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Differential Effects of Eicosaoentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) on Neurinal Precursor Cell Proliferation and Neurogenisis

Christopher Jenny

FLORIDA STATE UNIVERSITY

COLLEGE OF HUMAN SCIENCES

DIFFERENTIAL EFFECTS OF EICOSAPENTAENOIC ACID (EPA) AND DOCOSAHEXAENOIC ACID (DHA) ON NEURONAL PRECURSOR CELL PROLIFERATION AND NEUROGENESIS

BY

CHRISTOPHER JENNEY

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The members of the committee approve the thesis of Chris Jenney defended on July 16, 2009.

_____
Cathy Levenson
Professor Directing Thesis

_____
Jodee Dorsey
Committee Member

_____
Michael Meredith
Committee Member

Approved:

_____
Bahram Arjmandi, Chair, Department of Nutrition, Food & Exercise Sciences

_____
Billie J. Collier, Dean, College of Human Sciences

The Graduate School has verified and approved the above-named committee members.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

As much as 10% of the US population will experience at least one bout of depression within their lifetime. It has been reported that an increased time spent with major depressive disorder (MDD) results in a decreased volume in the hippocampus. This decreased volume is the result of apoptosis, or programmed cell death. In recent years it has become known that new neurons (neurogenesis) are continuously born in the hippocampus of humans. In fact, it now appears that antidepressant drug efficacy may be dependent on adult neurogenesis in the hippocampus.

At least six epidemiological studies have shown an inverse correlation between seafood intake and prevalence of mood disorders ($p<0.05$ or better). There is mounting evidence that this result is due to fish oils containing the long-chain, poly-unsaturated, omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Although extensive study has been undertaken using both of these omega-3s together, very little has been done to determine which fatty acid has the greater effect. Although previously thought to be readily interconvertible, there is mounting evidence that these two lipids are not treated equally in the body. EPA has produced greater cell proliferation over DHA in the B-lymphocyte cells, and DHA has even been known to cause a decrease at higher concentrations. Differences have also been reported in both molecular and behavioral outcomes.

This research tested the hypothesis that EPA facilitates proliferation and survival of neuronal precursor cells to a greater extent than does DHA. Human neuronal precursor cells were grown in the presence of EPA, DHA, and varying ratios of EPA and DHA to determine their dose-response relationships. While there were no large effects on proliferation or differentiation, EPA, but not DHA, protected cells from iron-induced oxidative stress. This protection appears to be, at least in part, the result of altered p53 translocation in EPA-treated cells. Future work will be needed to determine the role of this molecular protection in the antidepressant activity of EPA.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Depression and Neurogenesis

As much as 10% of the US population will experience at least one bout of depression within their lifetime[1]. Approximately 15% of people with severe depression eventually commit suicide.[2] It has been reported that an increased time spent with Major Depressive Disorder (MDD) results in a decreased volume of the hippocampus.[3] This decreased volume appears to be partially due to programmed cell death, or apoptosis. Apoptosis has been observed in the hippocampus of adult MDD patients.[4] However, in recent years it has become known that new cells are continuously born in the hippocampus.[5] This includes new neurons in humans.[6] Thus, it is also possible that the decreases in volume seen in MDD could be the result of a reduction in neurogenesis. It has also been shown that antidepressants' action to promote recovery from depressive disorder can include neurogenesis[7,8].

Neurogenesis is the combination of 1) proliferation of stem cells and precursors, and 2) differentiation of precursors to neurons. Successful neurogenesis also involves regulation of apoptotic processes that govern stem cell and neuronal survival.[9,10] The presence of cellular proliferation of precursors is important in that it is an indication of possible neurogenesis. It has been shown that an increased proliferation in the hippocampus can aid in depression recovery.[7,8]

Several factors have been identified as influences in neurogenesis. For example, increased glucocorticoid levels result in a decrease in neurogenesis.[11] Dietary restriction can produce increased neurogenesis,[12] via increased stem cell survival.[13] And, oxidative stress can cause neuronal apoptosis via the p53 tumor suppressor protein.[14]

Fish Consumption and Fish Oil Studies

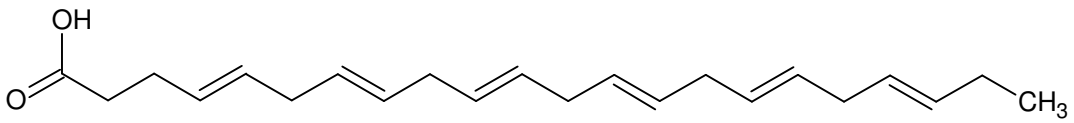
At least six epidemiological studies have shown a significant inverse correlation between seafood intake and prevalence of mood disorders ($p<0.05$ or better).[15] In one study of 3,204 Finnish adults, depressive symptoms and fish consumption were measured. After analysis, the likelihood of depression was significantly lower among frequent fish consumers ($p<0.01$).[16]

An extensive meta-analysis of double-blind, placebo-controlled clinical trials looked at various studies using the long-chain polyunsaturated, omega-3 fatty acids (FA) found in fish, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in relation to their effect on standardized scores on the Hamilton Depression Rating Scale (HDRS) or Children's Depression Rating Scale (CDRS) of depression in human patients. In all studies, participants were given capsules of DHA, EPA or a combination of the two.[2] The results suggest a striking biological difference between these two FA. Those studies that used DHA alone or a mixture containing predominately DHA observed no significant change in test scores, including one more recent follow-up study.[17] Meta-analysis of the data also revealed that although EPA alone had a significant effect in improving depression scores, a combination containing predominately more EPA than DHA resulted in significantly better scores.[2] Although no single study in the analysis tested various combinations and dosages of EPA and DHA, from this analysis, it appears that an EPA:DHA ratio of 2:1 may be most beneficial.

