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DHA, EPA and their combination at various ratios differently modulated Aβ$_{25-35}$-induced neurotoxicity in SH-SY5Y cells☆

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ABSTRACT

Omega-3 polyunsaturated fatty acids ($n-3$ PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been reported to prevent neurodegenerative diseases such as Alzheimer's disease (AD) in both experimental and clinical/epidemiological studies. However, whether DHA and EPA from natural products exert similar or different neuroprotective effects and how these $n-3$ PUFAs target cellular and molecular mechanisms associated with neurodegenerative disease pathogenesis are unknown. In the present study, we used amyloid-β (Aβ)$_{25-35}$-treated differentiated SH-SY5Y cells as a model of AD to compare the neuroprotective effect of DHA, EPA and their combination at various ratios. Administration of 20 μM Aβ$_{25–35}$ significantly decreased SH-SY5Y cell viability, the expression of nerve growth factor (NGF), its TrkA receptor, and the level of glutathione (GSH) and increased reactive oxygen species (ROS), nitric oxide, tumor necrosis factor (TNF)-α, brain derived neurotrophic factor (BDNF) and its TrkB receptor. Aβ$_{25–35}$ also increased the Bax/Bcl-2 ratio and the expression of Caspase-3 in these cells. Compared with the Aβ group, pretreatment with DHA/EPA significantly reduced cell death, especially at ratio of 1:1 and 2:1 DHA/EPA or pure DHA. However, the most efficient ratio for reducing changes in ROS and GSH and for decreasing TNF-α appeared at ratio of 1:2 and 1:1, respectively. The ratio of 1:1, 2:1 and pure DHA resulted in significant increase in the level of NGF. Furthermore, pure DHA was the most efficient for reducing Bax/Bcl ratio and Caspase-3 expression. In conclusion, DHA, EPA and their combination differently modulated Aβ$_{25–35}$-induced neurotoxicity in SH-SY5Y cells by exerting anti-oxidative, anti-inflammatory and neurotrophic effects.

1. Introduction

The neurodegenerative process in Alzheimer's disease (AD) is associated with progressive accumulation of intracellular and extracellular neurotoxic amyloid-β (Aβ) oligomers in the brain [1–3]. Excessive Aβ deposition may induce AD through oxidative stress and neuroinflammation. Aβ oligomers can activate microglia in vitro and in vivo [4], resulting in the production and release of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, both of which can cause neural degeneration. Elevated levels of ROS interfere with the actions of many key molecules including enzymes, membrane lipids and DNA, which leads to cell apoptosis or death [5,6]. Increased pro-inflammatory cytokine releases may stimulate neurons to increase production of Aβ and cause neuronal dysfunction and apoptosis [7,8]. Another hallmark of AD is decreased neurogenesis due to the dysfunction in neurotrophic signaling mechanisms [9]. In particular, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and their receptors in the brain are disrupted. Reduced BDNF expression in the brain is a common feature of AD [10]. In addition, Aβ peptides are able to interfere with BDNF

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; $n-3$ PUFAs, omega-3 polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ROS, reactive oxygen species; NO, nitric oxide; GSH, glutathione; TNF-α, tumor necrosis factor-α; NGF, nerve growth factor; BDNF, brain derived neurotrophic factor

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signal transduction pathways involved in neuronal survival and synaptic plasticity, hampering the transmission of neurotrophic responses [11].

Despite these findings, the etiology of AD remains unknown, and treatments that target AD are ineffective and often cause severe side-effects [12]. Most neurodegenerative diseases, including AD, are irreversible because the failure of neurogenesis and the increase in neuron death occurs before the clinical symptoms appear [13]. Thus, much effort is directed towards the discovery of neural pathways and their molecular mechanism that can be targeted by novel therapeutics to prevent AD. Natural substances with anti-oxidative and/or anti-inflammatory activity may provide effective treatments for the prevention of AD.

As key components of neuronal membranes, long-chain omega-3 polyunsaturated fatty acids ($n-3$ PUFA) play beneficial role in brain function [14]. Epidemiological studies have associated a low incidence of AD with a high blood content of dietary n-3 PUFAs [15]. Our previous studies have reported the effectiveness of dietary n-3 PUFAs, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), as potential treatment strategies for mental health and neuroinflammation-induced memory deficits [16–21] due to their anti-oxidative and anti-inflammatory properties [19–23].

However, the results from many studies are confusing or inconsistent. For example, in a pilot clinical trial, fish oil, mainly containing both EPA and DHA , treatment significantly improved cognitive functions in patients with mild cognitive impairment [24]. However, a randomized controlled trial indicated that increasing intake of DHA and EPA through supplementation in individuals with cognitive impairment no dementia (CIND) or AD was found negligible beneficial effect on their cognition or mood [25]. Another pilot study of ethyl-eicosapentaenoate (ethyl-EPA) in the treatment of AD patients found little difference between treatment and baseline condition, except for a small improvement in carer's visual analogue rating [26]. In depression treatments, pure DHA was reported ineffective, while pure EPA or higher EPA than DHA could markedly improve depressive symptoms [27]. In terms of anti-inflammation, EPA and DHA also exerted different effects on pro-inflammatory cytokines [28]. It seems that pure EPA or EPA-enriched $n-3$ PUFAs are more effective in the treatment of major depressive disorder [27,29,30], while pure DHA and DHA-enriched $n-3$ PUFAs may be better in preventing or improving age-related or AD-induced cognitive decline [31–33].

These discrepancies may come from that 1) different $n-3$ PUFAs may have different function in the brain; 2) the *n*-3 PUFAs used in many experiments contain variable mixtures of DHA and EPA; or 3) many experiments did not consider the interactions between different *n*-3 PUFAs. It has been reported that pretreatment with EPA, but not DHA, significantly decreased IFN-α-induced depression [27] and different DHA: EPA ratios have different effects on the spontaneously hypertensive obese rat model of the metabolic syndrome [34]. However, comparisons between pure DHA and EPA, or comparison of different DHA + EPA combinations have been rarely reported in both experimental and clinical investigations. Thus, the exact role of each fatty acid in neuroimmune modulation and neurogenesis, the interaction between EPA and DHA at certain effective ratios, and the best DHA: EPA ratios for improving brain disorders such as AD related pathological changes, remain unknown.

Therefore, the aim of the present study was to investigate the effects of DHA and EPA alone, as well as their combinations at various ratios of 1:1, 1:2 and 2:1 as found in sea seals, sea algae and fish oil or some market products respectively, on a cellular model of AD. The model was set up by $A\beta_{25-35}$-insulted differentiated SH-SY5Y cell. Following this, the effects of DHA, EPA and their combinations at 3 ratios on $A\beta_{25-35}$-induced changes in cell viability, oxidative stress (ROS, NO, and GSH) and neurotrophins (NGF, BDNF and their TrkA and TrkB receptors) were measured. Since TNF-α is a key "pro-neuropathic" cytokine [35] and can activate a pro-apoptotic factor JNK pathway and trigger cellular death signaling [36], TNF-α expression was measured to test the anti-inflammatory effect of DHA, EPA or their combination in the present study. Then, their abilities to regulate the expression of apoptosis-related genes (Bcl-2, Bax and Caspase-3) in the model were explored.

