Impact of Omega-3 Supplementation with Alpha-Linolenic Acid on Neuronal Cell Fatty Acid Status

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IMPACT OF OMEGA-3 SUPPLEMENTATION WITH ALPHA-LINOLENIC ACID
ON NEURONAL CELL FATTY ACID STATUS

by

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Abstract

A method of treating retinoic acid differentiated SH-SY5Y neuroblastoma cells with alpha-linolenic acid (ALA) in the free acid form has been developed in order to examine impacts of such supplementation on fatty acid composition of cells and on functional outcomes. Through Bovine Serum Albumin: ALA conjugation, an ALA treatment media was created and compared to the stock media which contained no detectible amounts of ALA. Results from fatty acid analysis of the control cells (n=3) and ALA-supplemented cells (n=3) showed a significantly higher content of ALA in ALA-supplemented cells (37.35 mol%) compared to control cells (<0.01 mol%), and a significantly higher amount of eicosapentaenoic acid (EPA) in ALA-supplemented cells (2.51 mol%) compared to control cells (<0.01 mol%). These results indicate that the method of media enrichment of ALA on SH-SY5Y neuroblastoma cells proved successful. The results also show that unenriched SH-SY5Y neuroblastoma cells incubated in 1% FBS are devoid of the essential fatty acids ALA and EPA, and that SH-SY5Y neuroblastoma cells are capable of converting ALA to EPA. This research provides the framework and basis to study the extent to which ALA supplementation affects functional outcomes, such as neurotransmitter release.

Background

Omega-3 fatty acids, particularly the long chain polyunsaturated fatty acids EPA and docosahexaenoic acid (DHA), are present in cell membranes and can influence biophysical properties of the membrane which can affect activity of transmembrane proteins.¹ For example, omega-3 fatty acids displace cholesterol in the membrane and can thus increase membrane fluidity. Considering that an optimal fluidity is needed for neurotransmitter binding and signaling within the cell, omega-3 fatty acids are essential for proper cell communication.² The omega-3
fatty acids ALA, EPA, and DHA and the omega-6 fatty acid arachidonic acid (AA) compete for the sn-2 position on phospholipids. The relative proportions of these fatty acids in the membrane can determine their availability as substrates for cyclooxygenases and lipoxygenases after phospholipase cleavage. The balance among these fatty acids, EPA, DHA, ALA, and AA, thus influences inflammation mediator autocoid production, such as eicosanoids and resolvins. Cell and organ function, and other biological processes, are thus impacted by the fatty acid composition of the membrane.¹

Omega-3 fatty acids are known to be present in the brain and impact function, however DHA is much more abundant than ALA and EPA. ALA can be converted to EPA and DHA through the enzymes Δ6-desaturase, elongase, and Δ5-desaturase (Figure 1). Unfortunately, this conversion rate is low, with less than 8% of ALA being converted to EPA, and less than 4% of ALA being converted to DHA. Because of the small amount of DHA and EPA found in adipose tissue, storage is limited for these fatty acids and continuous dietary intake is required to maintain adequate levels in tissues.¹

Figure 1: Desaturase and elongase pathway of omega-6 and omega-3 fatty acids adapted from Arterburn et al.¹
A review has shown that noradrenaline is important in the regulation of depression; possibly due to the neurotransmitter’s role in the regulation of cognition, motivation, and emotions. An insufficiency of omega-3 fatty acids has also been linked to depression due to their role in the development and function of the central nervous system. Treatment of differentiated SH-SY5Y neuroblastoma cells, a validated cell culture model for studying neuronal function, with the omega-3 fatty acid DHA was shown to increase basal noradrenaline release, whereas the omega-6 fatty acid AA showed no effect.

These results suggest a possible explanation for how omega-3 fatty acid status might impact depression. However, ALA has not yet been investigated for its effect on neurotransmitter accumulation in cell culture, and thus its potential role in affecting neurotransmitter associated mental disorders is not clear. Therefore, a method has been developed to supplement the SH-SY5Y cell line with ALA in order to investigate this question.

**Hypothesis**

The SH-SY5Y neuroblastoma cell line is a valid model to study neuronal function, and as such is a tool to study the effects of omega-3 and omega-6 fatty acid status on the physiology of these cells. For these studies we hypothesized that ALA containing media will increase cellular amounts of this fatty acid and potentially the elongation and desaturation downstream products: EPA and DHA.
Methods

Bovine serum albumin (BSA) and boron trifluoride (BF$_3$), were obtained from Sigma-Aldrich, St. Louis, MO. The Trypsin-LE Express and Dolbecco’s Modified Eagle Medium were obtained from ThermoFisher Scientific, Grand Island, NY.

**Media:** ALA free acid was obtained from Nucheck Prep and was conjugated with bovine serum albumin (BSA), combined with Dolbecco’s Modified Eagle Medium, and then filter sterilized.$^5$ For the ALA: BSA conjugation, a 20% BSA solution was first made which was created by sprinkling 5g BSA on top of 21.6mL cold Calcium-Magnesium Free (CMF)-PBS in a large beaker, allowing to dissolve at 4$^\circ$C overnight without stirring, and then adjusting the final volume to 25mL with CMF-PBS. The solution was then filter-sterilized and stored at 4$^\circ$C.

A 20mM ALA solution was prepared by first adding 5mL of ddH2O and 50µL of 1 M NaOH to a polypropylene Falcon tube. The solution was then warmed to 70$^\circ$C in a water bath. After warming to 70$^\circ$C 28mg ALA was then added to the solution and incubated at 70$^\circ$C for 20 minutes. The solution was inverted several times during incubation to mix. Another 50µL of 1 M NaOH was then added to completely solubilize the ALA. The solution was then incubated for a further 5 minutes at 70$^\circ$C and stored at -20$^\circ$C where it is stable for months.