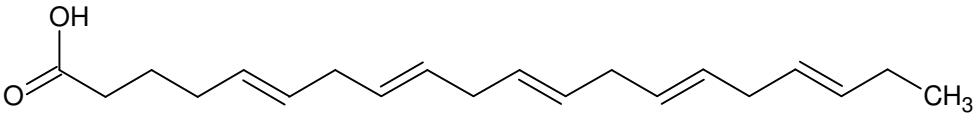
DHA

Docosahexaenoic acid (DHA) is a long-chain, polyunsaturated, omega-3 fatty acid. It is designated as 22:6n-3 in that it is 22 carbons long, with 6 *cis* double bonds (thus polyunsaturated), and the last double bond between the 3rd and 4th carbons from the omega end, that is, the end opposite the carboxylic acid functional group (Figure 1-1). Other biologically relevant unsaturated lipids have the last double bond 6 carbons from the end (omega-6) or 9 from the end (omega-9).

The adult brain consists of 50–60 % lipids, of which 35 % are mainly the long-chain polyunsaturated fatty acids (LCPUFA) arachidonic acid (AA, 20:4n-6) and DHA.[18] Fatty acids in human brain cellular membranes are very plastic and changeable. About 5% of AA and DHA in the mammalian brain are replaced daily utilizing plasma lipids.[19] More specifically, the primate cerebral cortex has the capability to adjust its fatty acid composition.[20] Dietary restriction of omega-3 FA across generations leads to reduced neuronal membrane DHA levels and impaired brain function in rats.[21] Others have observed that 10μM DHA in media of cell culture inhibits apoptosis in the HL60 human blood cell line.[22]



Docosahexaenoic Acid (DHA)



Eicosapentaenoic Acid (EPA)

Figure 1-1. Chemical structures of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)

EPA

Eicosapentaenoic acid (EPA, 20:5n-3) is also a long-chain, polyunsaturated, omega-3 fatty acid (Figure 1-1). Although DHA is the predominant membrane lipid, not all omega-3 fatty acid can be converted to DHA. The conversion of shorter-chain PUFA to EPA and DHA are highly limited,[15] with less than 1% being converted to DHA.[23] EPA incorporated into cells is not converted into DHA in B-lymphocyte (Raji) cells. EPA produced greater cell proliferation over DHA in the B-lymphocyte cells, and DHA has even been known to cause a decrease at higher concentrations.[24]

Neurogenesis and p53

The p53 tumor suppressor protein is an important transcription factor in cell-cycle regulation and apoptosis for the preservation of genetic integrity through tumor suppression. When activated it leads to cell-cycle arrest allowing for repair of DNA; however, if damage is too severe the p53 protein can lead to apoptosis. Under normal conditions, there is a low level of p53 present, mainly localized in the cytoplasm.[25] As a result of DNA damage or environmental stress, the p53 protein, which normally has a half-life of 15-30 minutes, is stabilized through dephosphorylation leading to conformational change, accumulation, activation and translocation to the nucleus.[26] The protein is also activated through acetylation as a reaction to environmental stress.[27]

Reactive oxygen species (ROS) have been shown to be involved in apoptosis.[28] Iron is a redox active element that can create reactive oxygen species. Iron has previously been used as a physiologically relevant oxidative stressor to induce apoptosis.

This research tested the hypothesis that EPA facilitates proliferation and survival of neuronal precursor cells to a greater extent than does DHA. In Aim 1 of this study, human neuronal precursor cells were grown in the presence of EPA, DHA, and varying ratios of EPA and DHA to determine their possible dose-response relationships in the promotion of cellular proliferation. Aim 2 of the study tested the effects of the various ratios of EPA and DHA to induce precursor differentiation to a neuronal phenotype. Finally, Aim 3 tested whether the omega-3 fatty acids promote survival of neuronal precursor cells when challenged with an oxidative stressor and whether EPA's effect on p53 is indicated as a possible mechanism in that survival.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture

Human Ntera-2 (NT2) neuronal precursor cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (GIBCO, U.S.), 10% Cosmic Calf Serum (HyClone, U.S.), 1% antibiotic/antimycotic, and 0.1% gentamicine at 37ºC with 5% CO_2 in Corning T-75 cell culture flasks.

Fatty Acid Treatment

Eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) were obtained from Sigma-Aldrich, aliquoted in ethanol and kept in glass under nitrogen at -20ºC. For proliferation studies, cells were plated at 70,000 cells per well in 6-well plates. After 24 hours, medium was replaced to remove redox active species and cellular debris. New medium containing fatty acids in concentrations of 0, 5μM, 10μM, 15μM and 20μM was replaced on cells for 24 hours; washed with TBS, removed from wells enzymically (Accutase, Sigma-Aldrich Corp. St. Louis, MO), and counted using a Cedex Hi-Res cell counter by Innovatis. Viable cell densities and percentage viability were logged.

