well plates and incubated overnight at 37ºC. To understand the mechanism of Aβ on the cell, 1µM $A\beta_{1-42}$ oligomer, labeled with fluorescein isothiocyanate (FITC- $A\beta_{1-42}$) was taken intervals of 24, 48, 72, and 96h by using the fluorescence microscope. Image processing and analysis in Java software (ImageJ) was used for evaluating the area of FITC-$A\beta_{1-42}$ uptake.

E. MTT Viability Assay

A methylthiazole diphenyl tetrazolium (MTT) assay, based on the determination of the metabolic activity, was used to assess the cell proliferation. SH-SY5Y cells were seeded at a concentration of 1.5 x 10^4 cells/well (100µl/well) in 96-well plates and incubated overnight at 37ºC. $A\beta_{1-42}$ Oligomer solutions, containing 1 µM peptide and various concentrations of donepezil in DMEM serum-free media, were incubated for 48h at 37ºC. To examine the effect of donepezil on the cells (see Fig. 1). After well washing by PBS, 5 mg/ml MTT in serum-free media was added (100µl/well) and incubated for 3h at 37ºC. MTT solution (85 µl) was then removed from each well and replaced with 50 µl of DMSO. Whole plates were then incubated 10 min at 37ºC. Absorbance reading was taken at 570 nm using a microplate reader (Infinite® M200, TECAN). Data are expressed as % viability compared to a 100% signal from untreated cells.

F. Low-Level Light Irradiation

The experiments were conducted with a LEDs which emits visible light with a specific wavelength of 660-nm. The cells were irradiated with a power density of 5mW/cm^2 for 10 min to achieve a total energy density of 3 J/cm^2. The light source was directly placed above the 6-well plate which was used for cell culture. All the experimental groups were designed to realize the effect of light over an irradiation time of 7 days. LEDs light treatment was given once a day.

G. Statistic

All data are presented as means ± SEM of five or more independent experiment. Statistical comparisons between groups were analyzed by one-way ANOVA. Two-tailed p-values less than 0.05 were considered statistically significant.

III. Results

A. Morphological Evidence for FITC-$A\beta_{1-42}$ in SH-SY5Y Cells

$A\beta_{1-42}$ (1µM) labeled with fluorescein isothiocyanate (FITC- $A\beta_{1-42}$) was incubated with the SH-SY5Y cells for 96h and then imaged using fluorescent microscopy at 24, 48, 72, and 96h interval (see Fig. 1).

The results show, 1µM FITC-$A\beta_{1-42}$ can attach the cells and uptake into the cells at 24h until 96h. An apparent morphological change was observed in the cells treated with 1µM FITC-$A\beta_{1-42}$. After 4 days, cells treated with 1µM FITA-$C\beta_{1-42}$ appeared severe morphological change, neurite retraction and cell body shrinkage (see Fig. 1H). Most cells had retracted their neurites, rounded up and formed clusters. These clusters remained attached to the FITC-$A\beta_{1-42}$. Many FITC-$A\beta_{1-42}$ were found within these clusters, which contained cells and cellular debris.

Moreover, FITC-Aβ_{1-42} can self-accumulate and increase its amount in a time-dependent manner. (see Fig. 1 E, F, G, and H).

