Title

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Journal

The Journal of nutritional biochemistry, 22（1）

Published

2011 Jan

URL

https://doi.org/10.1016/j.jnutbio.2009.11.005

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Docosahexaenoic acid withstands the Aβ$_{25-35}$-induced neurotoxicity in ~~SH-S5Y5~~ SH-SY5Y cells

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Received 12 April 2009; received in revised form 30 October 2009; accepted 6 November 2009

Abstract

Background: Docosahexaenoic acid (DHA, C22:6, n-3) ameliorates the memory-related learning deficits of Alzheimer's disease (AD), which is characterized by fibrillar amyloid deposits in the affected brains. Here, we have investigated whether DHA-induced inhibition of Amyloid β-peptide$_{25-35}$ (Aβ$_{25-35}$) fibrillation limits or deteriorates the toxicity of the human neuroblastoma cells (~~SH-S5Y5~~).

Experimental methods: In vitro fibrillation of Aβ$_{25-35}$ was performed in the absence or presence of DHA. Afterwards, ~~SH-S5Y5~~ cells were incubated with Aβ$_{25-35}$ in absence or presence 20 μM DHA to evaluate its effect on the Aβ$_{25-35}$-induced neurotoxicity by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)]-redox and TUNEL (TdT-mediated dUTP-biotin nick end-labeling) assay and immunohistochemistry. The level of Aβ$_{25-35}$-induced lipid peroxide (LPO) was determined in the absence or presence of oligomer-specific antibody. Fatty acid profile was estimated by gas chromatography.

Results: DHA significantly reduced the Aβ$_{25-35}$ in vitro fibrillation, as indicated by fluorospectroscopy and transmission electron microscopy. Aβ$_{25-35}$ decreased the MTT-redox activity and increased the apoptotic damage and levels of LPO when compared with those of the controls. However, when the SH-SY5Y cells were treated with Aβ$_{25-35}$ in the presence of DHA, MTT redox potential significantly increased and the levels LPO decreased, suggesting an inhibition of the Aβ$_{25-35}$-induced neurotoxity. DHA improved the Aβ$_{25-35}$ induced DNA damage and axodendritic loss, with a concomitant increase in the cellular level of DHA, suggesting DHA protects the cell from neurotoxic degeneration.

Conclusion: DHA not only inhibits the in vitro fibrillation but also resists the Aβ$_{25-35}$-induced toxicity in the neuronal cells. This might be the basis of the DHA-induced amelioration of Aβ-induced neurodegeneration and related cognitive deficits.

Keywords: Aβ$_{25-35}$ fibrillation; Docosahexaenoic acid; Neurotoxicity; ~~SH-S5Y5~~ cells

1. Introduction

Docosahexaenoic acid (DHA, C22:6, n-3), the predominant synaptosomal plasma membrane polyunsaturated fatty acid (PUFA) of the brain, is gaining ever more attention because of its protective [1] and preventive [2] effects on the impairments of memory-related learning ability in the Alzheimer's disease (AD) model animals including rats [1,2] and mice [3]. Epidemiological study also supports that plasma concentration of DHA is correlated with the AD symptoms [4]. AD is pathologically characterized by neuritic plaques and neurofibrillar tangles of amyloid beta peptides (Aβs) such as Aβ$_{1-42}$ and Aβ$_{1-40}$ [5]. After the proteolytic cleavage from membrane-bound amyloid precursor proteins (APP), the Aβ$_{1-42}$ deposited largely in the brain tissues of affected patients, while Aβ$_{1-40}$ is concentrated predominantly in the cerebrospinal fluids [6]. Though they are considered as the principal forms of Aβs, however, other short fragments of the Aβs might be involved in the pathogenesis of AD. Among them the short fragment Aβ$_{25-35}$ is of particular interest. This short sequence has been identified in the brains of aged patients Aβ$_{1-40}$ [7]. Aβ$_{25-35}$ is thus biologically active fragment of Aβ [8], indicating this short filament can render toxicity to neurons.

Xu et al. [9] reported that this truncated amyloid can exhibit equal potencies to that of the Aβ$_{1-40}$. What's more important is that Aβ$_{25-35}$, as being the terminal sequence of the Aβ$_{1-40}$ and/or Aβ$_{1-42}$, it may help in the understanding of the mechanism of fibrillation of the full length Aβs. We have recently reported in vitro studies that Aβ$_{25-35}$ is able to form fibrils [10] analogous to that of the full-length Aβ$_{1-40}$ [11] and that DHA can inhibit the fibrillation of both Aβ$_{25-35}$ [10] and Aβ$_{1-40}$ [11], thus suggesting Aβ$_{25-35}$ peptide can confer toxicity analogous to that of the full-length peptide in neurons. This toxicity may underlie the learning-related memory impairments of mice after the cerebroventricular infusion of Aβ$_{25-35}$ [12]. We recently found that DHA

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doi:10.1016/j.jnutbio.2009.11.005

inhibits the Aβ_{25-35} fibrillation [10]; however, DHA produced diffused and amorphous-type conformations. The question thus that remains to be confirmed whether these amorphous conformation further intoxicates the neuronal cells or whether inhibits the toxicity of these cells. The brain utilizes large amounts of DHA [13–15] and the level of DHA decreases in the hippocampus of AD patients [16,17], thus demonstrating that DHA have a significant role in the nurture of brain functions. Neuron lacks the ability to biosynthesize adequate DHA, thus DHA is taken into the neural cells from the extraneuronal medium after its release from the astroglial/cerebral capillary endothelial cells [18,19]. Thus, it is very likely that DHA inexorably endures an interaction with the extra-neuronally deposited amyloid fibrillar species, which render toxicity to neurons leading to neurodegenerations. Therefore, the study on the effect of DHA on the Aβ_{25-35}-fibriltion-induced neurotoxicity is of special significance. In this study we intended to prove whether the DHA-induced inhibition positively and/or negatively impacts the toxicity in the ~~SH-SY5Y Neuroblastoma cells~~.

SH-SY5Y

2. Materials and methods

2.1. Materials

(Minneapolis, MN, USA)

Aβ_{25-35} was purchased from the Peptide Institute (Osaka, Japan); thioflavin T (ThT) was purchased from Sigma-Aldrich (St. Louis, MO, USA); mouse antitubulin antibody (Tuj1) from the R&D Systems, ~~USA~~). Apoptosis Detection Kit, Millipore, ~~USA~~). Alexa 488-conjugated secondary antibody and Rabbit polyclonal anti-oligomer antibody (A11) were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical grade.

from

(Billerica, MA, USA)

2.2. Aβ_{25-35} preparation

Aβ_{25-35} was dissolved in 1,1,1,3,3,3- hexafluoro-2-propanol (HFP) at concentration of 500 μM to produce uniform, non-aggregated Aβ and immediately stored at −80°C after N_2 bath until use. At the day of use, the HFP-dissolved amyloid samples were initially spun down at 13,800×g, if any, then was blown by N_2 gas at ice-cold temperature and re-dissolved in the assembly buffer for aggregation study.