Immunocytochemistry for Differentiation

Immunocytochemistry was used to establish differentiation of NT2 precursor cells to NT2-N neuronal cells. Cells were plated on cover slips in 6-well plates at a density of 50,000 cells per well. The following day, cell medium was replaced with fresh medium containing EPA, DHA, or vehicle. After 72 hours treatment, cells were washed with PBS (+Ca, Mg), fixed with 4% paraformaldehyde for 10 minutes and washed three times with PBS. Cell membranes were made permeable with 0.2% Triton x-100 for 5 minutes at room temperature and washed three times with PBS. Cells were then pretreated with bovine serum albumin (BSA) for 15 minutes at room temperature prior to receiving antibodies. The mouse anti-human TuJ1 antibody (1:500) was used as a primary antibody to identify neural differentiation. Cells were treated with 1 mL

per dish overnight at 4ºC. Cells were washed three times with BSA for two minutes each and then pretreated for 10 minutes with BSA before receiving the secondary fluorescently labeled (Cy3) goat anti-mouse antibody for one hour at 37ºC. Cells were washed three times with PBS before receiving a fluorescent stain, DAPI (4',6-diamidino-2-phenylindole)(1:300). DAPI easily enters cells and binds tightly to DNA, labeling cell nuclei. Cells were treated with DAPI (1:300) for ~10 minutes and washed three times with PBS before mounting on slides with an anti-fade gelmount.

Iron Treatment

Cells were plated at 40,000 cells per well in 6-well plates which were scored with a grid pattern on the bottom of each well. After 24 hours, medium was replaced to remove redox active species and other cellular debris. New medium containing fatty acids was left on cells for 24 hours and replaced with new medium containing fatty acids again (n=2 wells each treatment) and 500μM iron (ferrous sulfate) for 72 hours. Every 24 hours, digital photographs (100x) were taken of three locations in each well. Scored bottoms assured photography recurred in the same locations. Photographs were printed and cells were hand counted.

Immunocytochemistry for p53

Cells were plated on cover slips in 6-well plates at a density of 40,000 cells per well. The following day medium was replaced with fresh medium containing 15μM EPA or its vehicle (ethanol). After 24 hours of lipid pretreatment, medium was replaced again with three treatments: 1) vehicle; 2) vehicle + 500μM Fe; or 3) 15μM EPA + 500μM Fe. After 72 hours treatment cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and washed three times with PBS(-). Cell membranes were made permeable with 0.2% Triton x-100 for 5 minutes at room temperature and washed three times with PBS. Cells were then pretreated with BSA for 15 minutes at room temperature prior to receiving antibodies. Cells were treated with a goat anti-human p53 protein antibody (1:500 in BSA) and incubated for 1 hour at 37ºC. Cells were then washed three times with PBS with 2 minutes agitation followed by a 5-minute incubation at room temperature with BSA prior to receiving the second antibody. Cells were incubated with a rabbit anti-goat antibody labeled with Cy3 for 1 hour at 37ºC followed by three washes with PBS with 2 minutes agitation. Cells were treated with DAPI (1:300) for 10 minutes

at room temperature followed by three washes with PBS. Cover slips were then removed from wells and mounted on standard microscopy slides using a gelmount with anti-fade agent. Cover slips were sealed with nail polish after 24 hours drying.

Statistical Analysis

All statistical analyses were performed utilizing GraphPad Prism (GraphPad Software Inc, La Jolla, CA). Data were analyzed by ANOVA with Bonforroni post hoc tests.

CHAPTER 3

RESULTS

Cellular Proliferation

EPA and DHA were individually tested on NT2 precursor cells at concentrations of 0, 5μM, 10μM, 15μM and 20μM and ethanol vehicle (n=6, two replicates). After 24 hours growth, DHA treated cells exhibited a dose-response relationship (Figure 3-1) with a significant increase in cellular proliferation at a concentration of 15μM ($p<0.05$). The EPA treated cells (Figure 3-2) showed significant increases in precursor proliferation at concentrations of 5μM and 15μM of the lipid (both $p<0.05$). The fatty acids were also tested in combinations of 5μM EPA with 10μM DHA, and 10μM EPA with 5μM DHA with no significant effect (results not shown).

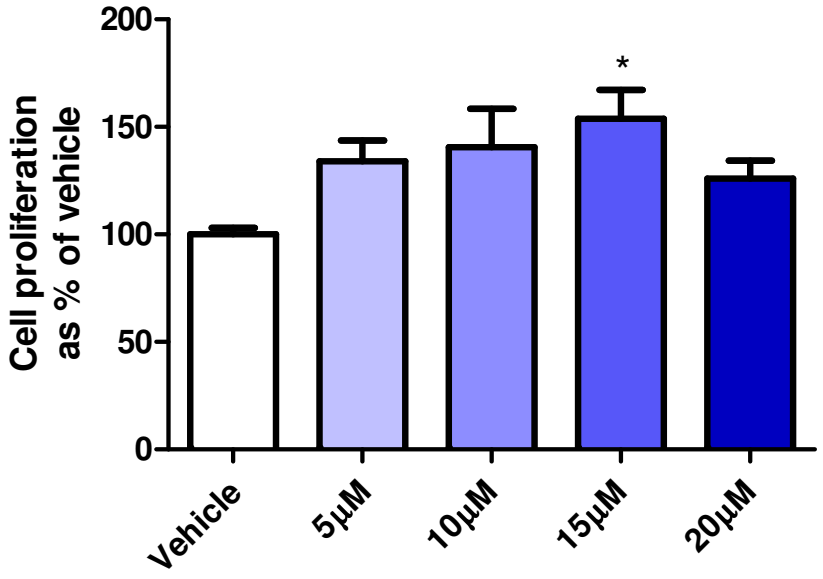


Figure 3-1. Precursor cell proliferation after 24 hours treatment with various concentrations of DHA. (n = 6 in two replicates) * is significantly different from vehicle at $p<0.05$

