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DUAL EFFECT OF DOCOSAHEXAENOIC ACID (ATTENUATION OR AMPLIFICATION) ON C22:0-, C24:0-, AND C26:0-INDUCED MITOCHONDRIAL DYSFUNCTIONS AND OXIDATIVE STRESS ON HUMAN NEURONAL SK-N-BE CELLS

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Abstract: Increased levels of C22:0, C24:0 and C26:0 were found in cortical lesions of patients with Alzheimer's disease (AD). So, it was of interest to precise the cytotoxic effects of these fatty acids, and to determine whether docosahexaenoic acid (DHA), described to prevent AD, can attenuate their eventual side effects. Human neuronal SK-N-BE cells were cultured in the absence or presence of C22:0, C24:0 or C26:0 (0.1-20 μM) without or with DHA (50-150 μM). C22:0, C24:0 and C26:0 induce an inhibition of cell growth, a loss of $\Delta\psi m$, an overproduction of reactive oxygen species (ROS), a decrease of reduced glutathione, and a lipid peroxidation. DHA attenuates C22:0, C24:0 and C26:0 induced-mitochondrial dysfunctions and/or cell growth inhibition measured with MTT whatever the concentrations considered, whereas it can either decrease or amplify (especially at 150 μM) ROS overproduction. C22:0, C24:0 and C26:0 have neurotoxic activities, and depending on its concentration, DHA attenuates or not fatty acid-induced side effects.

Key words: DHA, fatty acids, mitochondrial dysfunctions, oxidative stress, lipid peroxidation, SK-N-BE cells.

Introduction

The initiating event in Alzheimer disease (AD; OMIM # 104300) is related to the excess production of β-amyloid (Aβ) peptides in the brain (1). The excess of Aβ could be the consequence of abnormal processing of amyloid precursor protein (APP) via β- and ɣ-secretases (2) and/or to a reduced clearance of Aβ (3). In the presence of Aβ, a cascade of toxic events would be activated including a hyperphosphorylation of tau, a neural associated protein involved in microtubules stabilization and axonal transport (4). The hyperphosphorylation of tau (a major component of neurofibrillary tangles (NFTs)) favors microtubules disorganization (5).

The pathogenesis of AD is still poorly understood but some investigations support a linkage between lipid metabolism and AD (6). Alterations of cholesterol metabolism leading to abnormal levels of cholesterol oxide derivatives (also named oxysterols) in the central nervous system, such as 24S-hydroxycholesterol, may contribute to some neurological diseases including AD (7). Potential roles of 7-ketocholesterol and 7β-hydroxycholesterol resulting from cholesterol autoxidation (8), which could be favored by enhanced oxidative stress observed in brain of AD patients, have been suggested in AD (9). It is well known that Apolipoprotein E polymorphic alleles are main genetic determinants of AD risk: individuals carrying the ε4 allele are at increased risk of AD compared with those carrying the more common ε3 allele, whereas the ε2 allele decreases risk (10). Regarding the decline of peroxisomal function with age (11), which can affect lipid and oxidative stress homoeostasia (12), peroxisome may play a critical role in regulating cellular aging and in neurodegenerative diseases (13). Some lipid alterations observed in AD, concerning docosahexaenoic acid (DHA, C22:6 n-3), C22:0, C24:0, C26:0, and plasmalogens support the hypothesis of peroxisomal dysfunctions given that the β-oxidation or the synthesis of these lipids occurs, at least in part, in the peroxisome (13). Moreover, treatment of hippocampal neurons with Wy-14.463, a peroxisome proliferator, was able to prevent cell death induced by Aβ (14). C24:0 and C26:0 accumulations, increased in Aβ40 content, APP immunoreactivity and APP mRNA expressions were also observed in the cerebral cortex of rats treated with thioridazine, a peroxisomal β-oxidation inhibitor (15). In addition, a correlation analysis showed that the Aβ40 levels were positively correlated with the cortex C24:0 and C26:0 levels (15). In the brain of a transgenic mouse model of AD (Tg2576 mouse), biochemical and morphological modifications also support a role for peroxisome (16). In the hippocampus of these transgenic mice, the peroxisomal membrane protein ABCD3 and ACOX1 were induced (17). Interestingly, lipid analyses of cortical regions from AD patients revealed accumulation of C22:0, C24:0, C26:0 in patients with stages V-VI pathology compared with those modestly affected (stages I-II) based on the neuropathological Braak classification (18). The level of plasmalogens, which need intact peroxisomes for their biosynthesis, was decreased in severely affected tissues; the peroxisomal volume density was increased in some neurons in the gyrus frontalis at advanced AD stages (18). Therefore, the accumulation of C22:0, C24:0 and C26:0 in the cortex of AD patients could

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contribute to neuron dysfunctions via their ability to trigger various side effects involved in the physiopathology of AD such as activation of oxidative stress (19) and dysregulation of mitochondrial activity (20). Indeed, on various cell types of the central nervous systems, C22:0, C24:0 and C26:0 are able to trigger an overproduction of reactive oxygen species (ROS) associated with lipid peroxidation and mitochondrial changes which probably play key roles in cell death induction (21-23).

As there is no curative treatment for AD, many efforts are directed to develop alternative treatments including medical foods. DHA is a dietary essential (n-3) PUFA highly enriched in fish oils, which can also be synthetized from eicosapentanoic acid (EPA; C20:5 n-3) and linolenic acid (ALA; 18:3 n-3) (13). Then, DHA is esterified into phospholipids and delivered to the brain and retina. Neuroprotectin D1 (NPD1), the first identified stereoselective bioactive compounds of DHA (24), favours neuronal survival and downregulation of amyloidogenic processing (25). Based on the decreased activity of peroxisome with aging, which can reduce the intrinsic synthesis of DHA (26), and consequently of NPD1, medical foods enriched in DHA have some interests to prevent AD. However, some side effects of DHA (neuritic injury, astrocytosis, stimulation of oxidative stress) have also been reported (26, 27). In the context of fatty acids-induced neuronal damages, it was of interest to precise whether DHA can have beneficial or detrimental effects.