2.3. Preparation of DHA

Fifty milligrams of DHA dissolved in 200 μl ethanol (commercially available; Cayman Chemical, Arbor, MI, USA) was stored (in 5.0-μl aliquots) at −80°C until use. It was directly suspended in ultrapure water and used at desired concentration containing 0.002% ethanol. Only freshly prepared DHA was used.

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2.4. Aβ_{25-35} fibrillation

Prior to use in the cell culture, in vitro Aβ_{25-35} fibrillation was carried out as described previously [10,11] with some modifications. Hexafluoropropanol was blown from the Aβ_{25-35} stock-aliquot, and the peptide was immediately suspended in a desired volume of assembly buffer [~~(~~100 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl, 0.01% sodium azide~~)~~] at final concentration of 50 μM of Aβ_{25-35} with or without DHA. The final concentration of DHA was 20 μM. The reaction mixture was taken into oil-free polymerase chain reaction tubes (Takara Shuzo, Otsu, Japan), flushed with nitrogen gas to obviate any effect of atmospheric oxygen, and incubated at 37°C for 24 h. The incubation was stopped by placing the tubes on ice and then subjected to thioflavin T fluorescence spectroscopy.

2.5. Thioflavin T fluorescence assay of Aβ_{25-35}

After 24 h of incubation at 37°C for fibrillation, 40-μl aliquots from each tube were gently removed and mixed with 210 μl of 5 μM thioflavin T (ThT) in 50 mM glycine-NaOH buffer (pH 8.5) and subjected to fluorescence measurements (Hitachi F-2500 fluorescence spectrophotometer) at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 448 and 487 nm, respectively.

2.6. Transmission electron microscopy

After completion of Aβ_{25-35} fibrillation for 24 h at 37°C with or without DHA, an aliquot was used for electron microscopy. In brief, a 4-μl sample was placed on a copper grid, stained with 1% uranyl acetate, excess uranyl acetate was then removed from the grid using distilled water. Afterwards, the grid was air dried and examined under a Hitachi H-7000 transmission electron microscope with an operating voltage of 75 kV.

2.7. Cell culture

Human SH-SY5Y neuroblastoma cells were obtained from the European Collection Cell Culture and originally maintained in Ham's F12: Minimum Essential Medium Eagle (Sigma-Aldrich) (1:1) containing 15% fetal bovine serum, 50 IU/ml penicillin G and 50 mg/ml streptomycin in 6 cm culture dish (Corning, Corning, NY, USA) at a density of 2×10^5 cells per dish. The cells were passaged and cultured in 96-well plate at a density of 1×10^4 cells per well for 2 days. The culture medium was replaced to serum-free Opti-MEM (Gibco) supplemented with or without Aβ_{25-35} and 0.5 μM DHA. After 2 days of treatment, cells were used for MTT assay and immunofluorescence microscopy. We chose to conduct our in vitro studies in the absence of bovine serum albumin (BSA), because preliminary experiments showed that the presence of physiologic concentrations of BSA (i.e., 100 μg/ml or 0.01%) prevented the toxicity of ~~Abeta~~ treatment.

Aβ

2.8. MTT assay

The cytotoxicity of Aβ_{25-35} peptide was assessed by measuring cellular MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)]-redox activity, which detects active mitochondrial dehydrogenases of living cells to reduce MTT to a water insoluble blue formazan products. Cells at a density of 1×10^4/well were placed in 96-well plates with 100 μl of fresh medium. After 24 h, the medium was replaced with 100 μl of OPTI-MEM (Gibco BRL) serum-free medium and 10 μM Aβ_{25-35} peptide. The cells were incubated at 37°C in 5% CO_2 for 48 h, afterwards 10 μl of MTT (Dojudo) (5 mg/ml) was added to each well and the plate was incubated at 37°C for 4 h. The MTT solution was then removed, ~~DMSO~~ (100 μl) was added, and the plate was shaken for a few min and read at 550 nm with an enzyme-linked immunosorbent assay plate reader.

dimethyl sulfoxide

2.9. TUNEL assay

The apoptotic nuclei containing free 3′-OH termini were detected by using a TUNEL (TdT-mediated dUTP-biotin nick end-labeling) assay kit (ApopTag Red in situ, Apoptosis Detection Kit, Millipore) according to the manufacturer's protocol with slight modifications. ~~Aβ_{25-35} incubated cells with (Aβ_{25-35}+DHA) or without (DHA)~~ were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 and post-fixed with ethanol:acetic acid (2:1, v:v) for 5 min at -20°C. After incubating with the TUNEL reaction mixture, anti-digoxigenin conjugated with rodamine was added. The TUNEL-positive cells were detected by fluorescent microscope.

The cells

2.10. Cellular morphology study

For morphological immunofluorescence microscopy, cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed with 0.1 M Tris-buffered solution (TBS; pH 7.5), blocked with 3% normal goat serum (Dako Cytomation, Carpinteria, CA, USA) in TBS containing 0.3% Triton X-100 at room temperature for 60 min, and incubated with primary antibodies at 4°C overnight. The primary antibody was mouse anti-neuron-specific class III beta-tubulin (Tuj-1, 1:1000, R&D Systems). The cells were washed with TBS and incubated with Alexa Fluor 488-conjugated secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA) at room temperature for 60 min. To visualize nuclei, the cells were counterstained with 2 μg/ml propidium iodide (Dojindo laboratories). Finally, the cells were mounted with 80% glycerol, visualized under a fluorescent laser microscope (CLMS FV300, Olympus, Tokyo, Japan).

SH-SY5Y

2.11. ~~SH-SY5Y~~ cell preparation for lipid analyses

The cells were harvested and washed thrice with PBS containing protease inhibitors cocktail. Afterwards, the pellets were homogenized using 10 strokes in a dounce homogenizer and 10 passages through a 22-gauge syringe on ice. The samples were then directly used for the fatty acid composition and lipid peroxide (LPO) analyses.