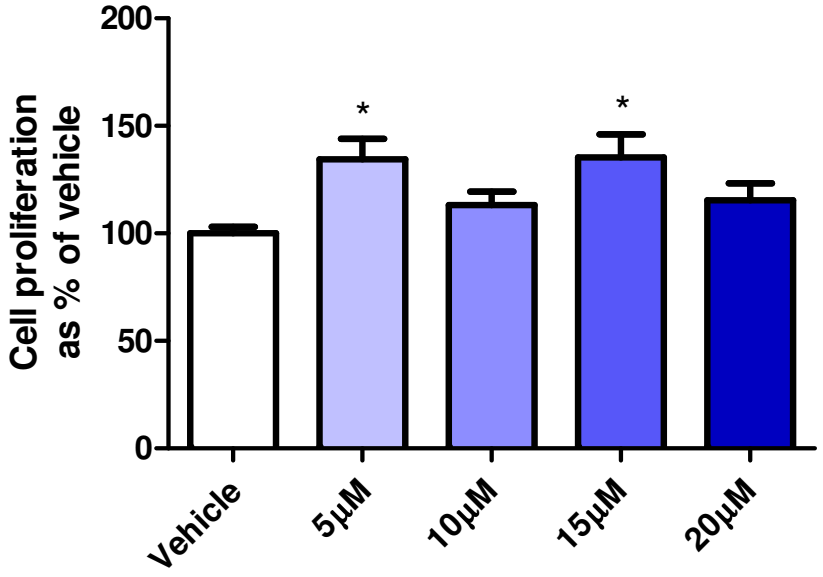


Figure 3-2. Precursor cell proliferation after 24 hours treatment with various concentrations of EPA. (n = 6 in two replicates) * is significantly different from vehicle at $p<0.05$

Cellular Differentiation

Human NT2 cells were treated with 5µM EPA, 15µM EPA, 5µM DHA, 15µM DHA, 5µM EPA with 10µM DHA, or 10µM EPA with 5µM DHA. Also included were regular and vehicle controls (n=4, two replicates). Analysis of cellular differentiation using the early marker TuJ1 and fluorescent microscopy showed no distinguishable differences between treatments (results not shown).

Iron Treatment

To test for a potential protective effect of the fatty acids against iron induced apoptosis, a series of experiments were undertaken, prior to fatty acid treatment, to determine a level of iron toxicity for NT2 precursor cells prior to differentiation to NT2-N cells. These series included one at 5µM, 10µM, 15µM, 20µM and 25µM iron, and repeated at 25µM, 50µM, 75µM and 100µM, iron and again at 100µM, 200µM, 300µM, 400µM and 500µM iron. It was not until a concentration of 500µM iron was reached before there was a considerable loss in cell number (figure 3-3) resulting in 50% death compared to control.

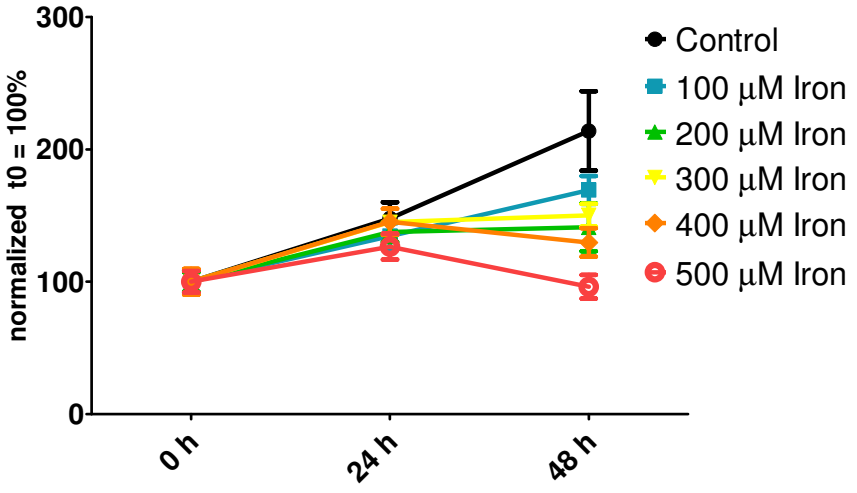


Figure 3-3. Treatment with 500µM Fe results in 50% death of NT2 precursor cells compared to control. (n = 6 in two replicates)

Once this concentration was determined, precursor cells were pretreated with fatty acids 24 hours prior to iron treatment. Iron treatment included a repeat of the corresponding fatty acid treatment. After 72 hours of iron or iron + lipid treatment there was a significant difference between the growth/survival of EPA-treated cells and cell survival from all other treatments (figure 3-4). Survival of EPA-treated cells differed from both Fe ($p<0.05$) and DHA ($p<0.001$) treated cells. Additionally, the 15µM EPA treatment was significantly different from both combinations of lipids ($p<0.05$) which included lower concentrations of EPA (<15µM). There was no statistically significant difference between cell survival in the DHA and Fe treated cells.

p53 Expression

Neuronal precursor cells were treated with 500µM iron or 500µM iron + 15µM EPA, and fluorescently stained for the p53 protein. Representative images are shown in Figure 3-5. A qualitative analysis of digital photos taken through fluorescent microscopy indicates an obvious decrease in extranuclear p53 in cells treated with EPA compared to non-EPA treated cells that were given iron only. Differences in nuclear p53 are less evident between treatments.