In the present study, on human neuronal cells SK-N-BE, we asked: i) whether C22:0, C24:0 and C26:0 known to trigger cytotoxic effects on different brain cells (21-23) were able to induce mitochondrial dysfunctions, cell growth inhibition, ROS overproduction, and oxidative stress damages (decrease of reduced glutathione (GSH); lipid peroxidation: increased levels of 4-hydroxynonenal and 7-hydroxycholesterols), and ii) whether DHA was able to prevent C22:0, C24:0 and C26:0 side effects. We observed i) that C22:0, C24:0 and C26:0 induce an inhibition of cell growth, mitochondrial dysfunctions, an overproduction of ROS, a decrease of GSH, and a lipid peroxidation, and ii) that DHA was able to attenuate C22:0, C24:0 and C26:0 induced-mitochondrial dysfunctions and/or cell growth inhibition, whereas ROS overproduction was either reduced or amplified.

Methods and Materials

Cells and cell treatments

Human neuronal cells (SK-N-BE) were seeded at 200,000-400,000 cells per well in 12-well microplates containing 1 mL of culture medium (Dulbecco's modified Eagle medium with L-glutamine (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Pan Biotech) and 1% antibiotics (Pan Biotech)). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and passaged twice a week.

Docosanoic acid (C22:0), tetracosanoic acid (C24:0), hexacosanoic acid (C26:0), and docosahexaenoic acid (DHA; C22:6 n-3) (Sigma-Aldrich) were solubilized in α-cyclodextrin (Sigma-Aldrich), and the maximal final concentration of α-cyclodextrin (vehicle) in the culture medium was 1 mg/mL (21-23).

The conditions of treatment with C22:0, C24:0 or C26:0 were the following: after plating SK-N-BE cells for 24 h, the cells were treated for 48 h with various fatty acids (0.1, 1, 5, 10, and/or 20 μM) in HAM's F-10 medium (Pan Biotech) containing 5% FBS in the absence or in the presence of DHA (50, 100, 150 μM). The concentrations of fatty acids were chosen according to measurements made on the plasma of healthy subjects (21, 28) and AD patients (23).

Evaluation of mitochondrial activity with the colorimetric MTT assay

The MTT assay was carried as previously described (21) on SK-N-BE cells plated in 12-well flat-bottom culture plates after 48 h of treatment with C22:0, C24:0 or C26:0. The MTT assay was used to evaluate the effects of C22:0, C24:0 and C26:0 on mitochondrial activity and/or cell growth. A microplate reader was used to record mitochondrial activity and/or cell growth at a wavelength of 570 nm.

Flow cytometric quantification of transmembrane mitochondrial potential with DiOC6(3)

Variations of the mitochondrial transmembrane potential ($\Delta\Psi$m) were measured with 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3) (Life Technologies), which allows the percentage of cells with low $\Delta\Psi$m to be determined. With DiOC6(3) used at 40 nM, mitochondrial depolarization is indicated by a decrease in green fluorescence collected through a 520/10 nm band pass filter. Flow cytometric analyses were performed on a Galaxy flow cytometer (Partec); 10,000 cells were acquired, and data were analyzed with Flomax (Partec) and FlowJo (Tree Star Inc.) softwares.

Flow cytometric measurement of reactive oxygen species after staining with DHE and H2DCFDA

Measurement of reactive oxygen species (ROS) contributing to oxidative stress was performed with DHE (dihydroethidium) or H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) on cells cultured for 48 h in 12 well plates in the absence or in the presence of C22:0, C24:0 and C26:0 without or with DHA.

DHE and H2DCFDA are useful probes for detecting ROS in cells (29, 30). To measure ROS production, cultured cells were incubated for 30 min at 37°C in the dark with DHE (Life Technologies) or H2DCFDA (Sigma-Aldrich) used at 2 μM and 10 μM final, respectively. At the end of the incubation time, the cells were trypsinized, resuspended in PBS, and the fluorescence of ethidium or 2',7'-dichlorodihydrofluorescein (DCF) resulting from oxidation from DHE and H2DCFDA, respectively, was measured by flow cytometry. The fluorescent signals of ethidium and DCF were collected through a 520/10 nm and a 580/10 nm band pass filter, respectively, on a Galaxy

flow cytometer. Fluorescent signals were measured on a logarithmic scale, 10,000 cells were acquired, and the data were analyzed either with Flomax or FlowJo softwares.

Quantification of intracellular reduced glutathione by fluorescence microscopy after staining with monochlorobimane

The RedOx status was also evaluated by the level of reduced glutathione (GSH) per cell by fluorescence microscopy. GSH was revealed by staining with monochlorobimane (MCB) (31). MCB, which reacts with GSH under the action of glutathione S-transferase, was prepared at 4 mM in 100% ethanol. It was added at 200 μM in cell suspensions adjusted to 106 cells/mL in PBS. After 30 min of incubation at 37°C, cell deposits were realized by cytocentrifugation. Cells were observed with an Axioskop fluorescent Zeiss microscope under UV light. The percentage of cells containing GSH (blue/green fluorescent cells) was determined on 300 cells.

Evaluation of lipid peroxidation by flow cytometric quantification of 4-hydroxynonenal

4-hydroxynonenal (4-HNE) produced by lipid peroxidation is a suitable marker of oxidative stress (32); it can react with various molecules giving 4-HNE adducts (33). To identify and quantify 4-HNE, and 4-HNE adducts by flow cytometry, cells were collected by trypsinisation, fixed in 2% paraformaldehyde diluted in PBS, incubated with blocking buffer (PBS, 0.05% saponin, 10% goat serum), washed in PBS, and incubated (1 h, room temperature) with a mouse monoclonal anti 4-HNE (Abcys) diluted in blocking buffer (2 μg/mL). Cells were further washed with PBS and incubated (1 h, room temperature) with a 488-Alexa goat anti-mouse polyclonal antibody (Life Science Technologies) diluted at 1/300 in blocking buffer. Cells were further washed and resuspended in PBS, and analysed on a Galaxy flow cytometer. The green fluorescence of 488-Alexa was collected with a 520/10 nm band pass filter and measured on a logarithmic scale; 10,000 cells were acquired, and the data were analysed with Flomax and FlowJo softwares.

