The Anti-oxident Effects of Docosahexaenoic Acid and Arachidonic Acid on Neuronal Cells and Astrogliocytes

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Abstract—**The production of reactive oxygen species (ROS) may cause severe cell damagement and apoptosis of neuronal cells and glial cells in the brain. The aim of this study is to investigate the neuronal protection by arachidonic acid (AA) or docosahexaenoic acid (DHA). Astroglioma C6, glioblastoma U-87MG and neuroblastoma IMR-32 were pre-treated with either DHA or AA and the reactive oxygen species (ROS) production and cell apoptosis were evaluated after pre-treated cells were pulsed by electrical stimulation. The results showed that DHA and AA both can significantly decrease ROS productions and cell apoptosis of C6, U-87MG and IMR-32 after electrical stimulation as compared with those of control cells without DHA/AA pre-treatments. Thus, DHA or AA supplementations may have neuro-protective effects by decreasing oxidative stress and cell apoptosis of neuronal cells and glial cells.**

Keywords- cell ability; ROS production; docosahexaenoic acid; arachidonic acid; neuronal cell; glial cell

I. INTRODUCTION

Docosahexaenoic acid (DHA; 22 : 6n-3) and arachidonic acid (AA; 20 : 4n-6) are highly concentrated in synaptic plasma membranes and essential for brain growth [1]. DHA is demonstrated increases membrane fluidity, improving neurogenesis, synaptogenesis [2], and scavenged the intracellular radical productions induced by H_2O_2 radical, $O_2 \bullet -$, and $\bullet OH$ in cultured retinal ganglion cells [3]. AA plays an important role as a precursor of prostanoids, leukotrienes and other lipoxygenase products. Previous studies have shown that DHA positively modulates PS biosynthesis and accumulation in neuronal cells, promoting survival, and inhibits apoptosis in a PS-dependent manner [1].

Astrocytes are star-shaped glia that respond to hold neurons and supply nutrients to maintain the composition of the extracellular milieu [4]. The aim of this study is to investigate the neuroprotective effects of DHA and AA in vitro. The reactive oxygen species (ROS) production and apoptosis of rat astroglioma C6, human glioblastoma U-87MG and neuroblastoma IMR-32 were evaluated after cells were pretreated with either DHA or AA and then pulsed by electrical stimulation.

II. MATERIALS AND METHODS

A. Cell treatment

The rat glioma cell line C6, human glioblastoma U-87MG and neuroblastoma IMR-32 were separatedly cultured in MEM medium (2 mM L-glutamine, 1 mM non-essential amino acids, and 1% penicillin- streptomycin) supplemented with 10% fetal bovine serum. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

For setting optimal voltage and impulse number per 50 ms during electrical stimulation for 50% cell viability of un-pulsed cells, C6 cells were treated with various voltages ranging from 0-100 V at 50 ms per pulse for consecutive 10 pulses or various pulse numbers ranging from 10-100 pulse at 5V/50 ms per pulse in triplicates in two independent experiments.

A stock solution of 2000 μM DHA and AA were prepared in MEM media with fatty acid free-bovine serum albumin (FFA-BSA MEM). The cells (1.6×10^4 cells/100μL/well) were pretreated with DHA or AA at a final concentration of 0.25 mM for 24 h in FFA-BSA MEM. After washed twice with the MEM, DHA/AA pre-treated cells were pulsed by 5 voltages with 25 pulse numbers (50 ms per pulse) as the electrical stimulation. (BTX ECM830, USA). The electrical pulsed cells were then re-cultured in the MEM for additional 50min at 37℃ in a humidified incubator containing 5% CO_2.

B. Cell viability assay

The cell viability was estimated using the commercially available methylthiazoletetrazolium (MTT) kit (Sigma Chemical Co., St. Louis, MO, USA). The resultant cells were treated with 50μL of MTT reagent at 37 °C, 5% CO_2 for 1h. After the supernatant was evacuated, 100μL of dimethyl sulfoxide (DMSO) was added to solve the formazan reduction product of MTT and then the absorbance was read at the 595nm using an ELISA reader in this colorimetric assay.

