

Effects of Zinc and DHA on the Epigenetic Regulation of Human Neuronal Cells

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Key Words

Zinc • Omega-3 fatty acid • DHA • M17 human neuronal cell • Histone H3 post-translational modification • Epigenetics

Abstract

Dietary intake of zinc and omega-3 fatty acids (DHA) have health benefits for a number of human diseases. However, the molecular basis of these health benefits remains unclear. Recently, we reported that zinc and DHA affect expression levels of histones H3 and H4 in human neuronal M17 cells. Here, using immunoblotting and densitometric analysis, we aimed to investigate the effect of zinc and DHA on post-translational modifications of histone H3 in M17 cells. In response to increase in zinc concentration, we observed increase in deacetylation, methylation and phosphorylation of H3 and decrease in acetylation. We also investigated the role of zinc in apoptosis, and found that zinc reduced the levels of the anti-apoptotic marker Bcl-2 while increasing the apoptotic marker caspase-3 levels, correlating with cell viability assays. Conversely, DHA treatment resulted in increase in acetylation of H3 and Bcl-2 levels and

decrease in deacetylation, methylation, phosphorylation of H3 and caspase-3 levels, suggesting that DHA promotes gene expression and neuroprotection. Our novel findings show the opposing effects of zinc and DHA on the epigenetic regulation of human neuronal cells and highlight the potential benefit of dietary intake of DHA for management of neurodegenerative diseases.

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Introduction

Docosahexaenoic acid (DHA) is an essential omega-3 fatty acid required for the development of human central nervous system and the continuous maintenance of neuronal cell function. The level of DHA in the brain has been shown to be important for learning ability and memory function in early life of rodents, baboons and humans [1]. The DHA composition of the brain decreases with age, which is thought to be a result of increased oxidative damage to the lipid membranes [2].

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Epidemiological studies have shown that patients with Alzheimer's disease (AD) have significantly lower levels of omega-3 fatty acids in their plasma phospholipids [3, 4].

An association between zinc and polyunsaturated fatty acids has been found in studies on zinc-deficient pregnant rats, which showed a whole-body depletion of maternal stores of both omega-3 and omega-6 polyunsaturated fatty acids (PUFA) [5]. We also demonstrated that a reduction of DHA level in the brain is associated with over expression of ZnT3, a transmembrane protein that is involved in the transport of zinc into synaptic vesicles [6]. Mice that were prone to developing Alzheimer's-like brain plaques, which lacked the zinc-transporter gene ZnT3, developed fewer and smaller plaques than Alzheimer's-prone mice with the gene [7], suggesting that zinc may contribute to the plaque formation in AD that depends in part on zinc released by nerve cells. Recent studies have indicated that excess zinc has a causative role in neuronal cell death or apoptosis in neurodegenerative disease, such as AD [8]. DHA, on the other hand, has neuroprotective properties against neurodegenerative diseases. It has been reported that the dietary supplement of omega-3 fatty acid may protect against Alzheimer's disease, through inhibiting amyloid plaque formation [9-11]. DHA was also observed to significantly increase neuronal survival by preventing cytoskeleton perturbations, caspase activation and apoptosis [11]. It has been shown that DHA prevents neuronal apoptosis by facilitating Raf-1 and Akt translocation/activation through its capacity to increase the phosphatidyl serine levels in neuronal cell membranes [12, 13]. Our recent data has shown that histones were key proteins that were affected by both zinc and DHA treatment [14]. The expression levels of histones H3 and H4 in cultured human neuronal M17 cells were down-regulated by zinc and up-regulated by DHA [14], suggesting a potential interaction between zinc and DHA in neurodegenerative diseases.

Histones are important carriers of epigenetic information. The dynamic structure of chromatin plays a significant role in DNA replication and regulation of gene expression. Chromatin structure is governed by post-translational modifications (PTMs) of core histone proteins at their N-terminal ends [15]. Some PTMs, or epigenetic markers, including acetylation and phosphorylation, are reversible and are often associated with increase in gene expression. Other PTMs, such as lysine methylation, are often found to be more stable, and participating in long term epigenetic maintenance [16].

Proper regulation of gene expression in the nervous system is not only controlled by the transcriptional machinery but is also subject to modulation by epigenetic mechanisms such as histone modifications. Abnormal epigenetic control in the brain is associated with mental retardation and neurodegenerative symptoms [17, 18]. In this study, we report, for the first time, the effect of zinc and DHA on post-translational modifications of histones, in particular histone H3, in human neuronal cells. Our results suggest that zinc and DHA alter histone PTMs and this may provide a potential mechanism by which DHA offers neuroprotection.

Materials and Methods

Cell culture

Human neuroblastoma M17 cell line was grown as a monolayer at 37°C with 5% CO₂ in T-75cm² flasks (Nunc, Roskilde, Denmark) and maintained in 10 mL of Opti-MEM media (a modified MEM (Eagle's media) with heat-inactivated 2.5% fetal bovine serum (FBS). Cells were allowed to grow to 90% confluency, which were then harvested or passaged using 0.025% trypsin/EDTA.

M17 cells were seeded at a density of 1 x 10⁶ cells /75 cm² flask and grown in media supplemented with docosahexanoic acid (DHA, Sigma Aldrich, MO, USA) in final concentration of 10 µg/mL (DHA-supplemented media with the addition of antioxidant, Vitamin E (0.05 µM/mL) was pre-incubated overnight to allow the DHA to conjugate with media proteins to allow delivery into cells) or without DHA and with (5 µM final concentration) or without ZnCl₂. To ensure that the differential expressions of histone PTMs was indeed zinc effect, the cell medium was firstly tested to have undetectable zinc content. After two days incubation, the cells were harvested, centrifuged at 1000xg for 5 minutes and cell pellet resuspended in 1X phosphate buffered saline (PBS). Each sample was divided into 3 aliquots, centrifuged at 14000xg for 5 minutes and cell pellets were stored at -80°C until needed for analysis.

