# ZINC AND DOCOSAHEXAENOIC ACID METABOLISM IN BRAIN CELLS

by

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BSc. (Hons.)

A thesis submitted in fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

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## GLOSSARY OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| Aβ | Beta amyloid |
| AD | Alzheimer's disease |
| ADK | Adenylate kinase |
| ADP | Adenosine 5'-diphosphate |
| AIF | Apoptosis-inducing factor |
| ALA | Alpha linolenic acid |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ANOVA | Analysis of variance |
| Apaf-1 | Apoptosis protease-activator factor 1 |
| APP | Amyloid precursor protein |
| ATP | Adenosine triphosphate |
| BACE1 | β-secretase |
| Bcl-2 | B-cell lymphoma 2 |
| BH3 | Bcl-2 homology domain 3 |
| $Ca^{2+}$ | Free ionic calcium |
| $[Ca^{2+}]_i$ | Intracellular free calcium |
| Caspase-3 | Cysteinyl aspartate-specific protease-3 |

| | |
|---|---|
| CBP | cAMP response element-binding (CREB) protein-binding protein |
| CHO | Chinese hamster ovary |
| CoQ10 | Coenzyme Q10 |
| Ct | Critical threshold |
| Cyt c | Cytochrome c |
| Da | Daltons |
| dATP | Deoxyadenosine triphosphate |
| DHA | Docosahexaenoic acid |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPA | Docosapentaenoic acid |
| DTT | Dithiothreitol |
| ECAR | Extracellular acidification rate |
| EDTA | Ethylenediamine tetratacetic acid |
| EPA | Eicosapentaenoic acid |
| $FADH_2$ | Flavin adenine dinucleotide |
| FBS | Fetal bovine serum |
| FCCP | Carbonylcyanide-p-trifluoromethoxyphenylhydrazone |
| GAPDH | Glyceraldehydes 3-phosphate dehydrogenase |

| | |
|---|---|
| $H_2O_2$ | Hydrogen proxide |
| HATs | Acetyltransferases |
| HDACs | Histone deacetylases |
| HMTs | Histone methyltransferases |
| HPLC | High performance liquid chromatography |
| HRP | Horseradish peroxidase |
| IAP | Inhibitor of apoptosis protein |
| JC-1 | 5,5',6,6'-Tetrachloro-1,1',3,3'.tetraethylbenzimidazolylcarbocyanide iodide |
| JNK | C-Jun N-terminal kinase |
| LA | Linoleic acid |
| $\Delta\Psi_m$ | Mitochondrial membrane potential |
| MAPK | Mitogen-activated protein kinase |
| MMP | Mitochondria membrane pores |
| MPT | Mitochondrial permeability transition |
| MRE | Metal response element |
| mtDNA | Mitochondrial DNA |
| MTs | Metallothioneins |
| MW | Molecular weight |
| NaCl | Sodium chloride |

| | |
|---|---|
| NADH | A reduced form of nicotinamide adenine dinucleotide |
| NADPH | β-Nicotinamide adenine dinucleotide phosphate |
| NgB | Neuroglobin |
| PBS | Phosphate buffered saline |
| PI | Isoelectric point |
| PKC | Protein kinase C |
| PL | Phospholipid |
| Ppm | parts per million |
| PTMs | Post-translational modifications |
| PUFA | Polyunsaturared fatty acids |
| OCR | Oxygen consumption rate |
| Omi/HtrA2 | Omi stress-regulated endoprotease/High-temperature requirement protein A2 |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| Rpm | Revolutions per minute |
| RT-PCR | Real time polymerase chain reaction |
| SD | Standard deviation |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |

| | |
|---|---|
| SEM | Standard error of the mean |
| Smac/DIABLO | Mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| ZIP | Zrt-Irt like proteins |
| $Zn^{2+}$ | Free ionic zinc |
| $[Zn^{2+}]_I$ | Intracellular free zinc ions |
| ZnT | Zinc transporter |

# PUBLICATIONS

**Peer reviewed publications:**

1. Cenk Suphioglu, **Nadia Sadli**, Damon Coonan, Loveleen Kumar, Damitha De Mel, Jessica Lesheim, Andrew Sinclair, Leigh Ackland (2010) Zinc and DHA have opposing effects on the expression levels of histones H3 and H4 in human neuronal cells. *Br J Nutr.* 103(3):344-51.

2. Cenk Suphioglu, Damitha De Mel, Loveleen Kumar, **Nadia Sadli**, David Freestone, Agnes Michalczyk, Andrew Sinclair, Leigh Ackland (2010) The omega-3 fatty acid, DHA, decreases neuronal cell death in association with altered zinc transport. *FEBS Lett*. 584(3):612-8.

3. Sean L. McGee, **Nadia Sadli,** Shona Morrison, Courtney Swinton and Cenk Suphioglu (2010) DHA protects against zinc mediated alterations in neuronal cellular bioenergetics. *Cell Physiol Biochem.* 28(1):157-62.

4. **Nadia Sadli,** Leigh Ackland, Damitha De Mel, Andrew Sinclair, Cenk Suphioglu (2012) Opposing effect of zinc and DHA on epigenetic regulation of human neuronal cells, *Cell Physiol Biochem*. 29(1-2):87-98.

5. **Nadia Sadli**, Colin J. Barrow, Sean Mcgee, Cenk Suphioglu (2011) The effect of DHA and Coenzyme Q10 against Abeta and zinc-induced mitochondrial dysfunction in human neuronal cells. *Neuropharmacol.* (Submitted 26 March 2012).

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6. **Nadia Sadli**, Nayyar Ahmed, M. Leigh Ackland, Andrew Sinclair, Colin J. Barrow and Cenk Suphioglu (2011). Effect of Zinc and DHA on Expression Levels and Post-Translational Modifications of Histones H3 and H4 in Human Neuronal Cells, In "Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring", Raymond Chuen-Chung Chang (Ed.), ISBN: 978-953-307-485-6, InTech publisher.

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1. **Nadia Sadli**, Leigh Ackland, Damon Coonan, Loveleen Kumar, Damitha De Mel, Jessica Lesheim, Andrew Sinclair, Cenk Suphioglu (2008). Zinc and DHA affect Histones H3 and H4 expression levels in human neuronal cells. *Life and Environmental Sciences (LES) Higher Degree by Research Student Symposium*, Deakin University, Geelong.

2. **Nadia Sadli**, Leigh Ackland, Damon Coonan, Loveleen Kumar, Damitha De Mel, Jessica Lesheim, Andrew Sinclair, Cenk Suphioglu (2009). The effect of

zinc and DHA on expression levels of novel genes in human neuronal cells. *Combio 2009 Conference*, Christchurch, New Zealand (awarded with travel grant to attend this conference).

3. **Nadia Sadli**, M. Leigh Ackland, Damitha De Mel, Andrew J. Sinclair, Cenk Suphioglu (2009). Opposing effects of zinc and DHA on epigenetic regulation of human neuronal cells. *Omega- 3 Fatty Acid workshop*, Deakin University, Melbourne.

# ABSTRACT

Zinc has been recognized as one of the most versatile of all the minerals in the human body. A large body of evidence has reported the critical role of zinc in human diet and that zinc deficiency is associated with numerous pathologies including growth retardation and delayed cognitive development. However, zinc has also been studied because of its potential role in neurotoxicity. This perspective stands apart from traditional biology of zinc, because it concerns the disruption of zinc homeostasis in the body, as opposed to the zinc-related cellular dysfunction, which is caused by either zinc excess or deficiency. Altered zinc homeostasis has been reported to contribute to the progression of Alzheimer's disease (AD) by inducing neuronal cell apoptosis and the formation of beta amyloid (Aβ).

Docosahexaenoic acid (DHA), the most abundant long-chain polyunsaturated fatty acid in the brain, plays a significant role in regulating cellular zinc uptake. It has been shown that consumption of DHA is associated with a reduced incidence of neurodegenerative diseases and neuronal apoptosis, which are possibly mediated by reduction in zinc toxicity. Therefore, the main focus of this thesis concerns the molecular interaction between zinc and DHA that may provide a potential molecular mechanism to explain the beneficial effects of dietary DHA against zinc-induced neurotoxicity caused by excess zinc fluxes.

**Chapter 2** aimed to characterize the effect of elevated $[Zn^{2+}]_i$ in mitochondrial function and whether DHA would protect against any zinc mediated alterations in bioenergetics. The results showed a decrease in cellular oxygen consumption rate in M17 human neuroblastoma cells, but not glycolytic rate in response to chronic zinc exposure, which was specific for neuronal cells. Zinc was also found to impair adenosine triphosphate (ATP) turnover, which was restored by DHA. These data have demonstrated that zinc disrupts bioenergetics at a point distal to the respiratory chain, which is restored by DHA.

Coenzyme Q10 (CoQ10), an essential cofactor involved in the mitochondrial electron transport chain, is well characterized as a neuroprotective antioxidant and like DHA it has been suggested as a potential therapeutic agent in AD. In **Chapter 3**, the effect of CoQ10 and DHA against Aβ- and zinc-mediated changes in the mitochondrial function was investigated. The results showed that CoQ10 may be protective against Aβ-induced alteration in basal respiration, ATP turnover, uncoupled respiration, maximal respiratory capacity, and mitochondrial membrane potential, whereas DHA had no significant effect on these mitochondrial parameters against Aβ toxicity. DHA, due to its direct molecular interaction with zinc, may specifically protect against zinc-mediated mitochondrial alterations in M17 neuroblastoma cells.

In **Chapter 4,** the aim was to investigate the interaction between zinc and DHA at the genomic and proteomic levels. In this study, histones were identified as the proteins

that were differentially expressed in response to zinc and DHA treatments. The proteomic analysis showed that expression levels of histones H3 and H4 were significantly decreased by zinc and increased following DHA treatment. It has been reported that histone and DNA synthesis are very tightly linked. Therefore, the pathophysiological levels of zinc may possibly inhibit DNA synthesis, which result in the reduced expression of histones in M17 cells. DHA has shown to increase histone expression back to the basal levels, indicating the ability of DHA to protect the cells and abolish the effect of zinc toxicity. Increased DHA may play significant roles in DNA synthesis and stability, which is therefore associated with the increase in histone protein levels.

Histones are also potentially important carriers of epigenetic information. In the next chapter, using M17 neuroblastoma cells, the effect of zinc and DHA on post-translational modifications (PTMs) of histones, in particular histone H3 was elucidated **(Chapter 5)**. In response to pathophysiological zinc concentration, there were significant increases in deacetylation, methylation and phosphorylation of histone H3 and significant decrease in acetylation of histone H3, all pointing towards possible gene silencing and apoptosis. Using Western blot technique, the level of anti-apoptotic Bcl-2 and pro-apoptotic caspase-3 were also evaluated. Indeed, zinc reduced the levels of the anti-apoptotic marker Bcl-2 while increasing the apoptotic marker caspase-3 levels. Conversely, increase in DHA resulted in significant increase in acetylation of histone H3 and Bcl-2 levels and significant decreases in

deacetylation, methylation, phosphorylation of H3 and caspase-3 levels, suggesting that DHA promotes gene expression and neuroprotection. The present findings have demonstrated that zinc and DHA have distinct epigenetic patterns, which suggest their opposing effect in the progression of neurodegenerative diseases.

In summary, several intracellular targets for $Zn^{2+}$-mediated neurotoxicity have been identified, which may provide the potential mechanism in which zinc toxicity leads to cellular apoptosis and neurodegenerative conditions. This thesis specifically emphasizes the direct interaction of DHA against zinc-induced mitochondrial dysfunction and involvement of bioenergetic regulation as a $Zn^{2+}$ toxicity target, which may be the initiator of oxidative stress, caspase cascade, alteration in epigenetic patterns and therefore gene expression in human neuronal cells.

# CHAPTER 1

## LITERATURE REVIEW ON MOLECULAR INTERACTION BETWEEN ZINC AND DOCOSAHEXAENOIC ACID (DHA) IN HUMAN NEURONAL CELLS

**PUBLICATION (Book chapter):**

**Nadia Sadli**, Nayyar Ahmed, M. Leigh Ackland, Andrew Sinclair, Colin J. Barrow and Cenk Suphioglu (2011). Effect of Zinc and DHA on Expression Levels and Post-Translational Modifications of Histones H3 and H4 in Human Neuronal Cells, "Neurodegenerative Diseases - Processes, Prevention, Protection and Monitoring", Raymond Chuen-Chung Chang (Ed.), ISBN: 978-953-307-485-6, InTech publisher.

## 1.1 NEURODEGENERATIVE DISEASES

As life expectancies are increasing and populations are ageing, neurodegenerative diseases have become a global issue [1, 2]. Neurodegenerative diseases such as Alzheimer's disease (AD) is the leading cause of dementia in the elderly, which is characterized by molecular changes in nerve cells that result in nerve cell degeneration and ultimately nerve dysfunction and cell death [3]. In 1995, Australia had a population of 18 million and 13,000 people were estimated to have dementia. It is predicted that Australia will have 25 million people in 2041, and 460,000 of these will have dementia [4]. In other words, while our total population will increase by 40%, our population with dementia will increase by more than three-fold [4].

The expected human lifespan is now longer than ever due to improved hygiene, the discovery of medicines such as antibiotics, and economic welfare. The consequences for this aging population are the increased incidence of age-related diseases. Therefore, treatments to prevent age-related neurodegeneration will have economic benefits as well as major impact on the quality of life of the patients [5]. A great deal is already known about the pathology of neuronal diseases, but the molecular mechanisms underlying many of these diseases remain unknown. Thus, more research is needed to find the cause and to improve the treatment methods for these significant mental health problems.

### 1.1.1 Risk factors associated with neurodegeneration

The most consistent risk factor for developing neurodegenerative disease is aging [6-8]. While it is possible to develop dementia early in life, the chances of developing it increases dramatically as people get older [9]. Although AD can strike people in their 30s, 40s, or 50s, the vast majority of cases of AD are diagnosed in people older than 65 [10-12]. A family history of dementia, gender (women are more likely to develop dementia than men), a head injury in the past [13], atherosclerosis, high cholesterol, hypertension, diabetes and high homocysteine levels, excessive alcohol and tobacco consumption, exposure to environmental substances and non-healthy diets are some of the factors likely to increase risk of dementia [14, 15].

While there are some risk factors that cannot be controlled, such as genetics or age, many risk factors can be managed through lifestyle changes or appropriate dietary intakes. These dietary and lifestyle interventions cannot stop people from developing dementia but they may reduce the risk [16-18]. The adequate omega-3 fatty acid intake is one example of dietary factors associated with a substantially reduced risk of neurodegenerative diseases [18-20].

## 1.2 PHYSIOLOGICAL ZINC IN THE NERVOUS SYSTEM

Zinc is the second most prevalent trace element in the body and is present in particularly high concentrations in the mammalian brain [21], including synaptic vesicles where it is tightly bound to intracellular proteins and zinc finger-containing transcription

factors [22]. The concentration of intracellular free zinc in the brain is thought to be very low under physiological conditions [22, 23]. However, it can rise to >300 nM in response to injurious stimuli [24].

Zinc plays an important role in growth and development, the immune response, neurological function and reproduction [25]. Zinc is also a constituent of many enzymes and is essential for the proper function of various enzymes including carbonic anhydrase [26], RNA polymerase, and superoxide dismutase [27].

The role of zinc in cognitive function has been studied extensively in both children [28] and the elderly [29]. Zinc deficiency during fetal life is associated with developmental delays and low serum zinc levels in elderly is linked with poor global cognitive function [30], particularly verbal function, and also increases stress [31, 32]. Zinc deficiency most often occurs when zinc intake is inadequate or poorly absorbed [27, 30, 33], when there are increased losses of zinc from the body or when the body's requirement for zinc increases [33]. Nonetheless, despite its importance, recent studies have revealed that excess zinc release in the pathological condition is toxic to the central nervous system. Moreover, disruption of zinc homeostasis has been implicated in several neurodegenerative diseases, such as AD [34, 35], where excess extracellular synaptic zinc was found to induce the formation of amyloid plaques, the characteristic feature of AD brains [36, 37]. These studies suggest the link between an altered neuronal zinc homeostasis and neurodegenerative disease progression.

### 1.2.1 Neuronal zinc distribution

Evidence from the literature suggests that there are three separate pools of zinc in the central nervous system: vesicular zinc, protein-bound zinc and free ionic zinc. Vesicular zinc is that which is sequestered in the pre-synaptic vesicles of a special class of zinc-containing neurons that are localized primarily in limbic, cerebrocortical and corticofugal system [38, 39].

The vesicles are accessible to zinc-specific permeable dyes and staining methods, and therefore the zinc residing within them can be histochemically stained [40]. Zinc accumulates in these vesicles because of the presence of a zinc- transporter-3 (ZnT-3)[41]. Upon synaptic stimulation, vesicular zinc is released along with glutamate into the synaptic cleft where it may regulate post-synaptic neuronal excitability [42, 43]. Although the vesicular zinc is only 5-15% of total zinc in the brain, it constitutes virtually 100% of the histochemically reactive zinc in the brain [44], which could be used as an indicator of neuronal cell injury.

The second pool of zinc is that which is bound into the structure of many zinc-containing enzymes in the brain. It has been reported that zinc ions are incorporated into these proteins at the time of synthesis [40], so this pool of protein-bound zinc is relatively stable and is only involved in the specific function of the zinc-containing enzymes. Since protein-bound zinc accounts for nearly 85-95% of zinc in the brain, the quantitative studies of zinc levels will be dominated by enzymatic zinc pool [40].

The third pool, is the intracellular free zinc ($[Zn^{2+}]_i$), which is located in the cytosol. Due to small amount of free zinc ions in the cells, it is difficult to study the $Zn^{2+}$ dynamics in the cell. However, it is estimated that under normal conditions, the $[Zn^{2+}]_i$ is approximately in the femtomolar to picomolar concentrations [23]. In fact, O'Halloran (2001) reported that there is no free zinc in the cell [23]. The rise and decline of $[Zn^{2+}]_i$ depends on the mobilization of zinc between storage sites, metal chaperons and zinc-bound proteins. While the amount and function of this free zinc ions are not completely understood, it is clear that the subtle increase in the level of $[Zn^{2+}]_i$ are lethal to neurons [23, 45].

### 1.2.2 Maintaining intracellular zinc homeostasis

Maintaining a constant state of intracellular zinc homeostasis is essential for normal cell function. It has been reported that intracellular zinc ion concentration $[Zn^{2+}]_i$ is maintained within the normal range (less than $10^{-12}$ M) by metal sensor proteins, zinc transporters, and zinc-binding proteins [23]. This extremely low free cytosolic zinc concentration is a reflection of the abundance of metal-binding components in the intracellular environment. These zinc regulatory systems act as switches that turn on or off depending on the condition of zinc excess or deficiency [23].

Metallothioneins (MTs) are a group of low-molecular-weight (6-7 kDa) intracellular proteins that bind to $Zn^{2+}$ [46]. These metalloproteins are involved in the storage, transport, and biological properties of not only zinc, but also copper, cadmium and

mercury. A study has reported that MTs may also protect against inflammation through their antioxidant potential, and by suppressing inflammatory cytokines, such as interleukin-1 beta [47]. Other studies have also demonstrated that MT knockout cells and animal models exhibit increased susceptibility to oxidative damage [48, 49], whereas MT overexpression confers resistance to heavy metal toxicity [50].

In addition to intracellular binding proteins metallothionein, zinc homeostasis in the brain is regulated and tightly controlled by zinc transporters, which are divided into two gene families: the ZnT proteins [solute-linked carrier 30 (SLC30)] and the Zip family [solute-linked carrier 39 (SLC39)][51, 52], to allow $Zn^{2+}$ influx with deficiency and to remove cytosolic $Zn^{2+}$ with toxic accumulations. ZnT and Zip proteins appear to have opposite roles in cellular zinc homeostasis. The function of ZnT transporters is to reduce intracellular cytoplasmic zinc by promoting zinc efflux from cells or into intracellular vesicles, while Zip transporters increase intracellular cytoplasmic zinc by promoting extracellular and perhaps, vesicular zinc transport into cytoplasm [53]. Table 1.1 lists the function of each zinc transporter that has been identified so far.

**Table 1.1: Family of ZnT zinc transporters.**

| ZINC TRANSPORTER | LOCALIZATION | TISSUE SPECIFICITY | FUNCTION | REFERENCE |
|---|---|---|---|---|
| **ZnT-1** | Plasma membrane | All cells | Extrude cellular $Zn^{2+}$ | Liuzzi et al. [54] |
| **ZnT-2** | Cytoplasmic vesicles | All cells | Store $Zn^{2+}$ | Liuzzi et al. [54, 55] |
| **ZnT-3** | Synaptic vesicles | Brain and testis | Store $Zn^{2+}$ | Cole et al. [56]; Smidt et al. [57] |
| **ZnT-4** | Luminal cell vesicle | Mammary gland | Induce lactation | Michalczyk et al. [55, 58] |
| **ZnT-5** | Vesicles | Pancreatic ß cells, ovary, testis | Sequestration of $Zn^{2+}$ | Ventine et al. [59]; kambe et al. [60] |
| **ZnT-6** | Golgi body | Brain, lung, small intestine, liver | Sequestration of $Zn^{2+}$ | Huang et al.[61] |
| **ZnT-7** | Cytoplasmic vesicles | Brain, lung, small Intestine, liver | Sequestration of $Zn^{2+}$ | Kirschke et al.[62] |
| **ZnT-8** | Vesicles | Pancreas, liver | Not yet known | Wijesekara et |

| | | | | |
|---|---|---|---|---|
| | | | | al.[63] |
| **ZnT-9** | Cytoplasmic and nuclear vesicles | Mammary gland | Induce lactation | Kelleher et al. [64] |

## 1.3 ZINC AND ALZHEIMER'S DISEASE (AD)

The pathogenesis of AD is associated with two main phenomena: hyperphosphorylation of the tau protein resulting in its accumulation in the neurofibrillary tangles and the formation of β-amyloid (Aβ) that is deposited early and selectively in senile plaques [65, 66].

Aβ is a 39-43 amino acid residue peptide derived from a large precursor protein amyloid precursor protein (APP) via endoproteolytic cleavage [67]. The aggregation and the conformational change of Aβ is strongly correlated with its neurotoxicity. Therefore, factors that promote the aggregation of Aβ may be involved in the pathologenesis of AD. One study has shown that APP expression is tightly regulated by zinc-containing transcription factors, which itself contains a zinc binding site [68]. Zinc has been reported to enhance the Aβ aggregation, mediated by its interaction with APP [69]. Apart from its interaction with zinc, APP has also been suspected to bind to copper and play a key role in regulating copper homeostasis [70]. Considering the low concentration of $[Zn^{2+}]_i$, a subtle increase is enough to initiate neurotoxicity and AD. It is possible that zinc may also influence the homeostasis of other trace metals such as copper and

aluminium, which have also been found to accelerate the aggregation of Aβ through APP interaction [71].

## 1.4 ZINC TOXICITY AND NEURONAL CELL DEATH

Based on the evidence described above, increase in $[Zn^{2+}]_i$ is toxic to the cells and can contribute to neurodegenerative diseases. Animal studies have shown that the detrimental effect of zinc accumulation could be overcome by treatment with a zinc chelator [72, 73], suggesting a reversible effect of zinc toxicity. Even though an unambiguous mechanism of $Zn^{2+}$-mediated neurotoxicity has not been delineated, several possible pathways have been identified. It is likely that more than one mechanism is involved in zinc-induced neurotoxicity, which converges or acts in parallel to cause cell death.

Apoptosis is an elimination process that plays an important role in cellular development. The activation of cell death program is regulated by many different signals that originate from the intra- and extracellular milieu. Apoptosis is characterized by DNA condensation, nuclear fragmentation, increase in plasma membrane permeability and cell shrinkage. Eventually, the apoptotic cell breaks into small membrane-surrounded fragments, which are cleared by surrounding cells [74]. All these events are tightly controlled and well organized. Dysregulation of apoptosis may lead to deviations from normal development and may cause or contribute to a variety of diseases, including cancer, neurodegenerative diseases and ischemic stroke (89, 90).

### 1.4.1 Mechanism of zinc-mediated toxicity leading to cellular apoptosis

It is well documented that oxidant exposure can trigger the release of $Zn^{2+}$ from intracellular stores [75], which may contribute to the initiation of cellular apoptosis and free radical accumulation [76]. The zinc-induced reactive oxygen species (ROS) production and cell apoptosis have also been found to overactivate protein kinase C (PKC), which is linked with calcium influx [77] and cell death [78]. This neuronal injury can be inhibited following application of PKC inhibitors or antioxidants [79], suggesting PKC-generated oxidative stress as one important mechanism in $Zn^{2+}$-induced neuronal cell death.

The mechanism of zinc-mediated apoptosis is considered relevant as many zinc-dependent transcription factors required for normal cell function are impaired during apoptosis [23]. Evidence also showed that zinc-induced apoptosis is mediated by caspase-dependent mechanisms. It has been reported that zinc treatment increases caspase-3 protein (pro-apoptotic marker) and reduces Bcl-2 (anti-apoptotic marker) expression levels in M17 neuroblastoma cells [80], indicating a direct involvement of zinc-mediated caspase cascade in cellular apoptosis.

Mounting body of evidence suggests that the dyshomeostasis of calcium ions may also be involved in the mechanism of zinc neurotoxicity. Upon increase in $Zn^{2+}$ levels, zinc is translocated through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-dependent $Ca^{2+}$ channel, where they induce the elevation of intracellular $Ca^{2+}$

of neurons [81]. The elevated level of intracellular $Ca^{2+}$ trigger various apoptotic pathways such as activation of calpain, caspases or other enzymatic pathways related to apoptosis [82, 83].

Zinc has also been reported to affect mitochondrial function by inhibiting glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (enzyme that involves in the glycolysis), and that pyruvate, the end product of glycolysis which acts as an energy substrate, attenuates zinc-induced death of neurons [84]. This suggests that inhibition of glycolysis and bioenergetic failure may be involved in the mechanism of zinc-mediated toxicity. My works have also demonstrated that pathophysiological levels of $Zn^{2+}$ causes loss of mitochondrial membrane potential ($\Delta\Psi_m$), enhances reactive oxygen species (ROS) production (Sadli et al. 2012 – *manuscript submitted*) and inhibits mitochondrial respiration [85], which may be due to inhibition of the electron transport chain. It has been reported that free zinc ions at about 100 nM are sufficient to depolarize mitochondria membrane potential and induce ROS production in neuronal cells [86]. This data shows the importance of maintaining zinc homeostasis, as the slight change in intracellular zinc ion concentration can lead to bioenergetic dysfunction. Based on the evidence described above, it has been suggested that mitochondria play an important role in regulating cell function and that there is no way to bypass mitochondria in the process of cell apoptosis and neurodegenerative conditions.

## 1.5 MITOCHONDRIA-MEDIATED APOPTOSIS

Mitochondria are the energy powerhouse of cells, which generate life energy in the form of ATP, the main energy currency of the body [87, 88]. Mitochondria consist of: 1). An outer membrane that encloses the entire structure, 2). An inner membrane that encloses a fluid-filled matrix, 3). The intermembrane space, 4). The inner membrane, which is elaborately folded with shelf-like cristae projecting into the matrix and 5). Mitochondrial DNA (mtDNA) [89]. The inner membrane contains 5 complexes of integral membrane proteins: 1). Nicotinamide adenine dinucleotide (NADH) dehydrogenase (Complex I), 2). Succinate dehydrogenase (Complex II), 3). Cytochrome c reductase (Complex III; also known as the cytochrome b-$c_1$ complex), 4). Cytochrome c oxidase (Complex IV), all make up the protein complex of the respiratory chain and 5). The ATP synthase (Complex V) [90]. These protein complexes manage the process of oxidative phosphorylation, which begins as complex I engages NADH and complex II engages Flavin Adenine Dinucleotide ($FADH_2$). Energy is released when electrons are transported from higher energy NADH/$FADH_2$ to lower energy $O_2$ [91]. Each enzyme strips away the pair of electrons from its substrate and passes them to coenzyme Q10 (CoQ10). CoQ10 is a small molecule that shuttle electrons from complexes I and II to complex III, while also providing potent antioxidant protection for the inner membrane oxidative phosphorylation complex [92]. CoQ10 is an important nutrient and CoQ10 deficiencies are associated with subnormal or pathological mitochondrial performance [93]. CoQ10 has also been shown to reduce the symptoms of neurodegeneration [94, 95].

During an apoptotic stimulus, the permeability of the mitochondrial membrane is disrupted, which is called the mitochondrial permeability transition (MPT). The MPT is characterized by rapid permeability of the mitochondrial membrane and release of apoptosis-mediator proteins from the intermembrane space into the cytosol. This membrane permeabilization implies the formation of pores or channels that cause the dissipation of the membrane potential across inner mitochondrial membrane, which is associated with disruption of the electron transport chain [90]. As a result of impaired electron transport chain, the ATP production is depleted and electrons escape from the respiratory chain to form ROS [96]. This ROS generation is intrinsic to the oxidative phosphorylation process and has potential to destroy the cells, usually beginning with the mitochondria themselves. As the mitochondria progressively lose their functional integrity, ever greater proportion of oxygen molecules are converted to ROS. Since free radical reaction self-propagate, additional ROS produced subsequently results in the destruction of fatty acids and cell-membrane phospholipids, as well as DNA fragmentation [97, 98]. Antioxidant enzymes, as well as the DNA and protein-repair enzymes themselves, can become damaged and no longer provide cellular protection. Mutations due to ROS attack can also accumulate along with malfunctioning molecules and other debris. Eventually, the cell is either crippled, killed outright (necrosis), commits suicide (apoptosis), or loses growth control and becomes cancerous [88].

Another consequence of the MPT can be swelling of the matrix and rupture of the outer membrane, allowing release of apoptotic mediator proteins from the mitochondria.

Western immunoblot detection of such proteins, such as cytochrome c (cyt c), adenylate kinase (ADK) and apoptosis-inducing factor (AIF) in cysotol and nuclei (the extramitochondrial compartment), indicates a sign of outer mitochondrial membrane permeabilization [99].

Proteomic analysis has also revealed that apart from cyt c and AIF, there are other numerous key proteins that are released from the mitochondrial membrane, which includes procaspase, Smac/DIABLO (mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein antagonist) [100], Omi/HtrA2 (high-temperature requirement protein A2) and Endonuclease G (enzyme contribute to apoptotic nuclear DNA damage in a caspase-independent way)[101, 102].