2. Materials and methods

2.1. Reagents

$A\beta_{25-35}$ (synthetic, > = 97% HPLC, Sigma Aldrich, CA, A4559) was dissolved in sterile double-distilled water at a concentration of 1 mmol/L stock solution, and aged at 37 °C for 4 d, and then stored at −20 °C before use. Docosahexaenoic acid (22:6 n-3, DHA, Sigma-Aldrich, CA, D8768) and Eicosapentaenoic acid (20:5 n-3, EPA, Sigma-Aldrich, CA, E6627) were obtained > 99% pure as sodium salts and dissolved in culture medium, then divided into aliquots under nitrogen stream and maintained at −80 °C until used.

2.2. SH-SY5Y culture and differentiation

SH-SY5Y cells were obtained from ATCC (CRL-2266, Lot. 61983120) and were cultured at 37 °C in vented 75-cm^2 flasks containing DMEM/F12 culture medium (Gibco®, Canada) supplemented with 10% fetal bovine serum (FBS, Gibco®, Canada) and 1% penicillin–streptomycin. SH-SY5Y cells were differentiated into fully human neuron-like cells by treatment with all-*trans*-retinoic acid (RA, Sigma Aldrich, Canada), at a final concentration of 10 μM in DMEM/F12 with 3% FBS (media changed every 2 days), for 7–8 days.

2.3. Experimental design

When the SH-SY5Y cells were differentiated into neuron-like cells with long axons compared to undifferentiated cells, they were used in the experiments. The optimal dose and culture duration of $A\beta_{25-35}$ and DHA or EPA were selected according to a significant attenuation of the decreased cell viability induced by $A\beta_{25-35}$. The effect of DHA and EPA at doses of 6, 12, 25, 50, 100 μM on cell viability was tested in the $A\beta_{25-35}$-induced AD model of differentiated SH-SY5Y cells. In the present study, cells were divided into seven groups treated with: (i) culture media only (control), (ii) $A\beta_{25-35}$ (culture media with $A\beta_{25-35}$), (iii) DHA + Aβ (pretreatment with DHA, then $A\beta_{25-35}$), (iv) EPA + Aβ (pretreatment with EPA, then $A\beta_{25-35}$), and (v-vii) DHA + EPA + Aβ (pretreatment with DHA + EPA at ratio of 1:2, 1:1 and 2:1 respectively, then $A\beta_{25-35}$). The SH-SY5Y cells were pretreated with DHA, EPA, their combinations or control solvent for 12 h before $A\beta_{25-35}$ administration. After the addition of $A\beta_{25-35}$, the cells were incubated for 24 h. Then the cell viability, the levels of oxidative stress factors, inflammatory cytokine TNF-a, neurotrophin NGF, BDNF and their receptors and apoptosis-related genes were measured (Fig. 1).

2.4. Measurement of cell viability by MTT assay and cytotoxicity by Lactate Dehydrogenase (LDH) assay

Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl) − 2,5-diphenyl- tetrazolium bromide (MTT) assay, which measures the mitochondrial dehydrogenase activity reflected cell viability and presented the number of active cells to some extent. A decrease in cell viability may reflect a decrease in cell proliferation rate or the occurrence of apoptosis or necrosis. Cells were seeded in 96-well plates and 90 μL of cell suspension (1×1.0^6 cells/mL) was added to each well. Following experimental treatments, cell viability was measured with MTT (ATCC) according to manufacturer's instructions. The optical density was measured at 570 nm using a microplate reader (BioTek, USA). The absorbance of the control group was considered as 100% of the cell viability.

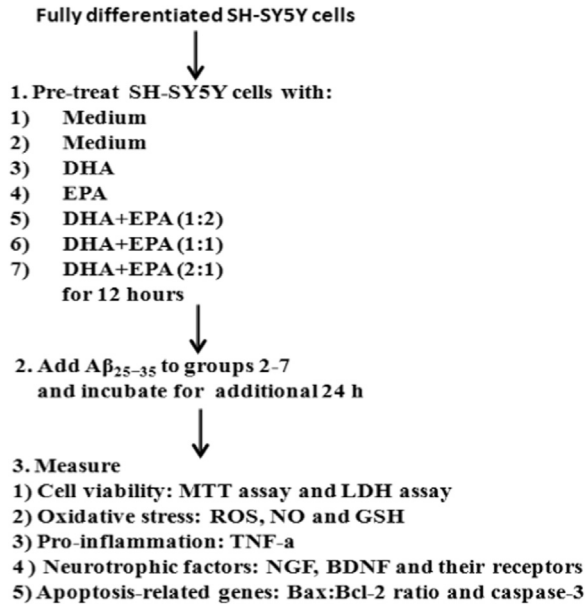

Fig. 1. An overview of experimental design.

The CytoTox-96 assay kit (Promega, Canada) was employed to evaluate cellular integrity damage by the total release of cytoplasmic lactate dehydrogenase (LDH) into the medium. This assay is based on a coupled enzymatic conversion from 2-p-(iodophenyl) − 3-(p-nitrophenyl) − 5-phenyltetrazolium chloride (INT, a tetrazolium salt) into a formazan product, and the enzymatic reaction is catalyzed by LDH released from cells and diaphorase in the assay substrate mixture. Absorbance was read at 490 nm by a microplate reader. The mean absorbance of each group was normalized to a percentage of the control value.

2.5. The measurement of oxidative stress by ROS, nitric oxide (NO) and glutathione (GSH) assays

SH-SY5Y cells were seeded in 96-well plates and 200 μL of cell suspension was added to each well. Following experimental treatments, the level of intracellular ROS was quantified with a fluorometric intracellular ROS kit (Sigma Aldrich) according to manufacturer's instructions. The fluorescence intensity was detected at lex = 650/lem = 675 nm by a fluorescence microplate reader (Reader Synergy HT, BioTek Instruments, USA). The intracellular level of NO production was determined by the Griess Reagent System (Promega, Canada) according to manufacturer's instructions. The absorbance was measured at 540 nm by a microplate reader. GSH concentration was measured with a glutathione assay kit (Sigma Aldrich) according to manufacturer's instructions. The fluorescence intensity was measured by a fluorimeter plate reader set at an excitation wavelength of 390 nm and emission wavelength of 478 nm.

2.6. Determination of gene expression with quantitative PCR

Differentiated SH-SY5Y cells were seeded in six-well plates and 2 mL of cell suspension was added to each well. Following experimental treatment, cells were harvested. The total RNA extraction method was the same as described by the manufacturer (RNeasy® Lipid Tissue Handbook). Complementary DNA (cDNA) was synthesized from RNA using the GoScript™ Reverse Transcriptase[(a)] (Promega, Canada). 2 μg RNA was used for first-strand cDNA synthesis. Nucleotide sequences of the primers for genes (Table 1) were obtained from NCBI's Nucleotide database and Primer Premier 6.0. After specificity validation in NCBI-nucleotide-BLAST, synthesis of primers was performed by Invitrogen Corporation. PCR reactions were prepared by Quantitect SYBR Green master mix (Qiagen) and by a Real-Time PCR Detection Systems (Bio-Rad, USA) CFX96™ Real-Time System. The PCR sequences consisted of an initial incubation for 5 min at 95 °C to activate the Hot-Star-Taq DNA polymerase, followed by 94 °C for 15 s (denaturing), 59 °C for 30 s (annealing), and 72 °C for 30 s (extension). After 38 cycles, a melting curve was generated for determination of primer specificity and identity. Gene expression levels were normalized to the RNA expression of housekeeping gene beta actin (relative quantification) with the △△CT correction.