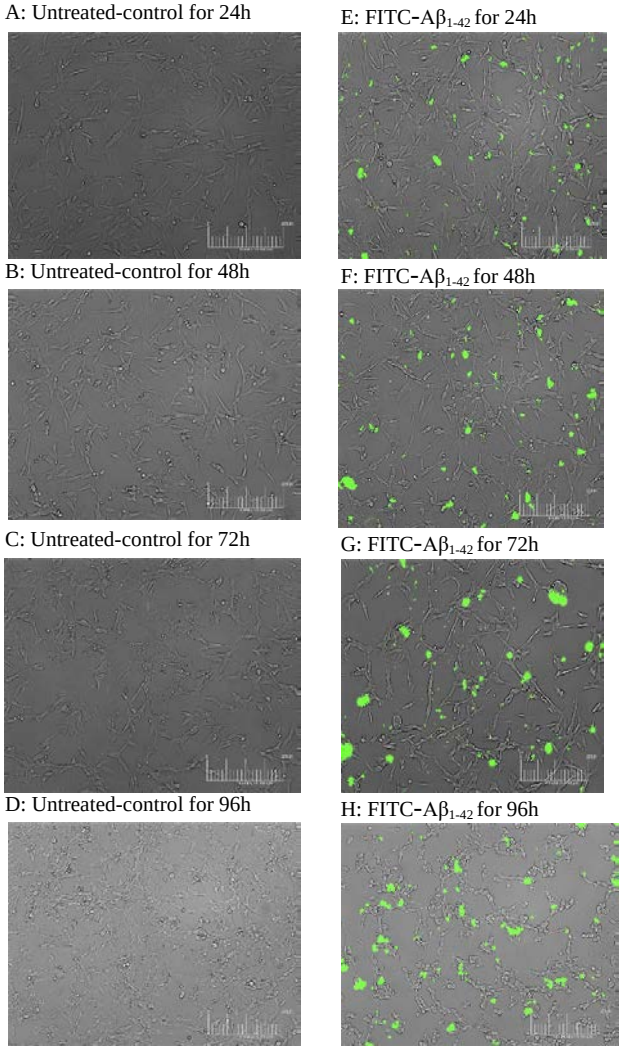

Figure 1. Cell morphology of SH-SY5Y cell incubated with FITC-Aβ_{1-42} for 24, 48, 72, and 96h. (A-D): FITC- Aβ_{1-42} non-treated cells. (E-H): cells treated with 1 μM FITC- Aβ_{1-42}.

B. *Neuroprotective Effect of Donepezil on Aβ_{1-42} - Induced Toxicity*

1) Determination of optimal treatment dose of donepezil for the assessing neuronal viability

To determine the optimal treatment dose of donepezil on neuronal viability, SH-SY5Y neuronal cells were treated with different concentrations of donepezil for 48h. In addition, cell viability was measured using MTT assays. As shown in Fig. 2, cell viability was gradually reduced with increasing concentration of donepezil. Cell viability was 76% at 1μM, 66% at 10μM, 63% at 50μM, and 56% at 100μM, respectively, as compared to non-treated control ($p < 0.05$, in MTT assay). At a concentration of up to 1 μM, donepezil did not have any significant effect on cell viability. Hence, the high concentration of donepezil resulting in loss of cell viability and may be less protection against Aβ_{1-42}-toxicity. Based on these data, 1μM could be selected as a candidate for optimal concentration because more than 70% viability was appropriate in the study of the neuroprotective effect of donepezil on Aβ-induced toxicity.

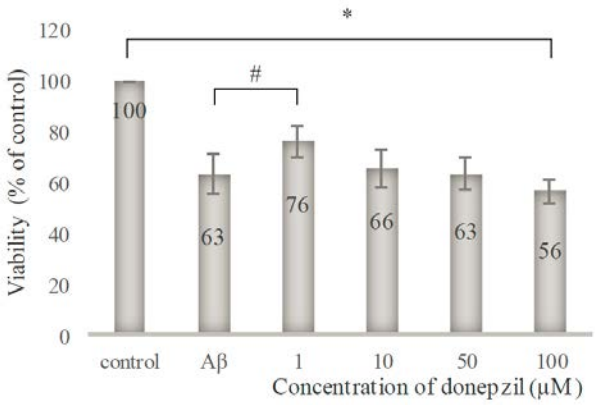

Figure 2. Cell viability of SH-SY5Y cells which exposed to Aβ and various concentration of donepezil for 48h. *P<0.05, significant from the untreated cells, #P<0.01, significant from the group treated with 1 μM Aβ_{1-42} alone.

2) Time- and concentration-dependent protection of neuronal cells from Aβ_{1-42} by donepezil

To determine the time and concentration dependence of neuroprotection provided by donepezil, SH-SY5Y neuronal cells were treated with donepezil (1μM) in three conditions; donepezil pre-treatment, simultaneous-treatment, and donepezil post-treatment. In the presence of 1μM Aβ_{1-42}, pre-treatment of SH-SY5Y cells with 1μM, and 5μM donepezil for 24h before exposed by1μM Aβ_{1-42} for 72h, and simultaneous-treatment between 1μM Aβ_{1-42} and 1μM donepezil for 72h gave significantly increase protection compared with Aβ_{1-42} alone. No protective effect was observed when the cells were post-treated with 1μM donepezil for 24h after exposed by 1μM Aβ_{1-42} for 72h (see Fig. 3). However, 1μM of donepezil pre-treatment has a highest protective effect on Aβ_{1-42}-induced toxicity thereby, 1μM of donepezil pre-treatment was selected as a candidate time and concentration of donepezil to be avoided for 1μM Aβ_{1-42} toxicity effect on neuronal cell death.