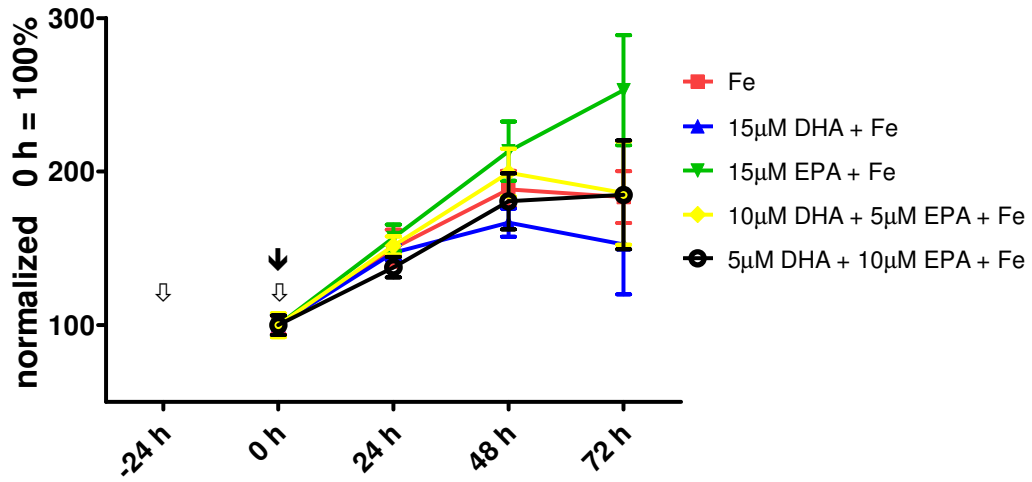


Figure 3-4. Pretreatment of NT2 cells with fatty acids for 24 hours followed by 72 hour treatment with 500μM Fe and repeat of FA treatment. Survival of 15μM EPA treated cells was significantly greater than DHA treated cells ($p<0.001$) and all other treatments ($p<0.05$) ($n = 12$ in two replicates)

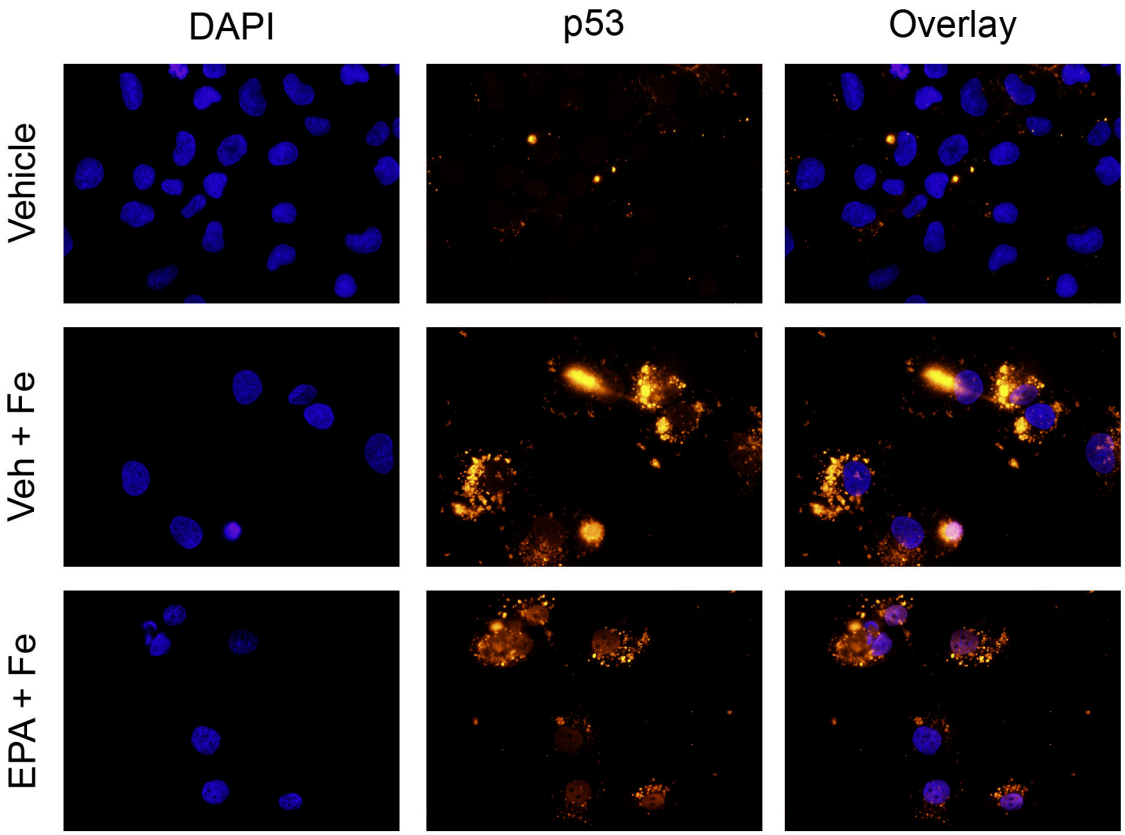


Figure 3-5. Cells pre-treated with EPA show a decrease in extranuclear p53 after iron treatment compared to cells treated with iron only.