Evaluation of lipid peroxidation by quantification of cholesterol oxide derivatives by gas chromatography coupled with mass spectrometry

Cholesterol oxide derivatives resulting from cholesterol autoxidation (7-ketocholesterol, 7β-hydroxycholesterol) (8), 7α-hydroxycholesterol (formed via CYP7A1 (34) but which can also arise from the conversion of 7α-hydroperoxycholesterol produced by free radical oxidation of cholesterol (8)), and total 7-hydroxycholesterols (7-ketocholesterol+7β-hydroxycholesterol+7α-hydroxycholesterol) were quantified as previously described as well as total cholesterol (22).

Statistical analysis

Statistical analyses were performed using WinSTAT® for Microsoft® Excel (version 2012.1) with the Mann-Whitney test. Data were considered statistically different at a P-value of 0.05 or less.

Results

Induction of mitochondrial dysfunctions and inhibition of cell growth in C22:0-, C24:0-, and C26:0 – treated SK-N-BE cells

The effects of C22:0, C24:0 and C26:0 (0.1-20 μM) on $\Delta\psi$m and cell growth were evaluated after 48 h of treatment by various complementary tests: MTT test; flow cytometric measurement of $\Delta\Psi$m with DiOC6(3); cell counting with trypan blue. Depending on the fatty acid considered, a more or less important dose-dependent decrease of MTT reduction was observed. The absorbance began to decrease significantly with C22:0 (0.1 μM), and with C24:0 and C26:0 (5 μM); similar absorbancies were obtained in control and vehicle-treated cells (Figure 1A). As MTT gives a blue color product (formazan) under the action of the mitochondrial enzyme succinate deshydrogenase, it provides information on cell proliferation and mitochondrial metabolism. To define whether the decrease in formazan production with C22:0, C24:0 and C26:0 is a consequence of reduced number of cells, and/or of mitochondrial dysfunctions, the number of viable cells was determined and $\Delta\Psi$m was measured. The increase of cells with depolarized mitochondria measured with DiOC6(3) became significant with C22:0 and C24:0 (1 μM), and with C26:0 (0.1 μM). Similar values of cells with depolarized mitochondria were obtained in control and vehicle-treated cells (Figure 1B). The decrease of viable cells determined by counting with trypan blue became significant with C22:0 (0.1 μM), and C24:0 and C26:0 (5 μM); similar number of cells was obtained in untreated cells (control) and vehicle – treated cells (Figure 1C).

Enhancement of reactive oxygen species production in C22:0-, C24:0-, and C26:0 – treated SK-N-BE cells

The effects of C22:0, C24:0 and C26:0 (5, 10, 20 μM) on intracellular ROS production were evaluated after 48 h of treatment. After staining with DHE and whatever the fatty acid considered, a marked increased of cells overproducing ROS, especially superoxide anions (HE-positive cells) [30], was observed at 10 and 20 μM and no difference was observed between untreated cells (control) and vehicle – treated cells (Figure 2A).

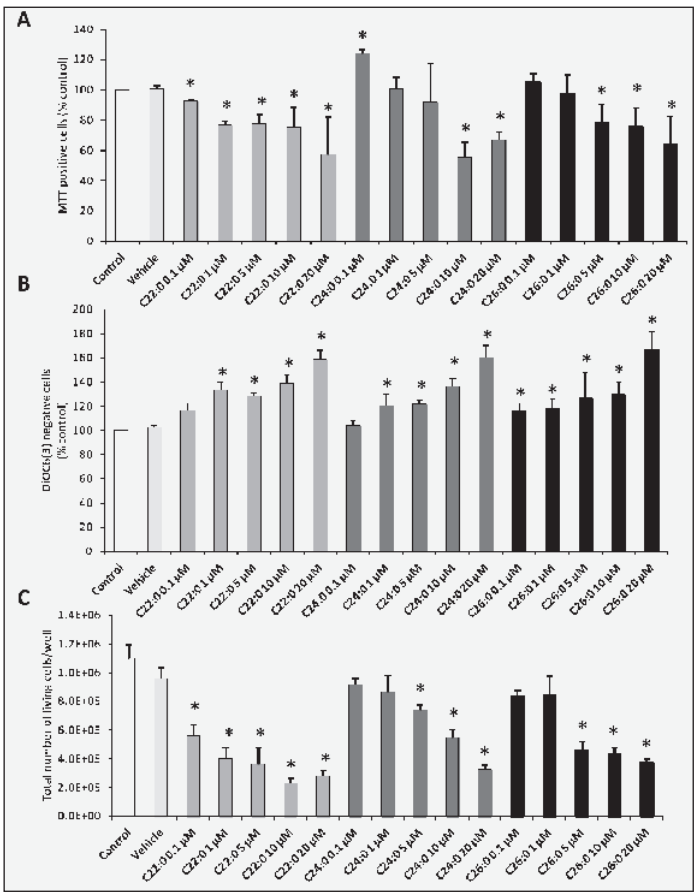
Evidence of oxidative stress damages in C22:0-, C24:0-, and C26:0 – treated SK-N-BE cells: decrease of intracellular GSH and induction of lipid peroxidation

The effects of C22:0, C24:0 and C26:0 (5, 10, 20 μM) on anti-oxidant defenses were evaluated after 48 h of treatment by measuring the intracellular level of GSH with MCB. Under

treatment with C22:0, C24:0 and C26:0, an important decrease of cells containing GSH (MCB positive cells) was observed. It was significant with C22:0 (5, 10 μM) and C24:0 (5, 10, 20 μM) whereas it was only significant with C26:0 (5 μM). Similar percentages of MCB positive cells were obtained in control and vehicle-treated cells (Figure 2B).