C. *Effect of Low-Level Light Therapy (LLLT) on Neuroprotection by Donepezil against 1 μM Aβ_{1-42} Neurotoxicity*

To examine the effect of LLLT on the neuroprotection by donepezil induced by Aβ_{1-42}. SH-SY5Y neuronal cells were treated with LLLT (660 nm, 5mW/cm^2, 3J/ cm^2) together with donepezil (1μM) for 24h before Aβ_{1-42} exposure. Then, both treatments continuous for 7 days. The viability increases significantly provided by donepezil treatment, LLLT treatment, and combination treatment of donepezil and LLLT; 111%, 104%, and 129% viability, respectively (see Fig. 4). In the presence of 1μM Aβ_{1-42}, both donepezil and LLLT treatment have a protective effect by increased the viability in response to Aβ_{1-42} by 51%, and 60%, respectively. Our data also indicate that combination treatment between donepezil and LLLT of SH-SY5Y cells provided the highest

increase in viability when compared with $A\beta_{1-42}$ alone in the absence of any treatments. Moreover, LLLT treatment also shown significantly increase cell viability resulted in 161% when compared to cells untreated control.

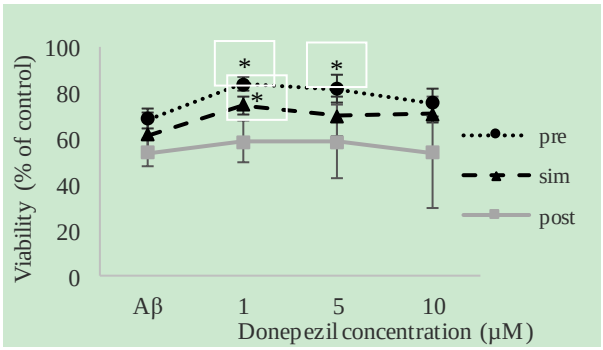

Figure 3. Effect of therapeutic donepezil on 1 μM $A\beta_{1-42}$ induced neuronal death. Cells were treated with several concentrations of donepezil; pre: donepezil pre-treatment of 24h before being exposed to 1μM $A\beta_{1-42}$ for 72h, sim: simultaneous treatment with donepezil and 1 μM $A\beta_{1-42}$ for 96 h., post: donepezil post-treatment of 24h after being exposed to 1μM $A\beta_{1-42}$ for 72 h. *P<0.05, significant from the group treated with 1 μM $A\beta_{1-42}$ alone.

IV. Discussion

The accumulation of Aβ is a key event in the pathogenesis of AD. Aβ-peptide is highly toxic to primary and other cell lines. The recent study examined the effects of a 39-43 amino acid peptide which self-aggregates in a β-pleated sheet conformation as Aβ, in a neuroblastoma SH-SY5Y cell line .These results suggest that the apoptotic cell is the key event involved in Aβ-induced cytotoxicity in SH-SY5Y cells [14], [15]. The accumulation and aggregation of $A\beta_{1-42}$ related to cellular toxicity and reduction of aggregation relate to the loss of toxicity [16]. In addition to, oligomeric $A\beta_{1-42}$ is higher toxic than monomeric $A\beta_{1-42}$ and more toxic than fibrillar $A\beta_{1-42}$ [17]. The important role that Aβ oligomers probably play in neuronal dysfunction [18]. Thus, in this study, $A\beta_{1-42}$ was used as a neurotoxin

In the present study, the SH-SY5Y neuronal cells were exposed to a 1μM FITC-$A\beta_{1-42}$ for 96h in order to observe the apoptotic cell and morphological changes characteristic of $A\beta_{1-42}$. Oligomeric FITC-$A\beta_{1-42}$ has presented to self-accumulation with increasing of time and induced SH-SY5Y neuronal cells death which supports accumulation and aggregation data. This result suggested that the possibility of a molecular interaction resulting in structure changing of SH-SY5Y cells and increasing of the amount of $A\beta_{1-42}$ oligomer. The result also confirms that apoptosis is the major event involved in $A\beta_{1-42}$-induced cytotoxicity in SH-SY5Y cells.