CHAPTER 4
DISCUSSION

Cellular Proliferation

In the present study, treatment with both FA resulted in significant increases in proliferation at concentrations of 15μM, however the two combinations of lipids totaling 15μM did not show significant results. This suggests that there is not a synergistic effect between the fatty acids, and there is no additive effect. In fact, the opposite effect was noted when the quantity of DHA exceeded EPA. At 5μM EPA alone, there was increased proliferation, yet with the addition of 10μM DHA (5μM EPA + 10μM DHA) there was no significant increase in proliferation. It is as if, in the presence of EPA, the DHA acts to nullify its effects.

This increased proliferation seen here by both EPA and DHA, combined with a lack of evidence for differentiation is consistent with the results of another study which looked at PUFA's effects on behavior and cellular changes in the hippocampus of mice.[29] The study compared a control diet containing only omega-6 PUFAs with an enriched diet containing both omega-6 and omega-3 fatty acids, including DHA (0.14% caloric intake) and EPA (0.35%). After six weeks on the enriched diet, mice showed significant improvement in the Forced Swim Test (FST, a behavioral model of despair predicting antidepressant effects), Tail Suspension Test (TST, another model used to evaluate antidepressant effects) and Novelty-Suppressed Feeding Test (NSFT, a model for anxiety), all suggesting the omega-3 fatty acids acted as an antidepressant. In a second step, mice were fed a diet enriched with a high level of omega-3 (70% omega-3 alpha-linolenic acid (ALA), 25% linoleic acid (LA) and 5% omega-9 oleic acid). After five weeks, supplemented-mouse behavior exhibited an antidepressant-like response. There was a significant decrease in immobility and significant increases in the duration of both swimming and climbing in the FST. In another round lasting only three weeks, a low dose of the antidepressant Imiprimine (10mg/kg) or the PUFA supplementation did not produce significant reduction of immobility in the FST; however, the combination of the two treatments together resulted in a significant additive effect.

At the cellular level, five weeks PUFA supplementation resulted in significant increases in hippocampal volume compared to placebo. Additionally, a significant increase in BrdU labeled cells was observed in the dentate gyrus (DG) of PUFA supplemented mice and neuronal differentiation was observed by immuno-labeled Neuronal specific Nuclear protein (Neu-N). The study did not test for differences between DHA and EPA and the differentiation noted was not significant. However an increase in hippocampal volume combined with increased cell proliferation in the DG was produced by omega-3 lipids. Without an increase in the number of neurons, the increased mouse hippocampal volume could be explained by either an increased proliferation of progenitor cells, an increase in non-neuronal cell types, an increase in size and complexity of existing neurons (synaptogenesis), or some combination thereof. For example, it has been shown that DHA supplementation will increase neurite length and complexity in rat hippocampal culture and a DHA deficient diet will shorten neurite growth in vivo.[30,31]

In another study, cell proliferation and gene expression were studied in B-lymphocyte cells treated with either EPA or DHA.[24] Significant increases in cell proliferation were noted for both FA; however, unlike the present study, there was a marked difference between proliferation of DHA vs. EPA treated cells, with EPA producing significantly greater proliferation ($p<0.05$) at concentrations of 12.5μM, 25μM, 50μM and 75μM. While EPA produced increased proliferation at all levels tested, DHA produced no difference at 50μM and a decrease in proliferation at 75μM.

DHA and EPA treatments resulted in marked differences in gene expression as well. Cells were treated with 25μM of either FA for 24 hours. Gene expression was determined by macroarray analysis. Results are summarized in Table 4-1.

As seen from Table 4-1, there was a distinct difference between DHA and EPA in the change of gene expression as compared to control. Although EPA induced a greater number of pro-apoptotic genes, this FA also produced a greater defensive response. It is worth noting that despite the greater pro-apoptotic gene expression of EPA treated cells, these cells were proliferating at a higher rate than DHA treated cells at the same concentration. This can be explained by the possible down-regulation of the tumor suppressor protein p53. As seen in the present study, EPA resulted in a decrease in extranuclear p53 in NT2 cells (further discussed below). In neuronal apoptosis, Bax has been shown to be a mediator of cell death and is p53 dependent.[32] Additionally, caspase-3 is linked to damaged DNA and apoptosis[33] and downstream

activation of caspases is prevented by inhibition of p53 in hippocampal neurons.[32] If EPA down-regulates p53 in B-lymphocyte cells as well as seen here in NT2s, this could explain why it results in greater proliferation despite the greater pro-apoptotic gene response it creates. Additionally, if EPA is inducing defensive genes in NT2 cells, as seen in B-lymphocytes, it would further explain the protective roll of EPA under oxidative stress seen in the present study.

Table 4-1. Fold-changes in gene expression of B-lymphocyte cells after treatment with 25μM DHA or EPA. (Verlengia R.et al., 2004)

Gene		DHA	EPA
Cell Cycle:			
CDK10	Cyclin-dependent kinase 10		+2.3
CCNE	G1/S-specific cyclin E		+3.4
Pro-apoptotic			
TGF-beta	Transforming growth factor beta	+3.4	
CD27LG	CD27 ligand		+4.7
BAX	BCL-2 –associated X protein membrane		+2.8
CASP3	Caspase 3		+2.1
BIK	BCL-2 interacting killer protein		+6.8
Anti-apoptotic			
Bcl2	B-cell leukemia protein 2	+3.9	
Bcl-xl	Apoptosis regulator bcl-x		+2.3
Defensive			
SOD1	Cytosolic superoxide dismutase 1		+2.2
GSTP1	Glutathione S-transferase pi		+4.2
HSP70-1	Heat shock 70 kDa protein 1		+2.5
Growth			
IGF1	Insulin-like growth factor		-5.3

Future work is needed to examine the role of EPA and DHA on hippocampal stem cell proliferation to further understand the mechanisms responsible for their action. It is possible that the effect of these FA is not only on neuronal precursors. In vivo, other cell types may be affected, such as synaptogenesis in post-mitotic neurons, supporting cells such as astrocytes, or possibly endothelial cells making up the blood-brain barrier. Results from the present study suggest the protective effect of FA in the diet[15,16] or from supplementation[2] seen in other studies

may not be entirely mediated via increased precursor proliferation, but may be from one of the other components of neurogenesis.