Figure 1

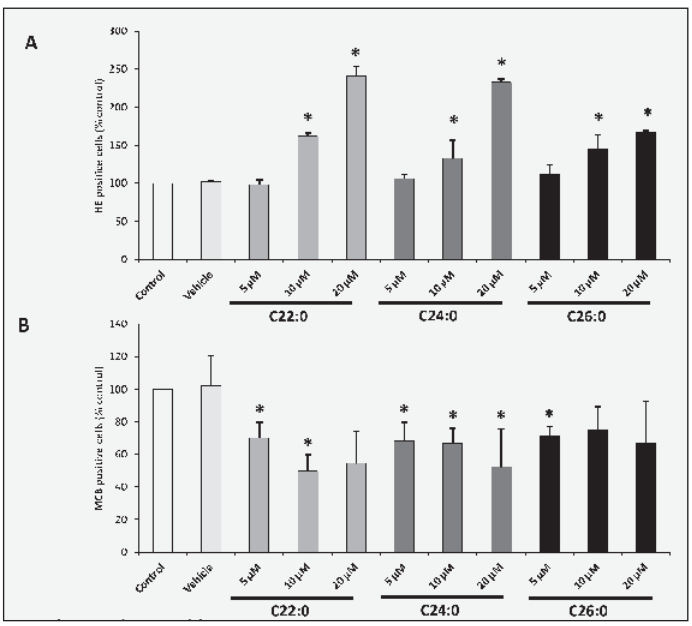
Evaluation of the effects of C22:0, C24:0 and C26:0 on mitochondrial activity and cell proliferation: MTT assay; measurement of transmembrane mitochondrial potential ($\Delta\Psi$m) with DiOC6(3); cell counting of viable cells with trypan blue. SK-N-BE cells were cultured for 48 h in the absence (control) or presence of α cyclodextrin (vehicle), or of C22:0, C24:0,or C26:0. In control and vehicle-treated cells, the percentage of cells with depolarized mitochondria (DiOC6(3) negative cells) were in the same range (5-10%). Data shown are mean ± SD from two independent experiments conducted in triplicates. Significance of the difference between vehicle- and fatty acid-treated cells is indicated by * (Mann Whitney test; * P<0.05)



Lipid peroxidation (fatty acid degradation, cholesterol oxidation) was evaluated by the intracellular level of 4-HNE and formation of cholesterol autoxidation products (resulting from oxidation of cholesterol at C7), respectively. Whereas increased cellular levels of 4-HNE were observed in C22:0, C24:0 and C26:0 (5, 10, 20 μM) - treated SK-N-BE cells, a significant difference was only found with C22:0 (20 μM) (Figure 3A). When compared to vehicle treated cells, significant increased of the ratio [total 7-hydroxycholesterols]/[cholesterol] was found in C22:0, C24:0 and C26:0 (10 μM) - treated SK-N-BE cells (Figure 3B). Significant increased of 7-ketocholesterol was also found with C22:0 and C24:0 (10 μM) but not with C26:0 (10 μM) (Figure 3C), and no significant increased of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and cholesterol were observed in C22:0, C24:0 and C26:0 (10 μM) - treated SK-N-BE cells (Figure 3D-F). Similar levels of 4-HNE, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, cholesterol and [total 7-hydroxycholesterols] / [cholesterol] were measured in control and vehicle-treated cells (Figures 3).

Figure 2

Evaluation of the effects of C22:0, C24:0 and C26:0 on superoxide anion production and reduced glutathione (GSH) content. SK-N-BE cells were cultured for 48 h in the absence (control) or presence of α-cyclodextrin (vehicle) or of C22:0, C24:0, or C26:0. The production of superoxide anion (HE-positive cells) was determined with DHE; data are expressed as % control (control cells: 5-10% of HE positive cells). The GSH content per cell was revealed with MCB; the percentage of MCB positive cells was determined (control cells: 95-100% of MCB positive cells). Data shown are mean ± SD from three independent experiments. Significance of the difference between vehicle- and fatty acid-treated cells is indicated by * (Mann-Whitney test; * P<0.05)



Attenuation of C22:0-, C24:0-, and C26:0– induced mitochondrial dysfunctions and/or cell growth by DHA

We determined whether DHA was able to attenuate cell dysfunctions induced by C22:0, C24:0 and C26:0. With the MTT test, marked attenuation of the side effects induced by C22:0 (Figure 4A), C24:0 (Figure 4B), and C26:0 (Figure 4C) were found. DHA used alone (50, 100, 150 μM) had no effect on mitochondrial activity and/or cell growth (Figure 4). These data show that DHA can attenuate C22:0-, C24:0- and C26:0-induced mitochondrial dysfunctions and/or cell growth inhibition.

Figure 3

Evaluation of the effects of C22:0, C24:0 and C26:0 on lipid peroxidation: measurement of the cellular level of 4-hydroxynonenal (4-HNE) and oxysterols oxidized at C7 (7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol). SK-N-BE cells were cultured for 48 h in the absence (control) or presence of α-cyclodextrin (vehicle) or of C22:0, C24:0, or C26:0. The intracellular content of 4-HNE was determined by flow cytometry; the mean fluorescence intensity (MFI) of 4-HNE positive cells was determined; data are expressed as % control. The cellular content of 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol and the ratio [total 7-hydroxycholesterols]/[cholesterol] were determined by GC/MS. Data shown are mean ± SD from three independent experiments. Significance of the difference between vehicle- and fatty acid-treated cells is indicated by * (Mann-Whitney test; * P<0.05)