Protein extraction and quantitation

Each sample was homogenized with 500 µL of lysis buffer (1% SDS, 10mM Tris HCl, pH 6.8). Samples were then sonicated on ice at 7 watts for 15 seconds, twice each using the Microson ultrasonic cell disruptor (Misonix, New York, USA) and centrifuged at 14,000xg for 5 minutes. Cell debris was then discarded and sample aliquots stored at -80°C until required. Quantification of the protein samples was performed using BCA protein assay kit (Perbio, Rockford, USA), following manufacturer's instructions. Optical density values for known bovine serum albumin (BSA) standards as well as 1:3 and 1:5 sample dilutions of unknown concentration were recorded on the Beckman DU 530 Life Science UV/Vis spectrophotometer at 595nm using Genesis Lite 3.03 computer software. Protein concentrations were obtained from standard curve of

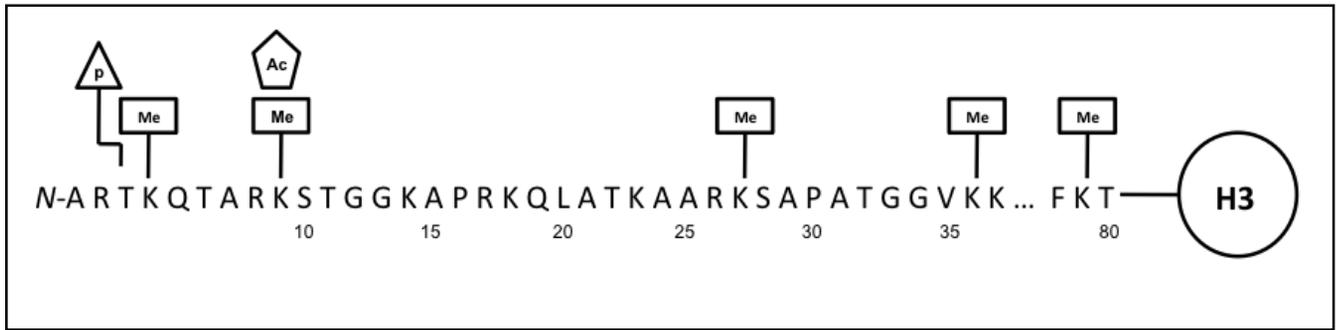


Fig. 1. Post-translational modification positions of histone H3 in human neuronal cell line M17 analyzed in this study. Ac, acetylation; Me, methylation; P, phosphorylation.

absorbance vs. protein concentration ($\mu\text{g/mL}$), using standards of known concentrations.

One-dimensional electrophoresis and Western immunoblot analysis

The level of each post-translational modification of histones in response to zinc and DHA treatments were analyzed using western blot analysis. Protein concentration of 25 μg , with total volume of 20 μl per well were subjected to 15% SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). After blocking with 1% (w/v) non-fat skim milk in 1x TBS for 1 hour at room temperature (RT), membranes were incubated overnight at 4°C with monoclonal primary antibody (diluted 1:1000) for anti-acetyl-H3 lys9; anti-HDAC1, 2, 3; anti-di-methyl-H3 lys4, lys9, lys27, lys36, lys79; and anti-phospho-H3 thr3 (Cell Signaling Technology Inc., MA, USA). After washing with 1x TBS solution three times for 5 minutes, membranes were incubated with goat anti-rabbit IgG horseradish-peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology Inc) with 1:2000 dilution for 1 hour at RT. Membranes were visualized using Immobilan Western Chemiluminescence HRP substrate (Millipore Corporation, CA, USA), according to manufacturer's instructions.

Developed membranes were stripped for 15 minutes at RT using 1 mL of Re-blot Plus-strong (Chemicon International, CA, USA) diluted 1:10 in MilliQ water, and re-probed for histone H3 using anti-histone-H3 monoclonal primary antibody (1:1000 dilution) followed by goat anti-rabbit IgG HRP conjugated secondary antibody (1:2000 dilution, Cell Signaling Technology Inc). In order to ensure equal protein loading in all wells, the membranes were re-probed for β -actin using mouse anti- β -actin (Sigma Aldrich, USA) primary antibody (1:4000 dilution) followed by anti-mouse HRP antibody (1:4000 dilution, Chemicon International) and developed as described above. To ensure specific antibody binding, negative control western blots (probed with detection/secondary antibodies only) were performed showing no binding (data not shown).

Apoptosis assay

Protein lysates (25 μg concentration with 20 μL total volume) were subjected to 15% (w/v) SDS-PAGE and transferred onto nitrocellulose membranes (Whatman), as described previously. The membranes were probed for apoptosis markers;

mouse monoclonal Bcl-2 (Abcam, Cambridge, UK) and active rabbit polyclonal Caspase-3 (Chemicon International). Both antibodies were prepared in 1:100 dilutions with 1x TBS, and membranes were blocked with 1% casein blocking-buffer prior to incubation with Bcl-2. No blocking was required for caspase-3. Antibodies were incubated with rabbit and mouse secondary antibodies (1:1000 for Bcl-2, 1:10,000 for caspase-3) and developed as described previously. All membranes were digitally captured using Fujifilm LAS-300, and the subsequent densitometry analysis of bands was done with the Fujifilm Multi Gauge V3.0 program (Fujifilm, Tokyo, Japan).

Statistical analysis

The data were analyzed using the SPSS programme, release 16.0 for Windows (SPSS, Chicago, IL, USA). The results were analyzed by Student's *t*-test to determine any statistically significant difference in signal intensity between zinc and DHA treatments. The statistical significance was set at $*p < 0.05$.