C. Apoptosis assay

The apoptosis of the resultant cells were determined using the commercial Apoptosis Kit (Invitrogen Corp.). In each well of the resultant cells, 5 μL Alexa Fluor® 488 annexin V and 1 μL 5 μM SYTOX® Green working solution were added and incubated at room temperature for 15 min. After the incubation period, 400 μL 1X annexin-binding buffer was mixed and then the fluorescence-staining apoptotic cells were immediately analyzed by using the flow cytometry (Coulter Epics Altra Flow Cytometry).

D. Detection of intracellular ROS accumulation

For evaluation of ROS production, the resultant cells were treated with 10μM of acetylated 2,7-dichlorofluorescein diacetate ($DCFH_2DA$) (Invitrogen Corp., Carlsbad, CA, USA), a fluorescent dye, for 30 min at 37°C. Then, the ROS production within epileptic cells stained with the $DCFH_2DA$ was determined using the flow cytometer (Coulter Epics Altra Flow Cytometry, Beckman Coulter, CA).

E. Statistical analysis

All results were expressed as mean ± SE. The significance of difference was evaluated with Duncan's multiple range test. A probability level of $p < 0.05$ was considered statistically significant.

III. RESULTS

A. Effects of DHA and AA on cell viability

The effects of increased electric tension and pulse number on cell activities of C6 exhibited a reduction in cell viability (Fig. 1).
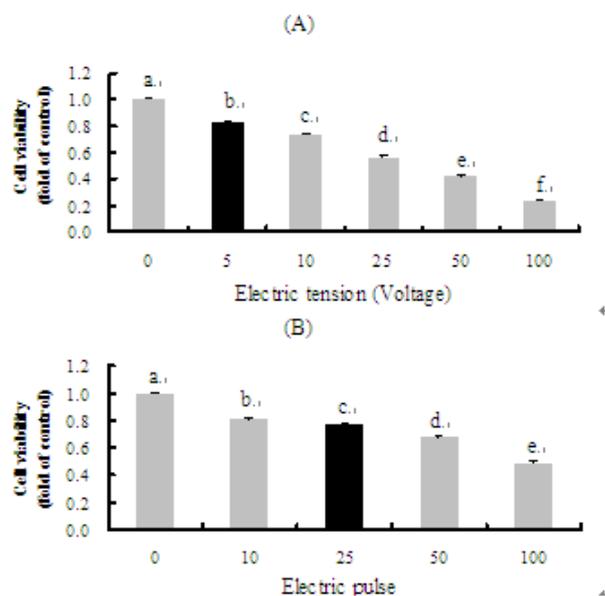

Figure 1. The effects of electric tension and pulse number on cell activities of astroglioma C6. Cells were treated with various voltages (V) ranging from 0-100 V at 50 ms per pulse for consecutive 10 pulses (A) and various pulse numbers ranging from 10-100 pulses at 5V and 50 ms per pulse (B). Data are expressed as mean ± SE from 3 replicates in two independent experiments. Means with the different letters are significantly different ($p < 0.05$).

The results showed that pre-treatment of 25 μM DHA were greater increased the cell viability of C6 and IMR-32 cells when compared to saline control group while 25 μM AA pre-treatment significantly increased the cell viability of C6, U-87MG and IMR-32 cells. Thus, both DHA and AA pre-treatment significantly increased the cell viability of C6 and U-87MG cells without electrical stimulations.

After the treatment of electrical impulse, 25 μM DHA significantly increased the cell viability of U-87MG and IMR-32 cells. However, pre-treatment by 25 μM AA only increased U-87MG cells viability; however, the cell viability of C6 and U-87MG significantly increased when Pre-treated by both DHA and AA (Fig. 2).