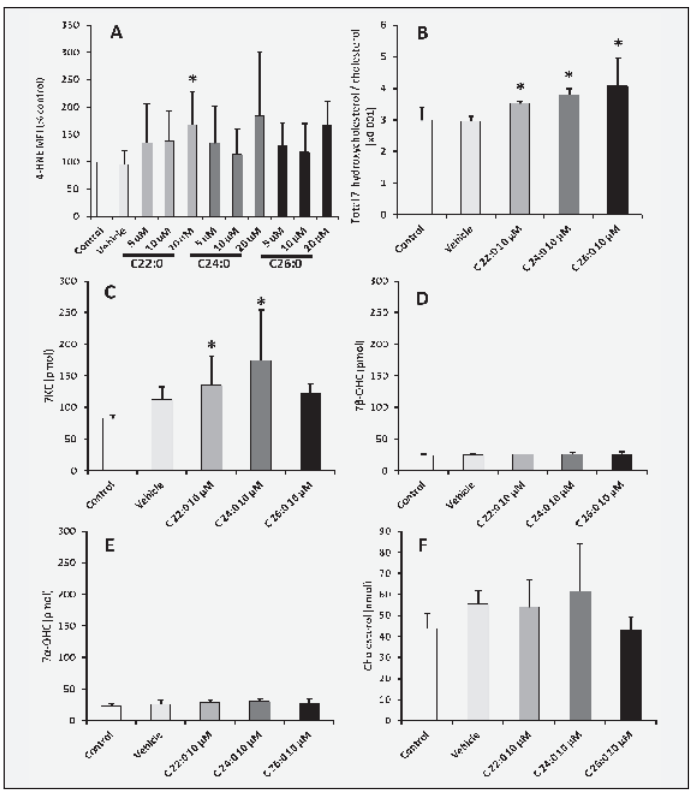
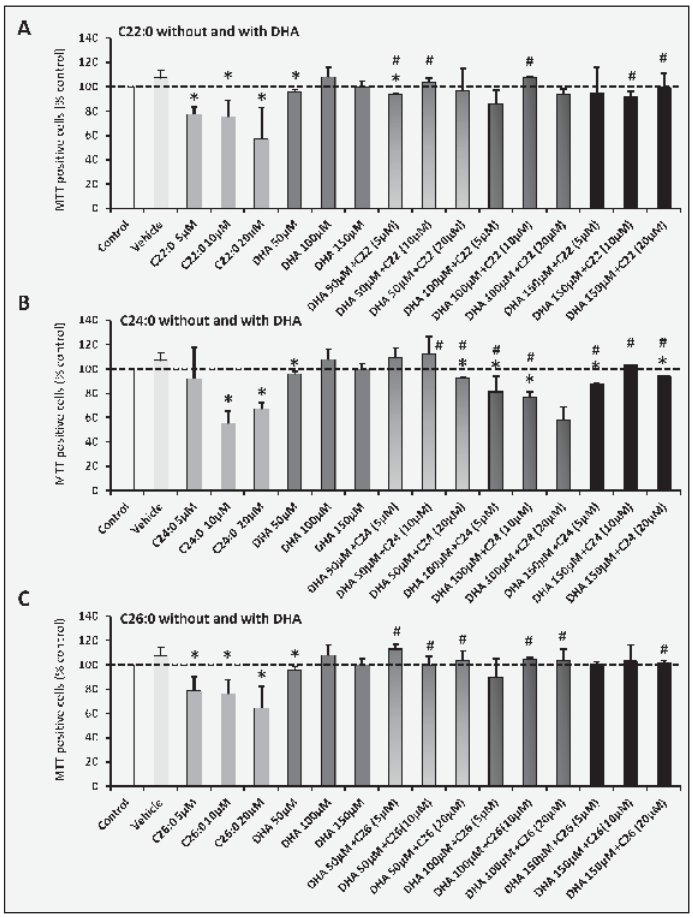


Figure 4

Evaluation of the effects of DHA on C22:0 -, C24:0 -, and C26:0 – induced mitochondrial dysfunctions and/or cell growth inhibition with the MTT test. SK-N-BE cells were cultured for 48 h in the absence (control) or presence of α-cyclodextrin (vehicle) or of C22:0, C24:0, or C26:0 without or with DHA. Data shown are mean ± SD from two to three independent experiments realized in triplicate. Significance of the difference between vehicle- and fatty acid-treated cells is indicated by * (Mann-Whitney test; * P<0.05). Significance of the difference between fatty acid-treated cells and (fatty acid + DHA)-treated cells is indicated by # (Mann-Whitney test; # P<0.05)



Attenuation or amplification of C22:0-, C24:0-, and C26:0–induced ROS overproduction by DHA

We attempted to determine whether DHA was able to attenuate C22:0-, C24:0-, and C26:0–induced ROS overproduction. After 48 h of culture in the absence or presence of C22:0, C24:0, and C26:0, associated or not with DHA, SK-N-BE cells were stained with H2DCFDA. Comparatively to untreated (control) or vehicle-treated cells, an overproduction of ROS was observed in fatty acids-treated cells: higher values of DCF positive cells (expressed as % control) were observed with C22:0 (Figure 5A), C24:0 (Figure 5B), and C26:0 (Figure 5C). When DHA was used alone, it

had no effect on ROS production at 50 and 100 μM; however, it was able to stimulate ROS production at 150 μM (Figure 5). DHA (50 μM) was able to reduce C24:0-, and C26:0-induced ROS overproduction; DHA (100 μM) was able to reduce C22:0-, C24:0-, and C26:0-induced ROS overproduction (Figure 5). DHA (150 μM) was most often not efficient on fatty acids-induced ROS overproduction, excepted with C24:0 (5 μM) (Figure 5). Thus, the ability of DHA to reduce C22:0-, C24:0- and C26:0-induced ROS overproduction depends on its concentration and on the fatty acid considered.