Results

In our recent initial studies, we found that zinc and DHA affected expression levels of histone H3 and H4 [14]. Here, we report the effects of zinc and DHA on the post-translational modification of histone H3, which has the longest N-terminal tail among all core histones (Fig. 1) and where most modification sites are found [19].

Effect of zinc and DHA on acetylation of histone H3 (K9)

In order to investigate the effect of zinc and DHA on the acetylation of histone H3 (K9) residue, M17 human neuronal cells were grown in culture medium in the presence and absence of zinc and DHA. We observed that in the presence of zinc and the absence of DHA (zinc effect), acetylated histone H3 (K9) levels were significantly decreased (Fig. 2) compared with the control (without zinc and DHA). On the other hand, in response to DHA in the absence of zinc (DHA effect), histone H3

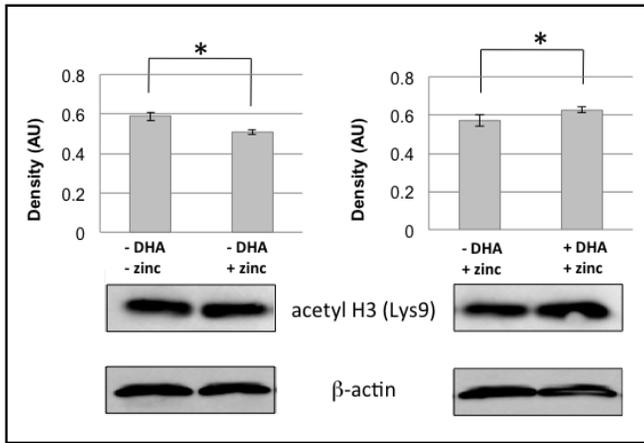


Fig. 2. Effect of zinc and DHA on acetylation of histone H3 (K9) in M17 human neuronal cells. Densitometric analysis, in arbitrary units (AU), of a western blot (with corresponding protein bands shown at the bottom) to quantitate acetylation of histone H3 (K9) in M17 cells following treatment with (+) and without (-) zinc (final concentration of 5 μ M) and with (+) or without (-) 10 μ g/mL DHA. Densitometric analysis show the values obtained from acetylated histone H3 (K9), compared with total H3 and normalized with β -actin expression level. The data are shown as means (n=3, *P<0.05).

(K9) acetylation levels were significantly increased (Fig. 2).

Effect of zinc and DHA on histone deacetylase (HDAC) 1, 2, 3

To characterize the effect of zinc and DHA on histone deacetylases, we performed western blot analysis to investigate the changes in expression levels of histone deacetylase (HDAC) 1, 2, and 3 using highly specific anti-HDAC1, 2 and 3 antibodies. As shown in Fig. 3A-3C, zinc (5 μ M) caused significant up-regulation of HDAC1, 2 and 3 compared with the control (without zinc and DHA), while DHA (10 μ g/mL) caused significant down-regulation of HDAC1, 2 and 3 (Fig. 3A-3C, respectively).

Effect of zinc and DHA on di-methylation of histone H3 (K4), (K9), (K27), (K36), (K79)

In order to assess the effect of zinc and DHA on di-methylation of histone H3 (K4), (K9), (K27), (K36), (K79), western blot analysis of methylation status were performed using human neuronal cells that were grown in culture medium in the presence and absence of zinc and DHA, as described previously. Concurrent analysis of di-methylation levels of histone H3 revealed that di-

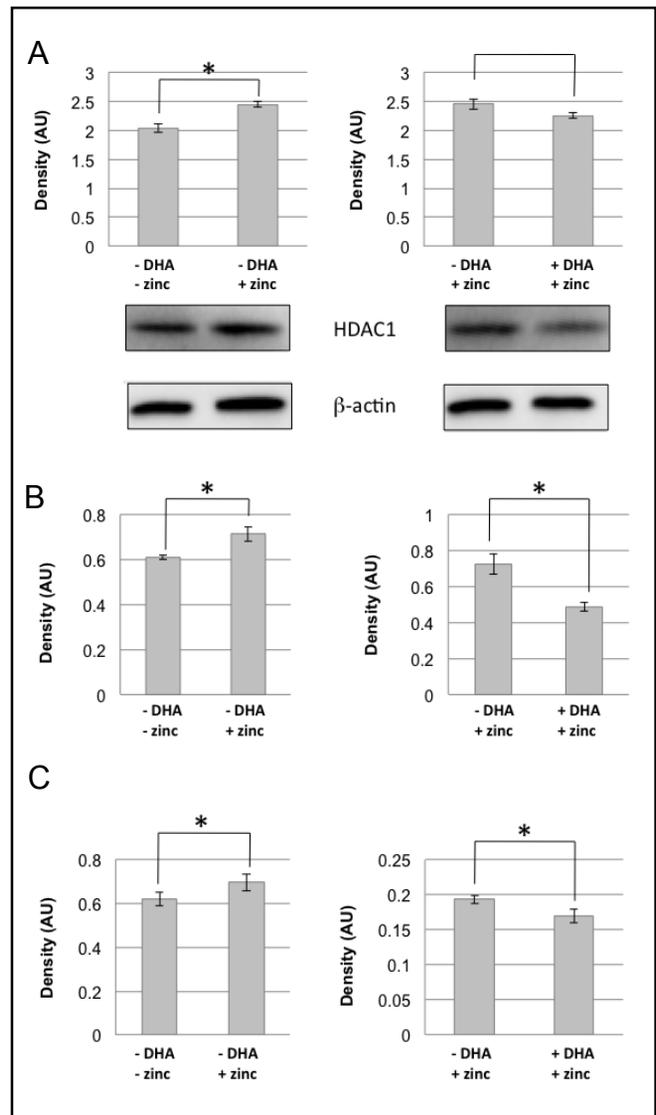


Fig. 3. Effect of zinc and DHA on histone deacetylases (HDACs) expression levels in M17 human neuronal cells. Densitometric analysis, in arbitrary units (AU), of a western blot (with corresponding protein bands shown at the bottom, for HDAC1) to quantitate the expression levels of HDAC1 (A), HDAC2 (B), and HDAC3 (C) following zinc (5 μ M) and DHA (10 μ g/mL) treatments. The results are shown as means (n=3, *P<0.05). The given values are the ratios of the density of HDAC1, 2 and 3 and the corresponding β -actin bands.