2.12. Fatty acid composition

Fatty acid composition was determined by the one-step analysis of Lepage and Roy (1986) [20] as described previously [1,2,11] using gas liquid chromatography.

2.13. LPO levels and protein

LPO concentration was assessed by the thiobarbituric acid reactive substances assay of Ohkawa et al. [21], as described previously [22]. Protein concentration was estimated by the method of Lowry et al. [23].

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2.14. Statistical analyses

Results are expressed as means±S.E.M. For two-group differences, data were analyzed by Student's *t* test. For more than two groups, the data were subjected to one-way analysis of variance (ANOVA), followed by Bonferroni post hoc comparisons. The statistical programs used were GBSTAT 6.5.4 (Dynamic Microsystems, Silver Spring, MD, USA) and StatView 4.01 (MindVision Software; Abacus Concepts, Berkeley, CA, USA). $P<.05$ was considered statistically significant.

3. Results

Fig. 1

3.1. Effects of DHA on in vitro $A\beta_{25\text{-}35}$ fibrillation and fiber morphology

Formation of $A\beta_{25\text{-}35}$ fibers at 50 μM was measured, alone and in the presence of 20 μM DHA. We found that $A\beta_{25\text{-}35}$ monomer at final concentrations of 50 μM incubated for 24 h in the assembly buffer had significantly higher thioflavin T fluorescence intensity. When the fibrillation was commenced in the presence of DHA (20 μM), the degree of fibrillation significantly decreased by about 43% (~~Fig. 1~~).

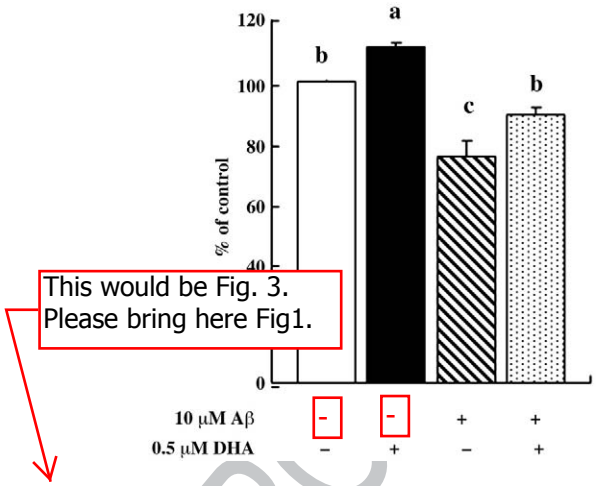
To confirm the inhibitory effect of DHA on the $A\beta_{25\text{-}35}$ fibrillation, Aβs fibrils with or without DHA (20 μM) were viewed under a transmission electron microscope. The control samples ($A\beta_{25\text{-}35}$ alone) exhibited abundant aggregated $A\beta_{25\text{-}35}$ fibrils both with a ribbonic and round morphology. Consistent with the ThT fluorescence data, the $A\beta_{1\text{-}42}$+DHA samples contained only very small amount of poorly defined fibrils, if at all. In the presence of DHA, the fibril contents were practically very poor and appeared as densely amorphous conformations (~~Fig. 1~~). The lengths of the $A\beta_{25\text{-}35}$ fibers were not determinable due to extensive branching; however, the widths were 5–6 nm.

Fig. 2

3.2. Effect of DHA on $A\beta_{25\text{-}35}$ induced cytoxicity

Fig. 3

As shown in the ~~Fig. 2~~, DHA alone had increased the MTT-redox potential as compared to that of the vehicle treated controls. The $A\beta_{25\text{-}35}$ significantly decreased (by >22%) the MTT-redox potential in the ~~SH-S5Y5~~ cells, whereas DHA had inhibitory effect on toxicity when fibrillation of $A\beta_{25\text{-}35}$ occurred in its presence, as indicated by the increase of MTT-redox potential in the $A\beta_{25\text{-}35}$+DHA cells.



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~~Fig. 2.~~ The cytotoxicity of $A\beta_{25\text{-}35}$ was assessed by measuring MTT-redox activity. The MTT assay measures cell survival. Cells were used at a density of 1×10^4/well. The absorbance of the untreated cells read at 550 nm was normalized to 100%. Data shown were from four experiments in quadruplicate determinations ($P<.05$).

SH-SY5Y

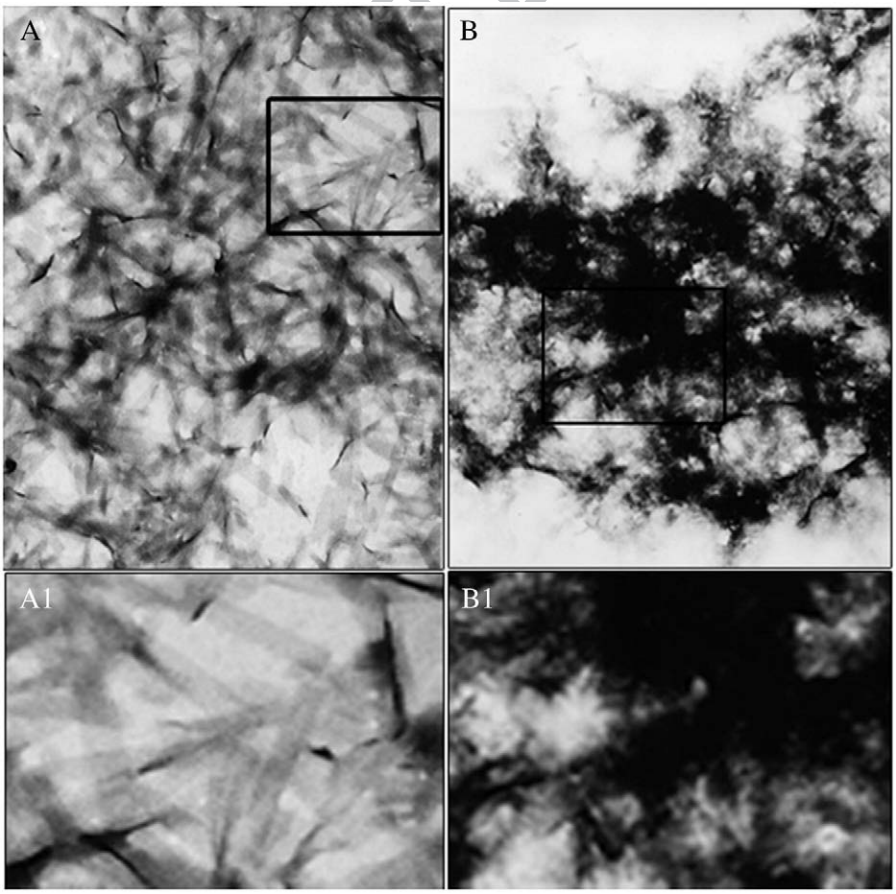

Fig. 2

Q3 ~~Fig. 1.~~ Representative transmission electron micrograph of the effects of DHA on the ~~Ab25-35~~ fibril morphology. $A\beta_{25\text{-}35}$ peptide (50 μM) was incubated in the absence (A) or presence (B) of 20 μM DHA for 24 h at 37°C; 4-μl of samples was subjected to 400-mesh grid, dried for 1 min, stained with 1% uranylacetate and subjected to visualization by electron microscope. The morphology of the control fibrils was structured and clear (A and its inset A1), while those of the DHA-treated samples had highly unstructured (B and its inset B1) and mostly amorphous type consistency.