Cellular Differentiation

As described previously, another factor in neurogenesis is increased progenitor differentiation to neurons. NT2 cells display many characteristics of precursor cells committed to the neuronal line.[34] Additionally, treatment with retinoic acid induces differentiation to a neuronal phenotype (NT2-N), as shown by expression of neuron-specific indicators such as Tau proteins[35] and Neuronal Class III β-Tubulin (TuJ1).[36]

To test for differentiation to NT2-N neurons in the present study, cells were treated with DHA and/or EPA then immuno-stained for TuJ1, a marker of early neuronal differentiation. The lack of any notable difference in TuJ1 expression from control or vehicle at any concentration or combination of FA suggests that the benefit of these omega-3 lipids is not coming from an increase in precursor cell differentiation to new neurons.

Oxidative Stress and p53

It has previously been shown that as little as 3μM iron will elicit p53-mediated apoptosis in differentiated neuronal cells in culture.[37] The concentration of 500μM used here is much higher than the 3μM used on the differentiated cells. Even though such a high concentration was needed to elicit a response, these data suggest that the protective effect of these FA comes at least in part through the action of EPA, but not DHA, working as a protectant against oxidative stress and the resultant apoptosis in neuronal precursor cells.

Treatment of NT2-N neuronal cells with oxidative stressors such as iron has caused increased nuclear p53, indicating that oxidative stress may be one cause of p53 and apoptosis.[14] In that experiment, using differentiated NT2-N neurons, cells were exposed to 5μM iron for 18 hours. Cells were immunostained for p53 and nuclei were labeled with DAPI. Results showed increases in p53 in the nuclei along with morphology consistent with apoptosis. The study also found similar results using copper as the oxidative stressor. Oligonucleotide arrays of mRNA from copper stressed cells revealed the expression of both pro- and anti-apoptotic genes, including the anti-apoptotic HSP70 seen above.

After showing a difference in the protective roles of EPA and DHA, the hypothesis was tested that EPA is protecting cells from apoptosis through down regulation of the p53 tumor suppressor gene. As described above, a previous study found a marked difference in effects on gene transcription between EPA and DHA, with EPA up-regulating almost three times more genes.[24] The p53 gene was not one of the tested genes. In the current experiment there was a difference between the iron only and iron + EPA treated cells with an obvious reduction in extranuclear p53 in EPA treated cells suggesting that EPA is protecting the cells from mitochondrial apoptosis.

In another mechanism of apoptosis, oxidative stress can lead to translocation of p53 protein to the mitochondria, p53-mediated apoptosis via permeabilization of the mitochondrial membrane and subsequent release of cytochrome c into the cytosol.[38] This was evidenced by a study of oxidative stress-induced apoptosis in glial cells. The rat C6 glioma cell line and rat primary cultures of astrocytes were exposed to H_2O_2-induced oxidative stress.[39] Through immunofluorescence analysis, p53 was detected almost entirely in the nuclei of both cell types during a 1 hour stress period. During a 24 hour recovery period p53 levels increased, with localization mostly in the cytoplasm. Immunofluorescence of cytochrome-c showed normal mitochondrial distribution during the stress period, but indicated progressively increasing mitochondrial apoptotic changes during the recovery period.

To further identify the location of p53, primary astrocytes were exposed to the 1 hour stress followed by 6 or 24 hours recovery. Mitochondria and p53 were stained for immunofluorescent microscopy. Results confirmed a mitochondrial localization of p53 after exposure to oxidative stress. In addition to this study, apoptotic cell death previously has been shown to increase mitochondrial ROS in the NT2 cell type as well.[40]

It is interesting to note that p53 may be acting primarily as a transcription factor in the nucleus of the differentiated form of this cell line, while this research indicates that p53 is acting via mitochondria in the undifferentiated form. Clearly, these two cell phenotypes behave rather differently, further evidenced by the 100-fold difference in iron concentration needed to induce oxidative stress. Without further comparison of the genetic expression between these two forms, it is difficult to hypothesize a reason for these observations.

Omega-3 Fatty Acids and Seretonin

Another possible influence of omega-3 fatty acids on cells may be via the neurotransmitter serotonin (5-hydroxytryptamine, or 5-HT). The class of antidepressant drugs termed selective serotonin reuptake inhibitors (SSRIs) are the most frequently prescribed medications for the treatment of depression.[41] They act to increase the synaptic level of serotonin available for binding to postsynaptic receptors. It has been shown that SSRIs significantly increase the number of neural precursor cells in the human DG in patients with MDD.[42]

In studies investigating the relationship between fatty acids and serotonin, it was shown that plasma DHA levels were significantly inversely correlated with the concentration of the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in human cerebral spinal fluid analyzed by gas chromatography-mass spectroscopy.[43] An EPA-supplemented diet significantly reversed most depression related indices induced by interleukin-1β (IL-1) as measured by the elevated plus maze and Morris water maze. Rats were administered IL-1 and fed basal diet supplemented with either EPA, or one of the omega-6 lipids AA or γ-linolenic acid (GLA, 18:3n-6). The EPA-fed rats also showed a significant increase in 5-HT brain concentration as measured by HPLC.[44]