methyated H3 (K4) and (K27), were significantly increased upon zinc treatment and significantly decreased with the addition of DHA (Fig. 4A, 4C). Di-methylated H3 (K79) also showed a significant increase with zinc treatment, but an even more significant increase was observed with DHA treatment (Fig. 4E). In contrast, zinc significantly decreased the expression levels of di-methylation of histone H3 (K9) and (K36) while DHA

Fig. 4. Effect of zinc and DHA on di-methylation of histone H3 in M17 human neuronal cells. Quantitative analysis of di-methylation level of histone-H3 (Lys4) (A), (Lys9) (B), (Lys27) (C), (Lys36) (D) and (Lys79) (E) by densitometric analysis, expressed in arbitrary units (AU), of a western blot (with corresponding protein bands shown at the bottom, for Lys4), following treatment with or without zinc (5 μ M) and DHA (10 μ g/mL). Densitometric analysis show the values obtained from di-methylation of histone H3, compared with total H3 and normalized with β -actin expression level. The results are shown as means (n=3, *P<0.05).

caused a significant increase (Fig. 4B, 4D). From these results, we can observe the different roles of methylation of histone H3 at distinct amino acids residues, which provides a variety of interpretation in regulating neuronal cell gene expression.

Effect of zinc and DHA on phosphorylation of histone H3 (T3)

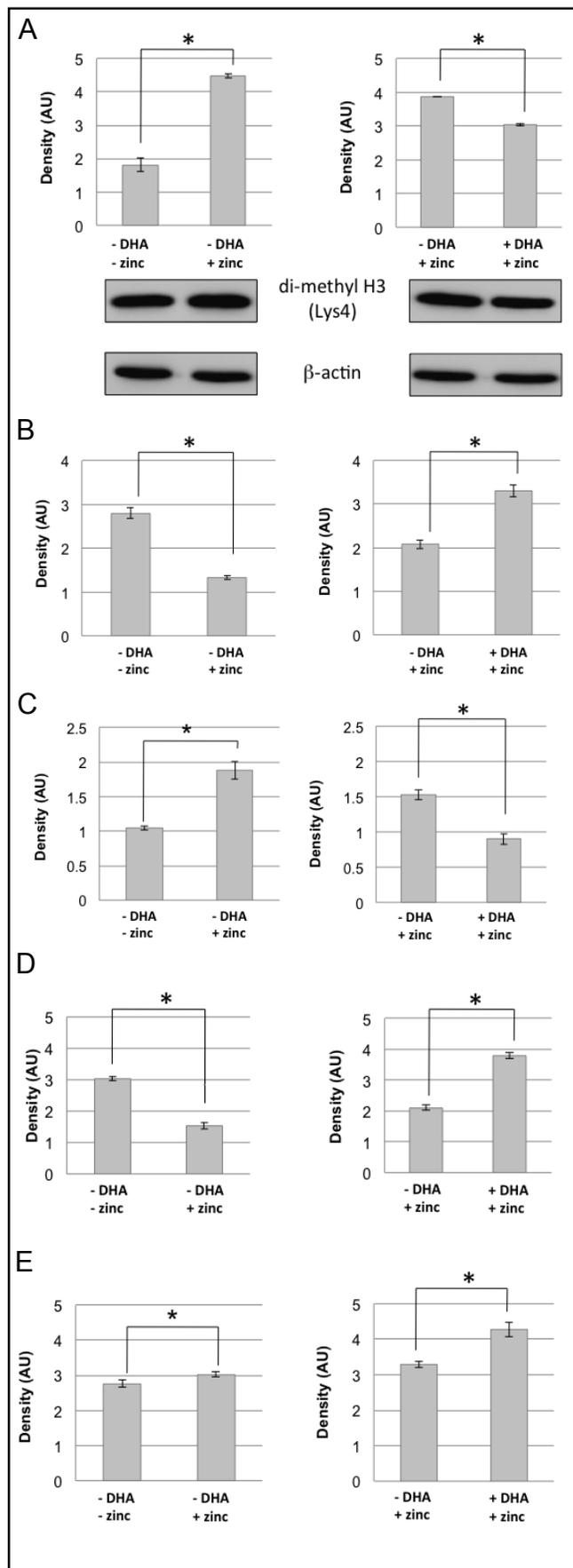
In this part of our study, we investigated histone H3 phosphorylation at Threonine 3, -11, and Serine 10, -28. However, only H3 (T3) phosphorylation was seen in M17 cells. Our results showed that phosphorylation of H3 (T3) was significantly increased upon zinc treatment and significantly decreased with DHA (Fig. 5).

Effect of zinc and DHA on Bcl-2 and caspase-3 expression levels

In this part of the study, we aimed to determine whether anti-apoptotic Bcl-2 and pro-apoptotic caspase-3 are involved in the cellular pathway affected by zinc and DHA in M17 cells, by investigating their expression levels using western blot analysis (Fig. 6A and 6B).

Our findings showed that both zinc and DHA opposingly modulated the levels of Bcl-2 and caspase-3 in M17 cells. Increase in zinc levels caused significant up-regulation of caspase-3 and down-regulation of Bcl-2 expression, while DHA treatment of M17 cells significantly increased expression levels of Bcl-2 and significantly reduced caspase-3 levels (Fig. 6A and 6B).