B. Cell apoptosis

The supplementation with 25 μM DHA or 25 μM AA significantly reduced the percentages of Annexin V^+ cells on untreated C6 and IMR-32 cells.

After the treatment of electrical impulse, pre-treatment by 25 μM DHA or 25 μM AA significantly reduced the percentages of Annexin V^+ cells on C6 and U-87MG cells when compared to saline control group (Fig. 3).

C. Modification of cellular ROS by treatment with DHA and AA

The DCF-DA fluorescence dye was used to detect the intracellular ROS accumulation in cells. The results indicated that the amount of ROS produced in U-87M and IMR-32 which pre-treated with 25 μM DHA or AA were significantly reduced before pulses stimulation.

After the treatment of electrical impulse, pre-treated by 25 μM DHAand AA significantly increased the cell viability of C6, U-87MG and IMR-32 cells (Fig. 4).

IV. DISCUSSION

Many previous studies have demonstrates that dietary restriction of n-3 fatty acids in the maternal diet lead to accumulation of DHA deficiency in the developmental period and results lower DHA content in the organs such as brain and retina. Further, DHA is reported to play a neuroprotective role against oxidative stress in retinal photoreceptors and culture neuronal cells [3-6]. In this present study, we demonstrated that DHA reduced the intracellular ROS productions and cell apoptosis induced by electrical stimulation in rat astroglioma C6, human glioblastoma U-87MG and neuroblastoma IMR-32 cells. C6 cells were supplemented with 25 μM DHA for 24h, the cell viability increased significantly as compared to saline control group. Several studies have demonstrated that both an increase in culture cells after DHA and AA supplementation. Previous work [6-7] reported that reduced-mitochondrial activity after 90 μM DHA pretreatment in C6 cells. In addition, the primary cultured cerebellar neurons with treatment of 90 μM DHA alone resulted in induction of ROS. Incubation of PC-12 or Neuro 2A cells supplemented with DHA showed no effective when the concentration from 1-25 μM, but when the concentration raised to a higher concentration (50μM), the fatty acids were be toxicity, and DNA fragmentation increased significantly [8]. Earlier study has indicated 25-50 μM DHA significantly enhanced neuronal viability, relative lower concentration of DHA (12.5 μM) did not show an obvious effect. In contrast, higher concentrations of DHA (100-200 μM) exerted the significant opposite effects by decreasing neuronal viability [9]. These results suggested that higher DHA concentration might be cytotoxicity to cultured neuronal cells. Cultured adult autologous bone marrow-derived mesenchymal stem cells supplemented with 40 μM DHA and 40 μM AA showed significantly enhanced neurite outgrowth with longer total neurite lengths (50-200 μm and higher) [10]. However, the addition of 10-30 μM AA resulted in a significant increase in both intracellular Ca_2^+ and Na^+ overload, resulting in cyclosporin A sensitive mitochondrial permeability transition pore opening, cytochrome *c* release, and induction of caspase 3-dependent neuronal apoptosis [11]. AA also cause intracellular Ca_2^+ overload and cellular damage to cardiomyocytes, probably through augmentation of lipid peroxidation of the cell membranes by free radicals [12]. Furthermore, AA cause increased cytochrome c release, activated cell cycle associated molecular such as p38, MAPK, JNK and p53 [13-16].

Our results used electrical pulses stimulation to induce cell oxidative stress but not necrosis, thus 5 voltages with 25 pulse numbers (50 ms per pulse) was be chosen for this study. The C6 cells exhibited reduce cell activities when increased electric tension and pulse number. C6, U-87MG and IMR-32 cells were supplemented with DHA or AA for 24 h, then treated by electrical stimulation. Results showed that 25 μM of DHA or AA increased cell survivability, relative

decreased percentage of cell apoptosis and ROS production induced by electrical stimulation. These results also suggested that continuous electrical pulses can cause cell oxidative stress and lead to cell apoptosis. In the other hand, astroglioma C6 and glioblastoma U-87MG are neuroglial cells and exhibited the similar cell activities, whereas neuroblastoma IMR-32, a neuron type of cell, exhibited lower cell activities than C6 and U-87MG, indicating that neuron are easily death caused by oxidative stress-induced injury.