Discussion

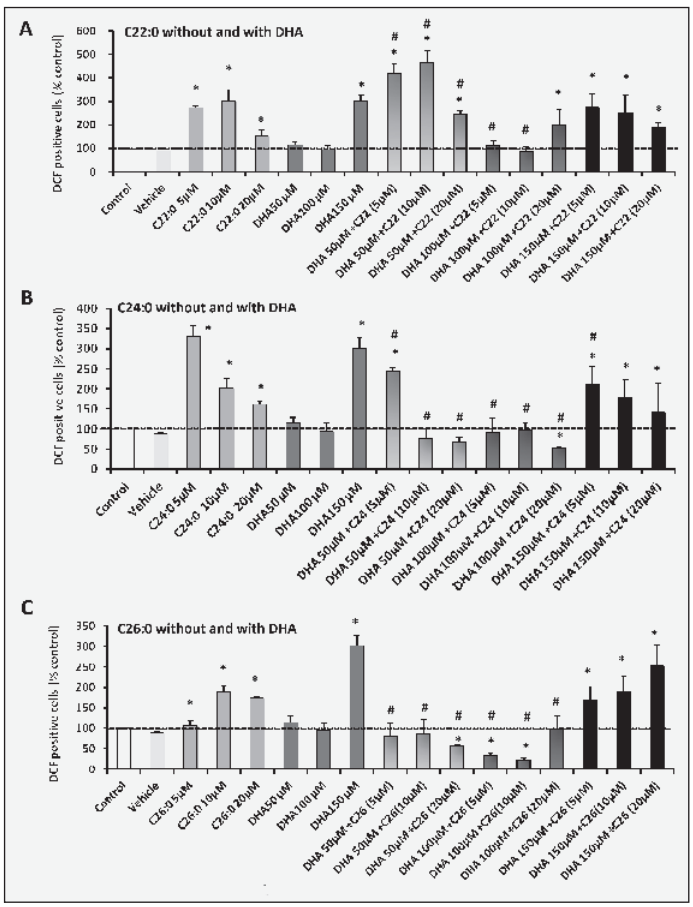
The pathogenesis of AD is still poorly understood but some investigations sustain a linkage between lipid metabolism and AD (7, 13). The accumulation of C22:0, C24:0 and C26:0 in cortical lesion of AD patients supports peroxisomal dysfunctions (13). As previous investigations performed on various cell types of the central nervous systems (especially glial cells) revealed that C22:0, C24:0 and C26:0 are able to trigger mitochondrial changes and rupture of RedOx homeostasia (21, 22), which are known to contribute to the development of AD (19, 20), it was of interest i) to precise the activities of these fatty acids on human neuronal cells and ii) to evaluate whether DHA, which has neuroprotective activities (25), can reduce some of their side effects. On human neuronal cells SK-N-BE cultured in the absence or in the presence of C22:0, C24:0, and C26:0 associated or not with DHA, we observed that C22:0 -, C24:0 -, and C26:0 - induced side effects (mitochondrial dysfunctions, oxidative stress) which can be, or not, attenuated by DHA.

Under treatment with C22:0, C24:0, and C26:0, in the range of concentrations found in the plasma of patients with different forms of peroxisomal diseases (28) and with AD (23), more or less pronounced side effects (in agreement with those described on 158N oligodendrocytes (21, 22)) are detected on human neuronal cells SK-N-BE: inhibition of cell growth, mitochondrial dysfunctions, and oxidative stress induction. The differences observed between the different fatty acids could depend on their incorporation and repartition in neutral lipids and phospholipids (22). As C22:0, C24:0 and C26:0 are able to induce various side effects on SK-N-BE cells, they could constitute potential cytotoxic compounds capable to contribute to neurodegeneration.

Under treatment with C22:0, C24:0 and C26:0, the decrease of MTT positive cells, giving information on mitochondrial activity and/or cell growth, and the reduced number of living cells measured by counting with trypan blue suggest substantial cellular damages and cell death induction. Such data are in agreement with those observed on 158N cells (21, 22). In this cell death process, the important loss of $\Delta\psi m$ support the hypothesis that mitochondria might contribute to the cytoxic effects of these fatty acids (23).

Figure 5

Evaluation of the effects of DHA on C22:0-, C24:0-, and C26:0–induced ROS overproduction after staining with H2DCFDA. SK-N-BE cells were cultured for 48 h in the absence (control) or presence of α-cyclodextrin (vehicle) or of C22:0, C24:0, or C26:0 without or with DHA. The fluorescence of 2',7'-dichlorodihydrofluorescein (DCF) resulting from oxidation of H2DCFDA was measured by flow cytometry (control cells: 5-10 % of DCF positive cells). Data shown are mean ± SD from two to three independent experiments realized in triplicate. Significance of the difference between vehicle- and fatty acid-treated cells is indicated by * (Mann-Whitney test; * P<0.05). Significance of the difference between fatty acid-treated cells and (fatty acid + DHA)-treated cells is indicated by # (Mann-Whitney test; # P<0.05)



We also confirm that C22:0, C24:0 and C26:0 induce ROS overproduction. Since H2DCFDA allows to detect a wide number of ROS (29), whereas HE is considered as more specific for superoxide anions (30), this can explain the differences observed between these two dyes on C22:0-, C24:0, and C26:0-treated cells. However, as we previously reported that extramitochondrial sources of ROS could be activated by these fatty acids, and could in turn contribute to inducing mitochondrial damages (23), it was of interest to precise the impact of oxidative stress at the cellular level. We show, in

agreement with data obtained on 158N oligodendrocytes (21), that C22:0, C24:0, and C26:0 cause a decrease of intracellular GSH measured with MCB. As GSH level has been measured with MCB, which binds to thiol groups by a reaction catalyzed by gluthatione S-transferases (31), the decrease in MCB positive cells observed under treatment with the fatty acids may be due to the lower glutathione S-transferase activity and/or to lower levels of GSH. In turn, lower levels of GSH could contribute to increase ROS-induced cellular damages such as lipid peroxidation which has been revealed on C22:0-, C24:0-, and C26:0-treated SK-N-BE cells by increased levels of 4-HNE, 7-ketocholesterol, and total 7-hydroxycholesterols. 4-HNE is a highly reactive aldehyde, which exhibits great reactivity with biomolecules (proteins, phospholipids, DNA) and generates a variety of intra- and inter-molecular covalent adducts which can contribute to amplifying cellular dysfunctions (33). So, this could further modulate the activity of various metabolic pathways involved in the regulation of inflammation, oxidation, and cell death, wich are hallmarks of brain lesions in AD (35). On the other hand, radical attack of cholesterol leading to intracellular accumulation of total 7-hydroxycholesterols (7-ketocholesterol + 7β-hydroxycholesterol + 7α-hydroxycholesterol), also considered as a sign of lipid peroxidation (8), was investigated. The increase of 7-hydroxycholesterols observed mainly depends on the increase of 7KC, excepted for C26:0. Since 7KC has a wide range of biological activities (stimulation of ROS production, induction of cell death) (36), this oxysterol could constitute a second messenger triggering oxidative stress and cell death. Noteworthy, as overproduction of ROS and lipid peroxidation observed on SK-N-BE cells evocates the data obtained on 158N oligodendrocytes (21), the present study supports that these side effects of C22:0, C24:0 and C26:0 do no depend on the neural cell type considered. These fatty acids could consequently lead to major brain damages. It was therefore of importance to determine whether DHA, which is considered as a neuroprotector (37), but which can also induce various side effects (26, 27), was able to attenuate the cytotoxicity of C22:0, C24:0, and C26:0.