In order to confirm the Bcl-2 and caspase-3 results, we conducted a cell viability assay (Fig. 6C), using trypan blue exclusion. Indeed, there was a direct correlation with the Bcl-2 and caspase-3 results, in which zinc treatment reduced and DHA treatment increased the M17 cell viability.



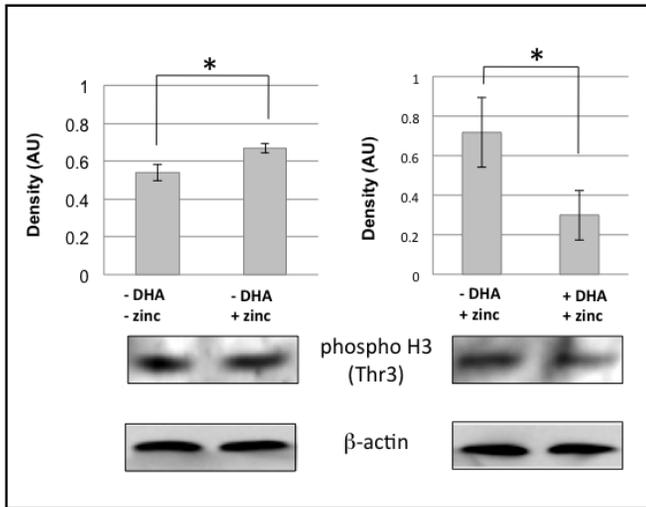


Fig. 5. Effect of zinc and DHA on phosphorylation of histone H3 (T3) in M17 human neuronal cells. Densitometric analysis, in arbitrary units (AU), of a western blot (with corresponding protein bands shown at the bottom) used to quantitate phosphorylation of histone H3 (T3), compared with total H3 and normalized with β -actin expression level, following treatment with or without zinc (5 μ M) and DHA (10 μ g/mL). The results are shown as means (n=3, *P<0.05).

Discussion

Effect of zinc and DHA on acetylation of histone H3 (K9)

Our results showed that zinc decreased acetylation of H3 (K9), suggesting the potential involvement of zinc in neurodegenerative disease through alteration of acetylation homeostasis in neuronal cells. During acetylation dyshomeostasis, transcriptional regulation may be affected and this has been implicated as the prime cause of several neurodegenerative diseases [17, 18].

On the other hand, DHA increased H3 (K9) acetylation, indicating the ability of DHA to normalize the histone H3 (K9) acetylation to the basal level (control) and abolish the effect of zinc. Although the molecular basis underlying neuroprotective effects by DHA in epigenetic regulation remains unknown, these results may suggest the contribution of DHA in neuroprotection through reinstating the altered acetylation homeostasis, which would possibly induce the expression of potentially neuroprotective genes. Indeed, we have shown here that DHA contributes to Bcl-2 up-regulation, thereby reducing neuronal cell death.

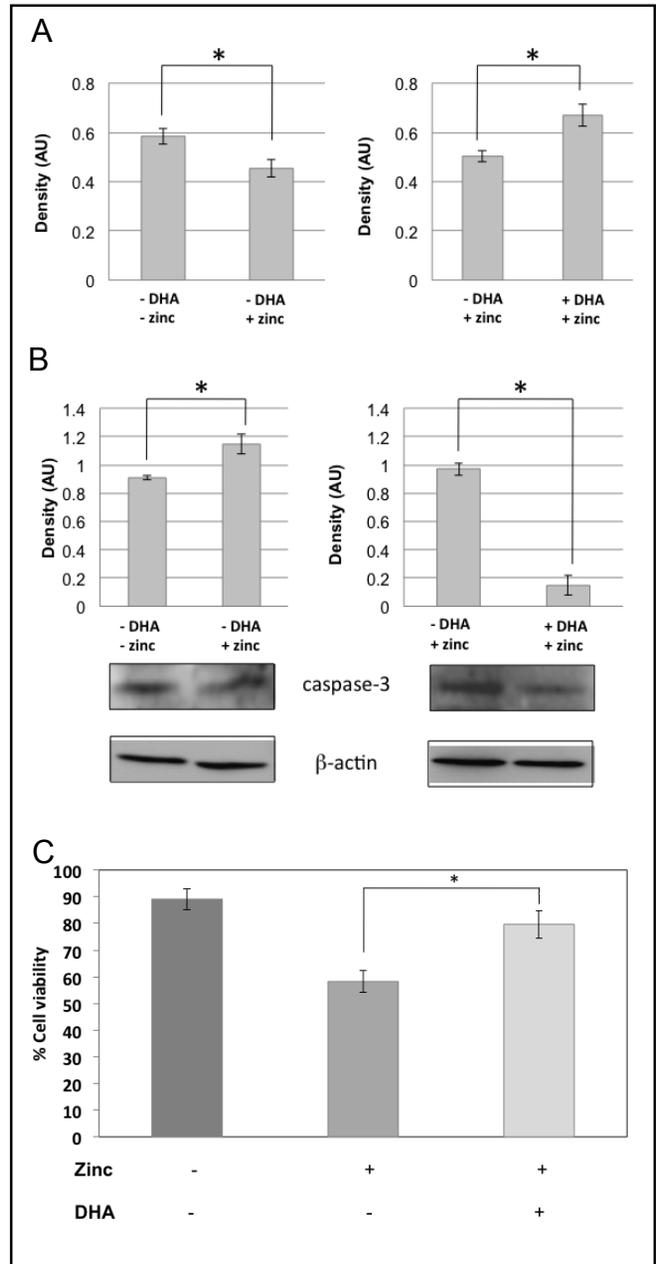


Fig. 6. Effect of zinc and DHA on Bcl-2 and caspase-3 expression levels and viability of M17 human neuronal cells. Densitometric analysis, in arbitrary units (AU), of a western blot (with corresponding protein bands shown at the bottom, for caspase-3) to quantitate anti-apoptotic marker, Bcl-2 (A) and apoptotic marker, caspase-3 (B) expression levels in M17 cells following treatment with (+) and without (-) zinc (final concentration of 5 μ M) and with (+) or without (-) 10 μ g/mL DHA, when compared with β -actin loading control. The data are shown as means (n=3, *P<0.05). (C) Viability of M17 cells after 48 hours treatment with or without zinc and DHA was determined by the trypan blue exclusion assay. The results were expressed as mean \pm SD of three independent experiments. Student *t*-test was used for statistical evaluation between treatment of zinc with and without DHA and a P value of <0.05 was considered significant (*).