In summary, the present study shows that DHA and AA significantly decrease ROS production and apoptosis of C6 astroglioma, U87-MG glioblastoma and IMR-32 neuro-blastoma with treatment of electrical stimulation and thus the supplement of adequate amounts of DHA and AA may be beneficial in the prevention of brain injury due to oxidative stress.

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Figure 2. The effects of DHA and AA on cell viability induced by electrical stimulation. Astroglioma C6 (A), glioblastoma U-87MG (B) and

neuroblastoma IMR-32 (C) were pre-treated without (control) or with 25μM DHA, 25μM and AA for 24h and then pulsed with electrical stimulation and detected by MTT assay. Data are expressed as mean ± SE from 3 replicates in two independent experiments. Means with the different letters are significantly different (p＜0.05).

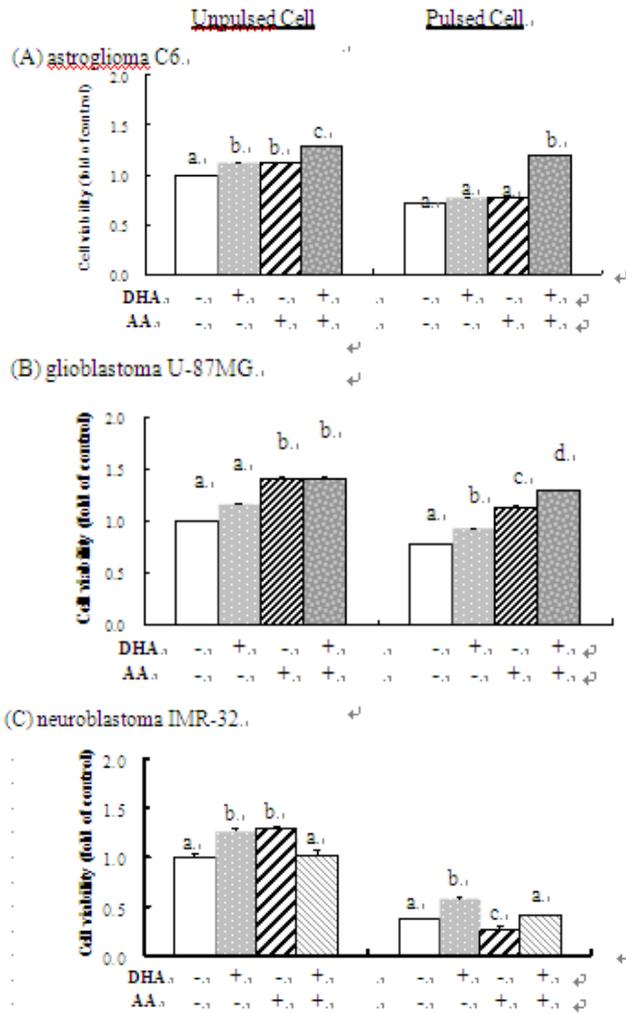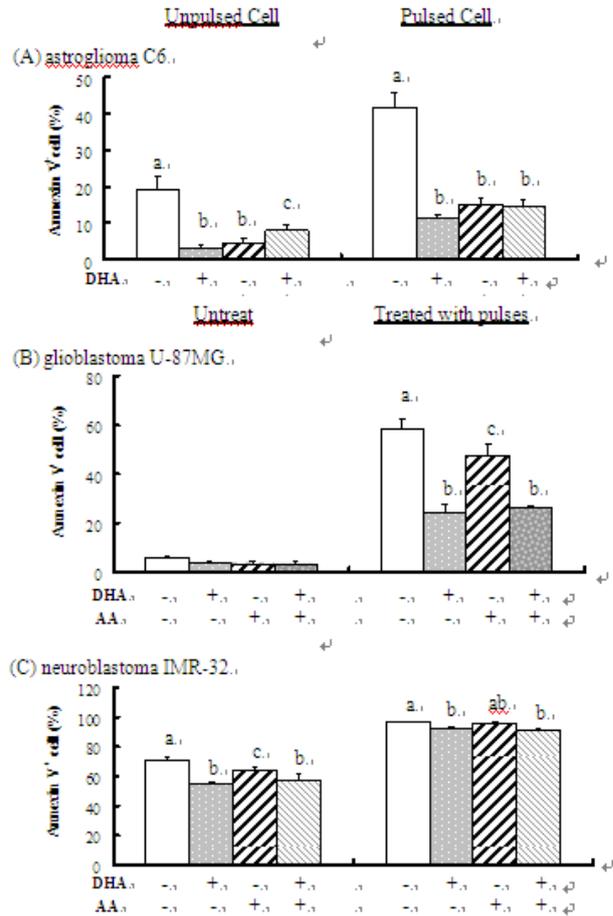Figure 3. The effects of DHA and AA on cell apoptosis. Astroglioma C6 (A), glioblastoma U-87MG (B) and neuroblastoma IMR-32 (C) were pre-treated without or with 25μM DHA, 25μM AA for 24h and then pulsed with electrical stimulation and apoptotic cells determined by flow cytometry were presented as percentage of annexin V+. Data are expressed as mean ± SE from 3 replicates in two independent experiments. Means with the different letters are significantly different (p＜0.05).
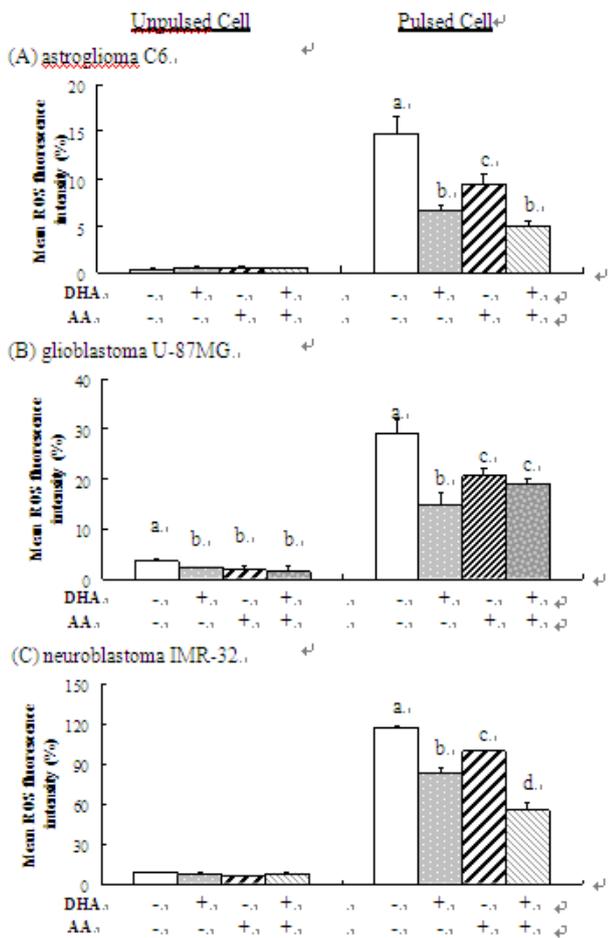
Figure 4. The effects of DHA and AA on ROS productions. Astroglioma C6 (A), glioblastoma U-87MG (B) and neuroblastoma IMR-32 (C) were pre-treated without or with 25μM DHA, 25μM AA for 24h and then pulsed with electrical stimulation. The intracellular ROS levels were represented by mean fluorescence intensity (MFI) of DCFH2DA. Data are expressed as mean ± SE from 3 replicates in two independent experiments. Means with the different letters are significantly different (p＜0.05).