The mechanism of $A\beta_{1-42}$ oligomer may bind to the plasma membrane to form a small annular structure which looks like membrane pore [14]. Alternatively, the $A\beta_{1-42}$ oligomer may impair membrane ATPase activity, thereby causing dysregulation of calcium homeostasis in the neuronal cells which promote the possibility of mitochondrial dysfunction and cell death [15], [16]. In the present report, it is shown that $A\beta_{1-42}$ oligomer decreases the viability of SH-SY5Y cells via mitochondrial function as reported by MTT assay (Fig. 2).

The results of this study provide evidence for the protective effects of LLLT, donepezil, and combination treatment of LLLT and donepezil against $A\beta_{1-42}$-induced toxicity in neuroblastoma SH-SY5Y cells, suggesting the possible therapeutic potential of donepezil in AD. In addition, the discoveries confirm the protective effects of LLLT in the same model and demonstrate an additive protection by the combination of LLLT and donepezil. Thus, the LLLT or the combination treatment of LLLT and donepezil may prove a therapeutic efficacy compared to donepezil alone. Interestingly, the effect of $A\beta_{1-42}$-induced toxicity could be significantly blocked by both donepezil and LLLT treatment. Moreover, the neuroprotective effect of donepezil was unaffected by LLLT, suggesting LLLT has the advantage of stimulation of neuronal cell viability to bring an optimal neuroprotective effect against $A\beta_{1-42}$-induced toxicity.

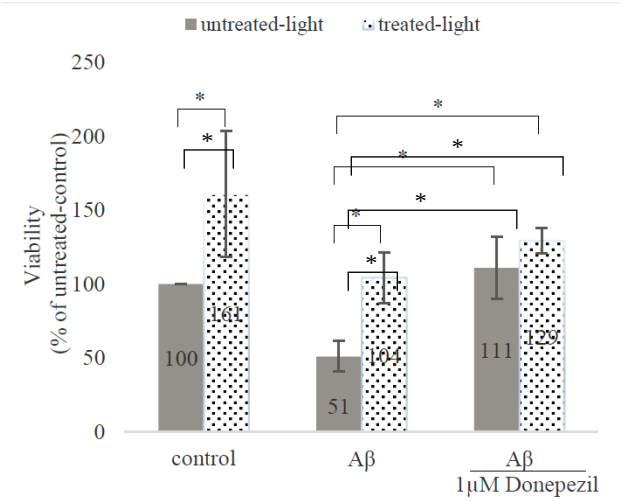

Figure 4. Effect of 1 μM donepezil, LLLT (660 nm, 5mW/cm^2, 3J/cm^2), and their combination of 1μM $A\beta_{1-42}$-induced neurotoxicity on neuronal viability. *P<0.01, significant from two indicated cells.

Neuroprotective effects of donepezil in general and its specific application in AD has been a subject of intense study. Previous studies reported that donepezil exerts its neuroprotective effect by activating PI3K-Akt in various cell types attributed to cognitive function [17], [18]. The dose-response effect of donepezil alone on SH-SY5Y cells viability is shown a dose-dependent decrease in donepezil's protective effects at a concentration above the 1μM threshold. The loss of protection at high concentrations could be explained as blocked the function of nicotinic acetylcholine receptors (nAChR), α7-nAChR mediates the neuroprotective effect of donepezil [19]. Moreover, it can be seen that therapeutic AChE inhibitors protect SH-SY5Y neuronal cells from $A\beta_{1-42}$-induced toxicity in a time- and concentration-dependent manner. The neuroprotective effect of donepezil increased in a pre-treatment time-dependent manner up to 24h, while concomitant treatment with $A\beta_{1-42}$ was lower effective, and post-treatment was not effective. Donepezil shows

concentration-dependent neuroprotective effects for 1μM, the percentage of viability higher than another concentration (Fig. 3). Thus, the effects of donepezil in this condition were presented as an additional increment of neuronal viability.