Effect of zinc and DHA on histone deacetylase (HDAC) 1, 2, 3

HDAC enzyme activity has been shown to increase in dying neurons due to loss of counterbalancing effect of HATs activity [20]. From our results, we propose that increase in zinc can also contribute to the neurodegenerative process through up-regulating HDACs enzyme expression levels, and therefore affecting histone deacetylation. The HDACs catalytic domain contains a Zn²⁺ ion, in the active site, which contributes significantly to its catalytic activity [21, 22]. X-ray crystallographic studies have shown that HDAC inhibitors can chelate zinc ions in the catalytic sites of HDACs and therefore block substrate access to the active zinc ions and inhibit the deacetylation reaction [22, 23].

It has been established that the isotopic selective inhibition of HDAC enzyme may be a potential treatment for neurodegenerative diseases. So far, the HDAC inhibitors investigated in treating neurodegenerative diseases are very limited and mainly focused on the well-established experimental drug trichostatin A (member of hydroxamic acid group) and the clinically used HDAC inhibitors sodium butyrate, valproic acid, phenylbutyrate and vorinostat, which belong to short chain fatty acid group that are known to be able to penetrate the blood-brain barrier [24]. Since DHA crosses the blood-brain barrier [25], our results suggest it may have neuroprotective characteristics that mimic the behavior of HDACs inhibitors.

Generally, increases in HDACs during neurodegenerative disease are associated with an increase in gene repression and transcriptional dysfunction of certain transcription factors (TFs) such as CREB, which is important in regulating the expression of pro-survival elements such as Bcl-2 [20, 26]. In this study, we showed how zinc contributed to dysfunctional acetylation homeostasis in M17 cells by up-regulating HDACs, which influence the reduction of HATs and consequently histone acetylation levels. DHA, however, was shown to re-establish the imbalance of acetylation homeostasis and therefore capable of correcting the down-regulation of specific genes caused by reduction in histone acetylation. The mechanism by which DHA inhibits the HDACs expression is unclear; perhaps DHA directly chelates zinc from catalytic sites of HDACs or hinders zinc binding to the enzymes. Indeed, it has been recently reported that DHA and zinc chelator act synergistically to kill tumor cells [27].

Effect of zinc and DHA on di-methylation of histone H3 (K4), (K9), (K27), (K36), (K79)

Based on our findings, we hypothesize that the increase in H3 (K4) di-methylation following zinc treatment, is likely to be due to overexpression in histone methyltransferase, SET9/7, which may result in 'hyperstabilization' and activation of nuclear p53 that then lead to the induction of cell-cycle arrest and apoptosis [28], a key feature in the pathology of neurodegenerative disorders, such as AD. On the other hand, DHA treatment of M17 cells caused significant decrease in di-methylation of H3 (K4), which possibly relates to the decrease in p53 protein activity, and subsequent inhibition of apoptosis. The molecular mechanism of zinc in regulating p53 activity by overexpressing the SET7/9 methyltransferases is unclear, however this study suggests that zinc may contribute to the regulation of cell apoptosis through increasing H3 (K4) methylation.

Several developmental disorders with abnormalities in nervous system have been linked to abnormalities in DNA methylation, such as Down's syndrome which is characterized by chromosome abnormality and associated with mental retardation [29]. Based on our results, it is possible that zinc may possibly be involved in neurodegenerative process through inhibition of H3 (K9) di-methylation and DNA methylation and consequently enhance DNA strand breakage that can also impair DNA repair system, resulting in genetic mutation and chromosome abnormality or possibly triggering apoptosis [30, 31]. DHA, on the other hand increased H3 (K9) di-methylation to basal level (control), indicating its neuroprotective ability to re-establish the effect of H3 (K9) hypomethylation caused by zinc treatment, which may in return normalize the DNA hypomethylation and therefore reduce genomic instability and chromosome structures, leading to a reduction/prevention of neurodegenerative diseases.

Di-methylation of histone H3 (K27) is generally associated with gene silencing [32-34] and may also play a vital role in transcriptional termination [19]. Based on our western blot analysis, zinc caused a significant increase in H3 (K27) di-methylation compared with the control (without zinc and DHA), while DHA caused a significant decrease in H3 (K27) methylation, restoring the basal level.

The role of H3 (K36) methylation is associated with the euchromatin region [35] at the individual gene level that leads to gene activation [35, 36]. H3 (K36) methylation

has been shown by various studies to play a role in mRNA synthesis in eukaryotic organisms through transcription elongation process, as well as its interaction with RNA polymerase II [37]. Reduction of H3 (K36) methylation decreases RNA polymerase II phosphorylation which then leads to a transcription elongation defect [38, 39]. The results obtained in this study showed a decrease in H3 (K36) di-methylation by zinc treatment and increase by DHA. The contribution of transcription elongation defect in the process of neurodegenerative disorders is still unclear. However, it is possible that zinc may contribute to neurodegenerative process through mediating changes in H3 (K36) di-methylation, which is functionally linked with mRNA synthesis and therefore regulation of gene expression. Our western blot analysis results also confirmed the reverse effect of DHA, which resulted in increase of H3 (K36) di-methylation in M17 cells. DHA, however, may have normalized the effect of zinc by phosphorylating RNA polymerase II and therefore increasing transcription elongation through increasing di-methylation of H3 (K36).