$A\beta_{25\text{-}35}$

3.3. Effect of DHA on the Aβ25-35-induced apoptosis

Aβ25-35-induced apoptosis in the SH-SY5Y cells, as indicated by the increased abundance of TUNEL-positive nuclei in these cells (Fig. 3). The characteristic nuclear fragment of the apoptotic cells was clearly observed in the SH-SY5Y cells. In addition, condensed nuclei and nuclear fragements were also found. The TUNEL-positive nuclei were significantly lower in the DHA+Aβ25-35-treated cells. Also, the TUNEL-stained nuclei were comparable between DHA-alone treated and the untreated control cells, indicating DHA did not induce an extra apoptotic stress in the SH-SY5Y cells.

3.4. Effect of DHA on the Aβ25-35 toxicity-induced cellular morphology

As shown in the Fig. 4, DHA alone significantly ameliorated the morphology of the SH-SY5Y cells, as compared to the vehicle-treated cells. The DHA-treated cells had well-viewed morphology with healthy axodendritic processes. However, a 48-h treatment of the Aβ25-35 prompted dramatic alterations in neuronal morphology. Most of the cells tended to lose their characteristic shape, acquiring an unnatural shape and showing few or no neuritic processes. They had lost the axodendritic processes. However, when the Aβ25-35-treated SH-SY5Y cells were examined after coincubation with DHA, the loss of axodendritic processes recovered with the appearance of well-defined sprouting processes (lower right), indicating an addition of DHA to the Aβ25-35+SH-SY5Y cells prevented the toxicity of Aβ25-35.

3.5. Effect of DHA on the fatty acid profile

As shown in the Table 1, the levels of saturated fatty acids palmitic and stearic acid and monounsaturated fatty acid oleic acid were not altered in either of the DHA or DHA+Aβ-treated cells in the absence or presence of oligomer-specific antibody (A11), when compared to those of the untreated controls. The levels of linoleic acid, arachidonic acid were significantly increased in the Aβ-treated cells. The levels of linolenic, eicosapentaenoic and docosapentaenoic acid were not affected. As expected, the levels of DHA were significantly increased in both the DHA and DHA+Aβ25-35-treated cells either in the absence (Aβ25-35+DHA) or presence of oligomer antibody (Aβ25-35+DHA+A11 cells). Finally, changes in the fatty acid profile resulted in a significant increase in the unsaturation index (USI) of DHA-treated cells.

3.6. Effect of DHA on the lipid peroxide levels of SH-SY5Y cells

The level of lipid peroxide (LPO) significantly decreased in the DHA-treated cells (~26%) when compared to those in the untreated controls. The level of LPO was significantly increased (>15%) in the Aβ25-35-alone-treated cells while the levels of LPO further increased (41%) in the Aβ25-35+DHA cells (Fig. 6A).

When the SH-SY5Y cells were incubated with Aβ25-35 in the presence of A11 (oligomer-specific antibody), the levels of LPO reverted to those of the untreated controls, and significantly decreased when compared with those of the Aβ25-35+DHA cells. The LPO level also decreased in the presence of A11 (in the Aβ25-35

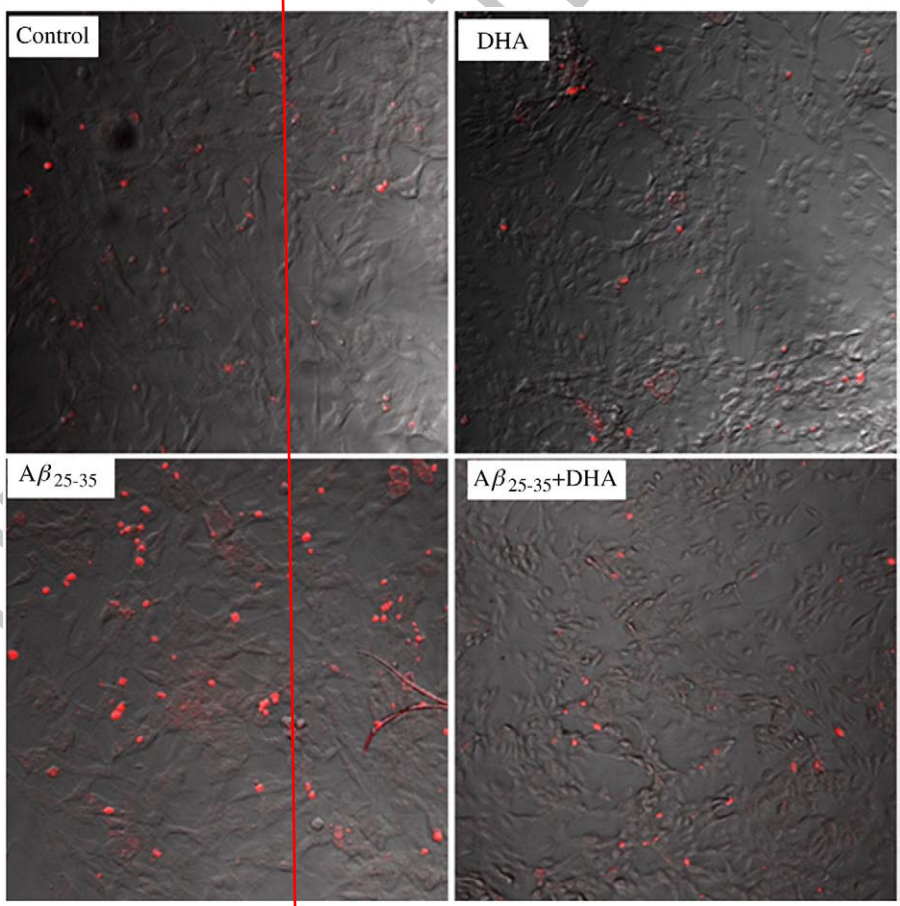

Fig. 3. Effect of DHA on the Aβ25-35-induced apoptosis. Representative fluorescence images of control (vehicle-treated) cells (upper left) and DHA-treated (0.5 μM) cells (upper right). TUNEL-stained nuclei (red) were increased after the treatment of SH-SY5Y cells with Aβ25-35 for 48 h, whereas, the DHA treatment of the cells (Aβ25-35+DHA) significantly reduced apoptosis, as indicated by the reduced number TUNEL-stained cells.