Table 1
Primer names and sequences.

Primer names	Sequences
hActin F	GATGAGATTGGCATGGCTTT
hActin R	CACCTTCACCGTTCCAGTTT
hTNF-αF	AGGTTTGGCCTCACAAGGAC
hTNF-αR	GCGGTAGGGACAGTTCACAG
hNGF F	ACCTTTCTCAGTAGCGGCAA
hNGF R	TGTGTCACCTTGTCAGGGAA
hBDNF F	GGAGACACATCCAGCAAT
hBDNF R	ACAAGAACGAACACAACAG
hTrkA F	TACAGCACCGACTATTACC
hTrkA R	ATGATGGCGTAGACCTCT
hTrkB F	CTATGCTGTGGTGGTGATT
hTrkB R	CCGAAGAAGATGGAGTGTTA
hBAX F	GGGGACGAACTGGACAGTAA
hBAX R	CAGTTGAAGTTGCCGTCAGA
hBCL 2 F	TCTAGGGGAGGTGGTAGGCT
hBCL 2 R	CTGAGCAAGTCAGAGACCCC
hCaspase 3 F	GACTCTAGACGGCATCCAGC
hCaspase 3 R	TGACAGCCAGTGAGACTTGG

Note: F: forward primer; R: reverse primer.

2.7. Analysis of protein expression by western blotting

SH-SY5Y cells treated as above were collected and spun down at 10.000 g for 10 min and lysed with a RIPA buffer (RIPA, Thermo Scientific). Lysis was aided by sonication and lysates was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected and aliquots containing 20–40 μg of protein were loaded after boiling and separated on 10% SDS PAGE gels at 100 V for 60 min in electrophoresis buffer. After running the gels, the proteins were transferred to Polyvinylidene difluoride (PVDF) membranes (Millipore, Bellerica, MA, USA). Blots were then washed in Tris-buffered saline–Tween-20 (TBST) for 5 min, followed by blocking (TBST and 5% non-fat milk power) for 1 h at 20 °C. Following blocking, blots were washed with TBST for 5 min and incubated with primary antibodies, including the rabbit origin polyclonal antibodies for Actin (Abcam, ab8227, 42 kDa, 1:4000), NGF (Abcam, ab52918, 27 kDa, 1:500), BDNF (Abcam, ab6201, 28 kDa, matured form, 1:200), TrkA (Abcam, ab59272, 85 kDa, 1:800), TrkB (Thermo fisher science, MA5-14903, 90–140 kDa, 1:1000), TNF-α (Abcam, ab9739, 17 kDa, 1:2500), Bcl-2 (Abcam, ab136285, 26 kDa, 1:5000), Bax (Abcam, ab32503, 21 kDa, 1:2000) and Caspase-3 (Abcam, ab44976, 32 kDa, activated forms, 1:500), overnight at 4 °C, followed by the secondary antibody, peroxidase (HRP)-conjugated anti-rabbit IgG (Abcam, ab6721, 1:5000), for 1 h at 20 °C. The blots were washed in TBS three times. Immunoreactive bands were detected by Clarity™ Western ECL Substrate Kit (Bio-Rad, Canada) on a ChemiDoc™ MP System with Image Lab™ Software (Bio-Rad, Canada). All target proteins were quantified by normalizing them to β-Actin re-probed on the same membrane and then calculated as a percentage of the control group.

2.8. Statistical analysis

Data were presented as mean ± SEM. Statistical analyses were

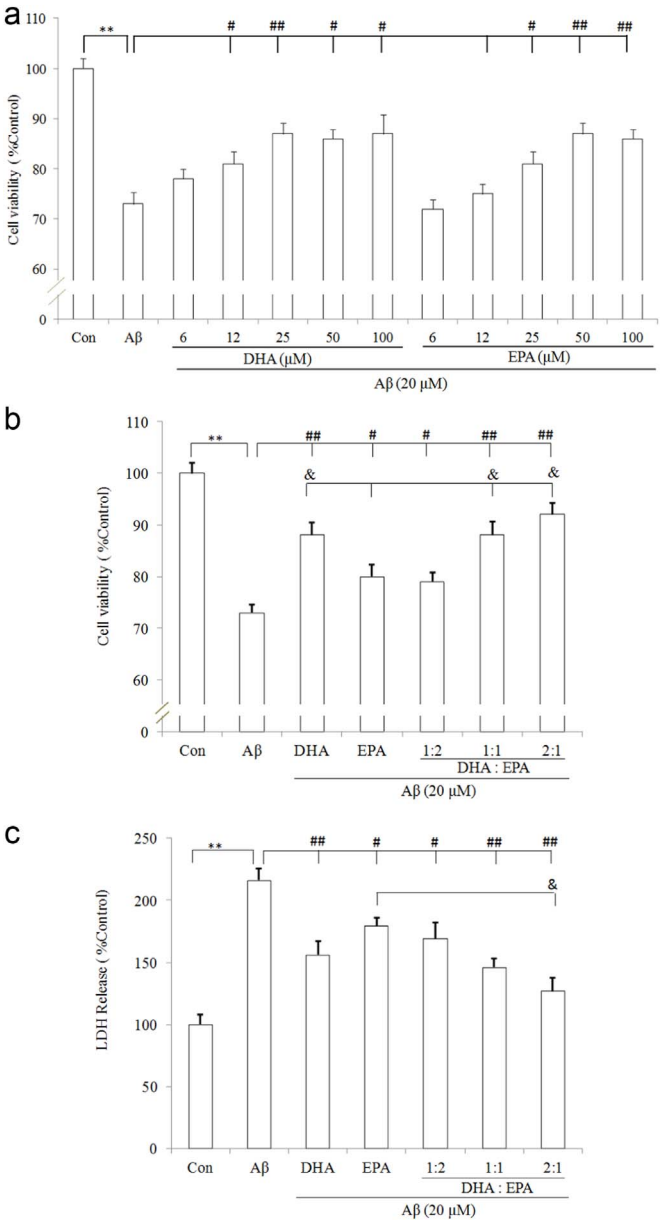

Fig. 2. Cell viability and cytotoxicity. (a): The effect of DHA and EPA at various doses on $A\beta_{25-35}$-induced neurotoxicity in SH-SY5Y. (b): Changes in cell survival rate after the treatment with 20 μM $A\beta_{25-35}$ for 24 h in the absence or presence of DHA, EPA and their combination at indicated ratio (c): Changes in LDH release after the treatment with 20 μM $A\beta_{25-35}$ for 24 h in the absence or presence of DHA, EPA and their combination at indicated ratio. Data were the mean value of 3 separate experiments (n = 4 in each experiment) and three mean values from independent experiment were used for statistics. $^{**}p < 0.01$ vs. control group; $\#p < 0.05$, $\#\#p < 0.01$ vs. $A\beta_{25-35}$ group; & Mean value was significantly different from that of the EPA group ($p < 0.05$, bc).

performed using IBM SPSS Statistics 22. When a one-way ANOVA indicated significant differences among groups, Tukey's *post hoc* test was used to determine the specific pairs of groups that were statistically different. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of DHA, EPA and their combination at various ratios on decreased neuronal cell viability induced by $A\beta_{25-35}$

As shown in Fig. 2a, administration with $A\beta_{25-35}$ at 20 μM for 24 h significantly decreased cell viability of differentiated SH-SY5Y cells ($p < 0.01$). However, pre-treatment with different concentrations of DHA (12–100 μM) or EPA (25–100 μM) significantly attenuated the reduction of cell viability caused by $A\beta_{25-35}$ in a dose-dependent manner and at DHA 12 ($p < 0.05$), 25 ($p < 0.01$) and 50–100 μM ($p < 0.05$) and at EPA 25 ($p < 0.05$) and 50–100 μM ($p < 0.01$) (Fig. 2a). Based on the above results, pure DHA (25 μM), pure EPA (25 μM) and their combinations at the ratios of 1:2, 1:1 and 2:1 (total 25 μM) were adopted in the following tests to test the hypothesis that DHA, EPA and their combinations at various radios may have different effect on neuroprotection.