It has been recently shown that H3 (K79) methylation is associated with DNA repair system [40, 41]. Our observation showed that zinc increases H3 (K79) di-methylation in neuronal cells while DHA causes further increase in H3 (K79) di-methylation. The enhanced increase of di-methylation of H3 (K79) by DHA may suggest different outcomes. The significant increase in H3 (K79) methylation by DHA treatment may indicate its neuroprotective effect by inducing DNA repair system. It has been reported that dietary omega-3 polyunsaturated fatty acids (PUFAs) are effective in attenuating oxidative stress-induced apoptosis and protecting the cells from DNA damage caused by ultraviolet radiation-induced p53 expression [42]. A reduction in p53 expression following dietary PUFAs is also anticipated to reflect the presence of less DNA damage and therefore allowing cell survival [42]. From this study, further increase in di-methylation H3 (K79) in response to DHA would possibly involve reducing p53 expression levels and therefore inhibition of cell apoptosis.

Effect of zinc and DHA on phosphorylation of histone H3 (T3)

Phosphorylation is involved in ‘methyl-phos’ mechanism, which is a concept where local phosphorylation could regulate binding of an effector protein of adjacent methylation sites [43] and neighboring modifications act together as ‘binary switches’. The phosphorylation of threonine (T3) regulates the binding

of chromodomain protein Chd1 to dimethyl H3 (K4), which has been associated with an “on” or “off” transcriptional state. Binding of Chd1 to methylated H3 (K4) destabilizes nucleosome and expose DNA for gene expression, which is antagonized *in vitro* by phosphorylation of neighbouring T3 [44]. This binding dissociation reduces the interaction with transcriptional elongation factors and therefore reduces gene transcription and subsequently induces apoptosis [44]. Our results show that phosphorylation of H3 (T3) is significantly increased upon zinc treatment and decreased with DHA. Increase in zinc levels may be involved in reducing gene transcription and increasing apoptosis through H3 (T3) phosphorylation, which in turn dissociate binding complex between H3 (K4) methyl group and its effector protein. However, DHA treatment reduced the phosphorylation of H3 (T3), which would increase Chd1 protein binding to dimethylated H3 (K4) and therefore increase recruitment of transcription elongation and gene transcription and subsequent reduction in apoptosis.

Effect of zinc and DHA on Bcl-2 and caspase-3 expression levels

Our observation that zinc increased caspase-3 and reduced Bcl-2 levels, suggests the potential occurrence of apoptosis of zinc-treated M17 cells, which is representative of neurodegenerative conditions such as AD, where intracellular zinc is elevated while DHA level is reduced. Conversely, the DHA treatment increased Bcl-2 but reduced caspase-3 levels, suggesting that DHA may exclusively activate the extracellular signal regulated kinase/mitogen-activated protein kinases (ERK/MPK) pathway to promote cell survival, which lead to the up-regulation of Bcl-2 and inhibition of caspase-3 activation [45]. The study by Akbar et al. [46] also showed the involvement of DHA in neuronal cell survival by driving Akt translocation, which results in activation of Bcl-2 and subsequent suppression of caspase-3 activity, leading to inhibition of apoptosis in neuronal cells. Our findings with Bcl-2 and caspase-3 highlight the importance of DHA in neuroprotection and zinc in apoptosis. Indeed, cell viability assays of M17 cells confirmed these results, in which zinc treatment reduced and DHA treatment increased the M17 cell viability.

Zinc toxicity has been reported to affect gene transcription, which we propose is mediated by alteration in the epigenetic patterns. Anti-apoptotic and pro-apoptotic markers were assessed in this study to look at the potential occurrence of cellular apoptosis, which we believe is also mediated by the change in the epigenetic

Fig. 7. Schematic representation of “zinc effect” on histone post-translational modifications. The schematic diagram shows the effect of zinc on histone H3 post-translational modifications and the potential downstream effects leading to neurodegenerative diseases, as supported by current literature (indicated by the superscript numbers which correspond to the references list). The findings of this study are shown by the shaded boxes.