Factors released from the mitochondria are crucial for the activation of pro-apoptotic signaling. During apoptosis, cyt c (an electron shuttle molecule) is released from mitochondria complexes with apoptosis protease-activator factor 1 (Apaf-1), cytochrome c/2'-deoxyadenosine 5'-triphosphate (dATP) and cytosolic pro-caspase-9 to form a caspase-activating complex called the apoptosome. Cyt c and dATP induce refolding of Apaf-1, which allows interaction with pro-caspase-9. In this way, pro-caspase-9 is activated, which subsequently cleaves and activates caspase-3 [103]. Depending on cell type and stimulus, caspase-3 can be involved in the activation of procaspase-8, procaspase- 6, pro-caspase-9 and BH3 (The Bcl-2 homology domain 3)-

domain-only subgroup of Bcl-2 family member (BID) that results in a feedback amplification of the apoptotic signal [104].

## 1.6 LINK BETWEEN CELLULAR APOPTOTIS AND NEURODEGENERATIVE DISEASES

A characteristic of many neurodegenerative diseases, such as AD, Parkinson's disease and stroke, is neuronal cell death [105, 106]. Central nervous system (CNS) tissue has very limited regenerative capacity and therefore, it is important to limit the damage caused by neuronal cell death [107, 108]. In recent years, the investigation regarding the contribution of caspases and neuronal apoptosis to neurodegenerative diseases has gained increasing attention. This evidence has been generated by using a variety of complementary approaches, including evaluating human tissue and using transgenic mouse and *in vitro* models [108].

Studies have shown the imbalance levels of pro-apoptotic (Bax, Bak and Bad) and anti-apoptotic Bcl-2 proteins [109, 110], as well as caspase-3 and -6 in the brain of AD patients [111]. In addition, immunohistochemical and biochemical studies reported the presence of active caspases and caspase-cleaved substrates around senile plagues and neurofibrillary tangles in neuron [112]. A marked co-localization of hyperphosphorylated tau, caspase-3 and caspase-6 in Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive neurons in the brainstem of AD patients [113] were also observed, indicating the potential involvement of apoptotic death in the etiology of AD.

Caspase-mediated apoptotic pathways have specifically been linked to the progression of AD, especially toward the formation of amyloid precursor protein (APP) and Aβ peptide production. Caspase-3-mediated APP stabilizes BACE1 (the β-secretase enzyme that is responsible for the cleavage of APP and the associated creation of Aβ), which lead to an increase in Aβ formation [114]. Studies also indicate that caspases have been implicated in the mechanism of tau-mediated neurodegeneration of AD [115]. According to this hypothesis, Aβ peptide was reported to promote neuronal pathological tau filament assembly by triggering caspase activation leading to tau cleavage, which in turn generate more tau pathological filaments [114] that further contribute to increase of cellular dysfunction in AD [116].

The balance between pro-apoptotic and anti-apoptotic pathways determine the outcome of cell death upon a stimulus. As long as protective proteins are present in high amounts, the balance will be in favor of cell survival. Therefore, understanding of cell survival mechanisms is essential to study the mechanism of zinc-mediated cellular dysfunction and the potential therapeutic treatment to inhibit zinc toxicity and cellular apoptosis.

Recently, the role of polyunsaturared fatty acids (PUFAs) docosahexaenoic acid (DHA) in the brain has been examined, as they are associated with the reduction of neurodegenerative diseases such as AD, and found to be neuroprotective against cell apoptosis through maintaining zinc homeostasis.

## 1.7 POLYUNSATURATED FATTY ACIDS (OMEGA-3 PUFAs)

### 1.7.1 Metabolism of PUFAs

Fatty acids (FAs) are carboxylic acids with a long side chain of hydrocarbons. FAs with only single bonds between adjacent carbon atoms are referred to as "saturated", whereas those with at least one C=C double bond are called "unsaturated" [117] (see Table 1.2 below for a list of common fatty acids).

**Table 1.2: List of common fatty acids arranged in different classes.**

| COMMON NAME | SCIENTIFIC NAME | MOLECULAR NAME | ABBREVIATION |
|---|---|---|---|
| Saturated fatty acids | | | |
| Lauric acid | Dodecanoic acid | 12:0 | |
| Myristic acid | Tetradecanoic acid | 14:0 | |
| Palmitic acid | Hexadecanoic acid | 16:0 | |
| Stearic acid | Octadecanoic acid | 18:0 | |
| Arachidic acid | Eicosanoic acid | 20:0 | |
| Behenic acid | Docosanoic acid | 22:0 | |
| Lignoceric acid | Tetracosanoic acid | 24:0 | |
| Monounsaturated fatty acids | | | |
| Vaccenic acid | 11-octadecenoic acid | 18:1n-7 | |
| Oleic acid | 9-octadenoic acid | 18:1n-9 | |
| Omega-3 polyunsaturated fatty acids | | | |

| α-linolenic acid | 9,12,15-octadecatrienoic acid | 18:1n-3 | ALA |
|---|---|---|---|
| Eicosapentanoic acid | 5,8,11,14,17-eicosapentaenoic acid | 20:5n-3 | EPA |
| Docosapentaneoic acid | 7,10,13,16,19-docosapentaenoic acid | 22:5n-3 | DPA |
| Docosahexaenoic acid | 4,7,10,13,16,19 – docosahexaenoic acid | 22:6n-3 | DHA |
| Omega-6 polyunsaturated fatty acids | | | |
| Linoleic acid | 9,12-0ctadecadienoic acid | 18:2n-6 | LA |
| γ-linolenic acid | 6,9,12-octadecatrienoic acid | 18:3n-6 | GLA |
| Arachidonic acid | 5,8,11,14-eicosatetraenoic acid | 20:4-6 | AA |
| n/a | 4,7,10,13,16-docosapentaenoic acid | 22:5n-6 | |

The PUFAs, which contains more than one double bond, can be classified according to the position of the last double bond closest to the terminal methyl carbon, such as omega-3 (n-3) and omega-6 (n-6) series PUFA. For example, docosahexaenoic (DHA) is an omega-3 (n-3) FA with 22 carbon atoms and six double bonds, usually in an all *cis* configuration (Fig. 1.1).

**Figure 1.1: Linear schematic diagram of Docosahexaenoic acid (DHA, 22:6n-3).**

As the degree of unsaturation in FAs increase, the melting point decreases, which confer the attribute of fluidity of n-3 PUFAs in cell membranes that influence many aspects of cell function [118]. EPA and DHA are synthesized from the n-3 precursor of α-linolenic acid (ALA, 18:3n-3) [119, 120], whereas the long chain n-6 PUFAs, such as arachidonic acid (AA, 20:4n-6), are synthesized from the predominantly plant-derived precursor linoic acid (LA, 18:2n-6)  [121]. ALA are predominantly available from plant oils such as canola, soybean and flaxseed [122]. Major source of LA are corn, sunflower oil and safflower oil [123]. The ability of enzymes to produce the omega-6 and omega-3 family of products of LA and ALA declines with age. Study showed that desaturase enzyme function in old rats was only 44% of the desaturase function in young rats [124]. Since DHA synthesis declines with age, as we get older our need to acquire DHA directly from diet or supplements increases. Figure 1.2 shows the desaturation and elongation cascade of omega-3 and omega-6 series PUFAs.

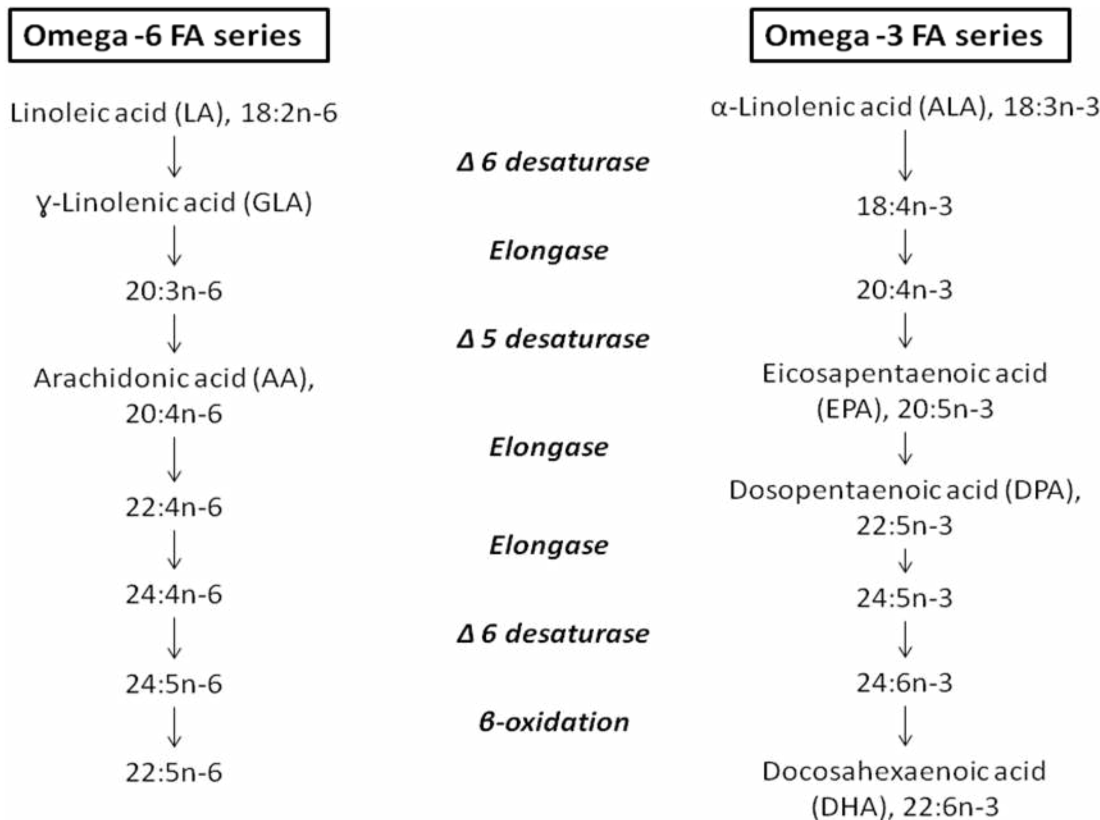



**Figure 1.2: Digrammatic representation of the omega-6 and omega-3 series metabolic pathway.** The essential precursors, α-linolenic and linoleic acid undergo a number of desaturations (addition of double bonds) and elongation reaction (addition of carbon atoms) and end up as long chain omega-6 and omega -3 PUFA. The enzymes for each step are written in *italics*.

### 1.7.2 Role of docosahexaenoic acid (DHA) in cellular function

Docosahexaenoic acid (DHA) is the predominant omega-3 fatty acid in the brain of mammals, which comprises up to 15% of the concentration of fatty acids in the nervous system [125]. Epidemiological studies suggest that dietary DHA, which is commonly found in fish [126], may modify the risk for certain neurodegenerative disorders [127]. Indeed, decreased blood levels of omega-3 FAs have been associated with several neurodegenerative conditions, including AD, schizophrenia and depression [128, 129]. Communities with regular consumption of fish have shown to possess reduced

prevalence of neurodegenerative disease and cognitive decline in general [128, 130, 131]. DHA can be linked with many aspects of neural function, including neurotransmission, membrane fluidity [132], ion channel [133], enzyme regulation [134] and gene expression [135]. DHA is found in breast milk, and may be required for early visual [136] and brain development in children [137, 138]. Furthermore, studies in animal models have provided support for the protective role of omega-3 FAs. For example, mice fed on diets high in omega-3 FAs were shown to improve in neurological function, such as better regulation of nerve cell membrane excitability [139], increased levels of neurotransmitters and higher density of neurotransmitter membrane receptors [140]. Hossain et al (1998) found that administration of DHA leads to the improvement in memory function and reduction in free radical levels while maintaining high level of antioxidant enzyme, indicating a role of DHA in antioxidant defense [141]. Study by Calon et al (2004) has reported that dietary DHA also protects the cells against apoptosis by decreasing caspase activity [142], while others have supported this finding by showing that the enrichment of dietary DHA prevents apoptosis under damaging conditions [143]. DHA also increases phosphotidylserine levels (PS) in neuronal membrane, which result in Akt translocation [144] and contributes to survival signaling by suppression of caspase-3 [145].

## 1.8 MOLECULAR LINK BETWEEN ZINC AND DHA IN NEURONAL CELLS

The alteration in both DHA and zinc homeostasis are key features of neurodegenerative disorders [146, 147]. A study by Jayasooriya et al (2005) has demonstrated the link

between an altered zinc homeostasis in the brain of rats fed on an omega 3-deficient diet [148]; this diet led to a significant decrease in brain DHA levels. Though these data have shown a relationship between DHA and zinc homeostasis, the basis of a molecular mechanism has not been elucidated.

This fundamental finding has been used to investigate the molecular mechanisms underlying this zinc and DHA interaction. Using human neuroblastoma cell line M17, the recent data have shown that DHA reduces cellular zinc uptake, possibly mediated by the zinc transporter ZnT3 followed by a significant reduction in pro-apoptotic marker, caspase-3 [45]. This indicates the effect of DHA deficiency in the progression of neurodegenerative disease, which is partly mediated by altered zinc fluxes. In M17 cells, the expression of these two zinc transporter families, including Zip1, Zip2, Zip3, Zip4, Znt1, ZnT2, ZnT3, ZnT4, ZnT5, ZnT6 and ZnT7 were detected [45]. ZnT3 has been the focus of the previous studies, as it is associated with brain zinc accumulation, as well as AD, the condition where the expression was found to be up-regulated [149].

As previously discussed, the alteration of zinc metabolism may play a significant role in cellular apoptosis, which is a key feature in the pathology of neurodegenerative disorders such as AD [150]. Using Western blot analysis of human neuroblastoma M17 cell line, a link between DHA treatment and inhibition of apoptosis was observed, where more than 66% reduction in active caspase-3 protein level was detected in cells treated with 20 μg/ml DHA, compared with the untreated control [45]. The suppression of

activated caspase-3 may be mediated by phosphatidylinositol 3-kinase-dependent pathway resulting in the phosphorylation of Akt and DHA may act through this pathway. Akbar et al (2005) reported the beneficial effect of DHA in neurosurvival through an increase in phosphatidylserine concentration, which resulted in translocation and phosphorylation of Akt suppressing the activation of caspase-3 [144]. Zinc, on the other hand, directly activates Akt by phosphorylation at Ser-473/Thr-308 in H1907 embryonic hippocampal cells, leading to activation of GSK-3beta and cell death [151]. This suggests that DHA inhibits apoptosis through decreasing intracellular zinc ion concentration, which leads to an increase in Akt activity and neuronal survival.

In summary, dietary DHA deficiency is associated with neurodegenerative conditions, which have been shown to be a factor in zinc toxicity. DHA also inhibits cellular apoptosis in M17 cells through decreasing cellular zinc uptake and reduction of ZnT3 mRNA and protein levels. Therefore, zinc homeostasis may play an important role in neuronal cell survival and altered zinc homeostasis may contribute to the development of neurodegenerative diseases.

This thesis focus only on the effects of DHA, which is based on the preliminary data that zinc fluxes is regulated by DHA in human neuronal cells. Other fatty acids such as EPA and DPA will be investigated in the future.

## 1.9 ZINC AND DHA INTERACTION MEDIATED BY HISTONES

The links between zinc and DHA interaction in gene regulation using proteomic and genomic analysis have also been investigated. Recently, histones were found to be regulated by both zinc and DHA [152]. Following this novel finding, it is important to gain an understanding on how these two nutrients involved in neuroprotection, which are mediated by histones.

### 1.9.1 Histones are involved in the assembly of chromatin

The activity of transcription is not only due to the regulation of DNA [153], but also to the structure of chromatin which plays a role in transcriptional regulation. Histones, the nucleosomal proteins, can be modified in several ways, which further affects the regulation of gene expression [154].

Histones are a group of conserved, highly basic proteins that are rich in lysine (K) and arginine (R) [155-158] (Table 1.3). Histones are involved in the assembly of chromatin through electrostatic interaction between the highly negatively charged polymeric DNA and the positively charged histones, which play a determining role in stabilizing the nucleosomes at physiological conditions [159]. About 85% of the DNA in chromatin is represented by uniform units, the nucleosomes, which are the complexes of DNA double helix with five histone proteins (H2A, H2B, H3, H4, and H1)[160]. The central part of the nucleosome is called the nucleosome core particle and consists of 147 bp DNA wrapped around the histone octamer which is formed from one (H3/H4)2 tetramer and

two H2A/H2B dimers [160, 161] (Fig. 1.3). The four core histones have similar isoelectric points (PI) and share a common structural motif called the histone fold, which facilitates the interactions between the individual core histones [162]. Flanking the core domains are the relatively *N*-terminal unstructured tail domains. The histone tails extend out from the face of the nucleosome and through the gyres of DNA superhelix into the area surrounding the nucleosome [160]. In contrast to the structural core histone proteins, histone H1 is associated into linker DNA, which connects the nucleosomes together, resulting in the formation of "beads-on-a-string" chromatin structure [163].

**Table 1.3: Characteristics of histones**. Molecular weight (MW) is given in Daltons (Da) and isoelectric points (PI) are shown.

| HISTONE TYPE | CLASS (AMINO ACID DISTRIBUTION) | M.W (Da) | SEQUENCE LENGTH | ISOELECTRIC POINT (PI) |
|---|---|---|---|---|
| H1 | Very lysine rich | 21,500 | 215 | |
| H2A | Lysine rich | 14,004 | 129 | 10.9 |
| H2B | Lysine rich | 13,774 | 125 | 10.3 |
| H3 | Arginine rich | 15,324 | 135 | 11.1 |
| H4 | Arginine rich | 11,282 | 102 | 11.4 |

### 1.9.2 Histone post-translational modifications (PTMs) and gene activities

In addition to nucleosome assembly, studies have found that histones are potentially important carriers of epigenetic information and therefore play significant role in

regulating gene activities, such as DNA damage repair, replication and transcription through post-translational modifications (PTMs)[164]. The *N*-terminal tail domain of the core histones serve as a substrate for histone modifying enzymes to introduce PTMs such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation [165, 166]. Some modifications such as acetylation and phosphorylation can modify the charge of the histone tails and therefore potentially influence the chromatin remodeling through electrostatic mechanisms. However, the primary mechanism by which the histone tail modification act is mainly through regulation of the binding of non-histone protein to chromatin [167-169].

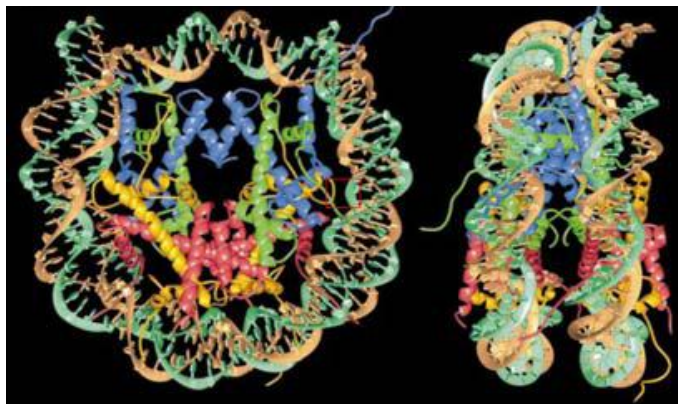


**Figure 1.3: Crystal structure of nucleosome** (Luger et al., 1997). Ribbon traces for the 146 bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B). Left: nucleosome particle viewed down the DNA superhelix axis. Right: perpendicular position to DNA [170].

PTMs of histones, alone or in combination, function to direct specific and distinct DNA-templated programs, and therefore give rise to “histone code“ hypothesis [171]. This hypothesis predicts that histone tail modification act as a binding site for recruitment of protein complexes to the nucleosome, which may further modify and regulate

chromatin accessibility [171]. The reversible acetylation and deacetylation of histone emerged to play an important role in chromatin modification [172] and gene activity regulation, which affect a variety of biological processes in response to internal and external signals, such as cell differentiation, growth and development [173]. The sites of acetylation include four highly conserved lysines in histone H4 (K5, K8, K12 and K16), five in histone H3 (K9, K14, K18, K23 and K27), as well as less conserved sites in histone H2A and H2B [174]. The acetylation acts by neutralizing the positive charge of the histone tails and weakens the electrostatic interactions between the histone and the DNA backbone [175, 176]. This allows the transcription activators and the basal transcription machinery to have better access to their specific binding sites in the gene promoter and enhancer sites [176], which therefore increasing gene transcription [172]. Recent studies have shown that when DNA is assembled onto the nucleosome with unacetylated histones, the general transcription factors cannot bind to the TATA box and the initiation region. By being unacetylated, the *N*-terminal lysines are positively charged and interact strongly with the DNA phosphates increasing the affinity of the DNA for the nucleosome. Therefore, a hyperacetylation of histone *N*-terminal tails is necessary in order for transcription factors to bind and initiate transcription [160, 165, 170].

The opposite effect of histone acetylation is deacetylation, also known as hypoacetylation status, which is associated with chromosome condensation, transcriptional repression and gene silencing [177]. This deacetylation state of histone

stabilizes the repressive state of the nucleosome through strengthening the electrostatic interaction between negatively charged DNA molecules and the positively charged lysine residues [178]. Basically, histone acetylation acts as a central switch that allows interconversion between permissive and repressive chromatin structures and domains, which govern gene transcription regulation and processes involving chromatin substrates, including replication, DNA recombination and repair [179].

Another PTM is the methylation. Specific methylation of lysine residues is known to occur on histone H3 at K4, K9, K27, K36 and K79 and on histone H4 at K20. Arginine methylation takes place on histone H3 at R2, R17 and R26, and on histone H4 at R3 [180]. Like histone acetylation, methylation can also modulate histone interaction with DNA, which then results in the alteration of nucleosomal structure and function. Therefore, it is involved in various biological activities, ranging from transcription regulation [181] to epigenetic silencing [182]. Lysine residues may be modified to either mono-, di- or trimethylated states, adding more complexity to this code [181].

It has been extensively reported that histone H3 at position K9 is a site for both acetylation and methylation, which lead to the formation of either euchromatin or heterochromatin, depending on the type of modification [180]. In general, methylation of histone H3(K4), -(K36) and -(K79) are correlated with euchromatin and transcriptional activation, while methylation of histone H3 (K9) and -(K27) and histone H4 (K20) are associated with heterochromatin and transcriptional repression [183]. Whether these

PTMs of histones have a role in setting the boundaries between heterochromatin and euchromatin, they also play important role in other biological activities such as DNA repair system [184], maintenance of DNA methylation [185] and apoptosis [186]. For instance, study by Huyen et al (2004) reported the involvement of histone H3 (K79) methylation in DNA repair by recruiting the DNA repair-associated protein, 53BP1, which specifically binds to methylated H3 (K79) [184]. Histone H3 (K9), apart from its association with heterochromatin region, is also required for the maintenance of DNA methylation [185]. The combination of enhanced histone and DNA methylation have been found to silence one of the copies of the X-chromosome in female mammals [187]. However, different histone modifications can possibly interact by antagonizing or cooperating with one another, which give various different outcomes in regulating cell activities [188]. Study by Nishioka et al (2002) reported the antagonizing function of H3 (K4) methylation (activation) and the H3 (K9) methylation (repression) in transcriptional control [189]. Furthermore, *in vitro* and *in vivo* studies have shown that methylation of H4 (K20) inhibits the acetylation of H3 (K16), which is a marker for hyperactive male X-chromosome in *Drosophilla* [190] and transcriptionally active chromatin in human cells [191]. Taken together, histone methylation may play significant role in gene regulation and therefore contributes to the epigenetic control of biological activities.

In addition to acetylation and methylation, phosphorylation is also involved in gene activities. Histone phosphorylation takes place at threonine 3 and 11 and at serine 10 and 28 residues and has been shown to be involved in cell cycle progress during mitosis,

meiosis [192], as well as transcriptional activation of immediate early-response genes, such as c-fos, c-myc and c-jun during interphase [193]. On the other hand, Enomoto et al (2001) suggested that histone phosphorylation, especially H2A, H3 and H1, is an early step of triggering DNA fragmentation in apoptosis [194].

### 1.9.3 Enzymes involve in histone post-translational modifications (PTMs)

Histone acetylation is a dynamic process that is regulated by two classes of enzymes, the histone acetyltransferases (HATs) and histone deacetylases (HDACs) [195]. HATs family is classified into two categories based on their subcellular distribution, type A and type B HATs [179].

The type A HATs are responsible in acetylation of histones within chromatin and therefore directly involved in regulating chromatin assembly and gene transcription [196]. Type A HATs consist of transcriptional activator Gcn5-related acetyltransferases (GNATs), PCAF (p300/CBP-associated factor), the transcriptional coactivator p300/CBP, MYST (for MOZ, Ybf2/Sas3, Sas2 and Tip60)-related HATs and TAFIID subunit TAF250, which have counterparts in eukaryotes including plants [196, 197]. Additional HAT family, nuclear receptor coactivator SRC-1 and ACTR are present in mammals but not in plants, fungi or other animals [198, 199]. Type B HATs are located in cytoplasm and are responsible in acetylation of free histones within cytoplasm, particularly at lysine 5 and 12 of histone H4, prior to histone incorporation into newly replicated chromatin [200]. Type B HATs include hat1p [201], ESA1 [202], tip60 [203] and SAS3 [204].

The discovery of cAMP response element-binding (CREB)-binding protein (CBP) and its homologue p300, as transcriptional co-activators that have intrinsic histone acetyltransferase (HAT) activity, suggest that histones also regulate DNA transcription through the recruitment of these co-activators [205]. Study by Janknecht (2002) also confirmed this finding by showing that P300/CBP interacts with multiple transcriptional regulators, which then facilitates the assembly of the basic transcriptional machinery [206]. Numerous studies have reported that synaptic activity in neuronal cells can potentially regulate the ability of CBP to function as transcriptional co-activator [207].

Conversely to HATS, histone deacetylases (HDACs) function to remove the acetyl groups that may be attached to histones of the nucleosome, which then inhibits access to the genes and therefore silencing of genes [208]. So far, 18 different HDACs have been identified and divided into three distinct enzyme classes [209, 210]. Class I HDACs, which are originally derived from the yeast RPD3, are found in the cell nucleus (HDAC1, 2, 3, and 8). In humans, class I HDACs share the same structure and are expressed in the nuclei of most cell lines and tissue types [210, 211], whereas class II subgroup (HDAC4, 5, 7, 9, and 10) are able to shuttle in and out of the nucleus, depending on the cellular signals, and are expressed in a small number of cell types [180]. Both class I and II have highly conserved catalytic domain but differ in overall composition and size [212]. Class III HDACs, share a common ancestry with yeast transcription repressor Sir2 (silent information regulator 2) and are composed of Sirtuin (SIRT) proteins 1–7[213]. The most recently described HDACs are Class IV, which is represented by HDAC11 [214]. So far,

very little is known about its function and regulation. HDAC1 and HDAC2 are often associated with large protein complexes such as Sin3, Mi2/NURD and CoREST, which demonstrate better deacetylases activity than HDACs components alone [215]. Each member of the HDAC family, however, may exhibit a different, individual substrate specificity and function *in vivo* [216] .

Histone methyltransferases (HMTs) are enzymes that catalyze the irreversible histone methylation on lysine residues. The HMTs family includes enzyme such as SUV39, which is specific for histone H3(K9)[217], SET1c and SET7/9 of H3(K4)[189], SET2 of H3 (K36) [218] and DOT1 of H3(K79)[219, 220]. The SUV39, the first HMTs discovered, acts on histone H3(K9) and is localized to transcriptionally silent heterochromatin sites [221]. A mechanistic connection between SUV39 and the histone binding protein HP1 (heterochromatin-associated protein 1) has been established recently [221], and is shown to be involved in the formation of heterochromatin [180]. Apart from their ability to covalently modify histones, some HMTs have also been shown to directly methylate the tumor suppressor, p53. Set9, which specifically methylates H3K4 [222], and has also been implicated in the regulation of p53. Methylation of p53 stabilizes and limits its localization to the nucleus [186]. More recently, Smyd2, which acts on H3K36 methylation, has also been directly linked to the regulation of p53 [178].

Accumulating evidence implicates the aberrant loss or gain of HMTs activity, which often leads to developmental defects and diseases. The HMTs SUV39 family member, SUV39H-deficient mice display severely impaired viability and chromosomal instabilities

that are associated with an increased tumor risk and perturbed chromosome interactions during male meiosis [223], suggesting the importance of HMTs as epigenetic regulators for mammalian development.

Histone H3 phosphorylation is catalyzed by the members of Aurora kinase family [224]. Immunocytochemical analysis revealed that Aurora-B co-localized with H3, catalyzed the phosphorylation of Ser10 from late G2 phase to metaphase and Ser28 from prophase to metaphase in mammals [225]. The phosphorylation of Thr(T)3 of histone H3, which is catalyzed by kinase haspin, also occurs during mitosis and plays an essential role in facilitating condensation and resolution of sister chromatids in late G2 and prophase [226].

### 1.9.4 Histone post-translational modifications (PTMs) and neurodegenerative diseases

As previously discussed, histone post-translational modifications (PTMs) play significant roles in regulating gene activities. Therefore, aberrant pattern of epigenetic regulation has been linked to the development of neurodegenerative diseases such as polyglutamine diseases (Huntington disease), Rubinstein-Taybi syndrome and AD [215].

During normal neuronal condition, HATs and HDACs, which are responsible for regulating acetylation and deacetylation respectively, remain in a state of balance which they counteract each other to ensure neurophysiological homeostasis. Such equilibrium

(Fig. 1.4A) is responsible for regulating gene expression leading to normal function of neuronal cell activity and memory formation [215, 227]. During neurodegenerative diseases, the acetylation homeostasis is altered when histone acetylation significantly decreases [228], reflecting dysfunctional acetylation-deacetylation apparatus (Fig. 1.4B). General loss of acetylating agent, would cause excessive increased in HDAC activity, which is then associated with transcriptional repression (Fig. 1.4B). Studies have reported that reduction in histone acetylation followed by the increase in HDACs activity or DNA methylation is common in many neurodegenerative disorders [229, 230].