Fig. 4
Fig. 4
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Fig. 5
SH-SY5Y
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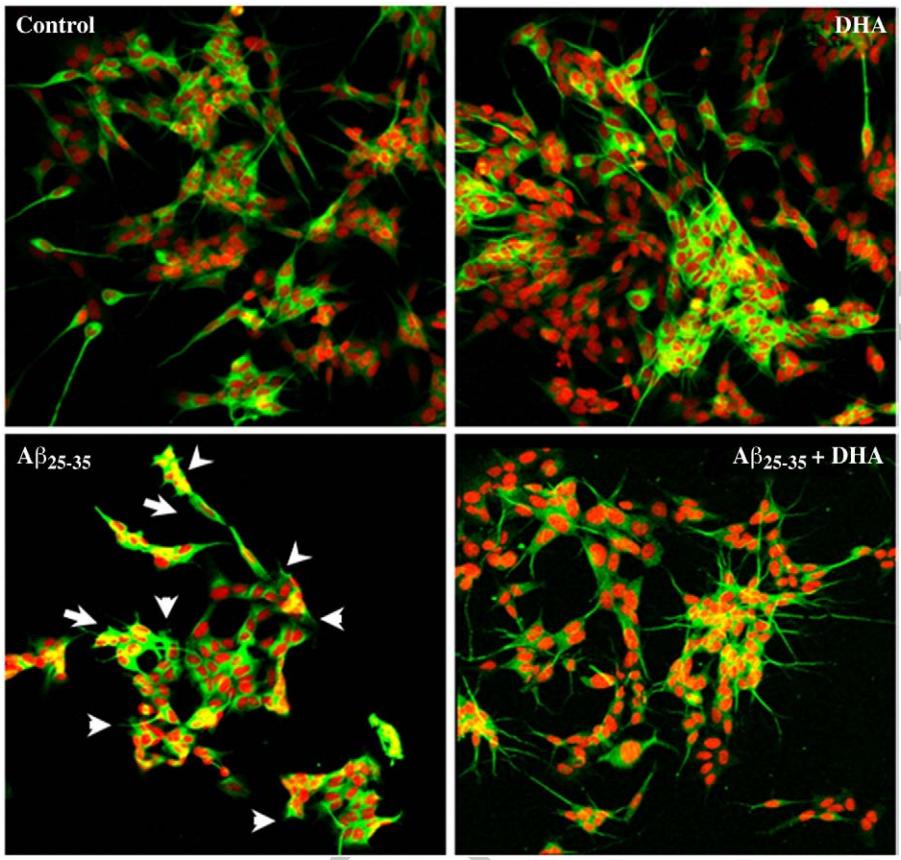

Fig. 5

Fig. 3

~~Fig. 4.~~ Fluorescence images of control (vehicle-treated) cells (upper left) and DHA-treated (0.5 μM) ~~SH-SY5Y~~ cells (upper right). Altered neuritic sprouting with dystrophic axodendritic systems are clearly observed after treatment with $A\beta_{25\text{-}35}$ for 48 h (lower left). DHA inhibited the toxicity; however, (as determined by the MTT assay in ~~Fig. 5~~) with the appearance of well-defined axodendritic sprouting processes (lower right). Fluorescent signals were then visualized by the confocal laser microscope system (CLSM FV300, Olympus, Tokyo, Japan) and processed by Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

SH-SY5Y

+DHA+A11 cells). A11 alone did not have any significant effect on oxidative stress. An alteration of the level of LPO was not significantly associated with the unsaturation index (Fig. 6B).

(data not shown)

4. Discussion

The purpose of the current study is to evaluate whether DHA can successfully inhibit the $A\beta_{25\text{-}35}$-induced toxicity in the human neuroblastoma cells ~~(SH-SY5Y)~~. The observation that DHA inhibits the $A\beta_{1\text{-}40}$-induced neurotoxicity and the memory impairments of the $A\beta_{1\text{-}40}$-infused Alzheimer's disease model rats [11] led us to hypothesize that DHA would ameliorate toxicity produced by the $A\beta_{25\text{-}35}$ peptide. While this is a hypothesis, currently, there is no direct experimental evidence to support the outcome of DHA on neurotoxicity and morphological deteriorations. Thus, we directly commenced $A\beta_{25\text{-}35}$ fibrillation in the cell culture media in the presence of DHA whether it positively impacts the neurotoxicity. DHA inhibited the in vitro fibril formation. Although the TEM data presented in ~~Fig. 2~~ illustrate that in the presence of DHA the $A\beta_{25\text{-}35}$ is transformed into an amorphous conformation rather than a fibril form, these amorphous structures, however, are not toxic; instead they render $A\beta_{25\text{-}35}$ less toxic; and otherwise, DHA could not have inhibited the toxicity in the ~~SH-SY5Y~~ cells. Here, we clearly demonstrate that that DHA inhibits the in vitro fibrillation of $A\beta_{25\text{-}35}$ with a concomitant inhibition of fibrillation-induced neurotoxicity of the ~~SH-SY5Y~~ cells.

Neurotoxicity in AD results from enhanced cellular processing of APP, interactions of Aβ with cell membranes itself, generations of [illegible]xygen species and abnormality in the protective response to [illegible]tress and/or susceptibility to apoptotic stimuli [24–29]. Therefore, we carried out two experiments to study the $A\beta_{25\text{-}35}$-induced cellular perturbation: suppression of cellular capacity to reduce MTT and induction of apoptosis. The MTT assay estimates the mitochondrial redox potential of live cells, in this case ~~SH-SY5Y~~ cells, and thus monitors cell condition and the cells in good physical shape reduce MTT, turning the redox dye from yellow to purple/blue,

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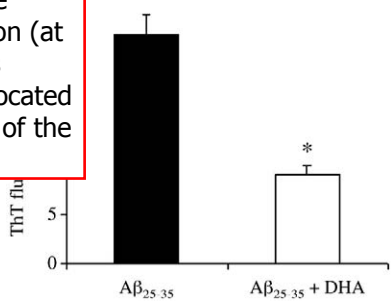
Fig. 1



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~~Fig. 5.~~ The effect of DHA on in vitro fibrillation of $A\beta_{25\text{-}35}$. For fibril formation, $A\beta_{25\text{-}35}$ peptides (50 μM) were incubated at 37°C for 24 h in the presence or absence of 20 μM of DHA. After completion of fibrillation, 40 μl of the sample was added to 210 μl of 5 μM ThT in glycine buffer (pH 8.5), and fluorescence intensity was measured at excitation and emission wavelengths of 448 and 488 nm, respectively. Results are means±S.E.M. (n=5). Significant difference at $^{*}P<.05$ (unpaired Student's t test).