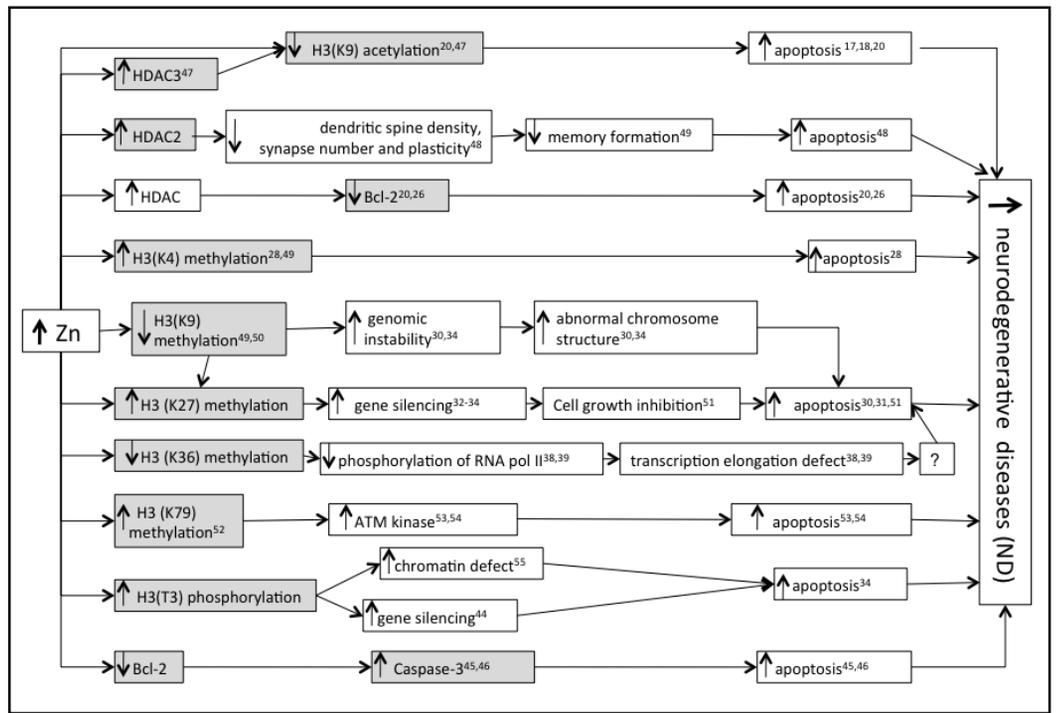
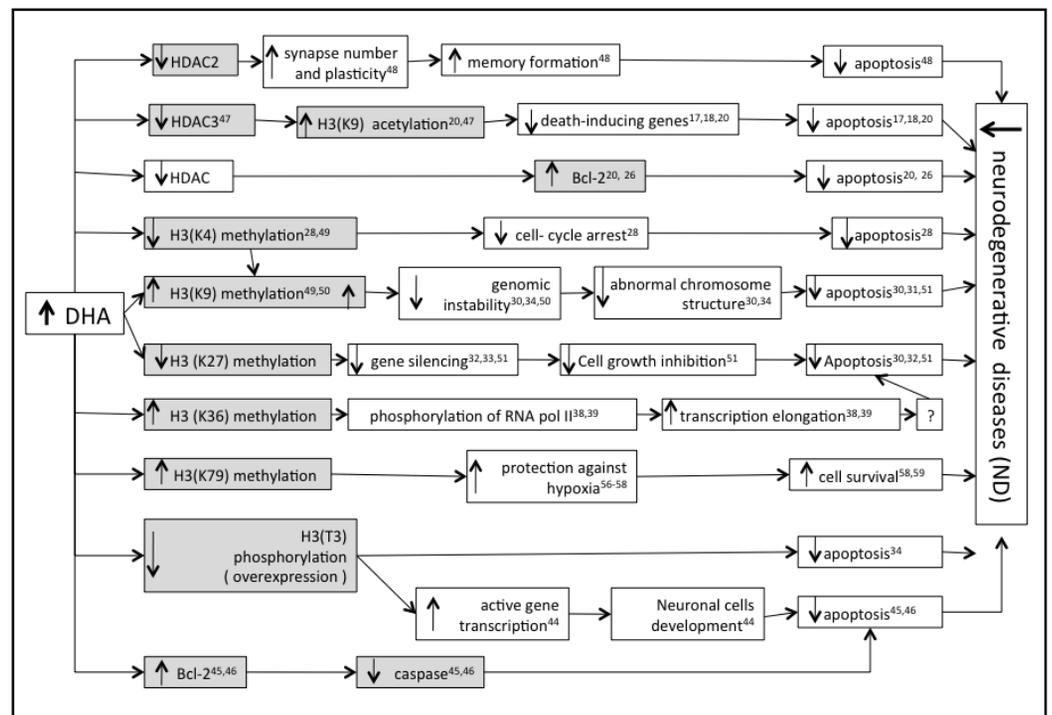


Fig. 8. Schematic representation of “DHA effect” on histone post-translational modifications. The schematic diagram shows the neuroprotective effect of DHA by histone H3 post-translational modifications and the potential downstream effects leading to reduction in neurodegenerative diseases, as supported by current literature (indicated by the superscript numbers which correspond to the references list). The findings of this study are shown by the shaded boxes.



patterns. Any other functions of the epigenetic changes and their influence in regulation of specific genes following zinc and DHA treatment were not tested as it was beyond the scope of this study. Here, we aimed to see whether or not DHA could restore zinc-induced

alteration of histone PTMs and to confirm that zinc and DHA indeed have direct interrelationships, mediated by histones and histone PTMs. Indeed, we will investigate how the epigenetic changes link to their functions in future studies.

Conclusions

Our key findings and their potential relevance in neurodegenerative diseases (e.g. zinc) or neuroprotection (e.g. DHA), as supported by current literature, are summarized in Fig. 7 and 8. Our data show that zinc reduced histone acetylation and increased HDACs, which represent a critical step in the apoptotic process, while our data showed that DHA reinstated the imbalance of acetylation homeostasis, indicating its potential neuroprotective ability to ameliorate neurodegenerative diseases. Histone methylation and phosphorylation were also significantly altered as a result of zinc and DHA treatment, and as a result we propose these two essential nutrients contribute to the epigenetic regulation of neuronal cell gene expression. However, it seems that zinc metabolism may somehow be dependent on DHA metabolism and vice-versa, and that DHA may normalize the effect of increased zinc levels in epigenetic alteration and therefore contribute to neuroprotection. From this study, we observed that zinc and DHA have distinct epigenetic patterns; this suggests they may have opposing effects in the progression of neurodegenerative diseases, such as AD. Such novel findings highlight the potential importance of dietary intake of DHA for the management and treatment of neurodegenerative diseases. In future

studies, it will be of interest to test how these epigenetic changes regulate the expression of anti-apoptotic or pro-apoptotic genes (e.g. p53). In addition, it will be important to assess the epigenetic effects observed in this study in other neuronal cells and in appropriate animal models.

Abbreviations

AD (Alzheimer's disease); AU (arbitrary units); BSA (bovine serum albumin); DHA (docosahexaenoic acid); ERK/MPK (Extracellular signal regulated kinase/mitogen-activated protein kinases); FBS (fetal bovine serum); HDAC (histone deacetylase); HRP (horse radish peroxidase); PBS (phosphate buffered saline); PTMs (post-translational modifications); PUFA (polyunsaturated fatty acid); RT (room temperature); TFs (transcription factors).

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