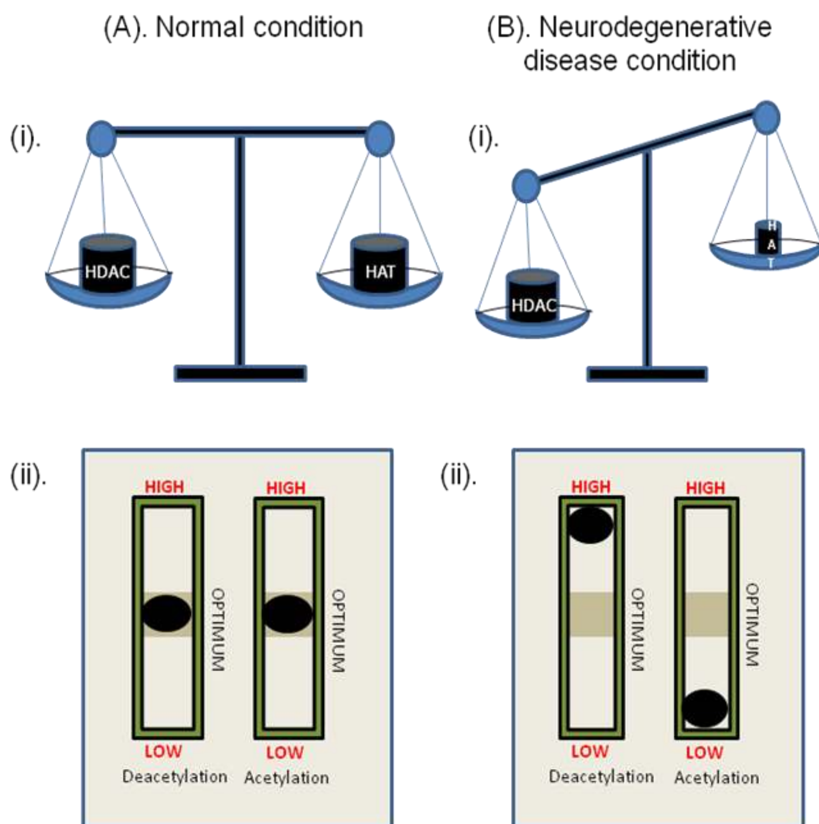



**Figure 1.4: Neuronal acetylation homeostasis.** (i) Weights on the balance represent the protein levels of HATs and HDACs. (ii) Enzymatic activity scale represents the activity and dark grey areas are physiologically optimal. (A) Under normal neuronal conditions, the level and activity of both HATs and HDACs are within their point of balance where they counteract each other to maintain internal equilibrium (homeostasis). (B) During neurodegenerative disease condition, acetylation homeostasis is altered resulting in the loss of HATs level and activity, which balance towards an excessive production of HDACs and subsequent increase in deacetylation.

In recent years, the increasing numbers of structurally diverse HDAC inhibitors have been identified with the potential to target specific brain regions and in cell-specific manner to reverse disorder-specific epigenetic dysregulation [231]. The HDAC inhibitors include: short-chain fatty acid (i.e. valproic acid) [232], hydroxamic acid (i.e. SAHA, TSA, oxamflatin) [233], cyclic tetrapeptides (i.e trapoxin, apicidin) [234] and benzamide (i.e

MS-275) [235]. These HDAC inhibitors are aimed to inhibit its enzymatic activity and to remove the repressive blocks from promoters of essential genes and therefore induce active gene transcription [215]. The X-ray crystallographic studies showed that this type of HDAC inhibitors act as a chelator of zinc ion in the catalytic site of HDACs, which therefore block the substrate access to the active zinc ion and subsequently inhibit the deacetylation ativity [236]. However, it is still uncertain whether certain neurodegenerative disorders are mediated by a specific HDAC.

Aging is also considered as the greatest risk factor for the development of the neurodegenerative diseases, where neuronal function declination and gene expression alternation could be detected in the aging human brain [237]. Studies have found the altered pattern of histone modification in aging cells, such as trimethylation of histone H4 at lysine 20, which was increased in kidneys and liver of the old-aged rat [238], and the level of H4 acetylation, which was decreased in the rat brain cortical neurons with age [239]. Several new methylated sites, such as H3 (K24), H3 (K128) and H2A (R89) were also detected in the study of aged mouse brain, however, no functional studies on these three sites have been reported [240]. It has been reported that in aging brains, most PTM sites were found on histone H3, which has the longest *N*-terminal tails amongst other core histones [240]. These studies suggest the importance of proper epigenetic modification in biological activities and neuronal cell development, while the altered epigenetic regulation leads to neurodegenerative diseases.

## 1.10 M17 CELL LINE AS A MODEL

Neurodegeneration is very difficult to study *in vivo*. Neuronal cells do not regenerate and cannot be observed or manipulated without removing them from the patients. For these reasons, *in vitro* models are very important options. An ideal cell line would possess similar characteristic as the *in vivo* neurons, while having the advantage of immortalization to ensure continuous supply of cells. Immortalized cells are also convenient to handle and experiments can be perfomed during continuous conditions in which biochemical process can be easily studied.

Throughout our studies, M17, a neuronal-derived, cell line was used. M17 cells were originally isolated from the bone marrow of a two year old male suffering from disseminated neuroblastoma (Global Bio-resource Center, 2007). Microscopic analysis shows that the cell type indicates a neuronal characteristic; being morphologically small in size and dense with triangular-shaped cell bodies. The advantage of this cell line is that it is of human origin, and by now, M17 cells constitute a well-studied and defined cellular system. The “in-house” results suggest that M17 cell line is a suitable model for studying the effects of zinc and DHA supplementation on bioenergetic function and gene regulation of neuronal cells throughout this study.

# <u>CHAPTER 2</u>

## DHA PROTECTS AGAINST ZINC-INDUCED ALTERATION IN MITOCHONDRIAL FUNCTION OF M17 NEUROBLASTOMA

**PUBLICATION:**

Sean L. McGee, **Nadia Sadli,** Shona Morrison, Courtney Swinton and Cenk Suphioglu (2010) DHA protects against zinc mediated alterations in neuronal cellular bioenergetics. *Cell Physiol Biochem.* 28(1):157-62.

## Summary

### Aim

Zinc accumulation in neuronal cells may result in mitochondrial destruction and therefore contributes to cellular apoptosis. The aim of the present study was to investigate the effect of zinc on mitochondrial function, and how it is affected by DHA supplementation in M17 human neuroblastoma cell line.

### Methodology

M17 neuroblastoma cells were treated with zinc (5 µM), DHA (10 µg/ml) or zinc and DHA in combination for 48 h. All bioenergetic and mitochondrial function analysis were performed using the Seahorse XF24 Extracellular Flux Analyser.

### Results

The results of the first study showed a decrease in cellular oxygen consumption, but not glycolytic rate, following chronic zinc exposure, which was specific for neuronal cells. This was due to impaired ATP turnover, without any other effects on mitochondrial function, and was restored by DHA. These data suggest that zinc disrupts bioenergetics at a point distal to the respiratory chain, which is restored by DHA.

## Conclusion

This chapter has demonstrated that chronic exposure of M17 neuroblastoma to moderate pathological levels of zinc impairs bioenergetics by inhibiting mitochondrial ATP turnover. Omega-3 fatty DHA has shown to protect against this effect and this was specific for neuronal cell line. It was also reported that the protective effect of DHA on neuronal cells in response to zinc toxicity would include restoration of metabolic processes.

## 2.1 INTRODUCTION

Impaired zinc homeostasis has a profound effect on neuronal cell viability, with zinc accumulation being a potent mediator of neuronal cell injury that is implicated in neurodegenerative diseases such as AD [21]. The mechanisms by which zinc impairs neuronal viability are thought to include impaired bioenergetics [241]. It is not surprising that mitochondria represent a target for zinc toxicity, bearing in mind their significant role in energy generation and metabolic function such as inhibition of the electron transport chain, uncoupling of oxidative phosphorylation, oxidation of mitochondrial DNA (mtDNA) and inhibition of mitochondrial DNA synthesis.

As previously described, increased in intracellular zinc accumulation in neuronal cells results in cell death. This zinc toxicity occurs due to the specific changes within the cells that lead to impairment of intracellular homeostasis and regulation, which can be considered as a comprehensive concept of zinc toxicity. It is interesting to note that, in all disorders, mitochondria play a key role. There are some critical biochemical mechanisms on how mitochondria-induced cell damage may be initiated, namely ATP depletion, overproduction of reactive oxygen species (ROS) and reduced oxygen phosphorylation, which will be analyzed in this chapter.

Zinc is thought to inhibit a number of metabolic pathways, including glycolysis [242], the tricarboxylic (TCA) cycle [243] and the mitochondrial respiratory chain [244]. However, much of this data has been gathered from *in vitro* reactions with purified metabolic

enzymes and studies of isolated mitochondria. Therefore, the role of zinc on metabolism in intact human neuronal cell lines is not entirely clear. Furthermore, many of these studies have used acute supraphysiological levels of zinc to induce bioenergetic dysfunction, such that the role of chronic zinc exposure at moderate pathophysiological levels on bioenergetics is also unclear.

In this chapter, the aims were to: 1. Determine whether zinc impairs bioenergetics in live M17 neuroblastoma cells; 2. Establish whether DHA can protect against any alterations in cellular bioenergetics; and 3. Determine whether the effects of zinc and DHA on bioenergetics are specific to neuronal cell lines.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Cell culture

M17 Neuroblastoma cells, obtained from Murdoch Children Research Institute, Royal Children Hospital were used in all experiments. M17 cells were originally isolated from the bone marrow of a two year old male suffering from disseminated neuroblastoma (Global Bio-resource Center, 2007). The M17 cells were grown at 37°C with 5% $CO_2$ with 100% humidity in Opti-MEM media (a modified MEM (Eagle's) media) with heat inactivated 2.5% Fetal Bovine Serum (FBS) supplementation. To determine whether the effects of zinc and DHA on bioenergetics are specific to neuronal cell lines, HaCaT human keratinocyte bioenergetics were also examined following chronic zinc exposure. HaCaT cell line was maintained in DMEM media supplemented with 10% FBS. Cultures

were split when they reached 80-90% confluency and the medium was replaced every two days with pre-warmed Opti-MEM or DMEM media.

#### 2.2.1.1 Cell thawing

Cryopreserved M17 and HaCaT cells in cryovials were removed from liquid nitrogen storage and placed into a covered water bath at 37°C until thawed. Quickly, cells were pipetted out into a 15 ml tube filled with 10 ml of pre-warmed Opti-MEM or DMEM growth media and then transferred to a 75 $cm^2$ culture flask. Cells were then incubated at 37°C in a 5% $CO_2$ humidified incubator. After 24 h, culture media was replaced in order to remove non-adherent cells and replenish nutrients.

#### 2.2.1.2 Passaging cells

After old medium was discarded, M17 and HaCaT cells were washed twice with sterile phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$ and 1.47 mM $KH_2PO_4$ (pH.7.3), to remove all traces of growth medium. The cells were harvested by adding 3 ml of 0.025% PBS-ethylenediaminetetraacetic acid (EDTA)-Trypsin to the culture flask, and solution was gently moved over the cells for 30 sec. The flask was placed in the 37°C and 5% $CO_2$ humidified incubator for approximately 1 min. The flask was then checked under a microscope to see if the cells had detached from the surface of the flask. Non-adherent cells were resuspended in 10 ml medium and 1 ml was transferred to a sterile 75 $cm^2$ tissue culture flask.

### 2.2.2 Zinc and DHA treatments

Docosahexanoic acid (DHA; Sigma Sldrich, MO, USA) stock solution was prepared in 100% (v/v) ethanol at a concentration of 100 mg/ml and the working solution was prepared by adding the stock to the Opti-MEM or DMEM media to get the final concentration of 10 µg/ml. DHA stock solution was stored at -20°C and the treatment media were prepared fresh before treating the cells. Before starting the experiment, DHA-containing media was pre-incubated overnight at 37°C to allow DHA to conjugate with media proteins, to allow delivery into cells.

Zinc (in the form of $ZnCl_2$) was added to the media on the day of the experiment to the final concentration of 5 µM. The extracellular zinc uptake was previously tested to confirm that zinc indeed enters M17 human neuroblastoma cells. It was observed that zinc does in fact get into cells as shown by our $^{65}Zn$ studies [45]. A range of zinc concentrations were also tested and anything above 5 µM caused significant death of cells, which therefore could not be used for analysis. Therefore, 5 µM was the concentration chosen for this study, which also represents the upper physiological levels. The growth media was also tested to have no zinc, so the observed results obtained in this thesis are indeed zinc effect.

M17 cells were treated with and without zinc (final concentration 5 µM) and DHA (final concentration 10 µg/ml) with the addition of anti-oxidant vitamin E (0.05 µM/ml) in every DHA treatment groups.

### 2.2.3 Bioenergetics analysis and mitochondrial function tests

All bioenergetic and mitochondrial function analysis were performed using the Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience, Billerica, USA).

M17 neuroblastoma and HaCaT cells were seeded into 24-well Seahorse V7 plates at 2.5 x $10^4$ cells/well. The following day, cells were treated with either vehicle, zinc (5 µM), DHA (10 µg/ml) or zinc and DHA in combination for 48 h. Prior to assay, cells were washed twice with assay running media (unbuffered DMEM, 25 mM glucose, 1 mM glutamine, 1 mM sodium pyruvate), before being resuspended in 675 µl of running media. Cells were equilibrated in a non-$CO_2$ incubator for 60 min prior to assay. The assay protocol consisted of repeated cycles of 2 min mix, 2 min wait and 4 min measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously through each measurement period by excitation of fluorophores for $O_2$ and $H^+$. This gives measurement of oxidative and non-oxidative metabolism, respectively.

Basal energetics were established after three of these cycles, followed by sequential exposure of the ATP synthase inhibitor oligomycin, the proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and the complex III inhibitor antimycin A, all to a final concentration of 1 µM. These compounds were introduced to the cell media by the Seahorse injection system. Three mix, wait and measurement cycles separated each compound injection (protocol is summarized in Fig.

2.1). Using these compounds as modulators of mitochondrial function, it is possible to determine a number of bioenergetic and mitochondrial parameters, including basal respiration, ATP turnover rate, proton leak, maximal and spare respiratory capacity (Fig. 2.2).

All treatment conditions were analysed as ten replicates, over at least two independent experiments and data was pooled to give average values for each treatment. At the completion of each assay, the assay plate was frozen at -80$^{o}$C, prior to determination of cell number in each well using the CyQuant kit (Invitrogen, Carlsbad, USA) according to manufacturer's instructions.

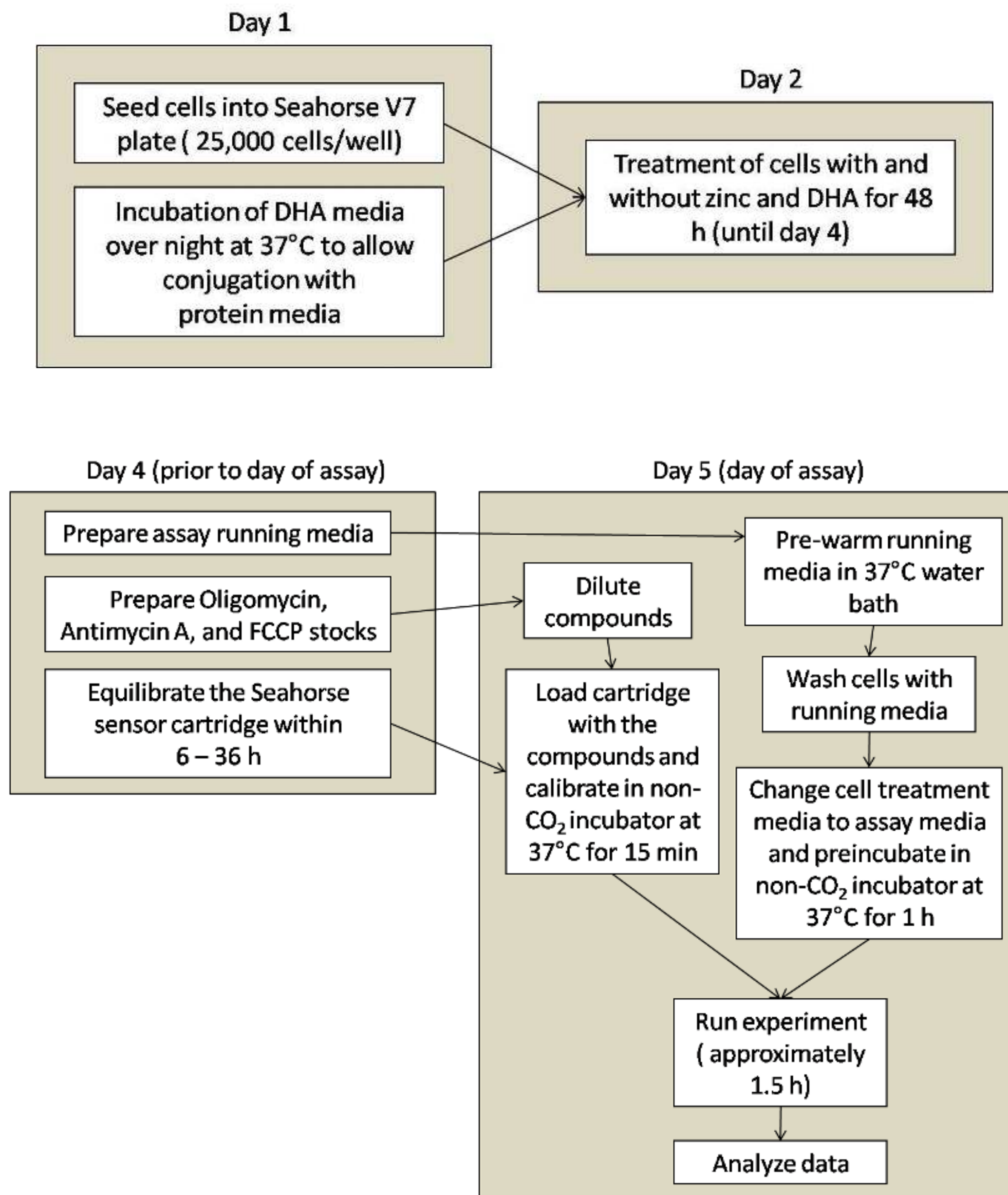



**Figure 2.1: Study design used to measure the bioenergetic and mitochondrial functions in M17 and HaCaT cells following zinc and DHA using XF Extracellular Flux Analyzer (Seahorse Bioscience).**

Bioenergetic experiment was prepared as illustrated. The treated (zinc, DHA or zinc and DHA in combination) and untreated cells were switched from treatment media to assay running media. Following baseline measurements, 75 µl of testing compounds (Oligomycin, FCCP, and antimycin A) prepared in assay running media in the cartridge were introduced to the cell media by the Seahorse injection system. After mixing, OCR and ECAR measurements were made.

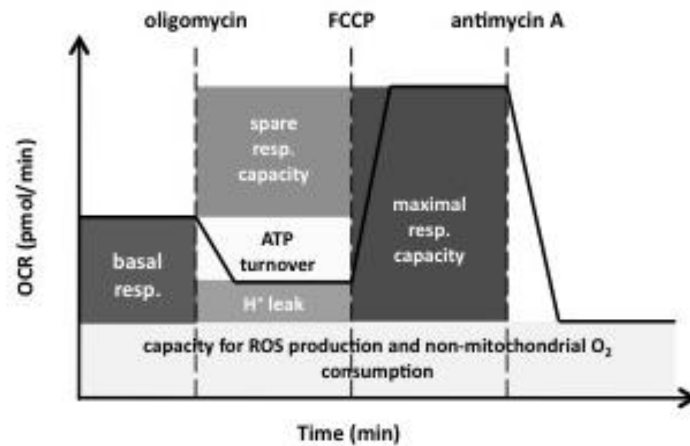



**Figure 2.2: Schematic diagram of the mitochondrial function test**. Multiple oxygen consumption measurements are made basally and after the injection of the ATP synthase inhibitor oligomycin, the proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) and the complex III inhibitor antimycin A. From these analyses, basal respiration, proton leak, ATP turnover, spare respiratory capacity and maximal respiratory capacity can be calculated.

### 2.2.4 Statistical analysis

All values are reported as means ± standard error of the mean (SEM) and were evaluated for statistically significant differences using analysis of variance (ANOVA) and Tukey post-hoc testing where appropriate. Differences between groups were considered statistically significant where $p<0.05$.

## 2.3 RESULTS

### 2.3.1 Zinc impairs basal cellular bioenergetics in M17 neuroblastoma, which is restored by DHA

The insight into physiological state of cells and alteration of cellular bioenergetic function can be studied through measuring the oxygen consumption rate (OCR), an indicator of mitochondrial respiration. Here, the extracellular acidification rate (ECAR)

was also determined. ECAR is the measure of lactic acid formed during glycolytic energy metabolism (in the absence of oxidative phosphorylation). Cellular OCR and ECAR are related to the flux through catabolic pathways used to generate ATP [245]. When the OCR is inhibited by toxins or drugs, ECAR would be increased as a result of an increase in glycolytic flux, as the cells attempt to recover the mitochondrial ATP lost due to inhibition of the electron transport chain, which is associated with reduction in OCR [246, 247].

OCR for M17 neuroblastoma throughout the bioenergetics analysis is shown in Figure 2.3A. Basal OCR and ECAR are shown in Figure 2.3B. Zinc significantly reduced basal OCR when compared with control cells (Figs. 2.3A and 2.3B), without altering basal ECAR (Fig. 2.3B), which is indicative of mitochondrial dysfunction without non-oxidative compensation. Basal OCR was not different in cells exposed to both zinc and DHA, suggesting that DHA can protect against zinc-mediated alterations in basal OCR (Fig. 2.3B). There was no effect of DHA exposure alone on cellular bioenergetics (Fig. 2.3B).

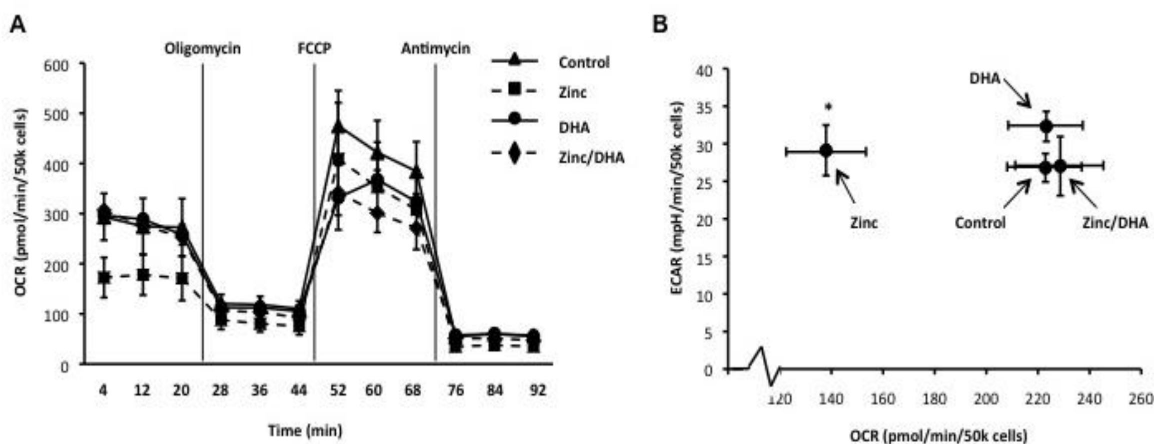



**Figure 2.3: Mitochondrial function and basal bioenergetics in M17 neuroblastoma.** Oxygen consumption rate (OCR) (A) throughout mitochondrial function testing, and basal OCR and extracellular acidification rate (ECAR) (B) of M17 neuroblastoma exposed for 48 h to either vehicle (Control), zinc, docosahexaenoic acid (DHA) or zinc and DHA together (zinc/DHA). All values are reported as means ± SEM (n=12-15 per group). *Denotes significantly different OCR from all other groups ($p<0.05$).

### 2.3.2 Zinc reduces oxidative ATP turnover, which is restored by DHA

To further examine why basal oxidative respiration was impaired following zinc exposure in M17 neuroblastomas, mitochondrial function in these cells was assessed. Zinc decreased oxidative ATP turnover, which was restored with co-exposure of DHA (Fig. 2.4A). Both zinc and DHA, either alone or in combination, did not significantly affect proton leak (Fig. 2.4B) and maximal respiratory capacity (Fig. 2.4C). However, as zinc decreased basal respiration without any significant effect on maximal respiratory capacity, zinc increased spare respiratory capacity when compared with all other treatments (Fig. 2.4D).

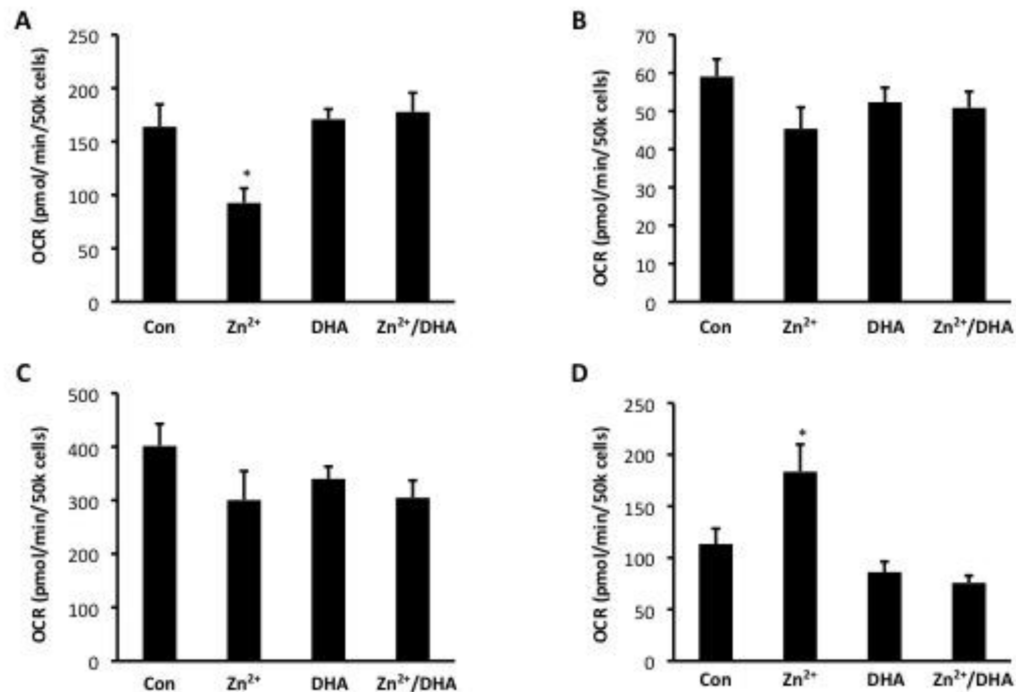



**Figure 2.4: Mitochondrial function parameters in M17 neuroblastoma**. ATP turnover rate (A), proton leak (B), maximal respiratory capacity (C) and spare respiratory capacity (D) were calculated from oxygen consumption rates of M17 neuroblastomas exposed for 48 h to either vehicle (Con), zinc ($Zn^{2+}$), docosahexaenoic acid (DHA) or zinc and DHA together ($Zn^{2+}$/DHA). All values are reported as means ± SEM (n=10 per group). *Denotes significantly different from all other groups ($p<0.05$).

### 2.3.3 Zinc and DHA have no effect on cellular bioenergetics in HaCaT keratinocytes

It has been proposed that due to their relatively high oxidative ATP demand and low spare respiratory capacity, neuronal cell bioenergetics, and in turn viability, are particularly sensitive to factors that induce mitochondrial dysfunction [248]. To determine whether the effects of zinc and DHA on bioenergetics are unique to neuronal cell lines, HaCaT keratinocyte bioenergetics following chronic zinc exposure were examined. OCR for HaCaT keratinocytes throughout the bioenergetics analysis are shown in Figure 2.5A and basal OCR and ECAR are shown in Figure 2.5B. This cell line

was used as they too have a relatively high oxidative ATP demand and low spare respiratory capacity, which was confirmed in our analysis (Fig. 2.5A). Zinc and DHA had no effect on basal keratinocyte bioenergetics (Fig. 2.5B), suggesting that the M17 neuronal cell line was more sensitive to the effects of zinc and DHA.

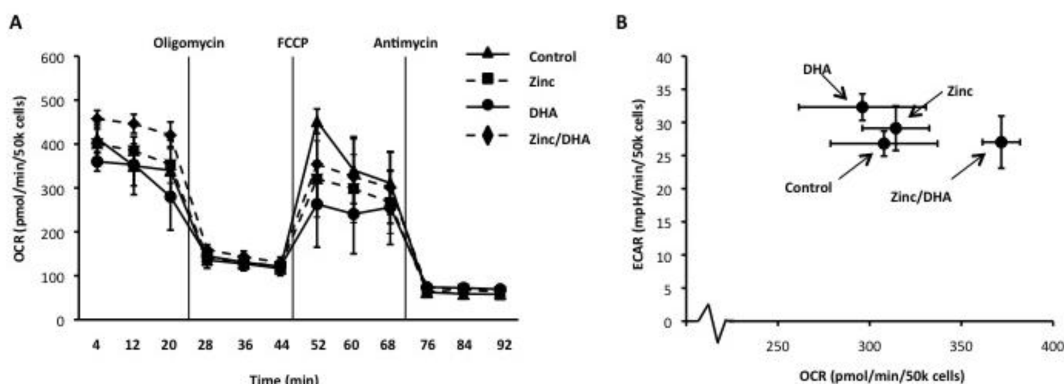



**Figure 2.5: Mitochondrial function and basal bioenergetics in HaCaT keratinocytes**. Oxygen consumption rate (OCR) (A) throughout mitochondrial function testing, and basal OCR and extracellular acidification rate (ECAR) (B) of HaCaT keratinocytes exposed for 48 h to either vehicle (Control), zinc, docosahexaenoic acid (DHA) or zinc and DHA together (zinc/DHA). All values are reported as means ± SEM (n=12-15 per group).

## 2.4 DISCUSSION

In this chapter, it was observed that chronic exposure of M17 neuroblastoma cells to moderate pathophysiological levels of zinc impairs cellular bioenergetics, through inhibition of basal respiration. The data showed that ATP turnover was the primary parameter of mitochondrial function that was impaired and that there was no compensatory response through anaerobic energy sources. This fits with previously published data showing that chronic zinc exposure can induce neuronal apoptosis through a decline in cellular ATP stores [84, 248], albeit at higher zinc concentrations. However, using indirect measures of metabolism, this study found that the primary

defect in bioenergetics was glycolysis [248]. There were no alterations in glycolytic rate, as measured by cellular proton production, using a bioanalyser that simultaneously measures both anaerobic and aerobic flux. While these differences could be due to the different analytical methods employed, the concentrations of zinc were also different. The present study used moderate pathophysiological zinc concentrations over a 48 h period. Importantly, it should be noted that cells were not exposed to zinc in the 60 min prior to, and throughout the assays. This suggests that chronic exposure to moderate zinc concentrations induces alterations in oxidative metabolism that are not readily reversible following withdrawal of zinc.