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mg protein

SH-SY5Y

0.3

Table 1
Effects of DHA (0.5 μM)-treatment on the fatty acid profile (μg/mg) of the SH-SY5Y cells with or without $A\beta_{25-35}$-oligomer-antibody

	PLA	STA	OLA	LLA	LNA	AA	EPA	DPA	DHA	USI
Control	30±2.5[a]	22±2.4	28±2.7	0.97±0.2[b]	0.1±0.0	7.2±0.5[a]	0.6±0.1	1.0±0.1[b]	2.3±0.3[d]	81±1.3[b]
DHA	24±2.7[b]	21±2.0	24±2.0	1.3±0.1[a,b]	0.1±0.0	5.5±0.4[b]	0.5±0.06	1.2±0.15[a,b]	5.0±0.05[a,b]	97±5.8[a]
$A\beta_{25-35}$	29±1.7[b]	19±1.9	28±1.5	1.7±0.2[a]	0.09±0.0	7.5±0.7[a]	0.6±0.10	1.2±0.1[a,b]	2.2±0.15[c,d]	86±3.0[b]
$A\beta_{25-35}$+DHA	26±1.0[b]	18±1.8	24±0.5	0.8±0.3[b]	0.1±0.0	5.7±0.4[b]	0.6±0.10	1.3±0.1[a,b]	4.3±0.4[b]	96±3.0[a]
$A\beta_{25-35}$+A11	27±1.1[b]	18±0.5	26±1.0	1.3±0.2[a,b]	0.1±0.0	7.2±0.5[a,b]	0.4±0.02	1.3±0.02[a,b]	2.8±0.06[c]	85±2.8[b]
$A\beta_{25-35}$+DHA+A11	25±2.5[b]	19±3	27±1.8	1.0±0.0[a,b]	0.09±0.0	6.3±0.9[a,b]	0.6±0.1	1.6±0.1[a]	5.7±0.4[a]	105±5.0[a]

Results are mean±SEM (n=3) of triplicate determinations. Values in the same column that do not share a common superscript[a–c] are significantly different at P<.05 (one-way ANOVA followed by Bonferroni post hoc test).
PLA, palmitic acid (C16:0); STA, stearic acid (C18:0); OLA, oleic acid (C18:1, n-9); LLA, Linoleic acid (C18:2, n-6); LNA, Linolenic acid (C18:3, n-3); AA, Arachidonic acid (C20:4, n-6); EPA, Eicosapentaenoic acid (C20:5, n-3); DPA, Docosapentaenoic acid (C22:5, n-3);
Unsaturation index was calculated as [(mole% of each (poly)unsaturated fatty acid X number of double bond(s) per fatty acid].

Please add "A11, oligomer-specific antibody.

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Fig. 4

(Fig. 3)

how less of a color changes. DHA alone MTT-redox efficiency in the SH-SY5Y cells when compared with that of the DHA-untreated cells, suggesting DHA can boost up the redox potential of the cells (Fig. 2). The decreased MTT redox levels in the $A\beta_{25-35}$-treated SH-SY5Y cells are consistent with other studies reporting that $A\beta_{25-35}$ inhibits the cellular reduction of MTT [30]. Considering it a measure of cell viability, the percent reduction of MTT was found to be significantly higher in the $A\beta_{25-35}$+DHA cells than in $A\beta_{25-35}$-incubated cells, indicating that the higher redox activity could be ascribed to DHA in the $A\beta_{25-35}$+DHA cells. Then, we carried out whether $A\beta_{25-35}$-induces

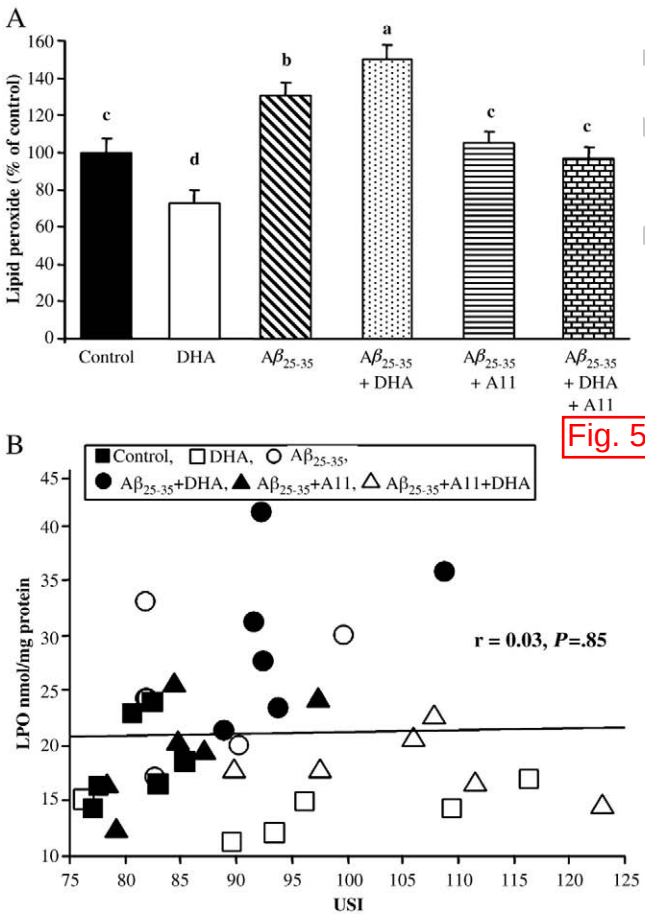

Fig. 6. (A) Effect of DHA in the presence of oligomers' conformation-specific antibody (A11). Results are mean±S.E.M., n=3 each with triplicate determinations. Bars with different letters are significantly different at [a-c]P<.05 (One-way ANOVA). B: Correlation between LPO content and USI of SH-S5Y5 cells.