Compared with the Aβ group, pretreatment with DHA, EPA and their combinations at 3 ratios all significantly inhibited $A\beta_{25-35}$-reduced cell viability ($p < 0.05$ or $p < 0.01$). Furthermore, pure DHA and the combination of DHA and EPA at the ratios of 2:1 and 1:1 were more effective than pure EPA alone in blocking $A\beta_{25-35}$-reduced cell viability ($p < 0.05$, Fig. 2b). The ordering of $n-3$ PUFA potency in blocking $A\beta_{25-35}$-induced reduction of SH-SY5Y cell viability was: 2:1 DHA/EPA ≥ 1:1 DHA/EPA ≥ DHA > EPA ≥ 1:2 DHA/EPA.

The protective effect of DHA, EPA and their various ratios against $A\beta_{25-35}$- induced cell death in SH-SY5Y cells was further confirmed by the LDH release assay. $A\beta_{25-35}$ significantly increased LDH release ($p < 0.01$), while DHA, EPA and 3 ratios of DHA/EPA reduced LDH release (all $p < 0.05$, Fig. 2c). From above results it may be drawn that DHA , EPA and their combinations can effectively protect the differentiated SH-SY5Y cells from $A\beta_{25-35}$-induced cellular damage in vary degrees, and the best DHA: EPA ratio for improving cell viability was 2:1.

3.2. Effects of DHA, EPA and their combination at various ratios on the changes of ROS, NO and GSH levels induced by $A\beta_{25-35}$

Fig. 3a shows that $A\beta_{25-35}$ markedly increased ROS fluorescence compared to the control group ($p < 0.01$). When compared to the $A\beta_{25-35}$ group, ROS fluorescence was significantly decreased by pure EPA ($p < 0.01$) and the combinations of DHA and EPA at the proportion of 1:2 ($p < 0.01$) and 1:1 ($p < 0.05$). Moreover, pure EPA and the combinations of DHA/EPA at 1:2 had a more pronounced effect on the $A\beta_{25-35}$-induced increase in ROS than DHA alone (both $p < 0.05$). The pure DHA group and 2:1 group did not significantly inhibit ROS increase compared to the $A\beta_{25-35}$ group (both $p > 0.05$). The $n-3$ PUFAs potency in decreasing $A\beta_{25-35}$-induced increased ROS fluorescence in SH-SY5Y cells was: 1:2 DHA/EPA ≥ EPA ≥ 1:1 DHA/EPA > 2:1 DHA/EPA ≥ DHA.

As shown in Fig. 3b, $A\beta_{25-35}$ significantly increased the level of NO compared to the control ($p < 0.05$). However, no significant differences in NO level were found in all the groups treated with $n-3$ PUFAs as compared to the $A\beta_{25-35}$ group.

The results in Fig. 3c show that there was a marked reduction in the GSH content in the $A\beta_{25-35}$-treated cells when compared to the control group ($p < 0.05$). While $n-3$ PUFAs significantly increased GSH content at all the proportions tested compared to the $A\beta_{25-35}$ group (all $p < 0.05$), especially pure EPA and DHA/EPA at the ratios of 1:2 (both $p < 0.01$). The potency of FAs to attenuate the decrease in anti-oxidant GSH in SH-SY5Y cells after the $A\beta_{25-35}$ administration was: 1:2 DHA/EPA ≥ EPA > 1:1 DHA/EPA ≥ DHA ≥ 2:1 DHA/EPA. Taken together, pure EPA and the combinations with higher dose of EPA and lower dose of DHA (e.g. DHA/EPA at 1:2) showed a more pronounced effect on anti-oxidation, including reduced ROS and increased GSH.

3.3. Effects of DHA, EPA and their combinations at various ratios on the $A\beta_{25-35}$-induced increased expression of pro-inflammatory cytokine TNF-α

When compared to the control group, the mRNA expression of TNF-α was significantly increased 4 h after $A\beta_{25-35}$ incubation ($p < 0.01$), but the increase in TNF-α protein expression was not found until 12 h after $A\beta_{25-35}$ administration ($p < 0.01$). DHA, EPA or their

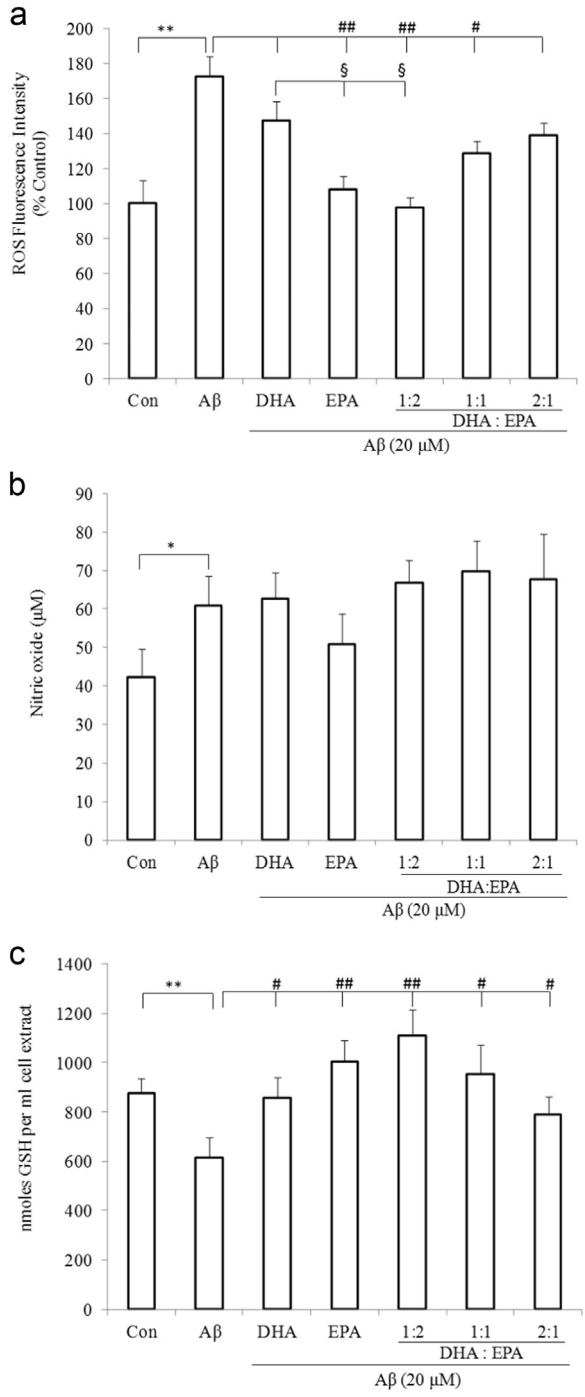

Fig. 3. The effects of DHA, EPA and their combinations at different ratios on the changes of oxidative stress and antioxidant response induced by $A\beta_{25-35}$. ROS (a), NO production (b) and GSH content (c) in fully differentiated SH-SY5Y cells treated with 20 μM $A\beta_{25-35}$ and/or DHA, EPA and their combination (total 25 μM) for 24 h. Results presented as mean ± SEM of three separate experiments (n = 2 in each experiment) and three mean values from independent experiment were used for statistics. *$p < 0.05$, **$p < 0.01$ vs. control group; #$p < 0.05$, ##$p < 0.01$ vs. $A\beta_{25-35}$ group. §Mean value was significantly different from that of the DHA group ($p < 0.05$, a).