The analysis of mitochondrial function localized the defect in basal respiration to a reduction in mitochondrial ATP turnover. This means that chronic exposure to moderate pathophysiological zinc concentrations induces a defect in mitochondrial metabolism that is distal to the respiratory chain. It has not been reported so far that zinc inhibits ATP synthase directly, or the availability of ADP for conversion to ATP by ATP synthase. Indeed, studies using isolated mitochondria with a wide range of zinc concentrations and various substrates for metabolism have found inhibition of the respiratory chain, most likely at the transfer of electrons between complexes II and III [249, 250]. However, zinc has also been found to dissipate the mitochondrial membrane potential through regulation of the mitochondrial transition pore [251]. As ATP synthase requires the proton gradient of the mitochondrial membrane potential to generate ATP [252], this mechanism could account for the impairment of ATP turnover induced by zinc. This

mechanism has also been associated with increased production of mitochondrial reactive oxygen species, release of cyt c and initiation of apoptosis [241]. These data suggest that inhibition of ATP turnover is the primary bioenergetic parameter modulated by chronic exposure to moderate pathophysiological concentrations of zinc and highlight the complex role that this transition metal has on both normal cell function, such as synaptic transmission, and pathological processes, such as initiation of apoptosis.

Data from the present study also shows that DHA is able to protect against reductions in oxidative ATP turnover. As dissipation of the mitochondrial membrane potential appears to be the mechanism by which zinc impairs ATP turnover, it is important to speculate on how DHA might protect mitochondrial metabolism. One of the major functions of DHA is its incorporation into membranes [253], where they can assist with membrane fluidity and function. It is possible that DHA may protect the transition pore in response to zinc through its role in membranes. Indeed, it has recently been shown that DHA can prevent mitochondrial permeability transition in the heart [254, 255]. However, the previous work has showed that DHA may impair cellular zinc uptake [45], which could contribute to its protective effects. Nonetheless, the data from the present study is the first to show that DHA normalizes cellular bioenergetics in response to chronic pathophysiological zinc exposure. As DHA has been shown to protect against neuronal apoptosis, part of this protective effect could be ascribed to protection against alterations in bioenergetics secondary to altered zinc transport.

A final aim of the present study was to examine whether the effects of zinc and DHA on cellular bioenergetics were specific to neuronal cells. To address this aim, anaerobic and aerobic metabolism in HaCaT keratinocytes was examined. Indeed, it has been proposed that neurons are particularly susceptible to apoptosis by insults that perturb bioenergetics due to their high ATP demand and relative low spare respiratory capacity [248]. HaCaT keratinocytes were used in the present study because of their similar properties (Fig. 2.5A). However, the data suggested that these cells were not sensitive to zinc mediated alterations in metabolism. A potential explanation for this finding could be altered zinc uptake in these cells when compared with neuronal cells. However, isolated liver mitochondria are also not sensitive to the effects of zinc on the transition pore [256], which could suggest that intrinsic differences in neuronal mitochondria make them more sensitive to this effect of zinc. As there was no alteration in metabolism following chronic zinc exposure in these cells, DHA had no effect on bioenergetics. These data are consistent with the fact that DHA had no effect on metabolism independent of zinc in M17 neuroblastoma. Furthermore, this data highlights the neuronal reliance on zinc for functions such as synaptic transmission, which could in turn make this cell type particularly susceptible to dysregulation of zinc homeostasis.

## 2.5 CONCLUSION

In conclusion, this study suggests that chronic exposure of M17 neuroblastoma cells to moderate pathological levels of zinc impairs bioenergetics by inhibiting mitochondrial

ATP turnover. The co-exposure of zinc with DHA protected against this effect and that this was specific for neuronal cell lines. It was observed that the protective effect of DHA on neurons in response to pathological stimuli also includes modulation of metabolic processes.

# CHAPTER 3

## NEUROPROTECTIVE EFFECT OF DHA AND COENZYME Q10 AGAINST Aβ- AND ZINC- INDUCED MITOCHONDRIAL DYSFUNCTION IN NEURONAL CELLS

**PUBLICATION:**

**Nadia Sadli**, Colin J. Barrow, Sean Mcgee, Cenk Suphioglu (2011) The effect of DHA and Coenzyme Q10 against Abeta and zinc-induced mitochondrial dysfunction in human neuronal cells. *Neuropharmacol.* (Submitted).

## Summary

### Aim

This chapter determined whether DHA and Coenzyme Q10 (CoQ10) have independent or additive effects against Aβ- and zinc-induced defects in energy metabolism and mitochondrial respiratory function in M17 neuroblastoma cells.

### Methodology

M17 neuroblastoma cells treated with Aβ (10 nM), zinc (5 μM), DHA (10 μg/ml in the presence of antioxidant vitamin E (0.05 μM/ml)), or CoQ10 (10 μM) alone and in combination after 24 h were subjected to following studies:

1. Using Seahorse Bioscience XF24 Extracellular Flux Analyzer to measure changes in all bioenergetics and mitochondrial function in M17 neuroblastoma cells.
2. To estimate the change in membrane potential ($\Delta\Psi_m$) across inner mitochondrial membrane using JC-1 assay.
3. Using Amplex red assay to examine the production of intracellular $H_2O_2$ in M17 cells.

### Results

**Study 1:** The results demonstrated a decrease in basal mitochondrial respiration in M17 cells without alteration in glycolytic rate, reduction in ATP turnover rate, uncoupled

respiration and maximal respiratory capacity in response to Aβ and zinc. CoQ10 has shown to have direct protective effect against Aβ-induced alteration in mitochondrial function, while DHA had no significant effect against Aβ toxicity. However, both CoQ10 and DHA, alone and in combination, have shown to protect against zinc-induced mitochondrial dysfunction.

**Study 2:** Significant reduction in $\Delta\Psi_m$ following Aβ was restored by CoQ10. DHA could not restore reduction in $\Delta\Psi_m$ caused by Aβ. On the other hand, CoQ10 alone did not have significant effect on $\Delta\Psi_m$ alteration in response to zinc, suggesting that CoQ10 was not directly protective against zinc-mediated alterations in $\Delta\Psi_m$.

**Study 3:** Aβ did not directly affect ROS production whereas zinc significantly increased superoxide anion levels. Our results show that combination of DHA and CoQ10 inhibit $H_2O_2$ production in M17 neuroblastoma cells.

**Conclusion**

DHA is specifically neuroprotective against zinc-triggered mitochondrial dysfunction, but does not affect Aβ neurotoxicity. CoQ10 has shown to be protective against Aβ-induced alterations in mitochondrial function.

## 3.1 INTRODUCTION

Previous data has shown that reduced DHA levels in AD brains were associated with an increase in intracellular zinc levels [148]. Zinc-mediated brain injury has been implicated as a neurotoxin in models of neurodegenerative disease [257]. However, the mechanism of zinc toxicity is unknown, but evidence suggests that zinc induces cellular apoptosis through inhibition of adenosine triphosphate (ATP) synthesis [21, 241], increase in the production of reactive oxygen species (ROS) and eventual loss of mitochondrial membrane potential $\Delta\Psi_m$ [241]. Recently, it has been reported that DHA protects against zinc-mediated alterations in human neuronal cell bioenergetics and mitochondrial function [85].

AD is characterized by the accumulation of amyloid-beta (Aβ)-containing plaques, hyperphosphorylated neurofibrillary tangles, neuronal death and synaptic loss [258, 259]. Impaired mitochondrial function [259] and a decrease in membrane potential $\Delta\Psi_m$ have also been recognized as early events within the Aβ toxicity cascade [260]. Coenzyme Q10 (CoQ10), a component of the mitochondrial electron transport chain, is well characterized as a neuroprotective antioxidant in human neuronal cells [261], as well as in animal models [262] and human trials of AD [263]. CoQ10 has membrane-stabilizing properties and also plays a vital role in ATP production [264]. Like the omega-3 FA DHA, CoQ10 levels are decreased with aging [265], and in a number of neurodegenerative conditions [266].

Although both DHA and CoQ10 have demonstrated neuroprotection in human neuronal cells, it is unclear whether the combined administrations of DHA and CoQ10 have additive effects against Aβ- and zinc-induced mitochondrial dysfunction. This chapter examined whether combined DHA and CoQ10 could preserve bioenergetics and mitochondrial function better than either DHA or CoQ10 alone, in response to Aβ and zinc toxicity in human neuronal cells.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Cell culture

As previously described, human neuroblastoma M17 cells were cultured in Opti-MEM media (Invitrogen), supplemented with 2.5% heat inactivated fetal bovine serum (FBS), in a humidified incubator at 37$^{o}$C with 5% $CO_2$. Trypsin/EDTA (0.05%; Gibco) was used for trypsinization.

For treatment analysis, the growth media was replaced with experimental media (Opti-MEM) treated with and without beta-amyloid ($A\beta_{1-42}$) peptide (final concentration 10 nM), zinc (final concentration 5 µM), CoQ10 dissolved in acetone (final concentration 10 µM) and docosahexaenoic acid (DHA) (final concentration 10 µg/ml; Nu Chek Prep Inc, Elysian, Mn, USA), in the presence of anti-oxidant vitamin E (final concentration 0.05 µM/ml).

Stock solution of DHA in ethanol was prepared as described in the previous chapter, stored at −20°C and pre-incubated in complete growth medium at 37°C overnight to allow protein conjugation. CoQ10 was a generous gift from Prof. Colin Barrow, School of Life and Environmental Sciences, Deakin University. The synthetic $Aβ_{1-42}$ peptide was purchased from Sigma Aldrich (MO, USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C until use. $Aβ_{1-42}$ peptide was pre-incubated in DMSO at 37°C overnight prior to experiments to allow aggregation. Unlike water that would promote a quick Aβ aggregation, dissolving $Aβ1_{-42}$ peptide in DMSO delays the protein aggregation, so pre-incubation prior experiment is necessary [267]. We initially tested a range of $Aβ_{1-42}$ peptide concentrations on mitochondrial function and cell viability. 10 nM $Aβ_{1-42}$ peptide in DMSO was chosen as our final concentration, as it induced mitochondrial dysfunction without reducing much cell viability. This concentration has previously been used to perturb mitochondrial function in other neuronal cell lines [268].

### 3.2.2 Seahorse XF-24 metabolic flux analysis

M17 neuroblastoma cells were cultured in Seahorse XF-24 (Seahorse Bioscience, Billerica, USA) plates at a density of 25,000 cells per well. Cells were treated with either the control vehicle, Aβ peptide (10 nM), zinc (5 µM), DHA (30 µM), or CoQ10 (10 µM), alone and in combination for 24 h, prior to analysis. On the day of analysis, cells were changed to unbuffered DMEM (DMEM base medium supplemented with 25 mM glucose, 1 mM sodium pyruvate, 1 mM GlutaMax, pH 7.4) and incubated at 37°C in a non-$CO_2$ incubator for 1 h. The assay protocol consisted of repeated cycles of 2 min mix,

2 min wait and 4 min measurement periods, with oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measured simultaneously through each measurement period by excitation of fluorophores for $O_2$ and $H^+$. This gives measurement of oxidative and non-oxidative metabolism, respectively. Three readings were taken after each addition of mitochondrial inhibitor before injection of the subsequent inhibitors. The mitochondrial inhibitors used were the ATP synthase inhibitor oligomycin (final concentration 1 μM), the proton ionophore carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP; 1 μM) and the complex III inhibitor antimycin A (1 μM). Mitochondrial function parameters were determined using these mitochondrial inhibitor compounds as modulators to determine a number of bioenergetic and mitochondrial function parameters, including basal respiration, ATP turnover rate, proton leak and maximal and spare respiratory capacity.

All treatment conditions were analyzed as ten replicates across 3 plates and data were pooled to give average values for each treatment. After the assays, plates were saved and protein concentrations for each well were measured. Mitochondrial function parameters were determined as described in Chapter 2 [85] and were normalized to cellular protein content.

### 3.2.3 JC-1 assay (inner mitochondrial membrane potential, $\Delta\Psi_m$)

Membrane potential across the inner mitochondrial membrane was estimated using the fluorescent indicator dye JC-1 (Invitrogen). M17 neuroblastoma cells were seeded into

black well/clear bottom 96-well plate at 25,000 cells/well, followed by incubation with Aβ (1 nM), zinc (5 µM), DHA (30 µM) or CoQ10 (10 µM) alone and in combination for 24 h. Stock solution of JC-1 was made up by dissolving JC-1 powder in DMSO at a final concentration of 5 mg/ml (kept in -20°C until needed). For a working solution, an aliquot of JC-1 dye was diluted 1:25 in OptiMem media. 5 µl of the working solution was added to each well and incubated in the dark for 10 min at 37°C. Cells were washed three times with 1x phosphate buffered saline (PBS). 100 µl of clear Dulbecco's Modified Eagle Medium (DMEM) without serum was then added in each well and fluorescence was measured using a FlexStation plate reader and analyzed using SoftMax Pro V5 software at 488 nm excitation and 522 nm and 605 nm emissions.

### 3.2.4 Intracellular reactive oxygen species (ROS) production (Amplex red assay)

In general, ROS are highly reactive and have short life time [266], which makes them difficult to measure directly in biological systems. Therefore, the indirect methods have to be used to assess levels of ROS. Amplex Red assay has shown to be a stable and sensitive assay, which is based on the non-fluorescent compound *N*-acetyl-3,7-dihydroxyphenoxazine [269].

Amplex red assay is used in this study to fluorometrically determine the Intracellular $H_2O_2$ production in M17 neuroblastoma cells (Amplex red reagent; Molecular Probes, Eugene, OR). Oxidation of Amplex red coupled by horseradish peroxidase (HRP) causes a

reduction of $H_2O_2$, which produces the red fluorescent oxidation product, resorufin [270].

To perform this assay, a reaction buffer (1X) was initially prepared from a 5X concentrate supplied by the kit, which was comprised of 0.05 M sodium phosphate (pH 7.4). A 10 mM stock solution of Amplex red was prepared by dissolving Amplex red reagent in dimethyl sulfoxide (DMSO). Several stock solutions were prepared according to the assay kit instructions. Horseradish Peroxidase (HRP) stock solution (10 U/ml) was prepared by dissolving HRP powder in 1X reaction buffer.

M17 neuroblastoma cells were seeded into black well/clear bottom 96-well plates at 25,000 cells/well (Corning Costar). Concentrations of each treatment were prepared and 50 µl of each treatment was added to the wells. The reaction was initiated by the addition of 50 µl of a working solution that contained 0.1 mM Amplex red reagent and 0.2 U/ml HRP in 1X reaction buffer. This resulted in a final concentration of 50 µM Amplex red reagent and 0.1 U/ml of HRP along with the various treatments in the reaction mix with a final reaction volume of 100 µl per well. Using a 544 nm excitation and a 590 nm emission, fluorescence was measured kinetically every 30 sec for 30 min using a FlexStation II 384 plate reader (Molecular Devices, Sunnyvale, CA). Results were analyzed by the Softmax Pro V5 software.

### 3.2.5 Statistical analysis

Data were presented as mean and standard error of the mean (SEM). When one-way ANOVA showed significant differences among groups, Tukey's *post hoc* test was used to determine the specific pairs of groups that were statistically different. A *p* -value less than 0.05 was considered statistically significant. Analysis was performed with SPSS 16.0 (SPSS Inc. Chicago, IL, USA).

## 3.3 RESULTS

### 3.3.1 Aβ induces mitochondrial dysfunction in M17 neuroblastoma cells, which is restored by CoQ10, but not DHA

It was shown in this study that Aβ significantly reduced basal OCR when compared with control cells without altering basal ECAR (Fig. 3.1A), indicating mitochondrial dysfunction without stimulating compensatory glycolysis. A reduction of OCR in response to Aβ treatment was restored by CoQ10 alone and in combination with DHA (Fig. 3.1B). However, DHA alone did not directly restore Aβ-mediated alteration in basal OCR (Fig. 3.1B). Similar results were observed for mitochondrial respiration due to ATP turnover (Fig. 3.1C), uncoupled respiration (proton leak) (Fig. 3.1D) and maximal respiratory capacity (Fig. 3.1E).

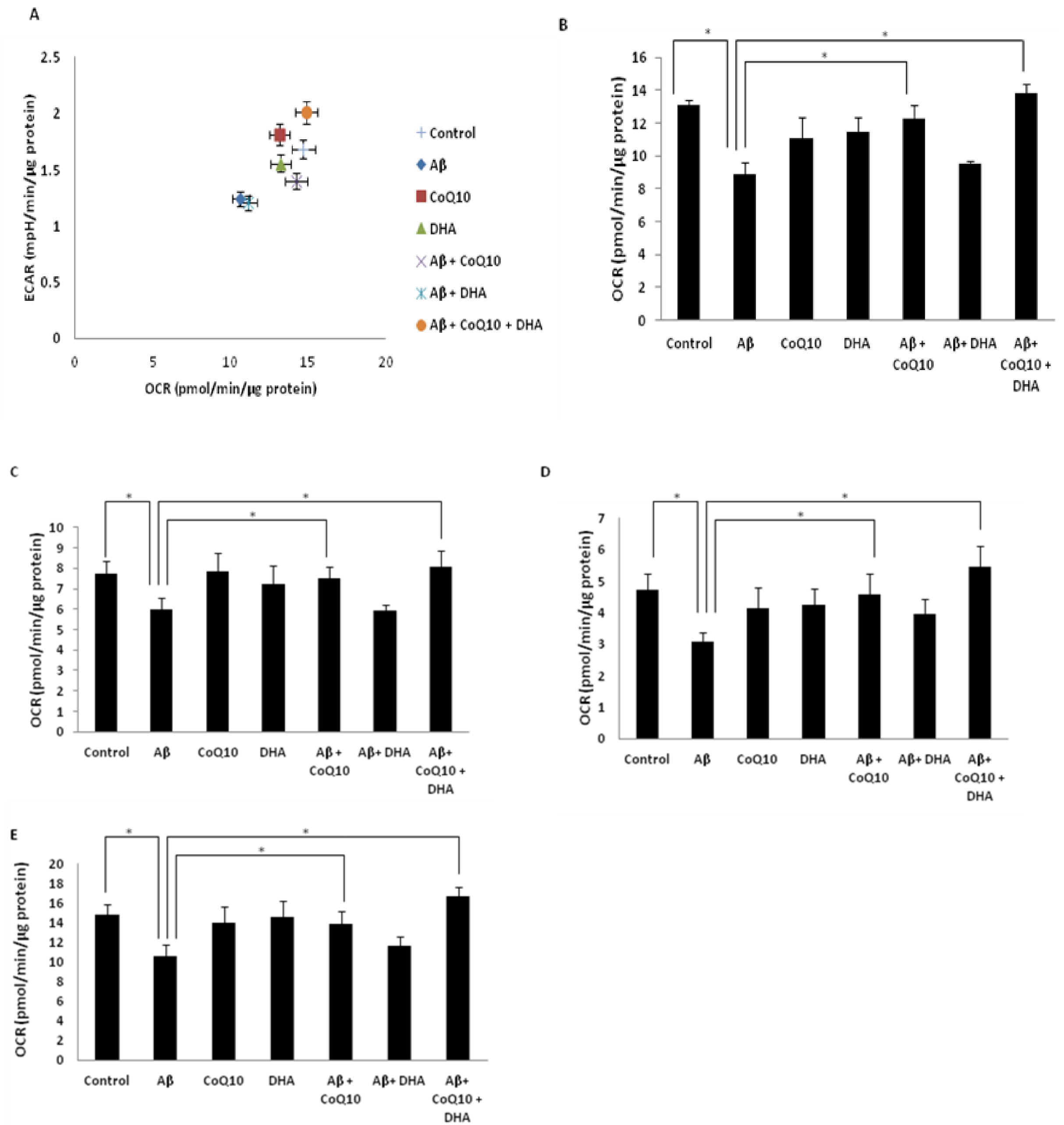



**Figure 3.1: Mitochondrial function and basal bioenergetics following Aβ treatment in M17 neuroblastoma cells.** Representative measurements of the basal OCR/ECAR (A) and OCR (B) in M17 cells relative to baseline rates. ATP turnover rate (C), uncoupled respiration (D) and maximal respiratory capacity (E) were calculated from oxygen consumption rates of M17 neuroblastoma cells treated with beta-amyloid (Aβ) peptide, docosahexaenoic acid (DHA) and Coenzyme Q10 (CoQ10), alone and in combination for 24 h. Values represent the means ± standard error of the mean (SEM) from n=12-15 per treatment group. One-way ANOVA (*p<0.05) versus corresponding Aβ-treated cells.

### 3.3.2 Zinc impairs mitochondrial bioenergetic function in M17 neuroblastoma cells, which is restored by CoQ10 and DHA, alone and in combination

To examine whether DHA and CoQ10 could protect against zinc-induced mitochondrial dysfunction in M17 neuroblastoma cells, basal OCR, as well as the combined OCR and ECAR response together, were measured as described before. This would produce a bioenergetic indicative of both mitochondrial respiration and glycolysis, respectively.

There was a significant zinc-induced decrease of the basal mitochondrial respiration in M17 cells without alteration in glycolytic rate (Fig. 3.2A). A significant reduction of OCR in response to zinc treatment was restored by CoQ10 and DHA, alone and in combination (Fig. 3.2B). The decrease in ATP turnover rate (Fig. 3.2C), and maximal respiratory capacity (Fig. 3.2E), were also restored by DHA and CoQ10, either alone or in combination. A decrease in uncoupled respiration was observed in zinc-treated cells, which was restored with either DHA alone or in combination with CoQ10. CoQ10 alone did not significantly affect uncoupled respiration (Fig. 3.2D).

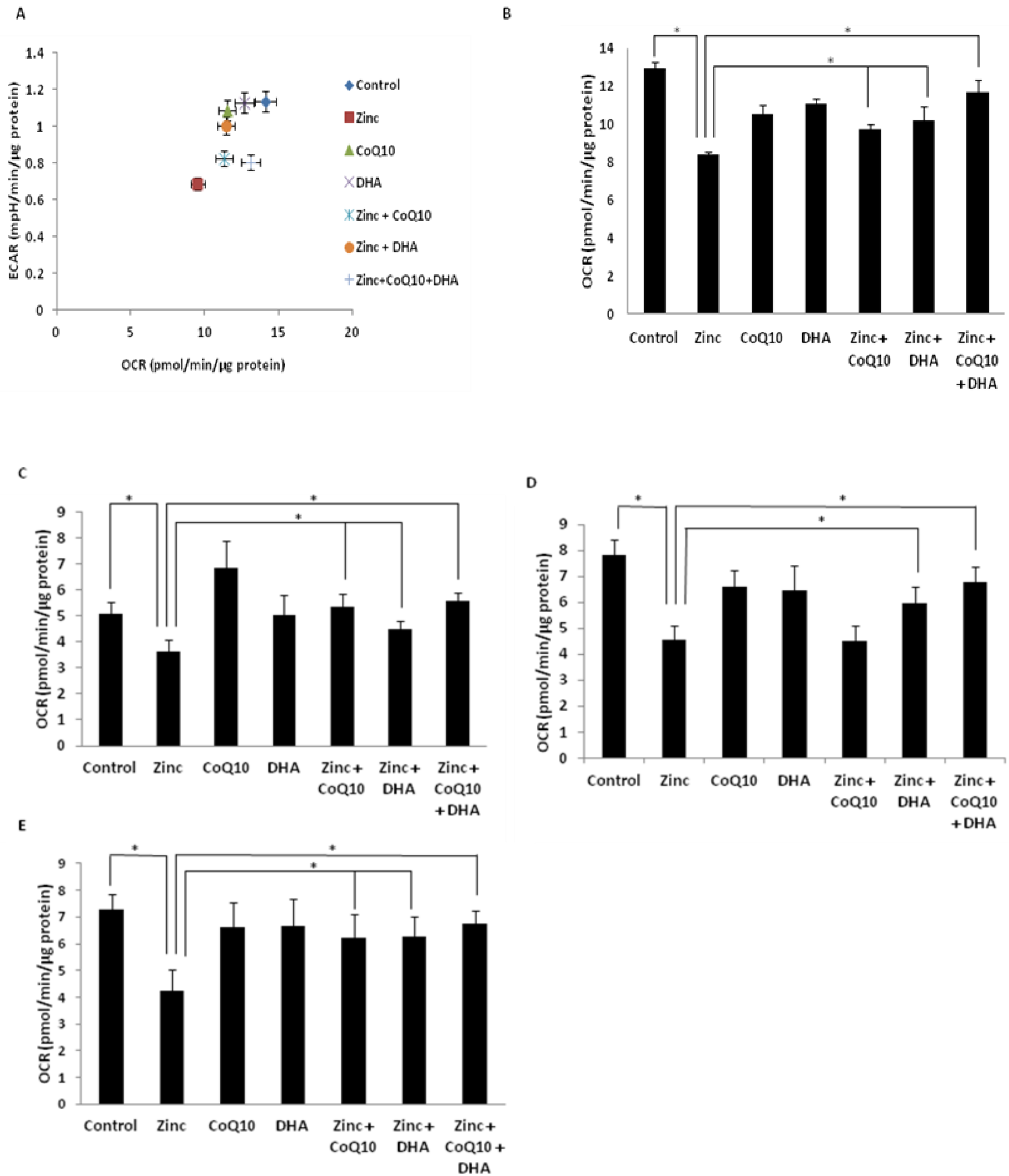



**Figure 3.2: Zinc effect on mitochondrial function and basal bioenergetics in M17 neuroblastoma cells.** Zinc-effect on mitochondrial function and basal bioenergetics in M17 cells. Basal OCR/ECAR (A) and OCR (B), as well as ATP turnover rate (C), uncoupled respiration (D) and maximal respiratory capacity (E) were calculated based on oxygen consumption rates of M17 cells exposed to zinc, docosahexaenoic acid (DHA) and Coenzyme Q10 (CoQ10), alone or in combination for 24 h. Values represent the means ± SEM from n=12-15 per treatment group. One-way ANOVA, (*$p<0.05$) versus corresponding zinc-treated cells.

### 3.3.3 The neuroprotective effect of DHA and CoQ10 against Aβ- and zinc-induced dissipation of the mitochondrial membrane potential $\Delta\Psi_m$ in M17 neuroblastoma cells

This study measured the mitochondrial membrane potential, which can be used as a broad indicator for mitochondrial damage [271]. Furthermore, it has been demonstrated that maintenance of the mitochondrial membrane potential is critical for neuronal cells survival [272], which also plays a crucial role in the induction of apoptosis [273].

Results showed a significant reduction in $\Delta\Psi_m$ following treatment with Aβ, which was restored by CoQ10, alone and in combination with DHA (Fig. 3.3A). DHA alone did not restore the reduction in $\Delta\Psi_m$ caused by Aβ. Zinc reduced $\Delta\Psi_m$, which was restored by the addition of DHA, but not CoQ10 (Fig. 3.3B). There were no additive effects of DHA and CoQ10 co-incubation, when compared with DHA alone, suggesting that CoQ10 was not protective against zinc-mediated alterations in $\Delta\Psi_m$.

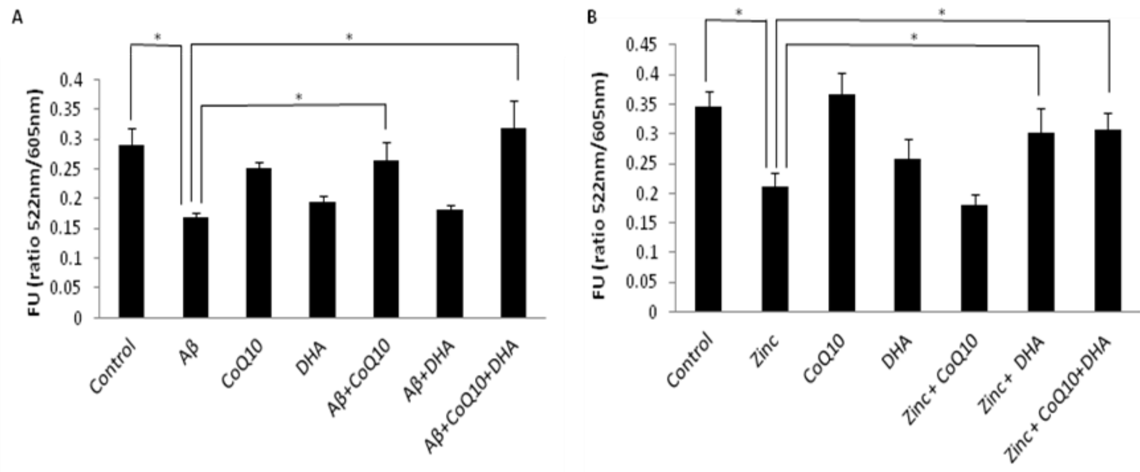



**Figure 3.3: Aβ and zinc effect on the mitochondrial membrane potential $\Delta\Psi_m$ in M17 neuroblastoma cells.** Aβ and zinc effect on the mitochondrial membrane potential $\Delta\Psi_m$ in M17 neuroblastoma cells. Effect of Aβ and zinc on the membrane depolarization of M17 cells mitochondria were measured by fluorescence plate reader using JC-1 assay. Aβ- (A) and zinc- (B) treated cells showed a reduction in $\Delta\Psi_m$ compared to the control. The cells were exposed to Aβ and zinc with and without DHA and CoQ10 for 24 h. Data represent mean and SEM of n=8 per treatment group with asterisks denoting significant differences between Aβ or zinc alone and co-exposure of DHA/CoQ10-treated cells. One-way ANOVA, (*$p<0.05$) versus corresponding Aβ or zinc-treated cells.

### 3.3.4 Zinc-induced mitochondrial dysfunction is associated with increased production of reactive oxygen species (ROS)

Altered $\Delta\Psi_m$ and increase in intracellular levels of $H_2O_2$ in neuronal cells are highly associated with cell apoptosis and the progression of AD [274]. Since both Aβ and zinc have shown to inhibit ATP turn over rate, uncoupled respiration and maximal respiratory capacity, their effect on ROS generation was then investigated in M17 neuroblastoma cells using Amplex red assay.

The effects of DHA and CoQ10 against oxidative stress induced by Aβ and pathophysiological levels of zinc were evaluated here. It was observed that Aβ did not directly affect ROS production when compared to the control cells (Fig. 3.4A), whereas zinc induced a small but statistically significant increased in $H_2O_2$ levels in M17 neuroblastoma cells (Fig. 3.4B). However, both DHA and CoQ10, as potent antioxidants have shown to reduce $H_2O_2$ production (Figs. 3.4A and 3.4B).