Fig. 5

an apoptosis via DNA fragmentation and whether such an apoptotic) could be intervened by DHA with the use of TUNEL assay.

As shown in the Fig. 3, $A\beta_{25-35}$ induced severe apoptosis. The $A\beta_{25-35}$(alone)-treated cells underwent nuclear condensation and segmentation, as indicated by the increased DNA strand breaks, which were detected by enzymatically labeling the 3-OH termini with modified nucleotides in TUNEL assay. These new DNA ends were typically localized in morphologically identifiable nuclei, and hence the numbers of the TUNEL-dye positive cells were higher in the $A\beta_{25-35}$-treated cells. Cultures exposed to DHA showed inhibition of $A\beta_{25-35}$-induced apoptosis, as indicated by the reduced number of TUNEL-positive nuclei in the $A\beta_{25-35}$+DHA cells. These findings thus further support the MTT data that DHA inhibits the $A\beta_{25-35}$-induced toxicity/apoptosis of the SH-SY5Y cells. The result of the decreased number of TUNEL-positive nuclei in the DHA-treated cells also is qualitatively consistent with our previous in vivo investigation [1], where we reported that dietary administration of DHA decreases apoptosis marker such as histone-associated DNA fragmentations in the cortical tissues of the $A\beta_{1-40}$-infused AD model rats.

With these aforementioned evidences of the inhibitory effects of DHA on the $A\beta_{25-35}$-induced cellular toxicity, we also have used immunohistochemical assays for the neuronal marker such as class III β-tubulin that takes part in the maintenance and changing of cell morphology. The DHA-incubated cells (DHA alone) demonstrated clearer axodendritic features with a healthier morphology than the vehicle-treated cells (controls). $A\beta_{25-35}$ caused severe axodendritic loss; also, floating debris in the culture media was more abundant, suggesting the degeneration of the cells (Fig. 4). The toxic effect of sistent with those of the Xu et al. [9] who also reported that $A\beta_{25-35}$ causes breakdown and dissolution of oligodendritic cellular processes and appearance of shrunken cell bodies. The coincubation of DHA with $A\beta_{25-35}$+SH-SY5Y cells clearly improved the morphological features of the cells. All these morphological results are thus, again, compatible with those of the MTT and TUNEL data that the $A\beta_{25-35}$-induced neurotoxicity is attenuated in the presence of DHA.

The content of DHA in SH-SY5Y cells shown in Table 1 was 2.15 ±0.25 mol%, which is considerably lower than in normal neuronal cells where DHA accounts for >10% of total fatty acids. The discrepancy of the lower basal DHA level in the SH-SY5Y cells may relate to the conditions of the cell culture, the differences in the innate capability of DHA to be incorporated into the membrane and the activities of the proteins/enzymes responsible for the translocation of DHA from the site of synthesis, the peroxisome. Reynolds et al. [31] have reported a highly significant deficit of DHA in SH-SY5Y compared with that in normal neuronal cells (rat synaptosomes, rat cerebellum and human cerebellum contain 15%, 20% and 18% DHA vs. 6% DHA in SH-SY5Y cells). The lower level of DHA in the cells of our study might be considered consistent with the fact that human neuroblastoma cells are profoundly deficient in DHA compared with

nontransformed neuronal tissue. In our study, the level of DHA increased ~3 times after supplementation with DHA, which is also consistent with the 3 times increase in DHA reported by Reynolds et al. [31].

It is unclear what concentration of free DHA might be routinely found in the brain or CSF; however, Pilitsis et al. [32] reported that the concentration of DHA in human cerebrospinal fluid is ~0.2 μM DHA. This is in the range of the 0.5 μM used to inhibit the amyloid-toxicity in the present *in vitro* ~~SH-S5Y5~~ experiments. Therefore, it (0.5 μM of DHA) could be presumed to act in the same way in the physiological condition. While this physiologically-relevant concentration of DHA (*i.e.* 0.5 μM of DHA) significantly inhibited the $A\beta_{25\text{-}35}$-induced toxicity in the ~~SH-S5Y5~~ cells, it did not, however, significantly affect in vitro $A\beta_{25\text{-}35}$ fibrillation and 10–20 μM of DHA was required to significantly inhibit it. This may relate to the differences in the in vivo and in vitro cell culture conditions and those of the environments, while DHA is directly incubated with $A\beta_{25\text{-}35}$ only. However the exact mechanisms required to be clarified.

DHA is a highly PUFA with six double bonds along its long axis. The unsaturation index was significantly increased in the DHA-treated cells (Table 1). The presence of double bonds in DHA renders it extremely sensitive to free radical damage during oxidative stress [33]. A DHA-enriched diet increases peroxidation in plasma and several tissues [34]. In contrast, DHA-supplemented human lymphocytes are less vulnerable to oxidative damage [35]. DHA at high doses (25–150 μM) shows anticancer effects in ~~SH-S5Y5~~ cells primarily by inducing oxidative stress (Lindskog et al. [36]). In the present study, we used 0.5 μM of DHA and demonstrated that ~~SH-S5Y5~~ cells respond differently to varying concentrations of DHA. In PC12 neuroblastoma cells DHA prevents apoptosis by down-regulating the caspase3 gene (Kim et al. [37]). Thus, the relation between DHA levels and oxidative stress remains controversial [38]. In the current study, the levels of LPO were significantly decreased in the DHA-treated cell as compared with those in the untreated control (Fig. 6A) and th[illegible] significantly increased in the DHA-treated cells, w[illegible] with the LPO ($r=0.03$, $P=.85$) (Fig. 6B). The oxidative stress imparted to ~~SH-S5Y5~~ cells may be attributable more to the oxidative effects of $A\beta_{25\text{-}35}$ than to the DHA-induced increase in the degree of unsaturation. We reported that DHA increases the Tuj1-positive cells in the primary neuron culture, during in vivo adult brain neurogenesis [39] and stem cell culture [40]. If DHA could have increased the oxidative stress, DHA would definitely reduce the number of ~~Tuj-~~positive cells (if oxidative stress is attributed to neurodegeneration); rather, it increased; demonstrating DHA did not introduce toxic oxidative insult in the present experimental condition. Consistent with this data, we also previously reported that DHA increases antioxidative enzymes such as catalase, glutathione peroxidase, glutathione reductase and reduced glutathione levels in the rat brain after dietary chronic administration of DHA [22]. A 2-day incubation of DHA significantly enriched this fatty acid in the ~~SH-S5Y5~~ membranes with a concomitant antiapoptic effect on them. Our results are consistent with those of the Kim et al. [41], who reported that DHA's antiapoptotic effect on mouse neuroblastoma cells apparently requires that it accumulates in cellular lipids. In contrast, addition of DHA and its increase in neuronal lipids augmented lipid radical formation and often enhanced susceptibility to oxidative stress [42,43]. However, our present results show that for ~~SH-S5Y5~~ cells, the protective effect of DHA prevailed, with its addition preventing oxidative-stress–induced apoptosis. Rostein et al. [44] reported that DHA inhibits the oxidative stress-induced apoptosis by activating the antiapoptotic proteins of Bcl-2 family.