combinations, significant attenuated both TNF-α mRNA and protein expression upon $A\beta_{25-35}$ insult (all $p < 0.05$). Furthermore, DHA and EPA at 1:1 ratio was more potent in reducing TNF-α protein expression than DHA or EPA alone ($p < 0.05$), suggesting that 1:1 DHA/EPA could exert a potential synergistic effect in terms of anti-inflammation (Fig. 4).

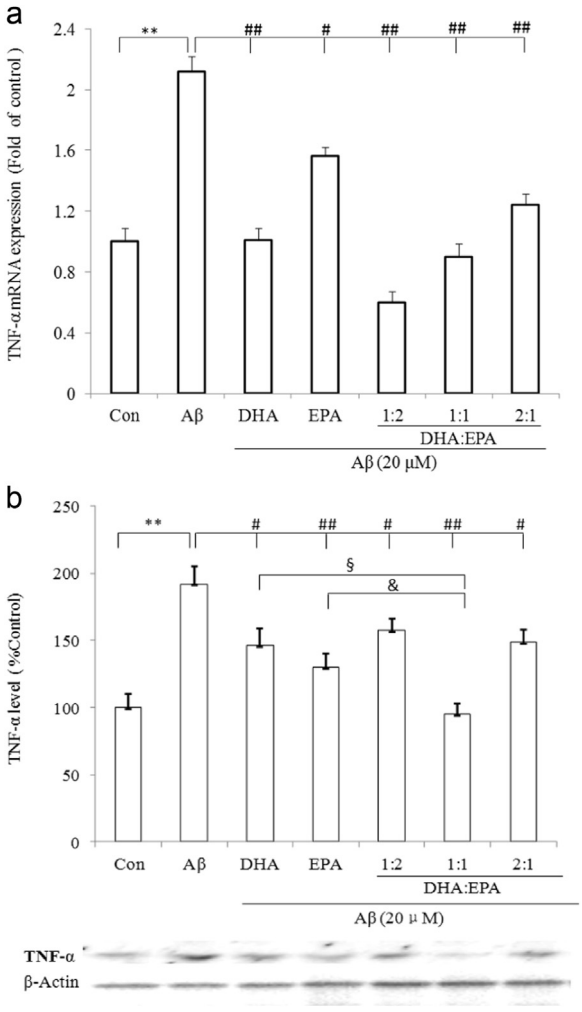

Fig. 4. The expression of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α). mRNA expression level (a) and protein expression level (b) of TNF-α, normalized to the corresponding level expression of housekeeping gene β-Actin, in differentiated SH-SY5Y cells treated with $A\beta_{25-35}$ (20 μM) and/or DHA, EPA and their combination (total 25 μM) for indicated times. Results presented as mean ± SEM of three separate experiments (n = 2 in each experiment) and three mean values from independent experiment were used for statistics. **$p < 0.01$ vs. control group; #$p < 0.05$, ##$p < 0.01$ vs. $A\beta_{25-35}$ group. & Mean value was significantly different from that of the EPA group ($p < 0.05$). §Mean value was significantly different from that of the DHA group ($p < 0.05$).

3.4. Effects of DHA, EPA and their combination at various ratios on the expression of neurotrophins: NGF and BDNF and their tyrosine kinase receptors

$A\beta_{25-35}$ significantly decreased NGF mRNA expression (at 4 h, $p < 0.01$, Fig. 5a) and NGF protein level (at 12 h, $p < 0.01$, Fig. 5c) as compared to the control group. Similarly, decreased expression of BDNF at mRNA level (at 4 h, $p < 0.01$, Fig. 5b), but increased BDNF protein level (at 12 h, $p < 0.01$, Fig. 5d) were found in $A\beta_{25-35}$ group. Pre-treatment with DHA alone, 1:1 and 2:1 DHA/EPA significantly increased the expression of NGF at mRNA and protein levels ($p < 0.01$, Fig. 5a, c). BDNF mRNA expression was significantly increased by pure DHA and 3 ratios of DHA/EPA compared to $A\beta_{25-35}$ group, but not pure EPA (Fig. 5b). BDNF protein expression was significantly reduced in the groups treated with EPA alone and 3 ratios of DHA/EPA ($p < 0.01$), but not in pure DHA group, when compared to $A\beta_{25-35}$ group (Fig. 5d). Taken together, pure DHA or the combination with higher dose of DHA are better than EPA to maintain the increased expression of both NGF and BDNF at mRNA and protein level (only pure DHA in BDNF protein, all $p < 0.05$).