Interestingly, our data bring evidences that DHA attenuates mitochondrial dysfunctions and/or cell growth whatever the concentrations used (50, 100, 150 μM), whereas overproduction of ROS (whatever the fatty acid considered: C22:0, C24:0 and C26:0) was only reduced at 100 μM. However, the ability of DHA (150 μM) to stimulate ROS overproduction, when used alone, or to amplify ROS overproduction in the presence of C22:0, C24:0 and C26:0 even at the lowest and physiological concentration tested (5 μM), reveals that DHA can also have side effects suggesting that it must be used with caution in order to prevent neurodegeneration without inducing additional damages. Based on the present study, the ability of DHA to preserve mitochondrial dysfunctions and/or cell growth, whatever the concentration used, supports the hypopthesis that DHA via its subsequent conversion in NPD1 (involving 15-LOX-1 or 15-LOX-like activities) can constitute a potent inhibitor of neuronal cell death and is able to counteract C22:0, C24:0 and C26:0-induced mitochondrial dysfunctions. According to the ability of DHA and of its precursor (EPA) to cross the blood brain barrier, to accumulate into the brain (38), and because of the high half-life of DHA in the human brain [39], neuroprotective effects of DHA against mitochondrial dysfunctions can be expected in vivo for some patients. As the ability of DHA (via NPD1 or other mechanisms) to counteract C22:0-, C24:0- and C26:0-induced-ROS overproduction is less efficient at high concentrations, it is hypothesized that DHA could inhibit the esterification of other fatty acids present at lower concentrations. This could favor an anarchic repartition of various free fatty acids in different cell compartments and subsequently trigger the activation of several side effects including an overproduction of ROS, which could result either from an enhancement of enzymatic activities involved in ROS production or from a decrease of anti-oxidative defenses.

Thus, we can hypothesize that the therapeutic efficiency of DHA could strongly depend on its posology, on the conditions of treatment, and of its packaging. In functional foods (especially in neutraceutical functional foods), it will be necessary to verify that other ingredients will not alter DHA activity. It is also important to consider that the intake of DHA is absent in human carrying APOE ε4 allele, the most important risk factor in AD (40). This dual effects of DHA revealed in the present study, as well as genetic factors, could explain, at least in part, the important differences on the benefits of DHA from one clinical study to another (27). Therefore, a better knowledge of DHA-associated side effects would greatly contribute to enhance its effectiveness to prevent and/or improve cognitive decline.

Our data obtained on SK-N-BE cells underline that C22:0, C24:0 and C26:0 inhibit cell growth, trigger a loss of $\Delta\psi m$, and induce oxidative stress (overproduction of ROS, decrease of GSH, lipid peroxidation). They also revealed that DHA attenuates C22:0-, C24:0-, and C26:0-induced mitochondrial dysfunctions and/or cell growth inhibition, whereas it can favor ROS overproduction. As mitochondrial dysfunctions, ROS overproduction and oxidative stress are hallmarks of AD, our data brings new evidences on the potential neurotoxicity of lipids (C22:0, C24:0, C26:0) in AD, and they highlight dual effects of DHA (attenuation or amplification) on C22:0-, C24:0-, and C26:0-induced side effects.

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References

1. Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, Petersen RC, Trojanowski JQ. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. Lancet Neurol. 2010;9, 119-128.