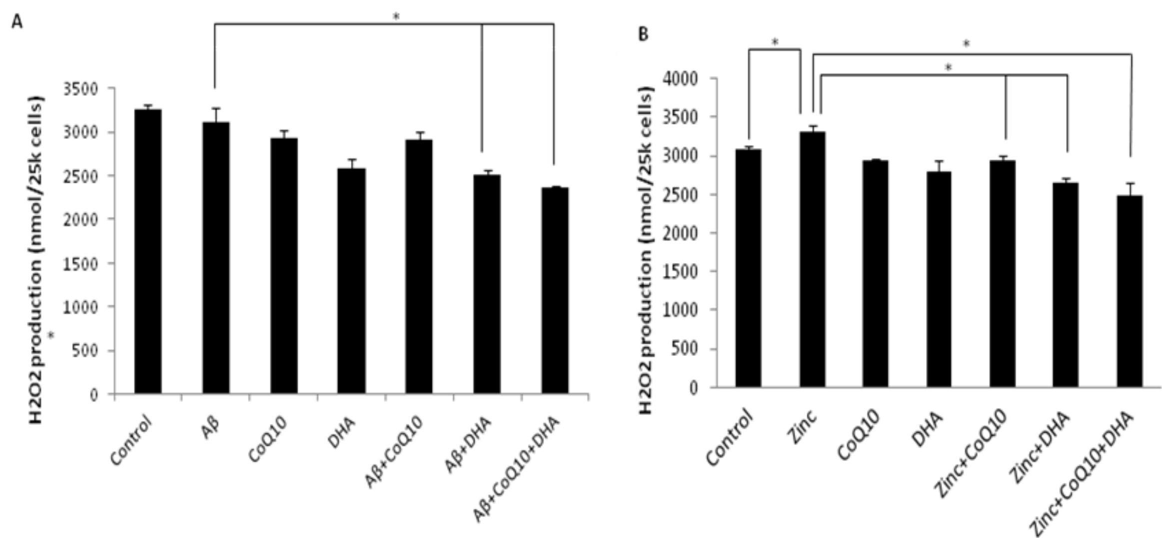



**Figure 3.4: Protective additive effect of DHA and CoQ10 against Aβ and zinc-induced $H_2O_2$ productions.** Neuroprotective effect of DHA and CoQ10 against Aβ- and zinc-induced $H_2O_2$ productions. The Amplex red assay to quantify ROS productions were performed in the absence/presence of Aβ, zinc, DHA and CoQ10, alone and in combination. Aβ (A) did not affect ROS production, whereas zinc (B) significantly increased ROS production in M17 cells. Data are expressed as mean and SEM of n=8 per treatment group with asterisks denoting significant differences between Aβ or zinc alone and co-exposure of DHA/CoQ10-treated cells.

## 3.4 DISCUSSION

### 3.4.1 Aβ and zinc impair basal respiration without alteration in glycolytic rate

In order to identify the mode of action of both CoQ10 and DHA on Aβ- and zinc-mediated bioenergetic alterations, oxygen consumption rate in M17 cells was measured using the Seahorse extracellular flux analyzer. The results showed a significant reduction in basal respiration without alteration in glycolytic rate following exposure to Aβ and zinc. This suggests that glycolysis may be the primary defect in bioenergetics.

### 3.4.2 Aβ and zinc treatments lead to impaired cellular bioenergetics and mitochondrial function

There were significant reductions in basal respiration, mitochondrial ATP turnover rate, uncoupled respiration and maximal respiratory capacity following Aβ and zinc treatments. This defect of the whole mitochondrial respiratory chain may possibly be due to the accumulated dysfunction of one or several mitochondrial chain complexes as a result of mitochondrial Aβ, as well as zinc uptake.

Regardless of the dysfunctional features of end-stage cell death, mitochondrial membrane permeabilization is frequently the decisive event between cell survival and death [260, 275]. Therefore, to further unravel the effect of Aβ and zinc on mitochondrial respiratory dysfunction, potential proxy of $\Delta\Psi_m$ and ROS production were measured in M17 cells. The data showed the depletion of $\Delta\Psi_m$ following Aβ and zinc treatment. This suggests that these Aβ-induced alterations in mitochondrial function

may be due to the ability of Aβ to permeabilize cellular membrane and therefore enter the mitochondria. A recent study by Pagani and Eckert (2011) has reported the deposition of Aβ in the outer and inner mitochondrial membrane [276], which may explain the increase in membrane permeability. In the outer membrane, Aβ may be present at the site where it could influence the interaction between mitochondria and anti-apoptotic marker Bcl-2 [276]. However, in the inner membrane, Aβ may interact with the important components of enzymatic activity, metabolic or antioxidant mechanisms and therefore inhibit their actions [276]. These interactions of Aβ would then affect mitochondrial respiration that would potentially impair cellular metabolism. Aβ has been shown to directly inhibit complex IV, but increase in complex III [277], which lead to bioenergetic impairment [260]. Taken together, the results suggest that following a decrease in $\Delta\Psi_m$ and in uncoupled respiration, which results in a futile proton cycle, Aβ exhibit an initial defect in mitochondrial function. This may be caused by inhibition of complex IV activity that is translated into a mitochondrial respiration deficiency with diminished ATP synthesis, which cannot be compensated by an increased activity of complex III.

Apart from decreased ATP turnover rate, maximal respiratory capacity and uncoupled respiratory, high physiological level of zinc has been reported to inhibit electron transfer [278], which could result in dissipation of $\Delta\Psi_m$ , as observed in this study. This loss of $\Delta\Psi_m$ contributes to the decrease in $\Delta\Psi_m$, which has been reported to be associated with release of cytochrome c and apoptosis-inducing factor (AIF) [241]. Aβ did not have much

effect on ROS production, whereas zinc significantly increased ROS production, which indicates that zinc directly caused oxidative stress in neuronal cells. Zinc has also been shown to instigate $H_2O_2$ production through tricarboxylic acid cycle (TCA) inhibition [279]. It is clear that zinc-mediated neuronal injury requires the mobilization and redistribution of zinc in the brain. Therefore, the strategies that prevent excessive zinc entry into the cells could ameliorate zinc-mediated cell death [280].

### 3.4.3 DHA may be directly protective against zinc-induced mitochondrial dysfunction, but not towards Aβ toxicity

Recently, it has been reported that DHA could protect against zinc-altered mitochondrial dysfunction in M17 cells [85]. Part of this effect could be due to the neuroprotective function of DHA in limiting cellular zinc uptake through decreasing ZnT3 zinc transporter expression levels [45, 148], which in turn inhibits zinc toxicity. The data show the ability of DHA to restore zinc-induced alteration in $\Delta\Psi_m$ and $H_2O_2$ production. It is believed that these effects are due to the neuroprotective effect of DHA against zinc-induced ROS production caused by oxidative stress, which therefore indirectly stabilizes $\Delta\Psi_m$.

It was observed that DHA alone did not significantly restore Aβ-mediated defects in the mitochondrial parameters assessed in this study. However, co-incubations of DHA in the presence of CoQ10 significantly restored Aβ-induced alterations to the same extent as CoQ10 treatment alone. These results suggest that DHA alone was not directly protective against Aβ-induced mitochondrial dysfunction.

### 3.4.4 The effect of CoQ10 against Aβ- and zinc-induced alterations in mitochondrial physiology

It was observed that CoQ10 could also interact with zinc and restore zinc-mediated cellular dysfunction. The finding is supported by a recent study that a decrease in CoQ10 and increase in zinc levels were observed in chronic obstructive pulmonary disease (COPD) patients [281], which probably result from the defense response of organism against zinc-mediated inflammation. There is no direct evidence in relation to zinc and CoQ10 interactions, however the results suggest that CoQ10 may be neuroprotective against zinc-induced mitochondrial dysfunction.

The results have demonstrated that CoQ10 also restores Aβ-induced alteration in membrane potential in neuronal cells, which is possibly caused by the ability of CoQ10 to inhibit the opening of mitochondrial permeability transition pore due to Aβ toxicity and therefore stabilize the membrane [261]. CoQ10 has also been reported to inhibit the aggregation of Aβ and therefore prevent its toxicity [282]. This may indicate a direct anti-amyloidogenic effect of CoQ10 in neuronal cells.

## 3.5 CONCLUSION

This study presents a novel finding on the effects of CoQ10 and DHA against Aβ- and zinc- mediated bioenergetic alterations in M17 human neuroblastoma cells. The results have shown that CoQ10 may have direct effect on Aβ-induced alterations in mitochondrial function, while DHA has no significant effect against Aβ toxicity. On the

other hand, DHA, due to its direct molecular interaction with zinc [45, 148, 152], may protect against zinc-mediated mitochondrial dysfunction. CoQ10 however has also shown to restore zinc-induced mitochondrial alteration to the same extent as DHA, suggesting the neuroprotective effect of CoQ10 against zinc toxicity.

# CHAPTER 4

## THE EFFECT OF ZINC AND DHA ON HISTONES H3 AND H4 EXPRESSIONS LEVELS IN HUMAN NEURONAL CELLS

**PUBLICATION:**

Cenk Suphioglu, **Nadia Sadli**, Damon Coonan, Loveleen Kumar, Damitha De Mel, Jessica Lesheim, Andrew Sinclair, Leigh Ackland (2010) Zinc and DHA have opposing effects on the expression levels of histones H3 and H4 in human neuronal cells. *Br J Nutr.* 103(3):344-51

## Summary


### Aim

Zinc and DHA have putative neuroprotective effects and these two essential nutrients are known to interact biochemically. This chapter aimed to identify novel protein candidates that are differentially expressed in human neuronal cell line M17 following zinc and DHA that would explain the molecular basis of this interaction.

### Methodology

Two-dimensional gel electrophoresis and mass spectrometry were applied to identify major protein expression changes in the protein lysates of human Ml7 neuronal cells that had been grown in the presence and absence of zinc and DHA. Proteomic findings were further investigated using Western immunoblot and real-time PCR analysis. Four protein spots, which had significant differential expression were identified and selected for in-gel trypsin digestion followed by matrix-assisted laser desorption ionization mass spectrometry analysis. The resultant peptide mass fingerprint for each spot allowed their respective identities to be deduced.

### Results

Two human histones variants H3 and H4 were identified as differentially expressed proteins following zinc and DHA. Both H3 and H4 were down-regulated by zinc in the absence of DHA (zinc effect) and up-regulated by DHA (DHA effect) in the presence of

zinc (physiological condition). These proteomic findings were further supported by Western immunoblot and real-time PCR analysis using H3- and H4-specific monoclonal antibodies and oligonucleotide primers, respectively.

**Conclusion**

The results suggest that dietary zinc and DHA cause a global effect on gene expression, which is mediated by histones. Such novel information provides possible clues to the molecular basis of neuroprotection by zinc and DHA that may contribute to the future treatment, prevention and management of neurodegenerative diseases.

## 4.1 INTRODUCTION

It was shown that the alteration in both DHA and zinc homeostasis are the key features of neurodegenerative disorders [148, 283, 284]. Previous study has demonstrated the link between a reduction of DHA in the diet of rats and over-expression of ZnT3, a transmembrane protein involved in transport of zinc into synaptic vesicles [148], suggesting a direct interaction between DHA and zinc. Although zinc is an important nutrient in high concentrations zinc is toxic and it induces formation of amyloid plaques and brain cell death, which are significant features of aging-related neurodegenerative processes like AD [285]. Since low levels of DHA and high levels of synaptic zinc are commonly seen in AD brains, these results suggest a likely synergy between zinc and DHA in AD pathophysiology.

Although previous data has shown a relationship between DHA and zinc homeostasis [45], the basis of the molecular interaction in genomic level has not been elucidated. Therefore, this chapter aimed to investigate the novel candidates that are differentially expressed in response to zinc and DHA in human neuronal cell line M17. Here, the results show for the first time that zinc and DHA affect the expression levels of histones H3 and H4 in the human neuronal cell line M17.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Cell culture

Human neuroblastoma M17 cells were maintained in Opti-MEM media as previously described. The cultures were growth at $37^oC$ in humidified air containing 5% $CO_2$. Cells were treated with zinc (5µM), DHA (10 µg/ml in the presence of 0.05 µM/ml antioxidant vitamin E) or zinc and DHA in combination for 48 h. The cell treatments were prepared as described previously.

### 4.2.2 Preparing cell lysates

M17 cells were seeded at a density of 1 x $10^6$ cells/$75cm^2$ flasks and grown in media supplemented with and without zinc and DHA. After 48 h incubation, the cells were harvested, centrifuged at 1,000 x g for 5 min and pellets were resuspended in PBS. Each sample was then divided into aliquots, centrifuged at 14,000 x g for 5 min and cell pellets were stored at -80°C until needed for analysis.

### 4.2.3 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cell pellets were resuspended in ZOOM protein solubilizer 1 lysis buffer (Invitrogen, CA, USA), disrupted by passing through a 21-gauge needle and sonicated by using a Microsone Ultrasonic cell disrupter (Misonix Incorporated, NY, USA), following the manufacturer's instructions. Samples were then centrifuged at 14,000 x g for 20 min at 4°C and stored in small aliquots at -80°C until needed for analysis.

Quantification of the protein concentration in cell lysates were performed using Quant-IT$^{TM}$ Protein Assay Kit (Invitrogen) and Qubit™ Fluorometer, following the manufacturer's instructions.

Isoelectric focusing (first dimension) was performed using pH 3-12 IPG strips (Invitrogen). Before the second dimension SDS-PAGE separation, IPG strips were reduced and alkylated with a solution containing 0.1 M Tris-HCL (pH 6.8), 20% glycerol, 10% SDS ,10% reducing agent β-Mercaptoethanol and a trace of Bromophenol Blue. Additionaly, the reduction step contained 1% DTT and the alkylation step 125 mM iodoacetamide. Proteins were separated according to their molecular weight (second dimension) on 4-20% Tris-Glycine ZOOM gels (Invitrogen) following the manufacturer's instructions. Following 2D electrophoresis, gels were fixed in 50% methanol and 7% acetic acid for 30 min, which then stained with SYPRO Ruby protein gel stain (Invitrogen) and incubated overnight at room temperature. The following day, gels were washed with 10% methanol and 7% acetic acid for 30 min. Gel images were captured using Fujifilm LAS-300 UV transilluminator (Fujifilm, VIC, Australia) to visualize protein spots.

### 4.2.4 Protein identification (Mass spectrometry analysis)

Proteins of interest were excised from each gel by automated robotic cutter and placed into sequential wells in a 96 well format plate and subjected to two rounds of destaining with 25 mM ammonium bicarbonate and dehydration with 50% acetonitrile. The gel plugs were then fully dehydrated at 37$^{o}$C prior to digestion with porcine trypsin for 16 h

at 37$^{o}$C. Digestion products were released from the gel plugs by sonication and acidified using 1% Trifluoroacetic acid (TFA). The peptides were then purified using a C18 zip tip according to the manufacturer's instructions (Varian, CA, USA) and eluted on to the ABI mass spectrometry target in α-cyano-4-hydroxycinnamic acid (HCCA) matrix. The resulting peptides were then analysed by Matrix-Assisted Laser Desorption Ionisation - Time of Flight/Time of Flight (MALDI-TOF/TOF) mass spectrometry using the ABI 4700 Proteomics Analyser (Applied Biosystems Inc (ABI), CA, USA). Samples were initially analysed in single MS mode to determine the peptide mass fingerprint of the sample. The ten most intense peptide peaks were then isolated, fragmented and analysed in tandem MS/MS mode to determine *de novo* amino acid sequence of the peptides selected. A combined protein score for the peptide mass fingerprint and ten most intense peptides was obtained using the Mascot bioinformatic search engine (Department of Primary Industries, VIC, Australia) to search the NCBInr, *Homo sapiens* database, mass tolerance 100 ppm and oxidation as a variable modification. Proteins were identified via their peptide mass fingerprint and deduced amino acid sequence determined by single MS and tandem MS/MS, respectively. Protein identity was only reported for samples that gave a significant ($P$<0.05) molecular weight search (MOWSE) score.

### 4.2.5 Western Immunoblot analysis

Cell lysates (described above) were resolved on 15% SDS-PAGE gels following our established techniques [286]. A molecular weight marker (Bio-Rad Laboratories,

Hercules, CA, USA) was employed for the confirming of protein transfer and the molecular weight orientation. For the electro-blotting of proteins onto a nitrocellulose membrane (Pall Life Science, FL, USA), a Tris-glycine "wet" electrophoretic transfer system containing 20% methanol was used (25 mM Tris, 192 mM glycine, and 20% methanol). The use of methanol was shown to increase the binding capacity of nitrocellulose for proteins [287]. After transfer of the proteins from the gel to the membrane, the remaining protein-binding sites on the membrane were blocked for 1 h at room temperature with 1% (w/v) non-fat skim milk in Tris buffered saline (TBS; 50 mM Tris.HCl, pH 7.4 and 150 mM NaCl) to avoid non-specific binding of the antibodies or detection reagents in subsequent steps. Immunoblots were then incubated overnight at 4°C with 1/1,000 dilution of monoclonal mouse anti-human H3 and H4 antibodies (Abchem, NSW, Australia). After intensive washing procedure with TBS, 1 h incubation was carried out with horseradish-peroxidase-conjugated 1:4,000 dilution secondary antibody for 2 h at room temperature followed by another wash with TBS. Antibody binding proteins were visualized using Immobilan Western Chemiluminesence HRP substrate (Millipore Corporation, CA, USA) according to manufacturer's instructions.

Developed membranes were stripped with Re-Blot Plus stripping solution (Chemicon, Temecula, CA) and were re-probed for β-actin using mouse anti-β-actin (Sigma Aldrich) primary antibody (1/5,000 dilution) followed by anti-mouse HRP antibody (Chemicon International) and developed as described above. Membranes were photographed using

Fujifilm LAS-300 (Fujifilm, Tokyo, Japan), and the subsequent densitometry analysis of bands was done with the Fujifilm Multi Gauge V3.0 program (Fujifilm, Tokyo, Japan).

### 4.2.6 RNA isolation and Real-time PCR analysis

M17 Cells treated with and without zinc and DHA (as described above) were harvested by centrifuging cell suspension at 1,000 x g for 5 min at 4°C. Supernatant was decanted and all remaining media was removed. Total RNA was isolated from cell pellers using RNeasy Mini-kit (Qiagen, Clifton Hill, Victoria, Australia). Cells were disrupted by adding 350 µl buffer RLT containing 1% β-mercaptoethanol and mixed thoroughly by vortexing vigorously. 350 µl 70% ethanol was added, mixed thoroughly by pipetting. The sample was applied to an RNeasy mini column placed in 2 ml collection tube. The tube was closed gently and centrifuged for 15 sec at 12,000 rpm. The flow-through was discarded. 700 µl buffer RW1 was pipetted into RNeasy mini column and again centrifuged for 15 sec at 8,000 x g to wash. The flow-through was discarded. 500 µl buffer RPE was pipetted onto the RNeasy column. The tube was closed gently and centrifuged for 15 sec at 8,000 x g. The flow-through was discarded. Another 500 µl buffer RPE was added to the RNeasy column. The tube was closed gently and centrifuged for 2 min at 8,000 x g. The RNeasy column was placed into a new 2 ml collection tube and centrifuged at 8,000 x g for 1 min. To elute, the RNeasy column was transferred into a new 1.5 ml collection tube. 50 µl RNase –free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was closed gently and centrifuged for 1 min at 8,000 x g.

Because this isolated RNA would be employed in real time -PCR, therefore DNA contamination in RNA template was not permitted. Even though RNeasy silica-membrane technology can eliminate most of DNA but DNase treatment still applied to ensure completely removal of DNA.

DNase treatment was undertaken according to instructions included with the Ambion (Austin, Texas) DNA-free™ kit. Briefly, a master mix of 0.1 volume of 10 x DNase I buffer and 1 µL rDNase I per sample was made up and was added to each sample. The samples were mixed and incubated for 20-30 min at 37°C. DNase inactivation reagent (0.1 volume) was added to each sample which was then thoroughly mixed. Samples were incubated for 2 min at room temperature and centrifuged at 10,000 x g for approximately 1.5 min. Aqueous RNA was transferred into a new tube and stored at -70°C until required. The RNA concentration and purity was spectrophotometrically measured on Nanodrop ND-1000 (NanoDrop Technologies, DE, USA). 1 µg of total RNA was reverse transcribed by using High-Capacity cDNA Reverse-Transcription kit (Applied BIosystem, Foster City, CA, USA). mRNA expression of Histone H3 and H4 were quantified, in triplicate by on 7500-Real Time PCR System (Applied BIosystem, Foster City, CA, USA) using 1 x SYBR green PCR master mix (Applied Biosystem, Warrington, UK) and specific primers to human histone H3 and H4. Primers were designed using Primer Express (Applied Biosystems, Foster City, CA). Target sequences used were sourced from the Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov). Potential cross reactivity of primers with other mRNA species was assessed by using the Basic Local

Alignment Search Tool (BLAST) and the Entrez Nucleotides Database. Primers were purchased from Geneworks and were designed as follows:

| GENE | FORWARD (5'→ 3') | REVERSE (5'→ 3') |
|---|---|---|
| Histone H3 | AATCGACCGGTGGTAAAGCA | TTGCGAGCGGCTTTTGTA |
| Histone H4 | TTATGAGGAAACTCGCGGAGTG | TGGCTGTGACTGTCTTGCGTT |

The housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. Expression levels for each sample were calculated using ΔCt method where the data were normalized by determining the difference in Ct values between H3 or H4 genes and GAPDH. The fold change was calculated as $2^{-\Delta\Delta Ct}$ where the ΔΔCt is the difference between the treated ΔCt and control ΔCt. Significance ($P<0.05$) was tested by the Student's *t*-test.

#### 4.2.7 Statistical analysis

The level of histone H3 and H4 protein and gene expressions in M17 cells treated with zinc with and without DHA were compared by Student's *t*-test using Statistical Package for the Social Sciences software (SPSS 16.0). *$P < 0.05$ was considered statistically significant.

## 4.3 RESULTS

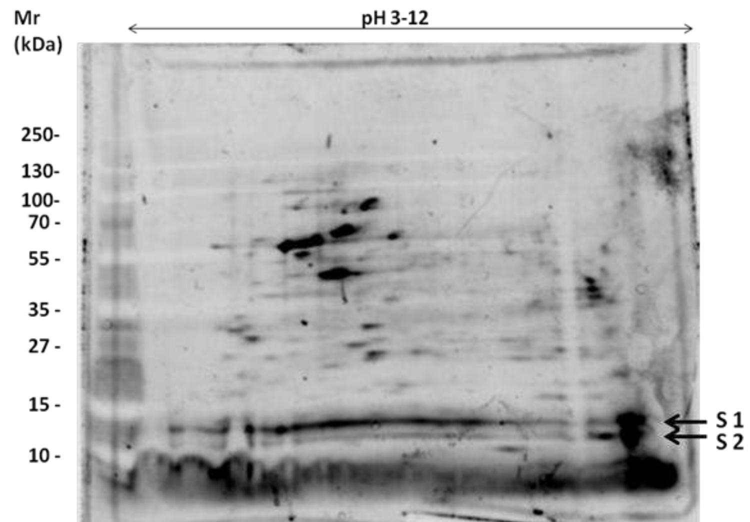
### 4.3.1 Proteomic analysis of human neuronal cells with and without zinc and DHA

The change of M17 neuroblastoma cells proteome treated with and without zinc and DHA was assessed using proteomic approaches, including 2D-electrophoresis and Western immunoblotting to identify novel proteins that are differentially expressed in response to zinc and DHA.

The effect of zinc alone on M17 cells proteins (Fig. 4.1) was first investigated. Many protein spots were significantly increased following treatment of zinc (Fig. 4.1B). There were two basic spots at around 11 and 15 kDa which showed a significant reduction in protein amounts (S1 and S2 in Fig. 4.1A) and these were chosen for mass spectrometry analysis.

The effect of DHA on M17 cells in the presence of zinc was also investigated by 2D-analysis (Fig. 4.2). A significant reduction in the number and level of protein spots with DHA treatment were observed, however the protein levels of two spots were significantly increased with the DHA treatment (S3 and S4 in Fig. 4.2B). These two spots occupied similar isoelectric point (*p*I) and molecular mass as those seen in zinc deficient cells (S1 and S2 in Fig. 4.1A). These spots were therefore selected for mass spectrometry analysis.

**(A). Control (-DHA -zinc)**




**(B). Zinc treatment (-DHA +zinc)**

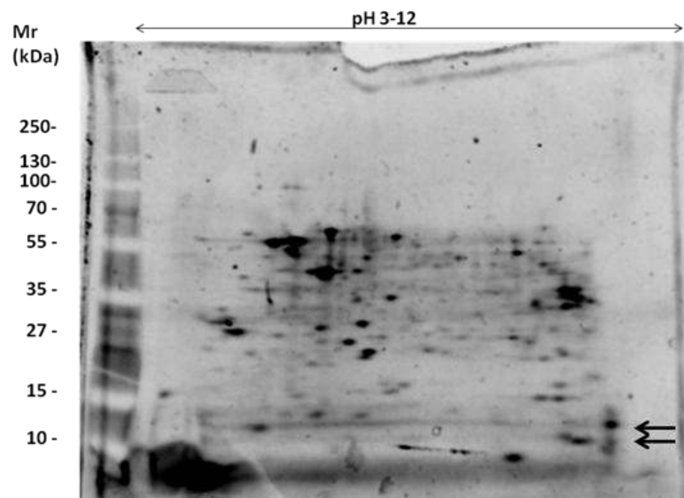
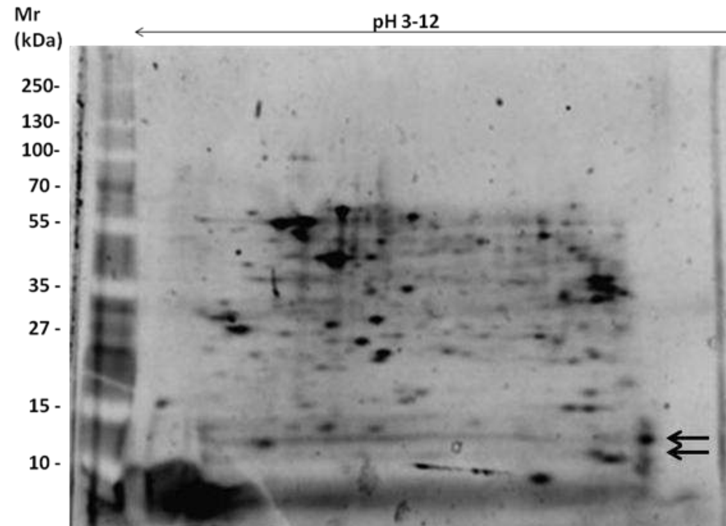



**Figure 4.1: Effect of zinc on protein expression in M17 neuroblastoma cells**.
2D image of the proteome expression of M17 cells grown in the absence of DHA without zinc (control) (A) and with zinc (final concentration of 5 μM) (B), highlighting proteins selected for identification. Proteins were separated first according to their pI using pH 3-12 IPG strips. In the second dimension, proteins were separated according to their molecular weight using 4-20% pre-cast SDS-PAGE gel. Five microlitres of molecular mass markers ($M_r$) were run concurrently and Gels were stained with SYPRO Ruby staining. Arrows indicate protein spots of significant difference which were subjected to mass spectrometer analysis (S1, S2).

**(A). Zinc treatment (+ zinc -DHA)**




**(B). Zinc and DHA treatment (+ zinc +DHA)**

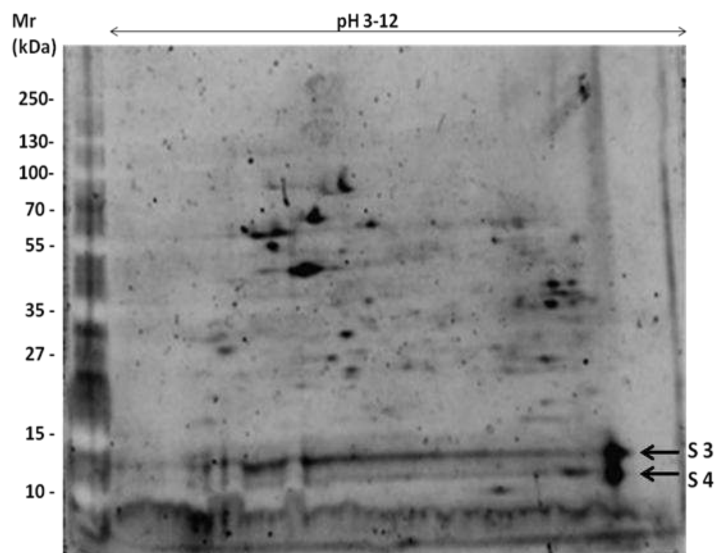



**Figure 4.2: Effect of DHA on protein expression in M17 neuroblastoma cells**.
Proteome expression of M17 cells grown in the presence of zinc (final concentration of 5 µM) without DHA (A) or with 10 µg/ml DHA (B). Five microlitres of molecular mass markers ($M_r$) were run concurrently and gels stained with SYPRO Ruby staining. Arrows indicate protein spots of significant difference that were subjected to mass spectrometer analysis (S3, S4).

The protein spots S1, S2, S3 and S4 (Figs. 4.1 and 4.2, respectively) were precisely excised by an automated robotic cutter, in-gel digested by trypsin and the peptide fingerprints subjected to mass spectrometric analysis. Upon *Homo sapiens* NCBI database searches, the identities of the spots were revealed with a score of 100% match (Table 4.1). Both S1 and S3 spots were identified as human histone H3 (*p*I 11.1, 15.3 kDa) while spots S2 and S4 were identified as human histone H4 (*p*I 11.4, 11.4 kDa) matching perfectly with the *p*I and molecular mass observed in the 2D gels (Figs. 4.1 and 4.2, Table 4.1).

| 2D PROTEIN SPOT | PROTEIN | SPECIES | ACCESION NO. | PROTEIN $M_r$ | PROTEIN *p*I | PROTEIN SCORE (%) |
|---|---|---|---|---|---|---|
| S1 | H3histone, family 3A | *Homo sapiens* | gi\|51859376 | 15346.5 | 11.14 | 100 |
| S2 | HIST2H4 protein | *Homo sapiens* | gi\|124504316 | 11370.4 | 11.36 | 100 |
| S3 | H3histone, family 3A | *Homo sapiens* | gi\|51859376 | 15346.5 | 11.14 | 100 |
| S4 | HIST2H4 protein | *Homo sapiens* | gi\|124504316 | 11370.4 | 11.36 | 100 |

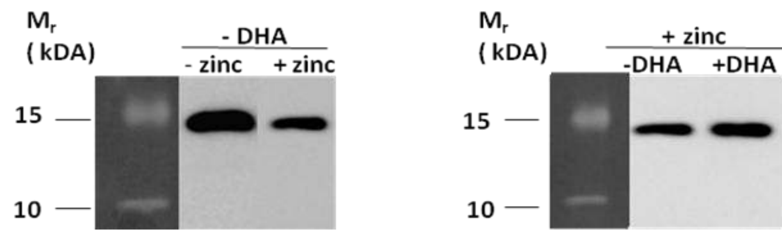
**Table 4.1: Protein identification by mass spectrometry.** Protein spots S1-S4 were excised from each gel by automated robotic cutter and subjected to trypsin digest followed by mass spectrometry analysis and submission of peptide fingerprints to *Homo sapiens* National Center for Biotechnology Information (NCBI) database searches. Proteins were identified via their peptide mass fingerprint and deduced amino acid sequence determined by single MS and tandem MS/MS, respectively. *p*I = isoelectric point; $M_r$ = molecular mass.