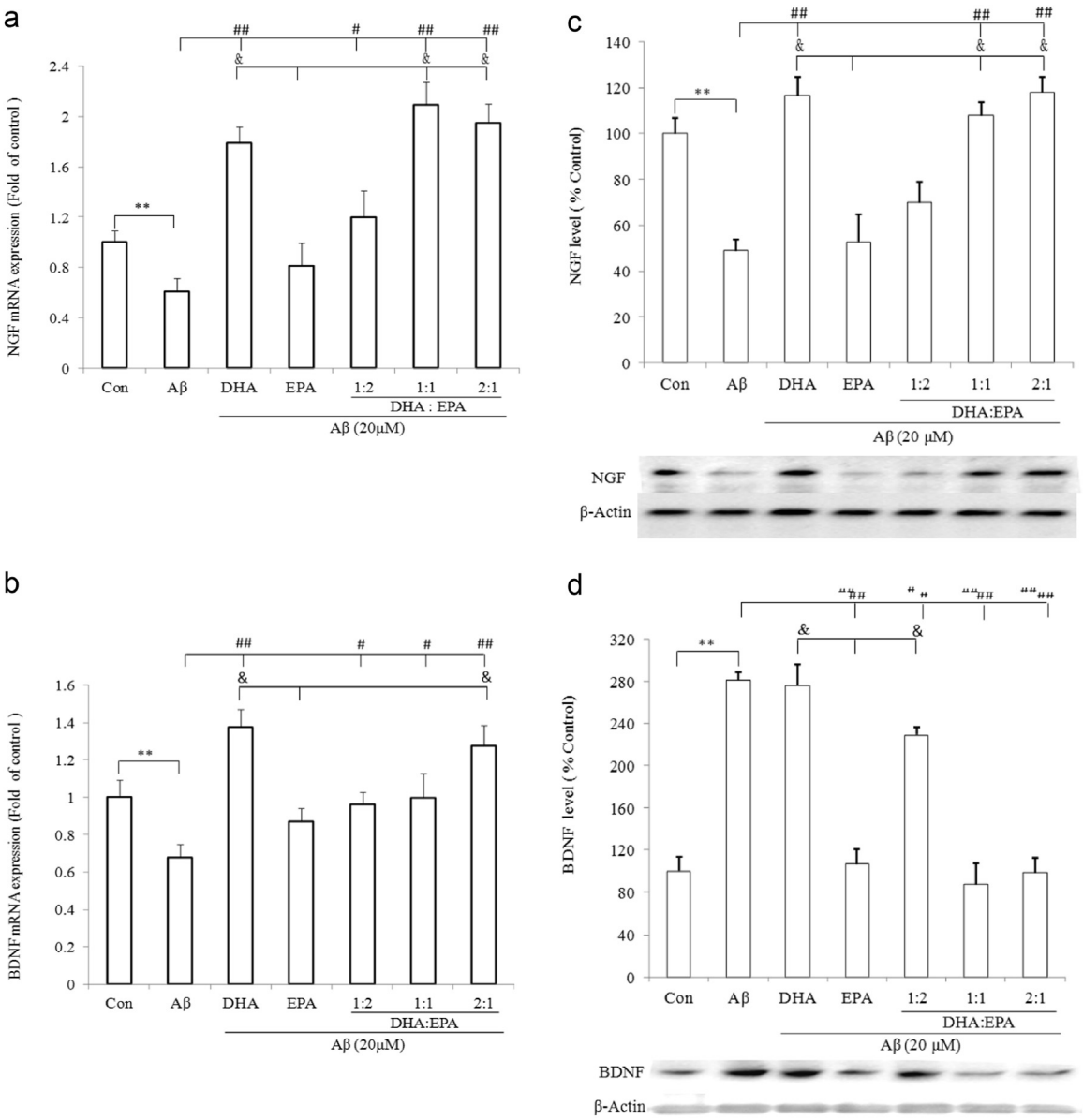

Fig. 5. The effect of DHA, EPA and their combination on the expression of neurotrophin genes: NGF and BDNF. NGF mRNA expression (a) and protein expression (c), BDNF mRNA expression (b) and protein expression (d), normalized to the expression of housekeeping gene β-Actin, in differentiated SH-SY5Y cells treated with $A\beta_{25-35}$ (20 μM) and/or DHA, EPA and their combination (total 25 μM) for 4, 8, 12, 24 h. Results presented as mean ± SEM of three separate experiments (n = 2 in each experiment) and three mean values from independent experiment were used for statistics. $^{**}p < 0.01$ vs. control group; $\#p < 0.05$, $\#\#p < 0.01$ vs. $A\beta_{25-35}$ group. Mean value was significantly different from that of the EPA group ($p < 0.05$).

24 h after $A\beta_{25-35}$ insult, TrkA protein expression ($p < 0.01$) was largely decreased, which was only reversed by the combination of DHA and EPA at 1:2 ration ($p < 0.05$, Fig. 6a). However, pretreatment with DHA, EPA or their combination at 1:2 and 2:1 all significantly reversed $A\beta_{25-35}$-induced increase in TrkB expression (both $p < 0.05$, Fig. 6b) compared to Aβ group.

3.5. Effects of DHA, EPA and their combination at various ratios on the expression of apoptosis-related genes: Bax, Bcl-2 and Caspase-3

The expression of gene Bcl-2 was significantly decreased 24 h after $A\beta_{25-35}$ administration, while this decrease was differently but significantly reversed ($p < 0.05$) by pretreatment with DHA, EPA and their various ratios. With regard to Bax, no significant change was found after 4–24 h $A\beta_{25-35}$ insult in all the experimental groups (data not shown). As compared to the control, the Bax: Bcl-2 ratio was significantly increased in cells administrated with $A\beta_{25-35}$ ($p < 0.01$). Pretreatments with DHA, EPA and their various ratios all significantly reduced the Bax: Bcl-2 ratio to the control level (all $p < 0.01$ except for EPA $p < 0.05$, Fig. 7a).

For Caspase-3, $A\beta_{25-35}$ significantly increased its protein expression ($p < 0.01$) when compared to the control. While pretreatment with DHA, EPA or their combinations at 1:2 and 2:1, but not 1:1, markedly attenuated the $A\beta_{25-35}$–induced increased Caspase-3 expression (all $p < 0.01$ except for 2:1 EPA/DHA $p < 0.05$, Fig. 7b). Taken together, DHA and EPA can both modulate the expression of genes related to apoptosis, such as Bcl-2 and Caspase-3, and DHA and DHA: EPA at 2:1 appeared to be more effective than other $n-3$ PUFAs tested.

4. Discussion

It has been reported that $n-3$ PUFAs improve or prevent neurodegenerative diseases in both experimental and clinical studies [13,33,37–39]. As essential membrane components, $n-3$ PUFAs

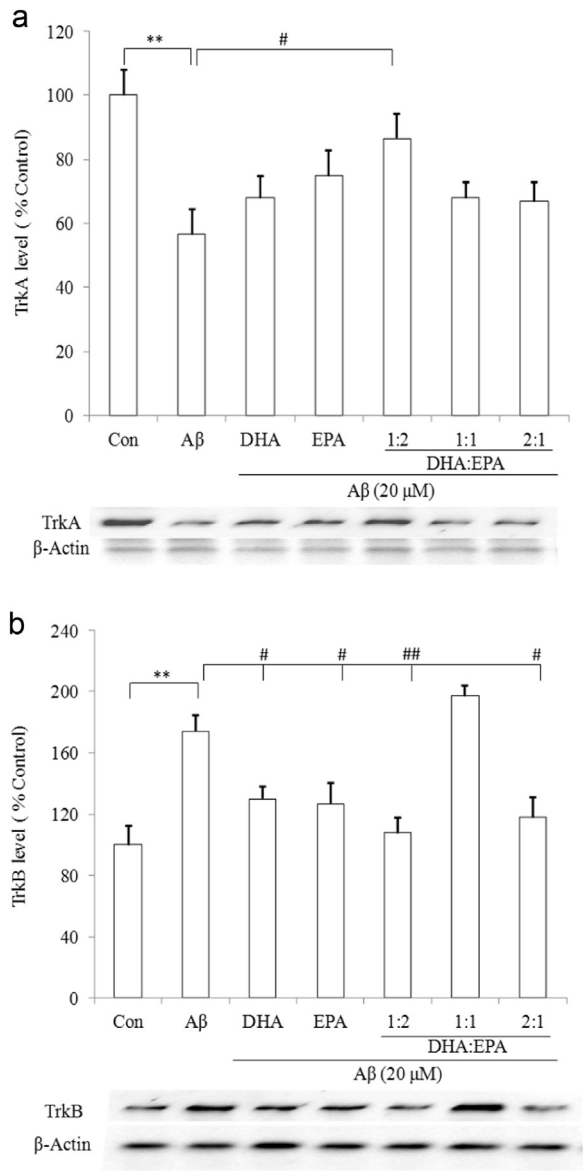

Fig. 6. The effect of DHA, EPA and their combination on the expression of neurotrophin receptors: TrkA and TrkB. TrkA (a) and TrkB (b) protein expression, normalized to the protein expression of housekeeping gene β-Actin, in differentiated SH-SY5Y cells treated with $A\beta_{25-35}$ (20 μM) and/or DHA, EPA and their combination (total 25 μM) for 24 h. Results presented as mean ± SEM of three separate experiments (n = 2 in each experiment) and three mean values from independent experiment were used for statistics. $^{**}p < 0.01$ vs. control group; $\#p < 0.05$, $\#\#p < 0.01$ vs. $A\beta_{25-35}$ group.