The mechanism by which DHA ameliorates the $A\beta_{25\text{-}35}$-induced neurotoxicity is not clearly understood. Amyloid-induced oxidative stress is a prominent feature of Aβ-mediated neuronal death [45]. Methionine (Met_{35}) residue of full-length amyloids is highly sensitive to oxidation [45,46]. The $A\beta_{25\text{-}35}$ also contains a Met_{35} at the C-terminal end. In an independent set of experiments, thus, we evaluated whether $A\beta_{25\text{-}35}$ and/or DHA induces the oxidative stress in the $A\beta_{25\text{-}35}$+DHA cells with the use of oligomer-antibody A11 (Fig. 7). A11 specifically binds with the conformation-specific oligomers, the on-pathway (during momomer into fibril transformation) intermediate amyloid toxic species. $A\beta_{25\text{-}35}$ again increased while DHA decreased the levels of LPO in the ~~SH-S5Y5~~ cells. In the $A\beta_{25\text{-}35}$+A11 or $A\beta_{25\text{-}35}$+DHA+A11 cells, the levels of LPO were significantly reduced to those of the controls. Thus, the increase in the LPO in the $A\beta_{25\text{-}35}$+DHA cells could not be ascribed to the effect (presence) of DHA rather it was due to $A\beta_{25\text{-}35}$, demonstrating toxic $A\beta_{25\text{-}35}$ oligomers contributed to the production of LPO. Notably, the antibody-alone did not have effect on the LPO levels of ~~SH-S5Y5~~ cells, thus confirming the effect of $A\beta_{25\text{-}35}$ on the oxidative stress. These findings thus rule out the possibility that DHA acts as a pro-oxidant in the present experimental condition rather it ameliorated the neurotoxicity mediated by the oxidative stress. Lukiw et al. [19] reported that DHA-derived docosahexatriene, namely, neuroprotectin D1, rather inhibits neurotoxicity induced by $A\beta_{1\text{-}42}$, which itself has strong oxidative potential [41,42]. Our results are also quantitatively consistent with those of Florent et al. [47] who reported that pretreatment with DHA reduces neuronal apoptosis in response to soluble $A\beta_{1\text{-}40}$. We point to the fact that DHA reduces the fibrillation of $A\beta_{1\text{-}40}$ [11] and $A\beta_{25\text{-}35}$ [10] by inhibiting at the levels of soluble oligomers of these amyloids. Amyloid oligomers but not the fibers correlate better with the neurodegeneration and symptoms of AD-related pathology [48]. Therefore, by inhibiting the formation and elongation of toxic $A\beta_{25\text{-}35}$-oligomers, DHA inhibits their oxidative insult and resultant neurotoxicity.

To examine whether the inhibitory effect of fatty acid on the $A\beta_{25\text{-}35}$ fibrillation is specific to DHA, we also evaluated the effect of stearic, arachidonic acid on fibrillation. Stearic acid increased amyloid [illegible]merization (Fig. 7). Arachidonic acid reduced polymerization but [illegible]educing effect was lower than that of DHA, suggesting that DHA is more potent than arachidonic acid in inhibiting $A\beta_{25\text{-}35}$ fibrillation. Thus, it is noted that the effect of fatty acids on $A\beta_{25\text{-}35}$ fibrillation is not specific to DHA. However, the (inhibitory) effect of other fatty acids on the $A\beta_{25\text{-}35}$-induced toxicity in ~~SH-S5Y5~~ cells needs to be determined. In summary, DHA is an essential brain nutrient and is required through out life for the well being of the brain functions. Deficiency of this PUFA declines the memory-related learning ability of the AD. Thus, the outcome of the effect of DHA on $A\beta_{25\text{-}35}$-induced

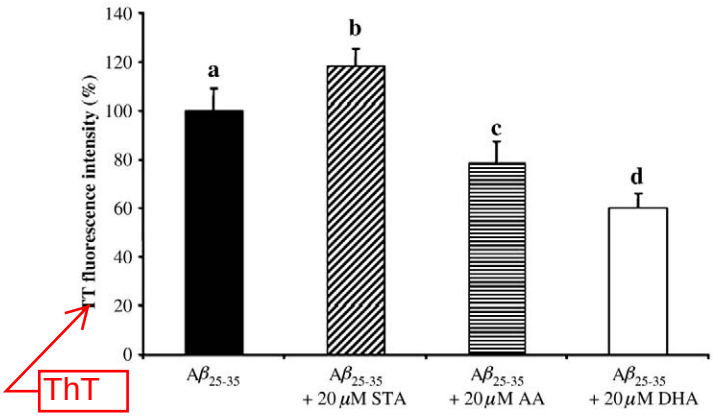

Fig. 7. Effects of fatty acids on the degree of amyloid fibrillation. STA, Stearic acid; AA, arachidonic acid; DHA, docosahexaenoic acid. Results are mean±S.E.M., each with duplicate determinations. $A\beta_{25\text{-}30}$ fibril formation was conducted similarly as described in the ~~Fig. 1~~. Bars with different letters are significantly different at $^{a\text{-}d}P<.05$ (One-way ANOVA).

Fig. 1

neurotoxicty has positive impact because Aβ_{25-35} retains the characteristics of its full length amyloids with regard to toxicity and the process of fibrillogenesis. Finally, the results of the present study clearly demonstrate that DHA inhibits the Aβ_{25-35}-induced neurotoxicity of ~~SH-S5Y5~~ cells and could thus be used to protect the neurodegeneration caused by cleavage products of full length amyloids such as Aβ_{25-35}.

SH-SY5Y

Acknowledgments

The authors gratefully acknowledge the contribution of Tsunao Yoneyama, Central Research facilities, Shimane University Faculty of Medicine for his enormous technical help in transmission electron micrographic studies. This work was supported in part by a grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (19500324. M.H.).

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