To validate the proteomic findings, human histones H3 and H4 protein and mRNA levels were investigated using Western blotting and real-time PCR analysis, respectively.

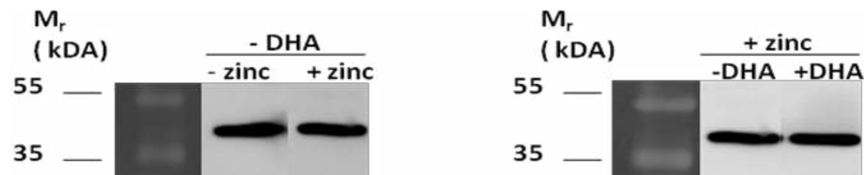
### 4.3.2 Western blot analysis of human histone H3 and H4

Using monoclonal antibodies specific to human histone H3 and H4, the same M17 total protein cell lysates as used in 2D analysis were subjected to Western blot analysis. In the absence of DHA, both histones H3 and H4 were down-regulated upon zinc treatment (zinc effect) (Figs. 4.3A and 4.4A), correlating with the 2D results (Fig. 4.1). In the presence of zinc and DHA (DHA effect), both histone H3 and H4 were up-regulated (Figs. 4.3A and 4.4A), again correlating with the 2D results (Fig. 4.2). To ensure that the observed changes in protein levels were not attributed to unequal protein loading of the wells of the gels, the same blots were probed with β-actin house-keeping protein, and this showed that there was equal protein banding intensities (Figs. 4.3B and 4.4B). Furthermore, densitometric analysis of the protein bands in relation to β-actin indicated significant difference in expression levels (Figs. 4.3C and 4.4C).

(A).




(B).




(C).

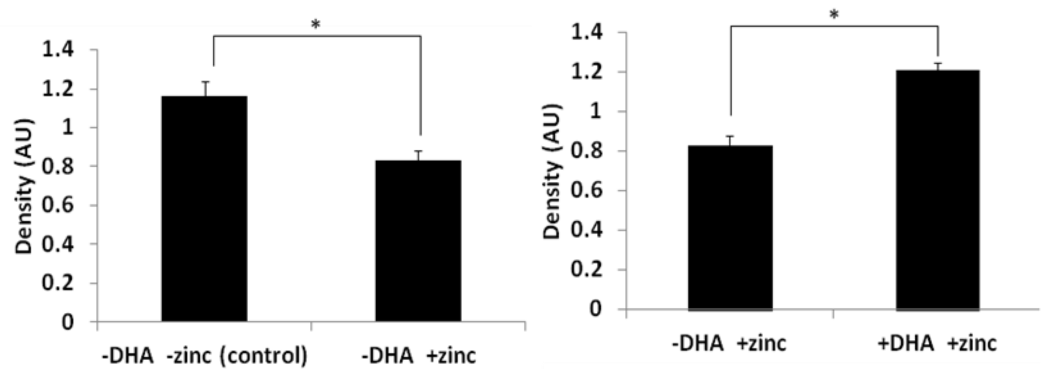
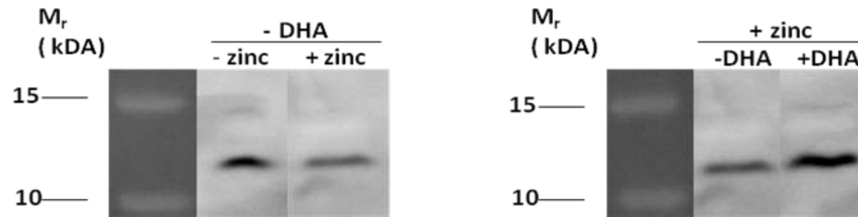
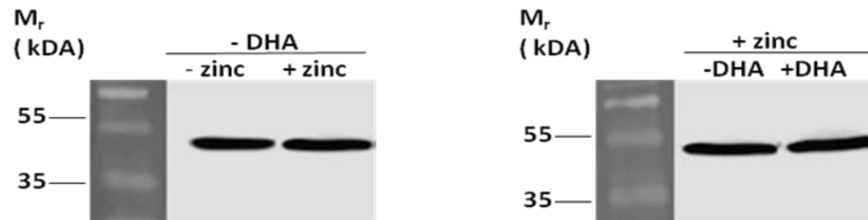



**Figure 4.3: Histone H3 protein expression levels following zinc and DHA treatments in M17 neuroblastoma cells.** Western blot analysis of H3 (A) and β-actin (B) expression in M17 cells grown in media supplemented with (+) or without (-) zinc (final concentration of 5 μM) and with (+) or without (-) 10 μg/ml DHA. (C) Densitometric analysis in arbitrary units (AU) are shown as means, with standard error represented as vertical bars. n=3, **P* < 0.05. Molecular mass protein markers ($M_r$) are indicated on the left of each gel.

**(A).**




**(B).**




**(C).**

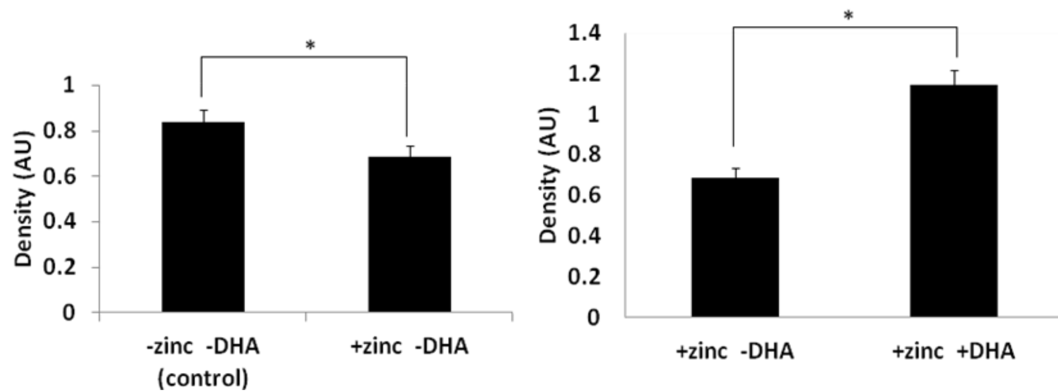
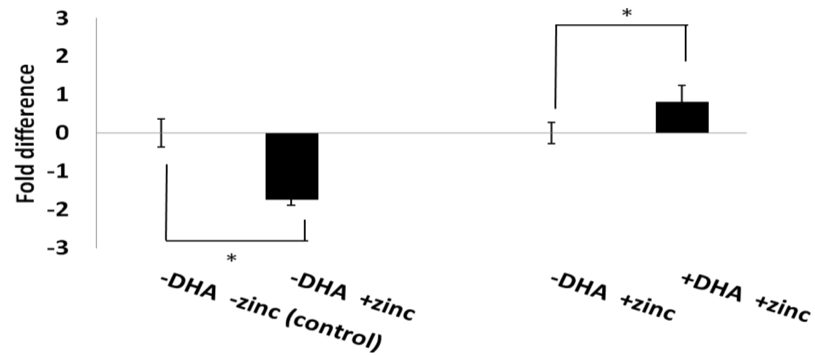



**Figure 4.4: Histone H4 protein expression levels following zinc and DHA treatments in M17 neuroblastoma cells.** Western blot analysis of H4 (A) and β-actin (B) expression in M17 cells grown in media supplemented with (+) or without (-) zinc (final concentration of 5 μM) and with (+) or without (-) 10 μg/ml DHA. (C) Densitometric analysis in arbitrary units (AU) are shown as means, with standard error represented by vertical bars. n=3, *$P$ < 0.05. Molecular mass protein markers ($M_r$) are indicated on the left of each gel.

### 4.3.3 Real-time PCR analysis of human histones H3 and H4

In order to investigate if changes in protein levels of histone H3 and H4 correlated with mRNA levels in M17 cells treated with and without zinc and DHA, real-time PCR analysis was performed. In the absence of DHA, both histones H3 and H4 showed significant down-regulation of mRNA levels in response to zinc treatment (-1.8 and -1.5 fold difference between treatment and control, respectively) (Fig. 4.5). In the presence of zinc, both histone H3 and H4 demonstrated significant up-regulation of mRNA levels in response to DHA (0.8 and 1.2 fold difference between treatment and control, respectively) (Fig. 4.5). Taken together, the changes observed in mRNA levels of histone H3 and H4, with zinc and with and without DHA (Fig. 4.5), correlated well with the changes observed in protein levels (Figs. 4.1, 4.2, 4.3 and 4.4).

**(A).**




**(B).**

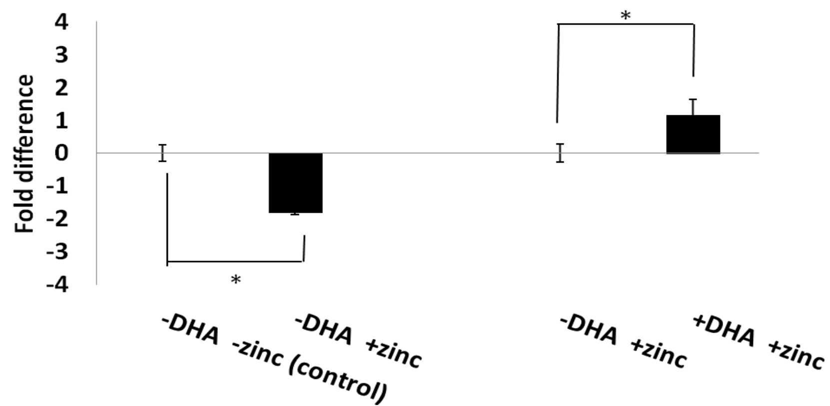



**Figure 4.5: Relative mRNA expression levels of histone H3 and H4 in M17 neuroblastoma cells treated with and without zinc and DHA.** Real-time PCR analysis of H3 (A) and H4 (B) expression in M17 cells treated with and without zinc and DHA. Negative values (less than zero) refer to down-regulation/reduction and positive values (more than zero) refer to up-regulation/increase in fold difference of histone H3 and H4 mRNA between the treatments. Fold difference is the difference in mRNA levels between – and + zinc (zinc effect) and – and + DHA (DHA effect). Data are shown as means $\pm$ SE, n=3. All statistical analysis was tested against a probability value (*P*) of $< 0.05$, compared to control (-DHA/-Zinc or -DHA/+Zinc).

### 4.3.4 Sequence analysis

Having demonstrated the effects of zinc and DHA on H3 and H4 protein and mRNA levels in M17 human neuronal cells, the sequences of these histones were then analyzed to reveal molecular details that would help to understand the connection between the histones H3/H4, zinc and DHA.

Comparison of nucleic and amino acid sequences between human H3 and H4 show minimal sequence identities. H3 and H4 have only 44.8% nucleic acid identity and only 20.6% amino acid identity (Table 4.2). It is interesting to note that there is 100% amino acid identity between human and mouse H3 and H4, such that similar effects of zinc and DHA on histones could be predicted in the mouse.

| **AA**<br><br>**NA** | **H3** | **H4** |
|---|---|---|
| **H3** | 100% | 20.6% (*34.8%)* |
| **H4** | 44.8% | 100% |

**Table 4.2: Nucleic (NA) and amino (AA) acid identity and similarity (*italic values in brackets*) between human histones H3 and H4.** Nucleic and amino acid sequences were analysed by EMBOSS pairwise alignment algorithms on the EMBL-EBI database (http://www.ebi.ac.uk/Tools/emboss/align/).

Since zinc had an effect on the expression levels of histone H3 and H4, 4,000 bp upstream from the start codon of these histones were searched for transcription binding motifs. Indeed, metal responsive elements (MRE) were identified in both H3 and H4 (Table 4.3). Both histones H3 and H4 are present on two different chromosome

clusters (eg. Clusters 1 and 2). For histone H3, one MRE is present in cluster 1 (located 2610 base pairs upstream from the start codon) while two MREs are present in cluster 2 (located 743 and 919 base pairs upstream from the start codon) (Table 4.3). For histone H4, two MREs are present in cluster 1 (located 1405 and 1995 base pairs upstream from the start codon) while no MRE was detected in cluster 2 (Table 4.3).

| HISTONE | TRANSCRIPTION BINDING MOTIF MRE TGCRCNC | CHROMOSOME LOCATION | NA POSITION FROM START CODON |
|---|---|---|---|
| H3 | TGCGCGC | 6p21.3 Cluster 1 | -2610 bp |
| | TGCGCGC, TGCGCGC | 1q21 Cluster 2 | -743 bp, -919 bp |
| H4 | TGCACCC, TGCACAC | 6p21.3 Cluster 1 | -1405 bp, -1995 bp |
| | X | 1q21 Cluster 2 | X |

**Table 4.3: Metal response elements (MRE), as transcription binding motifs, in histone H3 and H4.** Metal response elements (MRE), as transcription binding motifs, in H3 and H4 with their respective chromosome locations and nucleic acid (NA) position upstream from the start codon in base pairs (bp) identified from NCBI database searches (http://www.ncbi.nlm.nih.gov/sites/entrez). X – not present; R – bases A or G; N – bases G, A, T or C.

## 4.4 DISCUSSION

### 4.4.1 Zinc and DHA cause global effect on gene expression mediated by histones

Given what is known in regards to the protective effect of DHA [144, 288] and the deleterious effects of its deficiency on neuronal health [283, 289] basic questions remain about the underlying mechanisms. Previous studies have indicated a possible molecular interaction between zinc and DHA [45, 148]. Although an essential nutrient

[84, 290], an elevation of $[Zn^{2+}]_i$ may become neurotoxic [84], thus contributing to the formation of amyloid plaques and cell apoptosis which are significant features of aging-related neurodegenerative processes such as AD [285, 291].

This study was conducted to answer questions about possible mechanism behind molecular interaction between zinc and DHA, which is based on the well established data that both zinc and DHA play an important role in neuroprotection, perhaps even synergistically, by modulating gene and protein expression. In this study, histones, particularly H3 and H4, were discovered as proteins that were differentially expressed as a result of zinc and DHA supplementation, which provides evidence to support this hypothesis.

In the study reported here, both H3 and H4 were significantly down-regulated by zinc in the absence of DHA (zinc effect) and significantly up-regulated in M17 human neuronal cells following DHA treatment in the presence of physiological zinc levels (DHA effect). This is the first report showing that zinc and DHA regulate the expression levels of histones H3 and H4 in the M17 human neuronal cell line.

### 4.4.2 Possible mechanisms on the Effect of zinc and DHA on H3 and H4 expression

Both H3 and H4 possess multiple metal response elements upstream of their start codons, which suggest that the transcription of genes is under the control of zinc, possibly through metal binding transcription factors. Thus, the results indicate that zinc may inhibit/repress the transcription of histone H3 and H4 in M17 human neuronal

cells, resulting in lower histone protein levels, as observed in this study. Indeed, there are emerging studies that show a role for zinc-mediated transcriptional repression, rather than activation, through MREs [292]. It is has been reported that inhibition of DNA synthesis terminates histone protein synthesis indicating that histone synthesis and DNA synthesis are very tightly linked [293]. Although not investigated here, it is possible that zinc may also affect DNA synthesis and thereby result in the termination of H3 and H4 synthesis in M17 cells. Conversely, it was observed that DHA up-regulates expression levels of both H3 and H4 and abolishes the effect of zinc suggesting that there is an interaction between zinc and DHA in these neuronal cells. The mechanism for this interaction is not clear, however the end result is that zinc and DHA together may increase DNA synthesis.

These results are supported by previous studies, which found that zinc affects histone expression in the mouse thymus [294]. It has been demonstrated that the expression of a number of transcription/translation related factors, including H3 histone family 3A protein were influenced by zinc, showing that dietary zinc can also alter gene expression levels. In other study, rats fed a DHA-enriched diet for two months displayed many alterations in gene expression, among which H3 histone, family 3B (H3f3b) was identified [295]. The results obtained in this chapter are supported by other data in the literature, suggesting that zinc and DHA alter histone subunit expression, which in turn may alter the expression of many other genes. On a different note, effects of both zinc and DHA on histone expression may result from effects on the cell cycle. However, in

M17 human neuronal cell line, cells will be at different stages of the cell cycle and cell cycle effects cannot be ascertained.

Figure 4.6 summarizes the key findings from this chapter based on the observation that Zinc and DHA cause global effect on gene expression mediated by histones. Zinc inhibits transcription of histone H3 and H4 in M17 cells and therefore reduces histone protein and mRNA levels. Zinc may also affect DNA synthesis, which result in termination of H3 and H4 synthesis hence gene regulation. This leads to cellular apoptosis and neurodegenerative diseases (ND). DHA on the other hand, may induce DNA synthesis, through an increase in the level of histones H3 and H4. DHA may contribute to minimizing the onset of neurodegenerative disease through maintaining the integrity of DNA and histones H3 and H4 synthesis.

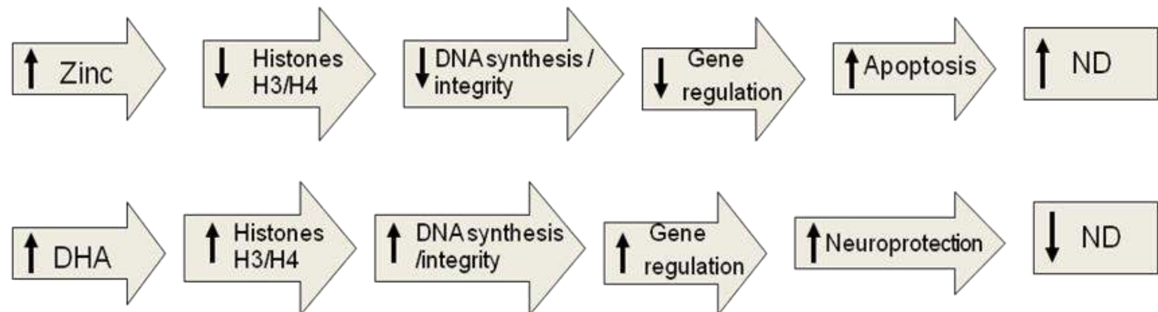



**Figure 4.6: Diagrammatic representation of key finding and potential outcomes.**
Zinc reduces Histone H3 and H4 expression levels, which then affect in reduction in gene regulation and therefore increase in apoptosis and neurodegenerative disease. On the other hand, DHA in the presence of zinc increases Histone H3 and H4 expression levels which also increase gene regulation and neuroprotection, therefore reduce the onset of neurodegenerative disease. ND = Neurodegenerative Diseases.

## 4.5 CONCLUSION

This chapter has shown for the first time that zinc and DHA alter expression of histone H3 and H4 in the M17 human neuronal cell line. This suggests that expression of H3 and H4 may be negatively controlled by zinc via multiple MRE [296]. Conversely, DHA may play a role in the up-regulation of H3 and H4 in nucleosome formation and gene expression. Under normal physiological zinc conditions, DHA may facilitate DNA synthesis resulting in increases in histone protein levels, as observed in this study. Thus, DHA may contribute positively to minimizing the onset of neurodegenerative diseases, through maintaining the integrity of neuronal cell DNA synthesis and histone H3 and H4 synthesis. Neurodegenerative diseases may involve the compromisation of the integrity of neuronal cells through inhibition of DNA synthesis with consequent termination of histone synthesis and subsequent apoptosis. Current research is underway to investigate the direct association of zinc with H3 and H4 and the affect of DHA on the post-translational modifications (PTMs) of histones. Understanding the molecular basis of the affect of zinc and DHA in neuroprotection may provide novel information on the treatment and management of neurodegenerative diseases.

# CHAPTER 5

## ZINC AND DHA HAVE OPPOSING EFFECT ON THE EPIGENETIC REGULATION OF HUMAN NEURONAL CELLS

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## Summary

### Aim

In this chapter, the aim was to investigate the effect of zinc and DHA on the post-translational modifications (PTMs) of histone H3 in human neuronal cells.

### Methodology

M17 human neuroblastoma cells were treated with and without zinc and DHA for 48 h. Immunoblotting and densitometric analysis of M17 cells were employed to determine changes in acetylation, deacetylation, methylation and phosphorylation of human histone H3 in response to zinc and DHA treatment. To investigate the potential occurrence of apoptosis in following zinc and DHA, anti-apoptotic Bcl-2 and pro-apoptotic caspase-3 expression levels were also analyzed using Western immunoblot analysis.

### Results

In response to pathophysiological levels of zinc, significant increases in deacetylation, methylation and phosphorylation of histone H3 and significant decreases in acetylation of histone H3 were observed, all pointing towards possible gene silencing and apoptosis. To further investigate the role of zinc in apoptosis, the levels of Bcl-2 and caspase-3 were measured. Indeed, 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 a significant increase in acetylation of histone H3 and Bcl-2 levels and a significant decrease in deacetylation, methylation, phosphorylation of H3 and caspase-3 levels, suggesting that DHA promotes gene expression and neuroprotection.

**Conclusion**

In conclusion, these 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 the control and management of neurodegenerative diseases.

## 5.1 INTRODUCTION

It was shown in the previous chapter that histone H3 and H4 expression levels in human neuronal cells were down-regulated by zinc and up-regulated by DHA [152], suggesting a potential interaction between zinc and DHA in neurodegenerative diseases. As a building block of nuclesosome core particles carrying epigenetic information, histones are not only essential for packaging DNA into eukaryotic cells, but also affect the interactions between DNA and other chromatin associated proteins [158, 297]. Histones are among the most highly conserved proteins in eukaryotes [158], emphasizing their important role in the biology of the nucleus. Histones are involved in regulating gene transcription through the diverse post-translational modifications (PTMs) on their *N*-terminal tails, which include acetylation, methylation, and phosphorylation [297].

Since histone PTMs are closely related with gene transcription, neurodegenerative disorders such as Huntington disease [298, 299], Parkinson's disease [300] and Alzheimer's disease [301, 302] can result from aberrant epigenetic regulations. In this chapter, the effects of zinc and DHA on PTMs of histones, in particular histone H3, were identified in human neuronal cells.

The potential occurance of neuronal apoptosis as a result of zinc toxicity was also investigated. Several apoptosis-regulatory genes are induced in apoptotic cells during neuronal injury and cellular toxicity [303]. Among these genes, caspase-3 [303] and Bcl-2 [304, 305] are the most effective apoptotic regulators, as they play a decisive role in

the occurrence of apoptosis. Using Western blot analysis, the change in the expression levels of caspase-3 and Bcl-2 in M17 neuroblastoma cells were also measured in order to see whether DHA is able to inhibit zinc-induced apoptosis.

The results suggest that zinc-induced alteration in histone PTMs may possibly contribute to cellular apoptosis, which is reduced by DHA, providing a potential mechanism that will establish the beneficial effect of DHA in neuroprotection.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Treatment reagents and preparations

Docosahexanoic acid (DHA, Sigma Aldrich, MO, USA), in final concentration of 10 µg/ml, was pre-incubated at 37°C in OptiMem media overnight to allow conjugation with media proteins. Anti-oxidant, Vitamin E (final concentration 0.05 µM/ml) was added to DHA treatment in order to stabilize DHA. Zinc (in the form of $ZnCl_2$; 5 µM final concentration) was added to the cells on the day of experiment. To ensure that the differential histone PTMs were indeed zinc effect, as done in the previous chapter, the cell medium was first tested to have no zinc content.

### 5.2.2 Experimental treatment of M17 neuroblastoma cells

M17 neuroblastoma cell cultures were maintained and treated with and without zinc (5 µM final concentration) and DHA (10 µg/ml final concentration) for 48 h following procedures described in previous chapters.

### 5.2.3 Cell viability experiment

Media was removed from cells cultured in 6-well plates, the cells washed with phosphate- buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 100 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) to remove traces of previous serum growth medium. 0.3 ml of 0.025% trypsin/EDTA was added and cells were suspended in 0.5 ml Opti-MEM media, which was then mixed with 0.4% trypan blue solution (diluted 1:2). The number of trypan blue-positive and negative-cells were counted on a haemocytometer using light microscope at 40x magnification.

### 5.2.4 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 sec, twice each using the Microson ultrasonic cell disruptor (Misonix, New York, USA) and centrifuged at 14,000 x g for 5 min. 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 dilutions of lystate samples 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 absorbance vs. protein concentration (µg/ml), using standards of known concentrations.

### 5.2.5 One-dimensional electrophoresis and Western immunoblot analysis

The level of each post-translational modification of histones in response to zinc and DHA treatment were analyzed using Western blot analysis. Protein concentration of 25 µg with total volume of 20 µl per well were run on 15% SDS–PAGE and transferred onto nitrocellulose membrane (Whatman, Dassel, Germany). After blocking with 1% (w/v) non-fat skim milk in 1 x TBS for 1 h at room temperature, membranes were incubated overnight at 4°C with monoclonal primary antibody with the dilution of 1:1,000 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). After washing with TBS solution three times for 5 min each, membranes were incubated with 1:2,000 dilution of goat anti-rabbit IgG horseradish-peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology Inc., MA) for 1 h at room temperature. Membranes were visualized using Immobilan Western Chemiluminesence HRP substrate (Millipore Corporation, CA, USA), according to manufacturer's instructions.

Developed membranes were stripped for 15 min 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 (1:1,000 dilution) followed by goat anti-rabbit IgG HRP conjugated secondary antibody (1:2,000 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 (1:4,000 dilution) followed by anti-mouse HRP antibody (1:4,000

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).

### 5.2.6 Apoptosis assay

Protein lysates (25 µg concentration with 20 µl total volume per well) were subjected to 15% (w/v) SDS-PAGE and transferred onto nitrocellulose membrane (Whatman), as described previously. The membranes were probed for apoptosis markers; mouse monoclonal Bcl-2 (Abcam, Cambridge, UK) and rabbit polyclonal antibody for active caspase-3 (Chemicon International). Both antibodies were prepared in 1:100 dilutions with 1 x 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 (1:1,000 for Bcl-2, 1:10,000 for caspase-3) and developed as described previously. All membranes were photographed using Fujifilm LAS-300, and the subsequent densitometry analysis of bands was done with the Fujifilm Multi Gauge V3.0 program (Fujifilm, Tokyo, Japan).

### 5.2.7 Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (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 alone and DHA treatments. The statistical significance was set at $*p < 0.05$.

## 5.3 RESULTS

In the previous chapter, it was observed that zinc and DHA affect expression levels of histone H3 and H4 [152]. Here the aim was to investigate the effects of zinc and DHA on the post-translational modifications (PTMs) of histone H3. Histone H3 has the longest *N*-terminal tail among all core histones (Fig. 5.1), and where most modification sites are found [306].

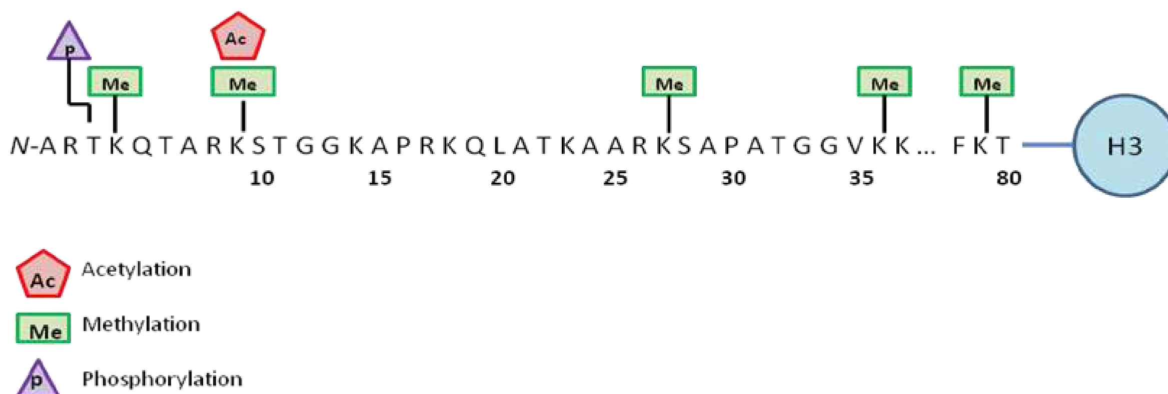



**Figure 5.1: Post-translational modification positions of histone H3 in human neuronal cell analyzed in this study.** Ac= acetylation; Me= methylation; P=phosphorylation.

### 5.3.1 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), M17 human neuronal cells were grown in culture medium in the presence and absence of zinc and DHA. In the presence of zinc and the absence of DHA (zinc effect), acetylated histone H3 (K9) levels were significantly decreased (Fig. 5.2). On the other hand, DHA in the presence of zinc, significantly increased histone H3 (K9) acetylation levels back to the control levels (without zinc and DHA) (Fig. 5.2).