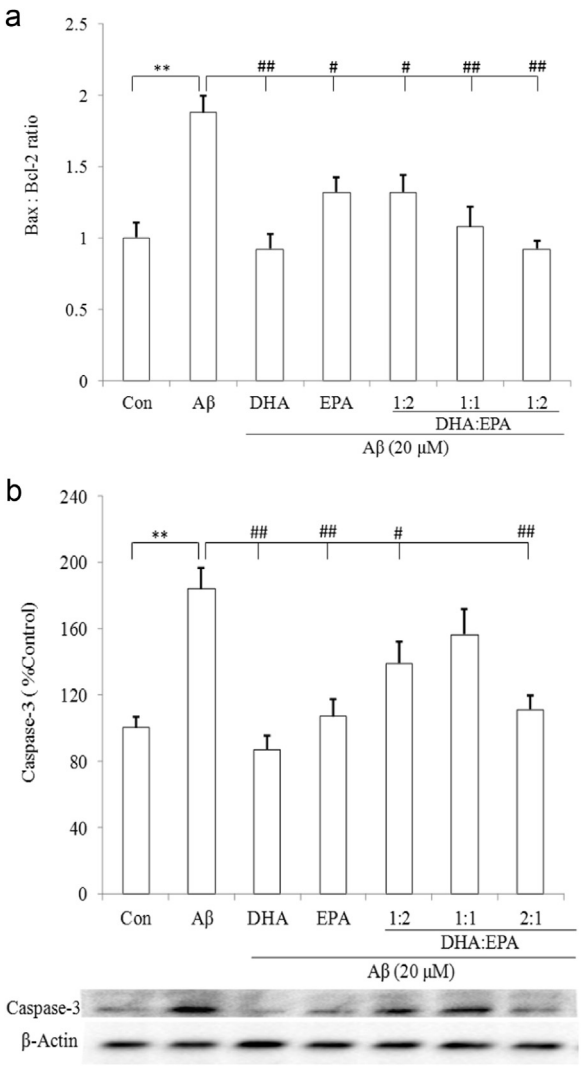

Fig. 7. DHA, EPA and their combination differently regulated the expression of apoptosis related genes: Bcl-2, Bax and Caspase-3. Bax: Bcl-2 ratio(a) and Caspase-3 (b) protein expression (normalized to the protein expression of housekeeping gene β-Actin) in differentiated SH-SY5Y cells treated with $A\beta_{25-35}$ 20 μM and/or DHA, EPA and their combination (total 25 μM) for 24 h. Results presented as mean ± SEM of three separate experiments (n = 2 in each experiment) and three mean values from independent experiment were used for statistics. $^{**}p < 0.01$ vs. control group; $\#p < 0.05$, $\#\#p < 0.01$ vs. $A\beta_{25-35}$ group.

benefit brain health by modulating brain and immune interaction [16] and apoptotic pathways [40], increasing membrane fluidity and reducing oxidative stress [41–43]. The present study, for the first time, has compared different roles of EPA and DHA, and then explored their potential synergistic actions at different ratios on inflammatory, oxidant and neurotrophic functions in the cellular model of AD. A summary and comparison of the results is presented in Table 2. According to these results, DHA, EPA and their various ratios can protect the viability of SH-SY5Y cells from $A\beta_{25-35}$ induced cytotoxicity in vary degrees. The mechanisms are involved in differently suppressing oxidative stress and TNF-α expression, regulating neurotrophin and their receptor expression and reducing neuron apoptosis. The following several findings gained in the present study are discussed.

4.1. Pure EPA or higher ratio of EPA is more effective in anti-oxidative stress than DHA

The current study found that $A\beta_{25-35}$ increased ROS and NO production and reduced GSH release from SH-SY5Y cells. In contrast, pure EPA or the combination of DHA and EPA at the ratio of 1:2 significantly reversed the increased ROS level. Previous studies have demonstrated that $n-3$ PUFAs have a potent ability to decrease oxidative stress in several pathological conditions [42–44]. We have also previously demonstrated that pure EPA exerts anti-oxidative and anti-inflammatory effects in the MPP + -induced model of Parkinson's disease and olfactory bulbectomized rat model of depression [13,37,40],. This anti-oxidative ability of $n-3$ PUFAs may be related to its improving mitochondrial dysfunction, which is the major source of ROS [45] or up-regulated nuclear factor erythroid 2-related factor 2 (Nrf2), a master transcriptional factor for the expression of various antioxidant genes [46], such as GSH. Accordingly, the present results confirmed that the reduced important anti-oxidant GSH content caused by $A\beta_{25-35}$ was significantly rescued by $n-3$ PUFAs, especially in pure EPA and the combination of

Table 2
A summary of effects of DHA, EPA and their combination at various ratios on the $A\beta_{25–35}$-induced changes in cell viability, oxidative stress, inflammatory response, neurotrophins and apoptosis related genes.

Category	Parameter	$A\beta_{25-35}$	DHA	EPA	DHA: EPA		
					1:2	1:1	2:1
Cell viability	MTT	↓↓	+ + &	+	+	+ + &	+ + &
	LDH	↑↑	–	–	–	–	– &
Oxidative stress	ROS	↑↑	NS	– §	– §	–	NS
	NO	↑	NS	NS	NS	NS	NS
	GSH	↓↓	+	+ +	+ +	+	+
Pro-inflammatory cytokine	TNF-α mRNA	↑↑	–	–	–	–	–
	TNF-α Protein	↑↑	–	–	–	– & §	–
Neurotrophins	NGF mRNA	↓↓	+ + &	+	+	+ + &	+ + &
	NGF Protein	↓↓	+ + &	NS	NS	+ + &	+ + &
	BDNF mRNA	↓↓	+ + &	NS	+	+	+ + &
	BDNF Protein	↑↑	NS &	–	–	–	–
Tyrosine kinase receptors	TrkA	↓↓	NS	NS	+	NS	NS
	TrkB	↑↑	–	–	–	NS	–
Apoptosis related genes	Bax/Bcl − 2	↑↑	–	–	–	–	–
	caspase − 3	↑↑	–	–	–	NS	–

When compared to the control group, "↑" $p < 0.05$, "↑↑" $p < 0.01$, significantly up-regulated or increased; "↓↓" $p < 0.01$, significantly down-regulated or decreased. When compared to the $A\beta_{25-35}$ group, "+" $p < 0.05$, "+ +" $p < 0.01$, significantly up-regulated or increased; "-" $p < 0.05$, "–" $p < 0.01$, significantly down-regulated or decreased; NS: not significantly changed. & Mean value was significantly different from that of the EPA group ($p < 0.05$). § Mean value was significantly different from that of the DHA group ($p < 0.05$).