Not only has it been shown that these FA have an influence on 5-HT, but also on at least one of its receptors. Long-term feeding of an omega-3-deficient diet (from weaning to 60 d) induced a significantly higher density of a serotonin receptor (5-HT_{2A}) in the rat brain compared with control.[45]

One possible mechanism of the lipids' effect on serotonin or its receptors may be through its influence on the neuronal membrane. With fish or fish oil consumption, EPA and DHA partially replace omega-6 FA, in particular AA.[46] It has been suggested that an imbalance between omega-3 and omega-6 FA may cause defects in neurotransmission by altering membrane fluidity.[47] DHA deficiency markedly alters membrane-bound enzyme and ion channel activities.[48] The resilience of plasma membranes also may be directly at risk via oxidative stress. Free radicals can assault the polyunsaturated FA of membrane phospholipids, resulting in what is termed lipid peroxidation.[49] Having a ready supply of PUFA available to replace damaged lipid may be another mechanism by which EPA is protecting cells from oxidative stress. Lipid peroxidation is also seen in neurodegenerative diseases.

Neurodegenerative Disease

The protective role of EPA may have implications for use in treating disease characterized by oxidative stress leading to massive neuronal death, such as neurodegenerative diseases like Parkinson's disease (PD) and Alzheimer's disease (AD), ischemia and traumatic brain injury. For example, in PD, high levels of iron result in oxidative stress and neurodegeneration.[50] One study found that chelation of iron significantly reduced ROS and the resultant cell loss in mice produced by administration of the Parkinson's-inducing agent MPTP.[50] The authors noted, however, that there are disadvantages to iron chelation. Some iron chelators lower serum and brain levels of vitamin B12. Also, iron is an essential nutrient in the brain needed for many biological functions such as synthesis of dopamine. Another study confirmed the presence of mitochondrial ROS in MPTP treated mice, but also found that severe iron restriction in cultured neurons led to evidence of apoptosis and expression of p53.[37]

The current study provides evidence that EPA can protect against oxidative stress, including that created by iron. This suggests that omega-3s could play a protective role in the treatment of neurodegenerative disease. The addition of EPA to chelation therapy may allow for better neuronal protection as well as reduce the need for excessive iron chelation. DHA may play a role as well. In a recently published review looking at omega-3s and neurodegenerative disease, the authors found much evidence for an important protective role of DHA in both PD and AD in epidemiological studies, postmortem studies and animal trials.[51]

Conclusions and Future Directions

There is mounting evidence that the long-chain, poly-unsaturated, omega-3 fatty acids EPA and DHA have a preventative and recuperative effect on MDD. The meta-analysis of clinical trials described above is strong evidence that EPA and DHA have a recuperative effect on depression.[2] These results along with those of the present study, demonstrate that EPA and DHA are distinguished in their effect on cellular metabolism. However, their unique mechanisms of action are not entirely understood. Although clinical trials have been undertaken using both of these omega-3s together, very little has been done to determine which fatty acid, EPA or DHA, has the greater effect.

This gap in knowledge presents a problem in understanding the usefulness of the observed beneficial effect of omega-3 oils. This work was undertaken to begin filling that gap.

Knowledge of their mechanism of action could be used in the creation of new treatments for those suffering MDD, PD or AD. This knowledge could be applied to nutritional therapy design and pharmaceutical development for treatment of depression, possibly other mood disorders, as well as neurodegenerative disease. Clearly EPA and DHA have varying roles in their effects on cells, and more needs to be understood about that difference. Of primary importance is to utilize the findings of this research in animal studies with the end goal to further this research in human clinical trials that could lead to dietary guidelines for neuro-protection and stimulating neurogenesis in the prevention and treatment of depression and neurodegenerative diseases.

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BIOGRAPHICAL SKETCH

Chris Jenney received his Bachelor in Business Administration from East Tennessee State University in 1989, where he was involved in extracurricular activities such as the Student Government Association, a professional co-ed business fraternity and a campus service organization. Chris went on to receive a Master's in International Business Studies in 1992 from the top-ranked graduate program at the University of South Carolina.

Chris has over ten years experience in operational and financial business management. Highlights include a sole proprietorship, Quality Assurance with General Motors in Venezuela, accounting systems & computer networks implementation, and nine years product development, international purchasing and import for top-line product manufacturers working with Asian factories (Hong Kong, Taiwan, China).

Along the way, Chris became very interested in nutrition, particularly dietary nutrients' effects on brain chemistry, mood and behavior. Believing that finding a life's passion is a rare occurrence that should not be ignored, Chris began studying toward a second career. In preparation, he enrolled in science courses at Florida Atlantic University, where for two years he volunteered in the lab of neuroscientist Dr Rui Tao, and taught recitation courses in General Chemistry and Organic Chemistry. He continued his studies at Florida State University receiving a Master's in Nutrition Science in 2009 while performing his thesis research under the guidance of Dr Cathy Levenson. Additionally, Chris had the pleasure of teaching Anatomy & Physiology Lab I and Anatomy & Physiology Lab II for the University's College of Human Sciences.

Chris will begin work toward a PhD in Neuroscience in the fall of 2009 in the College of Medicine at Pennsylvania State University's Milton S Hershey Medical Center. He intends to continue working in academia with hopes to further his research and continue teaching at university.