Neuroprotective effects of Low-Level Light Therapy (LLLT) has been subjecting of the present study. Several studies showed that LLLT has been used to treat the disease of regeneration limitation and promote cell proliferation. LLLT, as a relatively noninvasive technique that enhances both cell survival and proliferation [20]. LLLT uses different wavelengths of the visible and near-infrared (NIR) spectra. Many studies have attempted to understand the action of LLLT, as well as to determine the most appropriate, period of irradiation, energy density, and energy total [21]-[23]. Previous studies investigated the signaling pathway of LLLT with 660 nm responsible for anti-inflammation, probably because of an increase in cAMP level [24]. Furthermore, LLLT has been demonstrated to be useful for stimulating the proliferation of several cell types. Recent works provided an example of this situation, observing that cell viability increased after LLLT (660 or 780 nm, 2 to 6 J/cm^2) [25]. However, there are also reports that LLLT delivered at low fluences of light (both red and NIR) can inhibit apoptosis via Akt/GSK3beta signaling pathway [26]. In a seemingly antithetical model higher fluences of light can have deleterious effects on cells by inducing apoptosis via generation of high levels of reactive oxygen species (ROS) [27] and via the same Akt/GSK3beta signaling pathway [28], [29].

The mechanism of LLLT at the cellular levels is also related to the absorption of the light by components of the respiratory chain. It has been indicated that cytochrome c oxidase can be activated by LLLT, resulting in anti-neuroinflammatory and reduced neuronal cell death [30]. In this study, the cell proliferation values were assessed via MTT assay as the results of bioactivation applied to in vitro AD model at different treatments. Generally, it was seen that the cell viability rates were higher in the treatment groups than in the control groups. However, a lower cell viability was detected in those cells induced with A$\beta_{1\text{-}42}$. Percent of cell viability was less than 70% after treatment with A$\beta_{1\text{-}42}$, that means high toxicity occur. While LLLT at an energy density of 3J/cm^2 and irradiance of 5mW/cm^2 could reverse this effect through cell metabolic activation (Fig. 4). Moreover, the MTT assay showed that LLLT had stimulatory effects on the cellular viability of SH-SY5Y cells. Similarly, donepezil's protection against A$\beta_{1\text{-}42}$, a cell viability was not reduced by either A$\beta_{1\text{-}42}$ or LLLT. Hence, interpretation of the mechanism action of LLLT or donepezil in this paradigm is critical in the further development of appropriate synergistic therapies for neurodegenerative disease.

Although the present *in vitro* AD model finding suggest an added beneficial effect by combining donepezil with LLLT for AD therapy, further verifications of these results in primary cell cultures together with *in vivo* model are necessary. Furthermore, it should be noted that both donepezil and LLLT at higher doses may exert their own toxicity.

The results of this study also confirm that at least some of the neurotoxicity effects of A$\beta_{1\text{-}42}$ are mediated through disturbance with the cell-function as well as stimulation of the apoptotic cell death. However, A$\beta_{1\text{-}42}$ toxicity was blocked by donepezil and/or LLLT. Since A$\beta_{1\text{-}42}$-induced toxicity may be a major contributor to neuronal cell death, the current findings suggest possible utility of donepezil-LLLT combination in *in vitro* AD model. To our knowledge, this is the first documentation of such an effect by donepezil, LLLT, and donepezil/LLLT combination treatment against A$\beta_{1\text{-}42}$-induced toxicity in SH-SY5Y cells. The synergistic protective effects of donepezil and LLLT be beneficial in AD patients may be an alternative treatment in the future.

V. Conclusion

The current study demonstrates that neuronal toxicity in SH-SY5Y cells by A$\beta_{1\text{-}42}$ accumulation plays important roles in cellular model of AD. While donepezil increases SH-SY5Y cell viability, depending on the concentration and the time of treatment. In addition to the LLLT realizes its protective effect via activating metabolism of the cells, at an energy density of 3J/cm^2 and irradiance of 5mW/cm^2 increased the neuronal viability. Taken together, these findings demonstrate the importance of synergistic protective effects of donepezil and LLLT in SH-SY5Y cells against A$\beta_{1\text{-}42}$-toxicity.

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