2. Zhou ZD, Chan CH, Ma QH, Xu XH, Xiao ZC, Tan EK. The roles of amyloid precursor protein (APP) in neurogenesis: Implications to pathogenesis and therapy of Alzheimer disease. Cell Adh Migr. 2011;5, 280-292.
3. Kurz A, Perneczky R. Amyloid clearance as a treatment target against Alzheimer's disease. J Alzheimers Dis. 2011;24 Suppl 2, 61-73.
4. Cavallucci V, D'Amelio M, Cecconi F. Aβ toxicity in Alzheimer's disease. Mol Neurobiol. 2012;45, 366-378.
5. Iqbal K, Liu F, Gong CX, Grundke-Iqbal I. Tau in Alzheimer disease and related tauopathies. Curr Alzheimer Res. 2010;7, 656-664.
6. Grimm MO, Rothhaar TL, Hartmann T. The role of APP proteolytic processing in lipid metabolism. Exp Brain Res. 2012;217, 365-375.
7. Björkhem I, Cedazo-Minguez A, Leoni V, Meaney S. Oxysterols and neurodegenerative diseases. Mol. Aspects Med. 2009;30, 171-179.
8. Iuliano L. Pathways of cholesterol oxidation via non-enzymatic mechanisms. Chem Phys Lipids. 2011;164, 457-468.
9. Vaya J, Schipper HM. Oxysterols, cholesterol homeostasis, and Alzheimer disease. J Neurochem. 2007;102, 1727-1237.
10. Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol. 2013;9, 106-118.
11. Titorenko VI, Terlecky SR. Peroxisome metabolism and cellular aging. Traffic. 2011;12, 252-259.
12 Schrader M, Fahimi HD. The peroxisome: still a mysterious organelle. Histochem Cell Biol. 2008;129, 421-440.
13. Lizard G, Rouaud O, Demarquoy J, Cherkaoui-Malki M, Iuliano L. Potential roles of peroxisomes in Alzheimer's disease and in dementia of the Alzheimer's type. J Alzheimers Dis. 2012;29, 241-254.
14. Santos MJ, Quintanilla RA, Toro A, Grandy R, Dinamarca MC, Godoy JA, Inestrosa NC. Peroxisomal proliferation protects from beta-amyloid neurodegeneration. J Biol Chem. 2005;280, 41057-41068.
15. Shi R, Zhang Y, Shi Y, Shi S, Jiang L. Inhibition of peroxisomal β-oxidation by thioridazine increases the amount of VLCFAs and Aβ generation in the rat brain. Neurosci Lett. 2012;528, 6-10.
16. Cimini A, Moreno S, D'Amelio M, Cristiano L, D'Angelo B, Falone S, Benedetti E, Carrara P, Fanelli F, Cecconi F, Amicarelli F, Cerù MP. Early biochemical and morphological modifications in the brain of a transgenic mouse model of Alzheimer's disease: a role for peroxisomes. J Alzheimers Dis. 2009;18, 935-952.
17. Fanelli F, Sepe S, D'Amelio M, Bernardi C, Cristiano L, Cimini A, Cecconi F, Ceru' MP, Moreno S. Age-dependent roles of peroxisomes in the hippocampus of a transgenic mouse model of Alzheimer's disease. Mol Neurodegener. 2013;8: 8. doi: 10.1186/1750-1326-8-8.
18. Kou J, Kovacs GG, Höftberger R, Kulik W, Brodde A, Forss-Petter S, Hönigschnabl S, Gleiss A, Brügger B, Wanders R, Just W, Budka H, Jungwirth S, Fischer P, Berger J. Peroxisomal alterations in Alzheimer's disease. Acta Neuropathol. 2011;122, 271-283.
19. Cai Z, Zhao B, Ratka A. Oxidative stress and β-amyloid protein in Alzheimer's disease. Neuromolecular Med. 2011;13, 223-250.
20. Maruszak A, Żekanowski C. Mitochondrial dysfunction and Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry. 2011;35, 320-330.
21. Baarine M, Ragot K, Athias A, Nury T, Kattan Z, Genin EC, Andreoletti P, Ménétrier F, Riedinger JM, Bardou M, Lizard G. Incidence of Abcd1 level on the induction of cell death and organelle dysfunctions triggered by very long chain fatty acids and TNF-α on oligodendrocytes and astrocytes. Neurotoxicology 2012;33, 212-218.
22. Baarine M, Andréoletti P, Athias A, Nury T, Zarrouk A, Ragot K, Vejux A, Riedinger JM, Kattan Z, Bessede G, Trompier D, Savary S, Cherkaoui-Malki M, Lizard G. Evidence of oxidative stress in very long chain fatty acid—treated oligodendrocytes and potentialization of ROS production using RNA interference-directed knockdown of ABCD1 and ACOX1 peroxisomal proteins. Neuroscience 2012;213, 1-18.
23. Zarrouk A, Vejux A, Nury T, El Hajj HI, Haddad M, Cherkaoui-Malki M, Riedinger JM, Hammami M, Lizard G. Induction of mitochondrial changes associated with oxidative stress on very long chain fatty acids (C22:0, C24:0, or C26:0)-treated human neuronal cells (SK-N-BE). Oxid Med Cell Longev. 2012;2012:623257. doi: 10.1155/2012/623257.
24. Lukiw WJ, Cui JG, Marcheselli VL, Bodker M, Botkjaer A, Gotlinger K, Serhan CN, Bazan NG. A role for docosahexaenoic acid-derived neuroprotectin D1 in neural cell survival and Alzheimer disease. J Clin Invest. 2005;115, 2774-2783.
25. Stark DT, Bazan NG. Neuroprotectin D1 induces neuronal survival and downregulation of amyloidogenic processing in Alzheimer's disease cellular models. Mol Neurobiol. 2011;43, 131-138.
26. Tanito M, Brush RS, Elliott MH, Wicker LD, Henry KR, Anderson RE. High levels of retinal membrane docosahexaenoic acid increase susceptibility to stress-induced degeneration. J Lipid Res. 2009;50, 807-819.
27. Muntané G, Janué A, Fernandez N, Odena MA, Oliveira E, Boluda S, Portero-Otin M, Naudí A, Boada J, Pamplona R, Ferrer I. Modification of brain lipids but not phenotype in alpha-synucleinopathy transgenic mice by long-term dietary n-3 fatty acids. Neurochem Int. 2010;56, 318-328.
28. Takemoto Y, Suzuki Y, Horibe R, Shimozawa N, Wanders RJ, Kondo N. Gas chromatography/mass spectrometry analysis of very long chain fatty acids, docosahexaenoic acid, phytanic acid and plasmalogen for the screening of peroxisomal disorders. Brain Dev. 2003;25, 481-487.
29. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J. Immunol. 1983;130, 1910-1917.
30. Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. J Leukoc Biol. 1990;47, 440-448.
31. Hedley DW, Chow S. Evaluation of methods for measuring cellular glutathione content using flow cytometry. Cytometry 1994;15, 349-358.
32 Khatoon F, Moinuddin, Alam K, Ali A. Physicochemical and immunological studies on 4-hydroxynonenal modified HSA: implications of protein damage by lipid peroxidation products in the etiopathogenesis of SLE. Hum. Immunol. 2012;73, 1132-1139.
33. Grimsrud PA, Xie H, Griffin TJ, Bernlohr DA. Oxidative stress and covalent modification of protein with bioactive aldehydes. J Biol Chem. 2008;283, 21837-21841.
34. Pikuleva IA. Cholesterol-metabolizing cytochromes P450. Drug Metab Dispos. 2006;34, 513-520.
35. Singh S, Kushwah AS, Singh R, Farswan M, Kaur R. Current therapeutic strategy in Alzheimer's disease. Eur Rev Med Pharmacol Sci. 2012;16, 1651-1664.
36. Vejux A, Lizard G. Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. Mol Aspects Med. 2009;30, 153-170.
37. Dacks PA, Shineman DW, Fillit HM. Current evidence for the clinical use of long-chain polyunsaturated n-3 fatty acids to prevent age-related cognitive decline and Alzheimer's disease. J Nutr Health Aging. 2013;17, 240-251.
38. Ouellet M, Emond V, Chen CT, Julien C, Bourasset F, Oddo S, LaFerla F, Bazinet RP, Calon F. Diffusion of docosahexaenoic and eicosapentaenoic acids through the blood-brain barrier: An in situ cerebral perfusion study. Neurochem Int. 2009;55, 476-482.
39. Eckert GP, Lipka U, Muller WE. Omega-3 fatty acids in neurodegenerative diseases: focus on mitochondria. Prostaglandins Leukot Essent Fatty Acids. 2013;88, 105-114.
40. Vandal M, Alata W, Tremblay C, Rioux-Perreault C, Salem Jr N, Calon F, Plourde M. Reduction in DHA transport to the brain of mice expressing human APOE4 compared to APOE2. J Neurochem. 2014; 129:516-526.