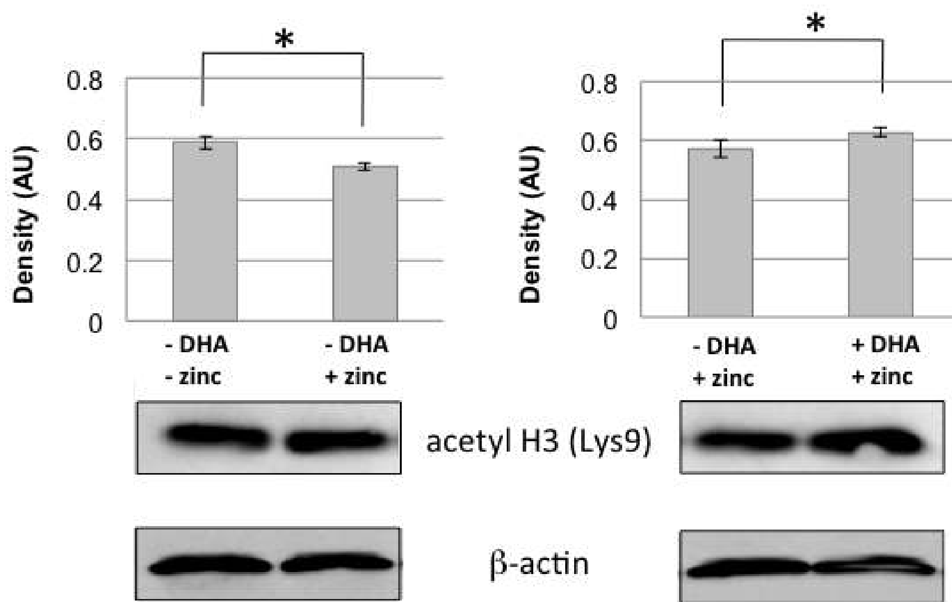
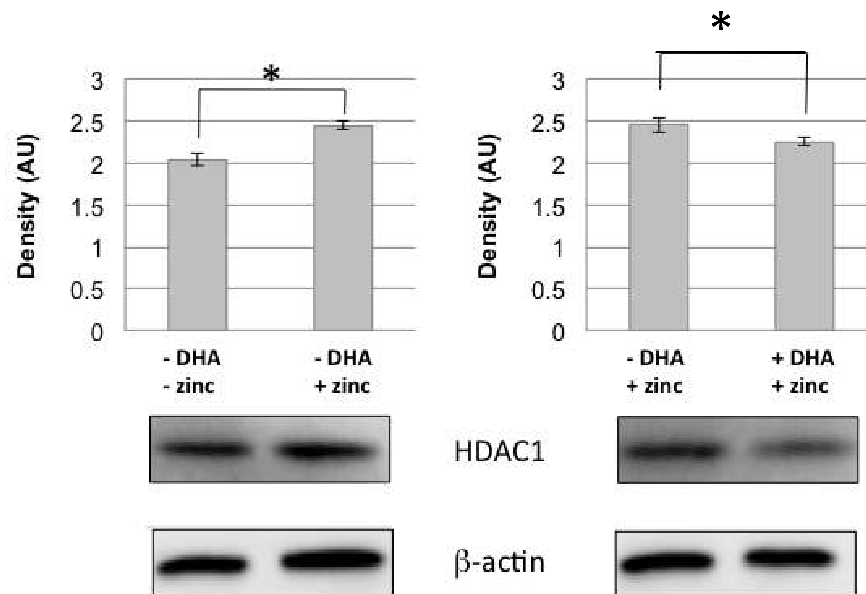



**Figure 5.2: Effect of zinc and DHA on acetylation of histone H3 (K9) in M17 human neuronal cells.** Densitometric analysis, in arbitrary units (AU), of a Western blot (with corresponding protein bands shown at the bottom) to quantitate acetylation of histone H3 (K9) in M17 cells following treatment with (+) and without (-) zinc (final concentration of 5 µM) and with (+) or without (-) 10 µg/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).

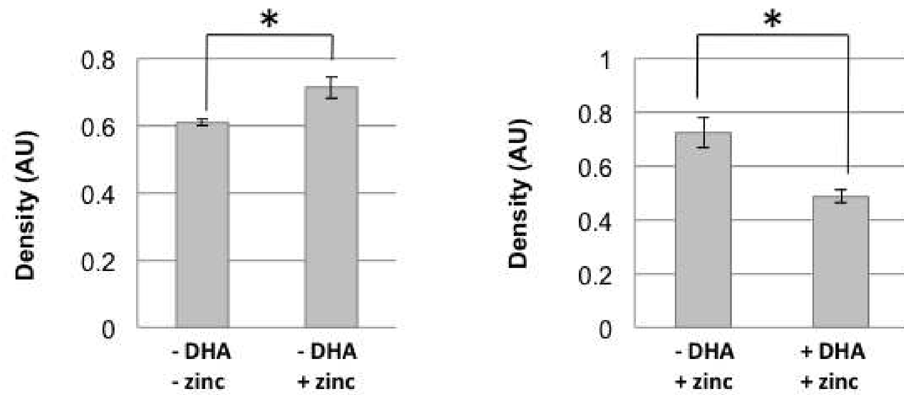
### 5.3.2 Effect of zinc and DHA on histone deacetylase (HDAC) 1, 2, 3

Western blot analysis was performed to investigate the changes in expression levels of histone deacetylase (HDAC) 1, 2, and 3, using highly specific anti-HDAC 1, 2 and 3 antibodies. Zinc caused significant up-regulation of HDAC1, 2 and 3 compared with the control (without zinc and DHA) (Figs. 5.3A- 5.3C), while DHA caused significant down-regulation of HDAC1, 2 and 3 (Figs. 5.3A-5.3C, respectively), as measured by densitometric analysis.

**A.**



**B.**




**C.**

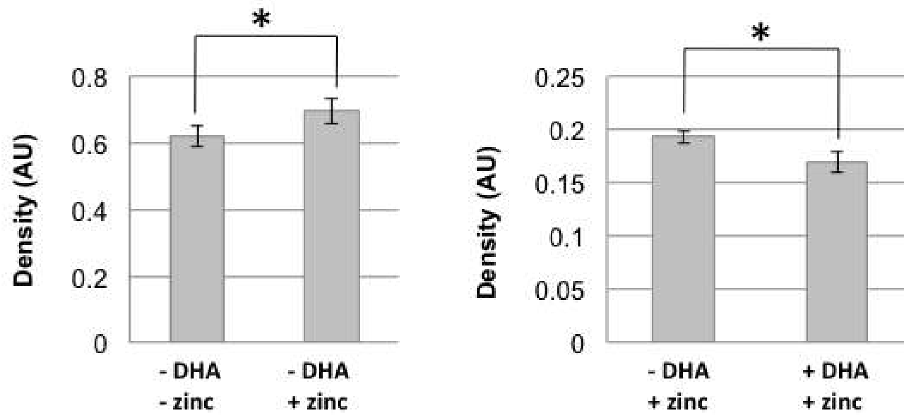


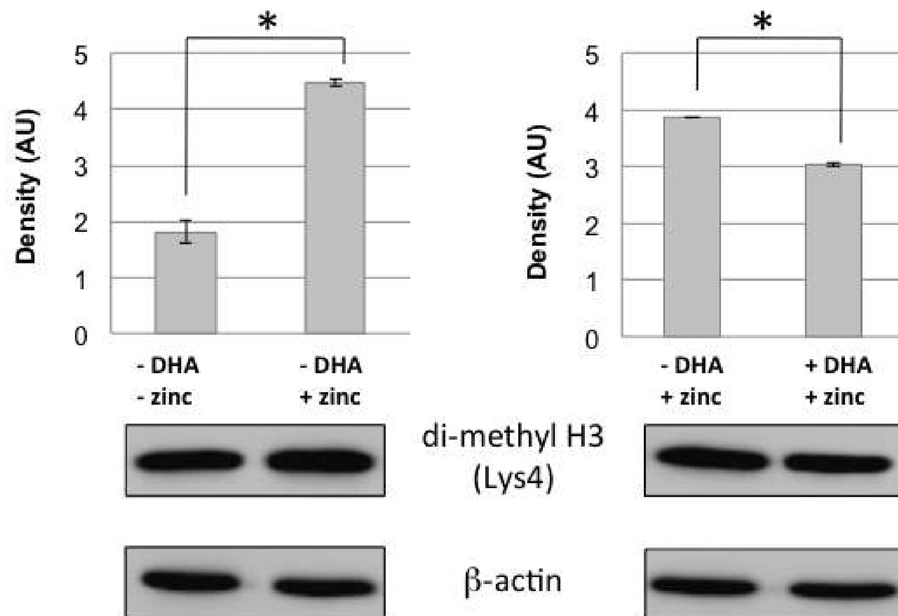

**Figure 5.3: Effect of zinc and DHA on histone deacetylases (HDACs) expression levels in M17 human neuronal cells.** Densitometric analysis, in arbitrary units (AU), of a Western blot (with corresponding protein bands shown at the bottom, for HDAC1) to quantitate the expression levels of HDAC1 (A), HDAC2 (B), and HDAC3 (C), following zinc (5 µM) and DHA (10 µg/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.

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

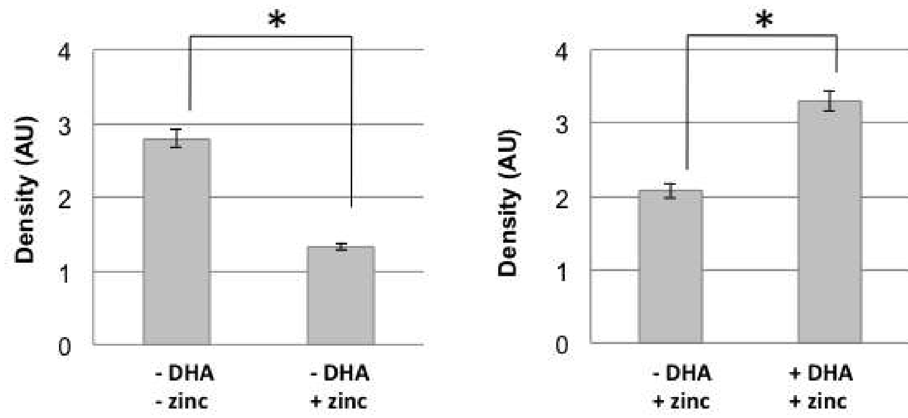
In order to assess the effect of zinc and DHA on dimethylation of histone H3 (K4), (K9), (K27), (K36), (K79), Western blot analysis was performed using human neuronal cells

that were grown in the presence and absence of zinc and DHA, as described previously. Concurrent analysis of di-methylation levels of histone H3 revealed that di-methylated H3 (K4) and (K27) are significantly increased upon zinc treatment and significantly decreased with the addition of DHA (Figs. 5.4A and 5.4C). Di-methylated H3 (K79) also showed a significant increase with zinc treatment, but an even more significant increase was observed with DHA treatment (Fig. 5.4E). In contrast, zinc significantly decreased the expression levels of di-methylation of histone H3 (K9) and (K36), while DHA caused a significant increase (Figs. 5.4B and 5.4D). These results demonstrated 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.

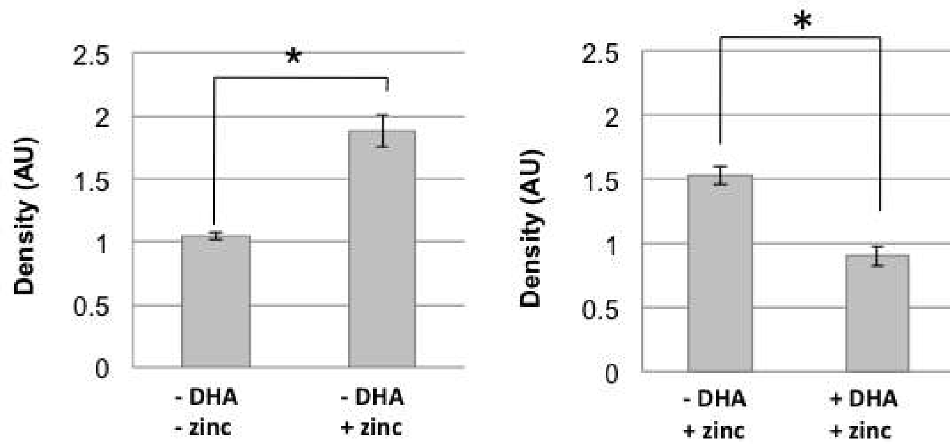
**A.**



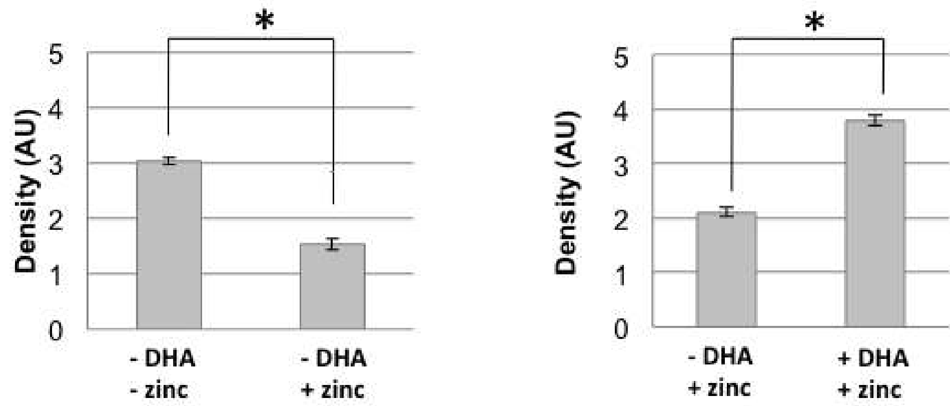
B.




C.




D.



**E.**

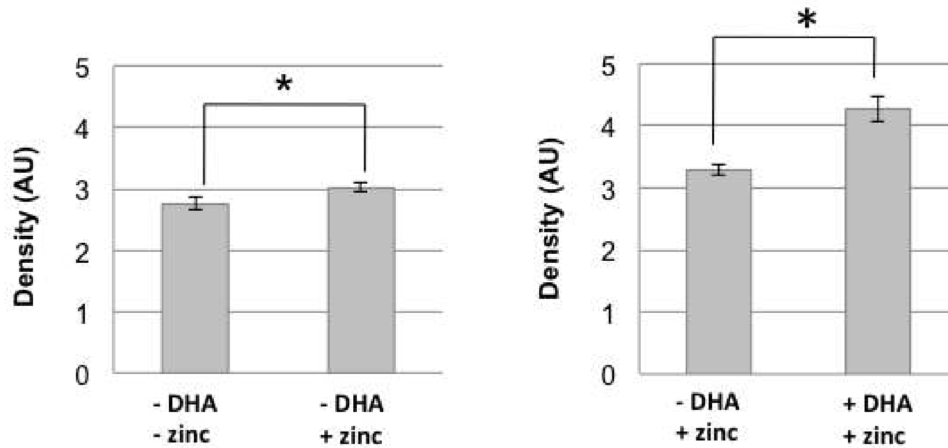



**Figure 5.4. Effect of zinc and DHA on di-methylation of histone H3 in M17 human neuronal cells.** Quantitative analysis of dimethylation levels of histone-H3 (K4) (A), (K9) (B), (K27) (C), (K36) (D) and (K79) (E) by densitometric analysis, expressed in arbitrary units (AU), of a Western blot (with corresponding protein bands shown at the bottom, for Lys4), following treatment with or without zinc (5 µM) and DHA (10 µg/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).

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

The effect of zinc and DHA on Histone H3 phosphorylation at Threonine 3, -11, and Serine 10, -28 were also investigated. However, only H3 (T3) phosphorylation was seen in M17 cells. The results showed that phosphorylation of H3 (T3) was significantly increased upon zinc treatment and significantly decreased with DHA (Fig. 5.5).

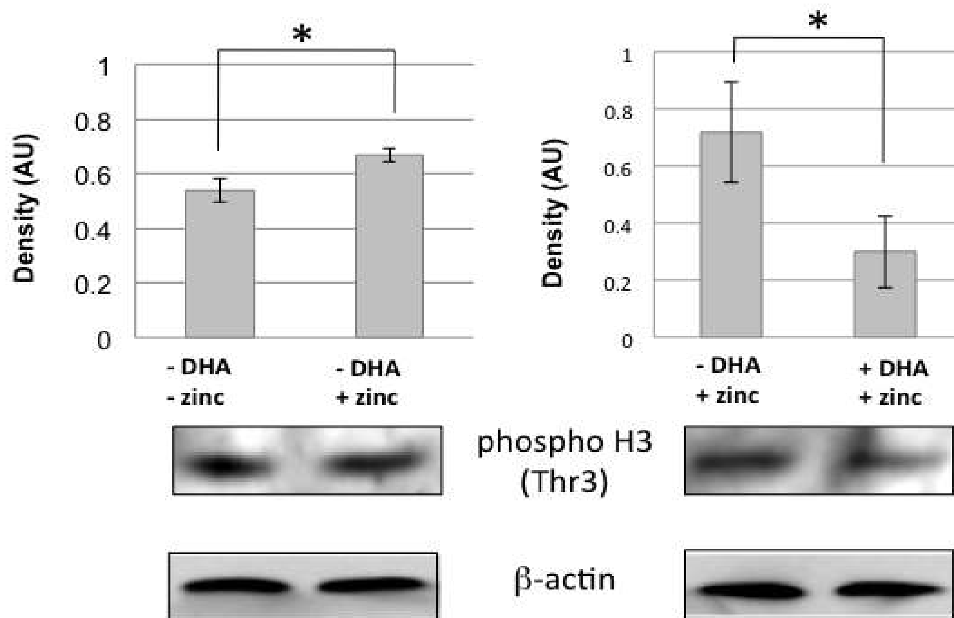


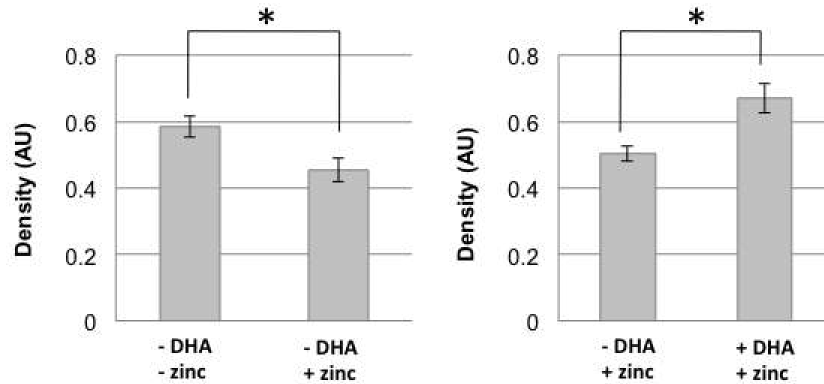

**Figure 5.5: Effect of zinc and DHA on phosphorylation of histone H3 (T3) in M17 human neuronal cells.** Densitometric analysis, in arbitrary units (AU), of a Western blot (with corresponding protein bands shown at the bottom) used to quantitate phosphorylation of histone H3 (T3), compared with total H3 and normalized with β-actin expression level, following treatment with or without zinc (5 μM) and DHA (10 μg/mL). The results are shown as means (n=3, *P<0.05).

### 5.3.5 Western blot analysis of caspase-3 and Bcl-2 activation in M17 neuroblastoma cells in response to zinc and DHA

The aim for this study was 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 (Figs. 5.6A and 5.6B). The results showed that both zinc and DHA opposingly modulate 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 (Figs. 5.6A and 5.6B).

**A.**




**B.**

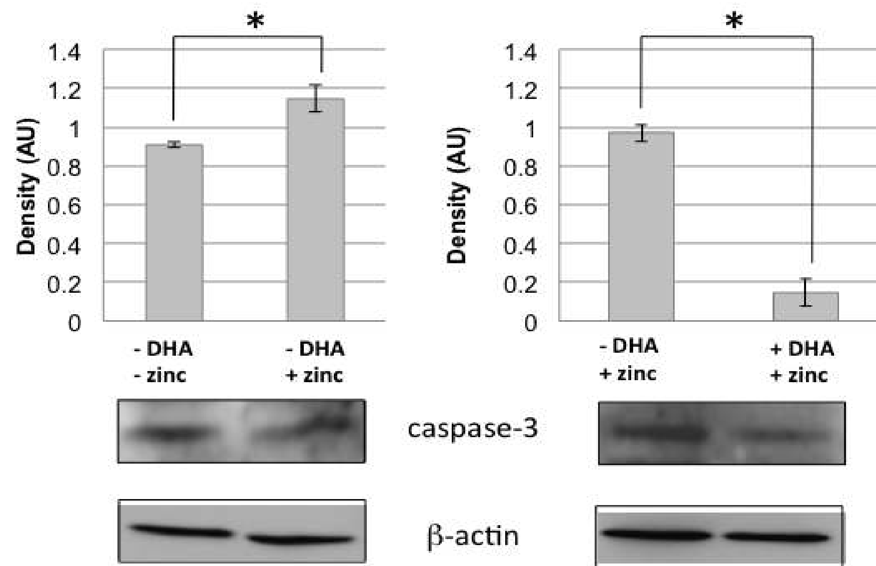



**Figure 5.6: Effect of zinc and DHA on Bcl-2 and caspase-3 expression levels in M17 neuroblastoma cells.** Densitometric analysis, in arbitrary units (AU), of a Western blot (with corresponding protein bands shown at the bottom, for caspase-3) to quantitate anti-apoptotic marker, Bcl-2 (A) and apoptotic marker, caspase-3 (B) expression levels in M17 cells following treatment with (+) and without (-) zinc (final concentration of 5 µM) and with (+) or without (-) 10 µg/ml DHA, when compared with β-actin loading control. The data are shown as means (n=3, *P<0.05).

### 5.4.6 DHA protects neuronal cells against zinc-induced cellular apoptosis

Cell viability assay using trypan blue exclusion was conducted in order to confirm the Bcl-2 and caspase-3 expressions results (Fig. 5.7). Indeed, there was a direct correlation with the Bcl-2 and caspase-3 results, in which zinc treatment reduced and DHA treatment increased M17 cell viability.

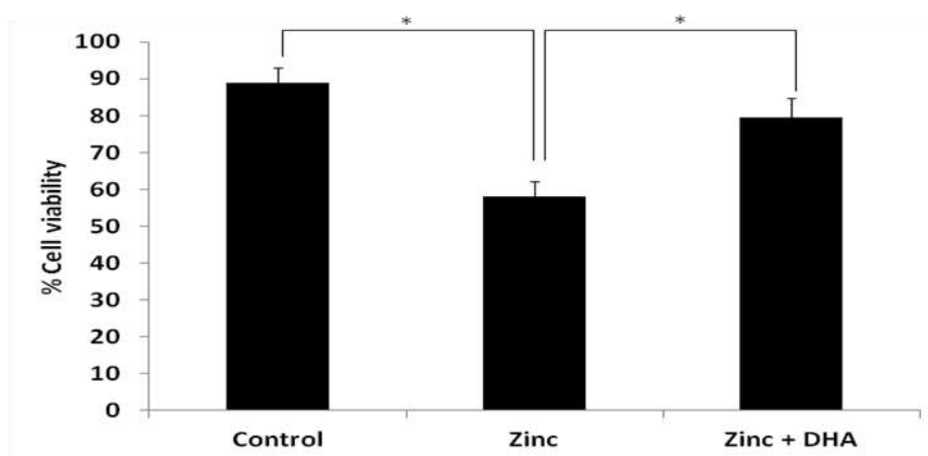



**Figure 5.7: Viability of M17 cells in response to zinc in the presence and absence of DHA after 48 h.** Cell viability was determined by the trypan blue exclusion assay. The results were expressed as mean ± SD of three independent experiments. Student's *t*-test was used for statistical evaluation between treatment of DHA with and without zinc and a *P* value of <0.05 was considered significant. (*) = significant.

## 5.4 DISCUSSION

### 5.4.1 The effect of zinc and DHA on acetylation of histone H3 (K9)

The 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 [215, 299]. The altered gene transcription may cause

opposite effects from normal gene regulation pattern, which is attributed to degenerative fate of neurons that subsequently reduce the expression of survival-associated genes by altered acetylation and, at the same time, stimulate the expression of death-inducing genes [215].

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 the neuroprotective effects of 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, it has been shown here that DHA contributes to anti-apoptotic, Bcl-2 up-regulation, thereby reducing neuronal cell death.

### 5.4.2 Effect of zinc and DHA on histone deacetylases (HDACs) 1, 2, 3 expression levels

HDAC enzyme activity has been shown to increase in dying neurons due to loss of counterbalancing effects of HAT activity [215]. The results suggest that the increase of zinc can also contribute to the neurodegenerative process through up-regulating HDAC enzyme expression levels, and therefore affecting histone deacetylation (Fig. 5.3).

The HDAC catalytic domain contains a $Zn^{2+}$ ion, in the active site, which contributes significantly to its catalytic activity [236, 307]. 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 [236, 308].

It has been established that the isotypic selective inhibition of HDAC enzymes 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 [309]. Since DHA crosses the blood-brain barrier [310], these data suggests that it may possibly have neuroprotective characteristics that mimic the behavior of HDACs inhibitors.

Generally, increase 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 [215, 311]. This chapter demonstrated 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 HDAC 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 [312].

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

The results suggest 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 which then lead to the induction of cell-cycle arrest and apoptosis [186], a key feature in the pathology of neurodegenerative disorders, such as AD. On the other hand, DHA treatment of M17 cells significantly decreased H3 (K4) di-methylation, 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, the results of this study suggest that zinc may contribute to the regulation of cell apoptosis through increasing H3 (K4) methylation.

Several developmental disorders with abnormalities in the 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 [313]. Based on our results, it is possible that zinc may be involved in neurodegenerative process through inhibition of H3 (K9) di-methylation, which is associated with reduction

in DNA methylation [153, 314]. This will consequently enhance DNA strand breakage that can also impair DNA repair system, resulting in genetic mutation and chromosome abnormality or possibly triggering apoptosis [153, 315]. 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 [316-318]. A recent study has reported the importance of H3(K27) di-methylation in cellular growth, where repression of H3(K27) methylation caused significant effect on cell cycle progression through G1 arrest, which led to cell growth inhibition and eventually apoptosis [319]. Based on 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) di-methylation, restoring the basal level. So far, the link between H3 (K27) di-methylation and neurodegenerative diseases is still unclear. However although not investigated here, it is possible that the increase in H3(K27) di-methylation caused by zinc leads to the gene repression as well as cell growth inhibition which resulted in cell death. Therefore, DHA can be proposed to reduce gene silencing by decreasing di-methylation of H3 (K27), which in turn induces cell cycle progression, cell growth and reduction in apoptosis.

The role of H3 (K36) di-methylation is associated with the euchromatin region [320] at the individual gene level that leads to gene activation [320, 321]. H3 (K36) di-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 [322]. Reduction of H3 K36) di-methylation decreases RNA polymerase II phosphorylation which then leads to transcription elongation defect [323, 324]. 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. Western blot analysis 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 also been recently shown that H3 (79) methylation is associated with DNA repair system [184, 325]. The rate of DNA repair is dependent on several factors such as the extracellular environment, cell types and how severe the damage is. Cells that have accumulated large amounts of damage can possibly lead to cell death or apoptosis. H3 (K79) methylation is required for the recruitment of the DNA-repair-protein p53 binding

protein (53BP1) that is shown to be a sensor of DNA double strand breaks (DSBs), which in turn activate ataxia telangiectasia-mutated protein (ATM) ( a DNA damage signaling kinase which then also mediates the activation of p53 (tumour suppressor)) through chk2 (checkpoint gene) and subsequently lead to cell cycle arrest or apoptosis [326, 327]. It was observed that zinc increases H3 (K79) di-methylation in neuronal cells while DHA causes further increase in H3 (79) di-methylation. The enhanced increase of di-methylation of H3 (K79) by DHA may suggest different outcomes. Indeed, zinc-induced neuronal death is associated by DNA DSBs [328], which is somehow mediated by H3 (K79) methylation. 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 [329]. A reduction in p53 expression following dietary PUFAs is also anticipated to reflect the presence of less DNA damage and therefore allowing cell survival [329]. From this study, further increase in di-methylation of H3 (K79) in response to DHA would may be involved in reducing p53 expression levels and therefore inhibit cell apoptosis (Fig. 5.4E).

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

Phosphorylation is involved in ‘methyl-phos’ mechanism, which is a concept that local phosphorylation could regulate binding of an effector protein of adjacent methylation sites [330], where neighbouring modifications act together as ‘binary switches’. The

phosphorylation of Thr3 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 the nucleosome and expose DNA for gene expression, which is antagonized *in vitro* by phosphorylation of neighbouring threonine 3 (Thr3) [331]. This binding dissociation reduces the interaction with transcriptional elongation factors and therefore reduces gene transcription and subsequently induces apoptosis [331]. The results show how 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 the binding complex between H3 (K4) methyl group and its effector protein. However, DHA treatment reduced the phosphorylation H3 (T3), which may increase Chd1 protein binding to dimethylated H3 (K4) and may therefore increase recruitment of transcription elongation, gene transcription and subsequent reduction in apoptosis, although this needs to be fully tested in future studies.

### 5.4.5 DHA inhibit cellular apoptosis through restoring zinc- induced alteration in caspase-3 and Bcl-2 expression levels in M17 cells

The 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, DHA treatment of M17 cells increased expression levels of

Bcl-2 and reduced caspase-3, suggesting that DHA may exclusively activate the extracellular signal regulated kinase/ mitogen-activated protein kinase (ERK/MPK) pathway to promote cell survival, which lead to the up-regulation of Bcl-2 and inhibition of caspase-3 activation [332]. The study by Akbar et al (2006) 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 [145]. The findings with Bcl-2 and caspase-3 highlight the importance of DHA in neuroprotection and zinc in apoptosis.

### 5.4.6 DHA protects neuronal cells against zinc-induced cellular apoptosis.

The viability of M17 cells in the zinc and DHA- treated groups and their corresponding controls were expressed as percentage (%) change. Approximately 34% decrease in M17 cell viability was observed following zinc treatment for 48 h. DHA has shown to inhibit zinc-induced toxicity by restoring cell viability. These findings are supported by previous findings in this chapter which indicate the neuroprotective effect of DHA in restoring zinc-induced alteration in epigenetics and subsequent cell death.

Zinc toxicity has been reported to affect gene transcription, which is proposed to be 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 is also believed to be mediated by a change in the epigenetic pattern. Any other functions of the epigenetic changes, and their influence in the regulation of

specific genes following zinc and DHA treatment, were not tested as it was beyond the scope of this study. Here, the aim was 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, how the epigenetic changes link to their functions will be investigated in future studies.

## 5.5 CONCLUSION

The 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 Figures 5.8 and 5.9. The data show that zinc reduced histone acetylation and increased HDACs, which represent a critical step in the apoptotic process, while 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 therefore proposed that these two essential nutrients may 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 increase neuroprotection. It was observed that zinc and DHA have distinct epigenetic patterns; this suggests they may have opposing effects in the progression of neurodegenerative diseases. 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 proapoptotic 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.

**Figure 5.8: 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. The findings of this chapter are shown by the shaded boxes. HDAC= histone deacetylase; CREB= cAMP response element-binding; Bcl-2= B-cell lymphoma 2; DSBs= double strain breaks; ATM= ataxia telangiectasia mutated, Chk2= checkpoint kinase 2; Chd1= chromodomain-helicase-DNA-binding 1.

**Figure 5.9: 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. The findings of this chapter are shown by the shaded boxes. HDAC= histone deacetylase; CREB= cAMP response element-binding; Bcl-2= B-cell lymphoma 2; Chd1= chromodomain-helicase-DNA-binding 1; RNA pol= Ribonucleic acid polymerase.