DHA and EPA at the ratio of 1:2. In addition, a couple of in vivo studies have reported that DHA and EPA were similar in terms of anti-oxidative effects [47,48]. However, our current findings appeared to differ from those from in vivo studies. According to our data, pure EPA and those with more or equal amounts of EPA were more effective in restoring the imbalance between oxidative and anti-oxidative factors than pure DHA or DHA-rich mixtures. This finding may confirm anti-oxidative function of fish oil, such as from *Salmon,* in which EPA ratio is higher than DHA.

It is well known that ROS and NO are increased in the AD brain [49–51]. However, NO is also a neurotransmitter, which may protect synapses by increasing neuronal excitability [52,53]. Thus, whether the Aβ-induced increase in NO acts as a neuroprotective or neurotoxic role is unclear. In this study, no any $n-3$ PUFA changed the $A\beta_{25-35}$-induced NO increase. If increased NO is neuroprotective, then $n-3$ PUFA may contribute to the self-protective ability of Aβ-insulted cells.

4.2. DHA and EPA have distinct but synergistic anti-inflammatory effects in cellular AD model

The contribution of chronic inflammation to neurological disorders has been extensively demonstrated [54–56]. The pro-inflammatory cytokine TNF-α can activate a pro-apoptotic JNK pathway that is involved in cell differentiation, proliferation and death signaling [57]. In the present study, $A\beta_{25-35}$ up-regulated the expression of TNF-α at mRNA and protein levels, which is consistent with the above findings. Previous studies have demonstrated that $n-3$ PUFAs, both DHA and EPA, can decrease the production of pro-inflammatory cytokines at different intensities [23,58,59]. Similarly, in the present study, DHA, EPA or their combinations all significantly inhibited the $A\beta_{25-35}$-up-regulated expression of TNF-α. The anti-inflammatory effects of $n-3$ PUFAs were related to their displacement of the production of proinflammatory $n-6$ PUFA and simultaneously generate anti-inflammatory resolvins (from EPA and DHA) [60,61]. The mechanism was reported to enhance signal transducer and the activators of transcription 6 (STAT6) phosphorylation, nuclear translocation of peroxisome proliferator-activated receptor gamma (PPARγ) and the DNA-binding activity of STAT6 [62]. Additionally, EPA acts as an inhibitor for cyclooxygenase (COX)2 during inflammation [63], the enzyme responsible for the metabolism of n-6 PUFA arachidonic acid (AA) and formation of eicosanoids (which, in turn, modulate the inflammatory/immune pathways). Thus, EPA and DHA may exert anti-inflammatory effect through different mechanisms and targets simultaneously. Interestingly, we for the first time showed that DHA and EPA at 1:1 was more potent than those of DHA or EPA alone in term of reducing TNF-α, suggesting that DHA and EPA may have synergistic effect to inhibit inflammation at this ratio.

4.3. Pure DHA or the combination containing more DHA is stronger than EPA to restore the changes in the expression of neurotrophins and their receptors

In a previous study, we demonstrated that neuroinflammation could markedly elevate APP expression, the precursor of Aβ in the hippocampus and cortex, as well as modulate the expressions of neurotrophins, such as NGF and BDNF [64,65]. In the present in vitro study, $A\beta_{25–35}$ differently regulated the expression of NGF and BDNF. The decreased expression of NGF and its TrkA receptor is in accordance with the reduced neuronal viability induced by Aβ. However, the significant increase of BDNF and its TrkB receptor in SH-SY5Y cells exposed to $A\beta_{25–35}$ were unexpected and inconsistent with a decrease in BDNF mRNA levels. The present data are partially in agreement with a previous in vitro study showing that the exposure of SH-SY5Y cells to $A\beta_{25–35}$ induced a significant increase of BDNF [66]. The increase in BDNF level possibly resulted from acute insults to cells induced by Aβ and might act as a compensatory response against amyloid toxicity. Since NGF protein expression was significantly increased by DHA alone and 1:1 and 2:1 DHA/EPA, while no significant change was found for BDNF expression in the DHA-treated group, pure DHA or combinations containing more longer-chain DHA may be better than EPA to promote the expression of neurotrophins. This action of $n-3$ PUFAs on neurotrophins is in line with previous studies [37,64,67,68]. N-3 PUFAs, especially DHA, may regulate the gene expression of neurotrophins through modulating DNA methylation at IGF2/H19 imprinted genes in promoter region of neurotrophin genes [69].

4.4. Both DHA and EPA attenuate changes in apoptotic gene expression and improve cell viability, but DHA is more effective than EPA

In the present study, $A\beta_{25–35}$ administration caused a decrease in SH-SY5Y cell viability and an increase in the pro-apoptotic Bax/Bcl-2 ratio and Caspase-3 expression. Both $n-3$ PUFAs can attenuate the imbalance between pro- and anti-apoptotic genes. The anti-apoptotic function of $n-3$ PUFAs is parallel to their protection of cell viability tested by MTT and LDH Except for above mentioned anti-inflammatory

and antioxidant function, neuroprotectin D1 derived from DHA may protect neurons from apoptosis by down-regulating β-secretase 1, while activating α-secretase ADAM 10 and sAPPα, and shift an amyloidogenic pattern to the non-amyloidogenic pathway in this AD model [70]. As a result, it is not surprising that pure DHA or combinations containing more DHA are more effective than pure EPA to improve cell viability and attenuate neuron apoptosis. This is also in line with the previous result that DHA is more potent than EPA in restoring neurotrophin dysfunctions.

Taken together, the results from the present study have, for the first time demonstrated that 1) both DHA and EPA attenuate neuron apoptosis and improve cell viability, while pure DHA or DHA at higher ratio are better than EPA in the protection of cell viability; 2) DHA and EPA have synergistic anti-inflammatory effects in the AD model; 3) pure EPA or EPA at higher ratio are more effective in anti-oxidative stress, while pure DHA or DHA at higher ratio are more powerful than EPA for improving neurotrophic systems. These differences between DHA and EPA may be due to the different targets of DHA and EPA on signaling pathways, or on the expression of certain genes, particularly on those enzymes related to cellular survival, such as inflammation, antioxidant enzymes and neurotrophic system. It has been reported that DHA and EPA differently influent signal transduction pathways related to cell growth, thereby affect different biological processes [71], the release of proinflammatory cytokines by peripheral blood mononuclear cells [39], and the expression of genes involved in lipid metabolism [72]. The results of the present study will shed light on the application of nutritional intervention for PUFAs in different diseases which process different pathological mechanisms.

The limitations of the present study are that 1) the same dose of unsaturated fatty acids with the similar chain length should be added into the control group, such as arachidic acid or behenic acid, to avoid the energy effect; 2) longer exposure time of SH-SY5Y cells to $A\beta_{25–35}$ should be studied, which confirms the dynamical changes in the expression of BDNF in response to $A\beta_{25–35}$; 3) *In vivo* investigations should be carried out to explore the effect of DHA, EPA and their combination at different ratios on cognitive behavior and neuropathologies in AD animal models.

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Author contributions

Cai Song applied the grant, directed and designed the experiments, as well as wrote the manuscript; Yongping Zhang performed the experiments, analyzed the data and wrote the manuscript; Richard E Brown contributed to discussion, edition and revision of the manuscript; Ping-Cheng Zhang contributed to data interpretation; Yuntao Zhao analyzed the data; Xianghong Ju contributed to analytical methods.

Disclosure statement

The authors declare that there are no actual or potential conflicts of interests involving them or the institutions with which they are affiliated.

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