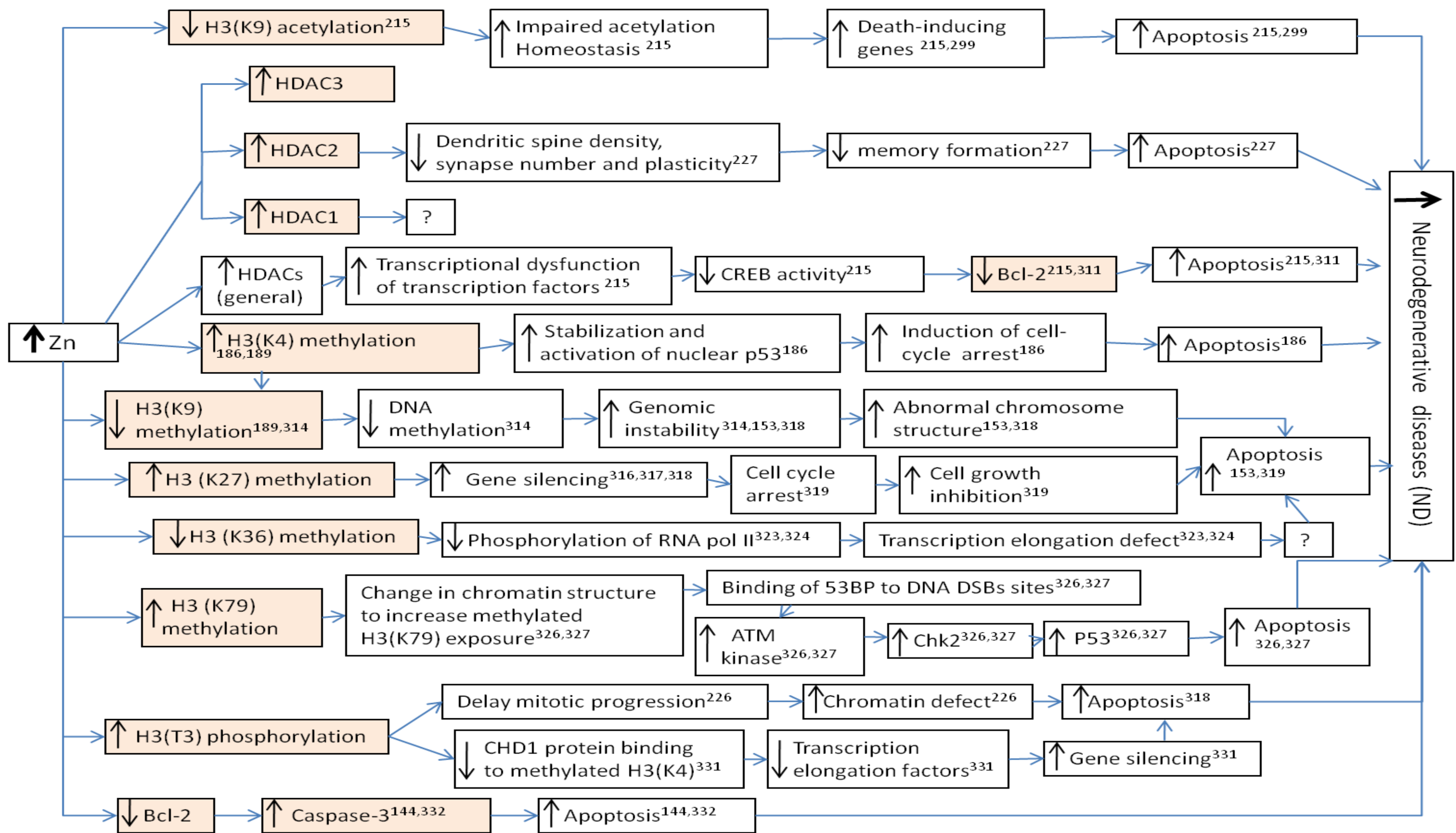



**Figure 5.8**

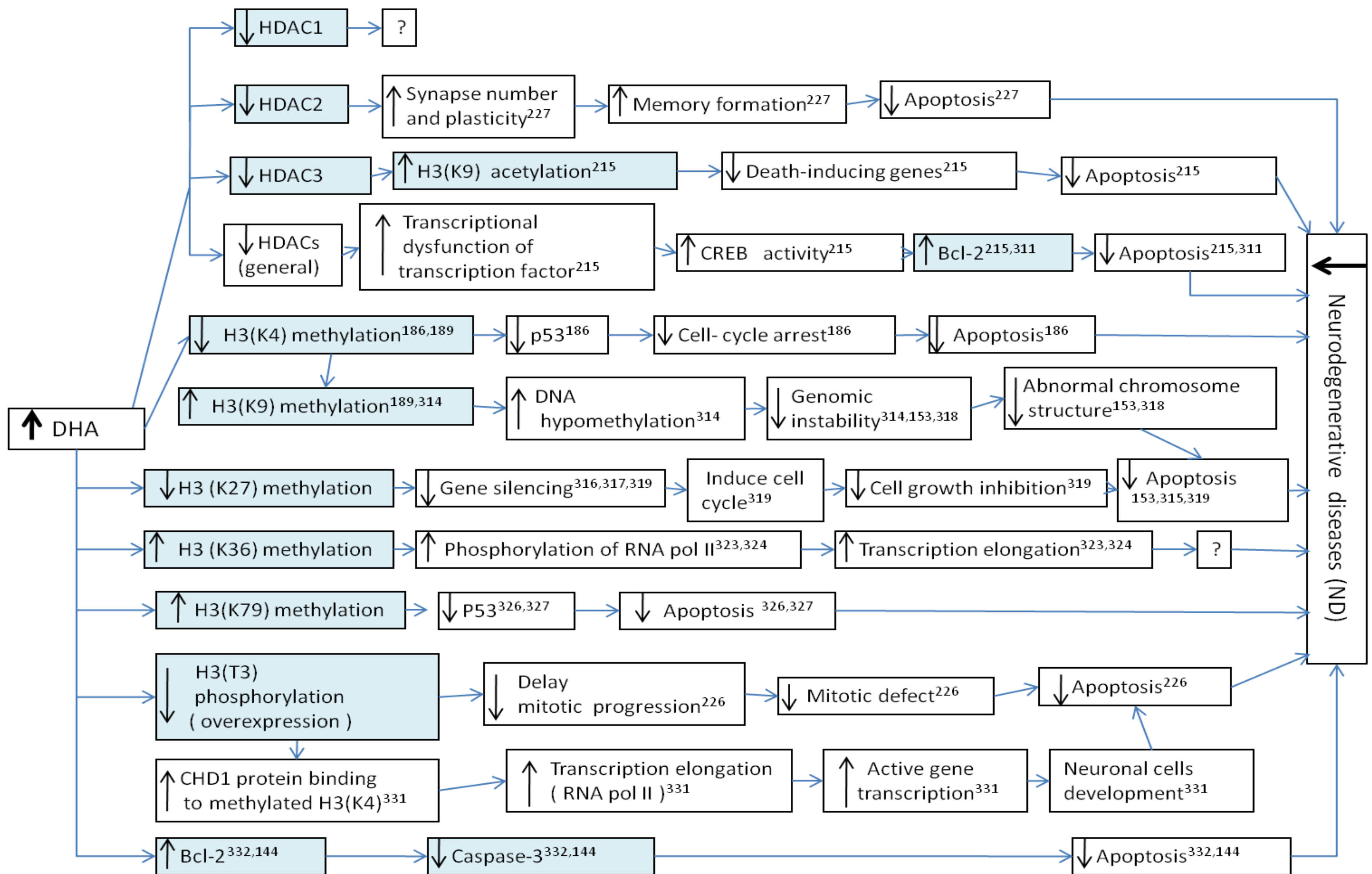



**Figure 5.9**

# CHAPTER 6

## GENERAL DISCUSSION AND FUTURE DIRECTIONS

## 6.1 INTRODUCTION

This thesis covers several aspects of zinc and polyunsaturated fatty acid DHA interactions in human neuronal cells, which focus on the neuroprotective effects of DHA in restoring zinc-mediated alteration in cellular bioenergetic functions, epigenetic patterns and gene expressions. The potential pathways of zinc toxicity in human neuronal cells are extracted into a summary to which our findings are related. This discussion will also include the potential therapeutic intervention of zinc-induced neuronal injury with DHA and a look into possible future directions for zinc and DHA molecular interactions.

This chapter provides a general discussion of the thesis, describing the findings and possible links between each result chapter in relation to:

1. Identifying the potential mechanism and effect of mitochondrial zinc toxicity as well as the neuroprotective effect of DHA in restoring cellular bioenergetic dysfunction following zinc treatment;
2. Determining whether DHA and CoQ10 have independent or additive effects against Aβ- and zinc-induced defects in energy metabolism and mitochondrial respiratory function in M17 neuroblastoma cells;
3. Using proteomic analysis to identify the differentially expressed proteins in response to zinc and DHA treatment in human neuronal cells;
4. The involvement of zinc and DHA in the epigenetic modification of histones, which therefore affecting gene expressions in human neuronal cells.

To elucidate the possible mechanisms in which DHA restores and inhibits zinc-induced cellular dysfunction, the signaling pathway involved in zinc toxicity will be investigated first. This could provide the insight into apoptosis of human neuronal cells and neurodegenerative conditions induced by altered zinc homeostasis and the potential therapeutic mechanisms of DHA.

## 6.2 MITOCHONDRIAL DYSFUNCTION MAY BE THE EARLY EVENT OF ZINC-INDUCED TOXICITY IN HUMAN NEURONAL CELLS

Perhaps the best understanding of the role of altered zinc homeostasis (caused by excess zinc efflux) in mitochondrial dysfunction comes from the study of zinc toxicity in altering bioenergetic functions, which lead to the reduction in $\Delta\psi_m$, along with the capacity to synthesize ATP production (Chapters 2 and 3). Uncoupled respiration and maximal respiratory capacity are also decreased (Chapter 3) following zinc, indicating that this $Ca^{2+}$-dependent process produces a major functional defect in the electron transport chain [333].

The disruption of mitochondrial function eventually leads to a cell death cascade involving the release of pro-apoptotic molecules and ROS [334]. This is consistent with the results showing that hydrogen peroxide, $H_2O_2$ were significantly increased following zinc treatment in M17 neuroblastoma cells, which suggest the potential occurance of neuronal cell death as a consequence of elevated zinc ions (Chapter 3).

Zinc is thought to cause neuronal cell injury by targeting mitochondria and cellular energy metabolism. While it is obvious that zinc can disrupt mitochondrial function, it is unclear whether zinc acts externally or if mitochondria import zinc into the matrix. Given that mitochondria are semiautonomous organelles that are capable of transcription and translation and therefore require zinc in such process. It is possible that the mitochondrial membrane possess transporters responsible for regulating zinc uptake. Studies suggested that zinc may enter mitochondria and produce immediate effect within 24 h incubation (Chapter 3), perhaps mediated by mitochondrial calcium uniporter [280, 335].

Zinc may enter mitochondrial matrix where they start to interact with endogenous target molecules, causing mitochondrial dysfunction. The biochemical changes induced by zinc toxicity, such as ROS generation, depletion of ATP as well as a decrease in $\Delta\psi_m$ (Chapter 3) may possibly be considered as causative factors for an abrupt increase in the mitochondrial inner membrane permeability, which is believed to be due to the opening of mitochondrial membrane transtition pores [241, 244]. This then causes an alteration in proton influx into mitochondrial matrix, which leads to a rapid dissipation of membrane potential and reduction in ATP turnover rate, as observed in my study (Chapter 3). This condition has been reported to be associated with mitochondrial swelling and release of protein from intermembrane space such as cytochrome c. This in the end may cause a complete failure of maintenance of neuronal cell function and possibly culminating in cell death.

The possible zinc-induced neurotoxic mechanism in mitochondrial function is shown in Figure 6.1. It is important to note that although the model presented here is broadly accepted by current literature, the function of membrane transition pores, and the release of apoptosis-mediated proteins such as cytochrome c were not experimentally tested in this thesis. Therefore, this potential mechanism induced by zinc is still hypothetical.

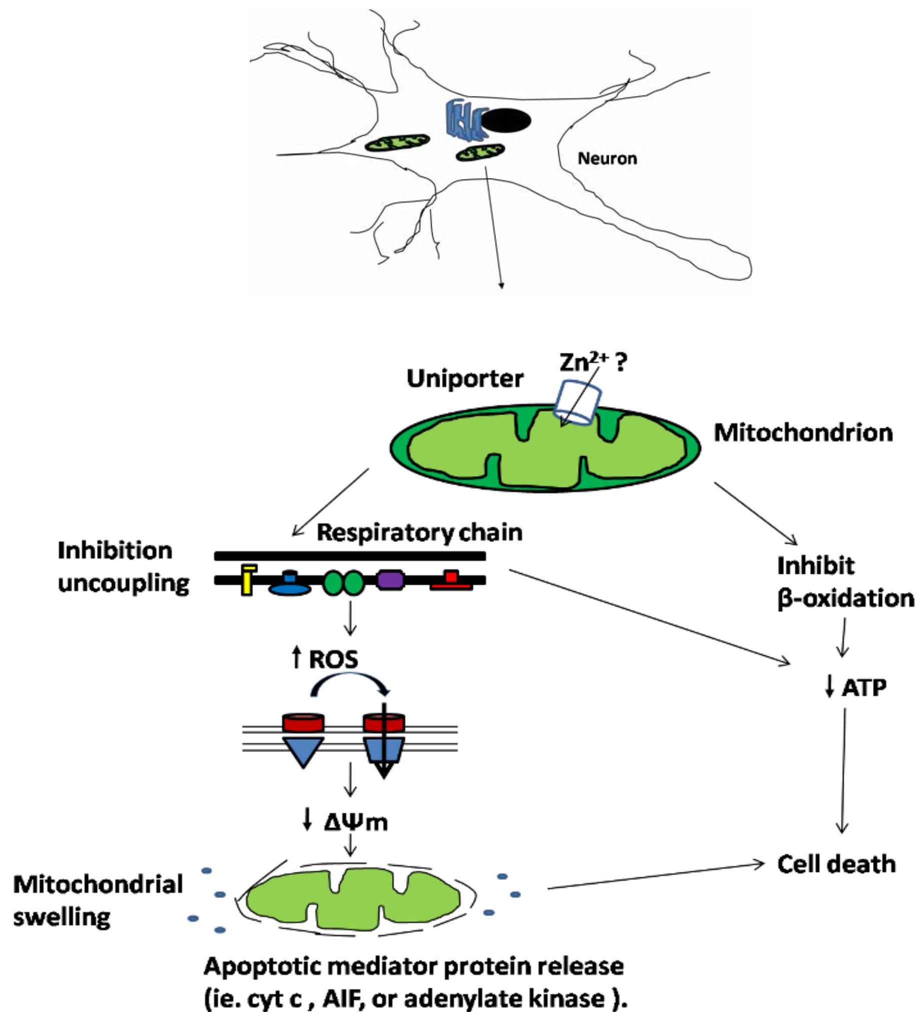



**Figure 6.1: Possible neurotoxic mechanisms induced by zinc toxicity in mitochondria**. Cellular injury and neurodegenerative disease such as AD are characterized by cellular dysfunction, which mitochondrial destruction is the early event. Increase in $[Zn^{2+}]_i$ is associated with an increase transport of $Zn^{2+}$ into mitochondria, which is possibly mediated by mitochondrial uniporter. $Zn^{2+}$ can inhibit both β-oxidation and the respiratory chain, leading to a decrease in mitochondrial and cellular ATP levels. Additionally, the uncoupled oxidative phosphorylation in combination with the inhibition of the electron transport, increases the generation of reactive oxygen species (ROS) leading to permeabilization of the mitochondrial membranes by opening of the mitochondrial permeability transition pore, which is located in the inner and outer mitochondrial membrane. The osmotically driven influx of water results in an increase in mitochondrial volume and a rupture of the outer mitochondrial membrane. Proteins from the intermembrane space (i.e. cyt c) can be released into the cytoplasm and activate apoptotic pathways. Cyt c = cytochrome c; AIF = apoptosis-inducing factor; $\Delta\Psi_m$ = membrane potential; ATP = adenosine triphosphate.

## 6.3 ZINC-MEDIATED MITOCHONDRIAL DYSFUNCTION MAY LEAD TO AN ALTERED GENE REGULATION

As previously mentioned, mitochondria are the major producers of ROS in the cells, which are generated as by-products of aerobic respiration and various other catabolic and anabolic processes [336]. Hydrogen peroxide ($H_2O_2$), which is converted from superoxide anion ($O_2^-$), is increased following pathophysiological levels of zinc in M17 cells (Chapter 3). Once produced, ROS react with proteins and nucleic acids causing oxidative damage to these macromolecules [337, 338]. It has been reported that ROS readily attack DNA and generate a variety of DNA lesions, such as oxidized DNA bases and DNA strand breaks, which ultimately lead to genomic instability [339].

In Chapter 4, proteomic analysis showed that histones were the key differentially expressed proteins in response to zinc and DHA interaction. Zinc treatment decreased histones protein and mRNA levels in M17, which may be associated with the reduction in DNA synthesis (Chapter 4). These data may suggest a link between zinc-induced mitochondrial dysfunction and accumulation of ROS, which have the tendency to disrupt DNA synthesis and therefore inhibit histone transcription. Insufficient ATP synthesis due to zinc-induced bioenergetic dysnfunction (Chapters 2 and 3) may also account for the inhibiton in DNA and histone synthesis in M17 cells (Chapter 4).

Disturbance of mitochondrial function following zinc supplementation in M17 cells has the potential to affect nuclear DNA, which can in turn affect the genomic function. This hypothesis is supported by a study that showed a link between aberrant production of ATP from oxidative phosphorylation and inhibition in nuclear DNA synthesis in mammalian cells [340]. This is also consistent with the data in Chapter 4 on the reduction of histone expression levels following zinc treatment, in which their synthesis may be associated with inhibition of DNA synthesis (Chapter 4). The results suggest that not only does ROS accumulation in the cells lead to a significant reduction in histone mRNA and protein levels, but zinc-mediated ATP depletion may also affect histones and DNA synthesis and may therefore affect gene transcription.

## 6.4 ZINC-MEDIATED MITOCHONDRIAL DYSFUNCTION MAY BE ASSOCIATED WITH ALTERED EPIGENETIC PATTERNS

Chapter 5 showed that zinc supplementation in M17 cells reduced acetylation, which is associated with increased gene transcription [341, 342] and increased histone deacetylases (HDACs) enzyme, which confers repressed gene transcription (deacetylation) [343]. ROS accumulation as a result of mitochondrial dysfunction has been shown to regulate both genetic and epigenetic cascades underlying altered gene expression in disease condition [98]. ROS-related $H_2O_2$ causes epigenetic alteration such as DNA damage methylation, which is mediated by accumulative change in chromatin structure. This epigenetic perpective on the free radical theory

of development was supported by Hitchler and Domann [344] through oxidation of DNA and histone methyltransferases (HMT).

The activity of enzymes and co-factors involved in epigenetic control, such as HDACs and HMTs, are linked to glycolysis and oxidative phosphorylation [344]. Redox status may also influence the function of epigenetic enzymes and the proteins that bind to their products [344]. From this, it is apparent that the epigenome relies heavily upon mitochondrial function.

A schematic diagram on how each of the epigenetic alteration regulate gene expression that lead to cellular apoptosis and neurodegenerative condition is shown in Figure 5.8 (Chapter 5). This discussion chapter also sought to expand upon current literature regarding the zinc-induced metabolic defects and epigenetic alteration (Fig. 6.2).

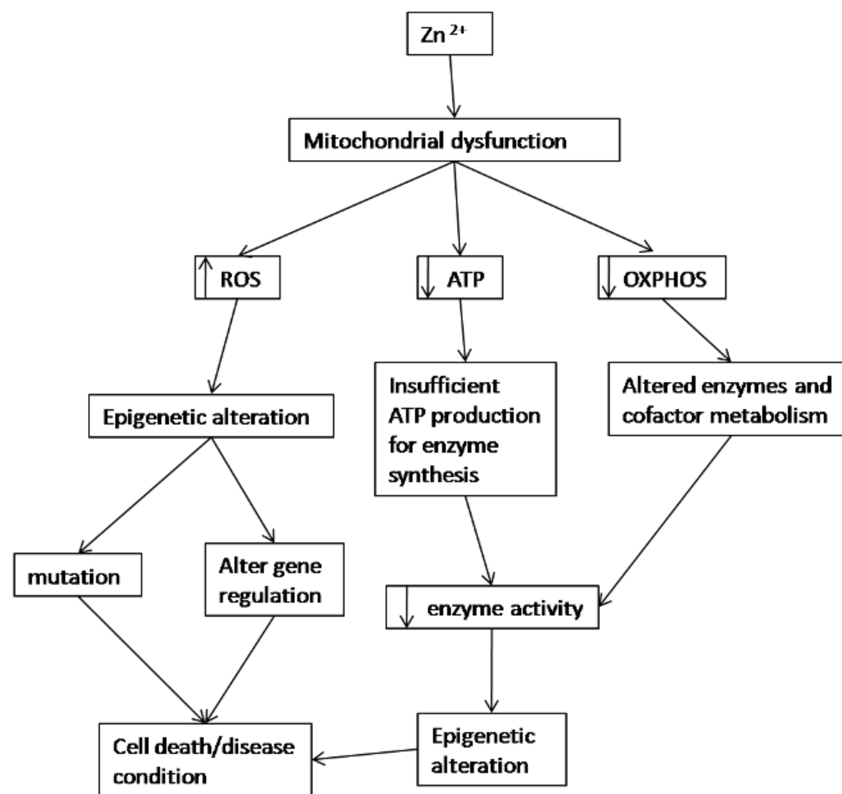



**Figure 6.2: Schematic model representing the effect on zinc-induced mitochondrial dysfunction and alteration in epigenetic function**. ROS accumulation in cells leads to epigenetic alteration, which may induce mutation and alteration in gene transcription that subsequently lead to cell death or neurodegenerative conditions. Insufficient ATP production, which is required for epigenetic enzyme synthesis, may also result in epigenetic dyshomeostasis. Inhibition in oxydative phosphorylation may affect enzyme and co-factor metabolism, leading to reduction in enzyme activity and consequently alteration in the epigenetic control. $Zn^{2+}$= zinc ions, ROS= reactive oxygen species, ATP= adenosine triphosphate, OXPHOS= oxidative phosphorylation.

## 6.5 MECHANISM OF NEUROPROTECTIVE DHA AGAINST ZINC TOXICITY

For every results chapter presented in this thesis, the neuroprotective effects of DHA against zinc toxicity were examined. High levels of oxidative stress, disruption of the electron transport chain and reduction in gene regulation are sufficient to cause apoptosis of neuronal cells. It was observed that DHA has the ability to restore zinc-

induced cellular dysfunction, which suggests the reversible effect of zinc toxicity. Each DHA-mediated neuroprotective mechanism presented in this thesis could be considered as a potential target for therapeutic intervention against zinc toxicity.

### 6.5.1 DHA inhibits $Zn^{2+}$ accumulation and toxicity in neuronal cells

Chapters 2 and 3 demonstrated the ability of DHA to protect against alteration in mitochondrial dysfunction following zinc treatment. Part of this effect could be due to neuroprotective function of DHA in regulating $[Zn^{2+}]_i$ in the cells, in which DHA inhibits excess zinc uptake through decreasing zinc transporter expression levels in M17 neuroblastoma cells [45]. Data also showed the ability of DHA to restore zinc-triggered ROS production, which is mainly due to its free-radical scavenging action as supported by *in vitro* and *in vivo* studies [345, 346]. In addition to direct attenuation of ROS, DHA has been found to restore ATP turnover rate and alteration in proton influx and therefore inhibit mitochondrial membrane potential and apoptosis (Chapters 2 and 3). Based on these studies, it is important to note that DHA may specifically restore zinc-induced mitochondrial alteration, but not directly interact with mitochondrial dysfunction following Aβ. Chapter 3 showed that DHA has no significant effect on Aβ-mediated ATP turnover, uncoupled respiration, maximal respiratory capacity and mitochondrial membrane potential.

### 6.5.2 Anti-apoptotic effect of DHA

DHA may maintain the integrity of the mitochondrial membrane and prevent the release of apoptosis mediators from the mitochondria and therefore block the apoptotic caspase cascade. The results showed that DHA treatment in M17 cells increases anti-apoptotic Bcl-2 and decreases caspase-3 expression levels (Chapter 5). This suggests that following DHA, pro-apoptotic c-jun *N*-terminal kinase (JNK) may be inactivated, followed by "turning off" downstream target c-Jun, which inhibit the cleavage of the key effector protease caspase-3, as observed in the study, therefore blocking the execution of apoptosis and prevention of nuclear DNA fragmentation, the molecular hallmark of apoptosis [347-349].

DHA may prevent apoptosis, by restoring the reduction of histone protein and mRNA levels back to the basal level and abolish the effect of zinc in inhibiting histone synthesis and therefore affecting DNA synthesis (Chapter 4). DNA fragmentation could also be possibly inhibited by neuroprotective DHA in attenuating ROS accumulation due to zinc toxicity. As previously mentioned, the ability of DHA in restoring zinc-mediated bioenergetic defects may be the first initiator in inhibiting neurotoxicity and cell apoptosis.

The recovery in ATP turnover rate following DHA may also provide the link between sufficient energy production and enzyme synthesis required for epigenetic regulation. Zinc and DHA have been reported to have opposing effects in epigenetic

regulation in M17 cells. Chapter 5 showed that DHA was able to restore epigenetic alteration following zinc treatment, which according to current literature has downstream effects in inhibiting apoptosis and neurodegenerative conditions. Specifically, DHA was shown to decrease HDAC expression and therefore enhance histone acetylation [215], which have been reported to be associated with neuroprotective gene expression, reduction in intracellular calcium release and stabilization of mitochondrial membrane potential [350].

Other studies also suggest the involvement of HDAC inhibitors against several mental disorders. Neurodegenerative disease and depression are the pathological conditions in which the increase in histone acetylation and reduction in HDACs are the valid therapeutic targets [215, 351]. Figure 5.9 showed the neuroprotective affect of DHA against epigenetic alteration in M17 neuronal cells and the down stream effects leading to cellular survival, based on the current literature. ROS prevention by DHA may also contribute to normal epigenetic regulation, as the ROS accumulation has been reported to attack DNA and consequently alter base structure leading to mutation and cell apoptosis [98, 352]. As a source of cellular energy function, increase in ATP production by DHA may also restore the epigenetic pattern by inducing effective enzymatic activity, which is required for epigenetic enzyme synthesis.

Taken together, there are several ways in which zinc toxicity leads to cellular dysfunction. However, it seems that there is no way to bypass mitochondria in the process of cell apoptosis and neurodegenerative conditions. Mitochondria are pivotal for extrinsic and intrinsic apoptotic pathways, which also play a central role in cell survival. DHA has been shown to restore zinc-triggered alteration in mitochondria, which may also mediate neuroprotection in other signaling mechanisms, such as epigenetic regulation. Finally, this thesis has provided the evidence that zinc homeostasis and neuroprotective effect of DHA play an important role in regulation of cell survival, which highlights the essential need for further investigation in this field.

## 6.6 CONCLUSION

The following conclusions can be drawn on the basis of the present findings regarding the possible mechanism of zinc toxicity in human neuronal cells and the ability of DHA to restore zinc-mediated cellular dysfunction.

1. Increase in oxidative stress and mitochondrial dysfunction may be the early signals of zinc-induced neurotoxicity and apoptosis. This bioenergetic impairment caused by zinc leads to the loss of mitochondrial transmembrane potential, which may subsequently lead to the formation of membrane transition pores and release of caspase activators. DHA is able to restore mitochondrial alteration induced by zinc toxicity, which account for the ability

of DHA to stabilize membrane potential, induce anti-oxidant effect to inhibit ROS production and preserve overall mitochondrial function.

2. Mitochondrial dysfunction in response to zinc may also be associated with reduction in histone expression levels and therefore affecting DNA integrity in neuronal cells. DHA has been shown to be neuroprotective against zinc toxicity by increasing histone protein and mRNA expression levels back to basal levels, indicating the ability of DHA to abolish the effect of zinc and stabilize DNA. Zinc also affects histone PTMs, which is possibly mediated by epigenetic enzyme dyshomeostasis and ROS production that subsequently alter DNA structure and function. Zinc specifically reduces histone acetylation and increases HDACs expression, which therefore inhibit gene transcription, whereas DHA restores acetylation dyshomeostasis and therefore increases gene regulation. This altered acetylation represents the condition of neurodegenerative diseases and apoptosis, mediated by zinc toxicity.

3. CoQ10 may be more directly protective against Aβ-triggered mitochondrial dysfunction than DHA. DHA, due to its direct molecular interaction with zinc, may directly protect against zinc-mediated mitochondrial alterations.

4. DHA may offer neuroprotection against zinc-induced toxicity and apoptosis that are multifactorial and may act through multiple intracellular signaling

mechanisms. Each mechanism presented in this thesis could be considered as a potential target for therapeutic intervention of neurodegenerative diseases.

## 6.8 FUTURE DIRECTION

The accumulation of endogenous oxygen radicals generated in mitochondria, ATP impairment and the consequent oxidative modification of biological molecules have been implicated to be responsible for the zinc-induced toxicity. It appears that these alterations lead to the formation of mitochondrial permeability transition pores that link the neuronal oxidative stress and overall mitochondrial dysfunction leading to cellular apoptosis. In this thesis, polyunsaturated fatty acid DHA is suggested to be neuroprotective against zinc toxicity in human neuronal cells. However, more extensive research is required to investigate the effects of DHA against zinc toxicity in relation to mitochondrial function and gene activities as there are still more research that needs to be conducted, such as:

1. It would be interesting to look at whether zinc-induced apoptosis is actually mediated by the formation of mitochondrial membrane pores, since in our current study the zinc-induced mitochondrial membrane pores formation is still hypothetical. This could be achieved by perhaps measuring the cytosolic release of mitochondrial apoptotic mediator proteins, such as cytochrome c or apoptotis inducing factor (AIF), as a result of mitochondrial membrane

pores, which are the key events in the mitochondria-dependent apoptotic pathway.

2. Other fatty acids such as the short chain omega-3 and the long chain omega-6 should be investigated in the future to ensure that the effects observed in our studies are indeed DHA-specific.

3. *In vivo* studies using an established animal model or primary cultured cells would be useful to clinically address the effects of zinc and DHA interaction.

4. To further examine whether DHA could restore zinc-induced mitochondrial dysfunction mediated by specific mitochondrial ion channels or proteins that contribute to mitochondrial membrane pore regulation, and thus may be useful for the therapeutic inhibition of bioenergetic failure and cell death.

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