ALA: BSA conjugation (500uM ALA, 2:1 ALA: BSA molar ratio) was done by slowly adding 250µL of 20mM ALA warmed to 37$^\circ$C via a water bath to 750µL of room temperature 20% BSA. The ALA:BSA solution (1.0mL) was then added to 49mL of culture media to make a concentration of 100uM ALA as this concentration is in the range of omega-3 supplementation used in previous studies.$^5$ The ALA culture media was then filter sterilized and stored at 4$^\circ$C and used within the week in prevention of oxidation that would occur over time.
Cells: Cells were plated at 2,000,000 cells per flask. Control SH-SY5Y neuroblastoma cells (n=3) were supplemented with 10ml of differentiating media containing 10µl of retinoic acid. ALA SH-SY5Y neuroblastoma cells (n=3) were supplemented with 10ml of differentiating media containing 10µl of retinoic acid plus 100µM of ALA conjugated with BSA. Cells were cultured for three days, and media was not replaced during this incubation period. Cell media was aspirated and 5mL of Trypsin-LE Express was used to detach cells from plate. Five mL of differentiating media was then added to the flask to neutralize the action of Tyrp-LE Express, and then transferred into 15mL Falcon tubes and spun at 1690 rpm for 10 minutes to pellet cells. After aspiration of the Tryp-LE Express and media solution, the cells were then rinsed with 500µL of PBS twice, and then harvested into 1mL ddH₂O.³

Fatty Acid Analysis: Lipids were extracted from the cell lysate via the modified Folch procedure and converted to methyl esters as previously described.⁶ This procedure starts with adding 3mL of chloroform/methanol (2:1) with 0.001% BHT to the suspended cells in 1ml ddH₂O, vortexing, and transferring the lower organic phase to a new tube. The extraction was repeated twice to ensure optimal recovery of fatty acids. The combined organic phases were then evaporated to dryness via nitrogen gas. Saponification and methylation was carried out by suspending the dried sample with 200 µL of 2N KOH in MeOH and heating at 100°C for 5 minutes. After cooling, 400 µL BF₃ solution was then added and heated again to 100°C for 5 minutes and then let to cool. After cooling, 1mL hexane was added and vortexed. After vortexing, 3mL of saturated NaCL was added and vortexed once again. The top hexane layer was then transferred to a GC vial, and the sample was run on a gas chromatography machine. The fatty acid content was analyzed through comparison of retention times to a Supelco-37 standard (Sigma-Aldrich).
Dopamine Analysis of Cells: Flasks prepared identically to the fatty acid analysis experiment were made, and the cells were suspended in 1 mL 0.01M HCL. The control (n = 3) and ALA samples (n = 3) were analyzed by high performance liquid chromatography (HPLC), using a C18 column in mobile phase containing 20 mM phosphate buffer pH 2.5 with 3% (v/v) methanol at a flow rate of 0.7 ml/min.

Results

In these studies, SH-SY5Y cells were first treated with retinoic acid to induce a change in phenotype to more closely approximate the appearance and functionality of neurons. One characteristic that is commonly used to document this phenotypic change is the development of neurite-like projections.

The undifferentiated SH-SY5Y cells (Figure 2A) are round and lack projection of neurites. The differentiated SH-SY5Y cells (Figure 2B) are less round with more definition, have projections of neurites, and exhibit the beginnings of a neuronal network. Differentiated cells were used in this study as differentiated SH-SY5Y cells have a phenotype more typical of a neuron present in the brain, and thus are a more accurate biological model of brain cells.

Control Cells and Differentiated Cells

Figure 2: Undifferentiated SH-SY5Y cells (A) and differentiated SH-SY5Y cells (B).
As previous studies used sodium salts directly dissolved in media with ethanol, we tested an alternate method of conjugation of the free fatty acid to BSA to enrich the media.\textsuperscript{5,7} This approach was found effective, as ALA made up 52\% of total fatty acids in the differentiating media with ALA (Figure 3). In contrast, no detectible amount of ALA in the differentiating control media was found. Additionally, there was a lower amount of linoleic acid in the differentiating media with ALA in comparison to the differentiating control media.

**Confirmation of ALA Enrichment of Fatty Acids in Cell Media**

![Fatty acid analysis](image.png)

Figure 3: Predominant fatty acids in differentiating media (n=1) and differentiating media (n=1) with alpha-linolenic acid.

Fatty acid analysis of the control and ALA cells revealed a 58\% lower (p<0.05) content of oleic acid in ALA cells (12.41 mol\%) compared to control cells (28.28 mol\%). Also, a 91\% lower (p<0.05) content of elaidic acid was observed in ALA cells (0.73 mol\%) compared to control cells (8.33 mol\%). The most abundant fatty acid present in ALA-treated cells was alpha-linolenic acid (37.35 mol\%), and this fatty acid was absent in control cells (<0.01 mol\%).
There was a significantly higher (p<0.05) content of eicosapentaenoic acid in ALA cells (2.51 mol%) compared to control cells (<0.01 mol%) (Figure 4).

The results thus indicate that the differentiated SH-SY5Y cell line appear to have poor omega-3 status with use of 1% FBS media in reference to no detectible amounts of ALA and EPA present in the control cells. However, some DHA was present in these control cells (0.58 mol%). The method of supplementing SH-SY5Y cells with ALA supplemented media was shown to be effective given the pronounced increase in the content of ALA. The significantly higher proportion of EPA in ALA cells compared to control cells suggest that the ALA from the media that the cells uptake can be converted through the elongase and desaturase pathway to produce EPA. However, there was no significant increase in DHA, adding to the evidence of the weak conversion rate of ALA to DHA.

*Impact of ALA Treatment on Fatty Acid Proportion in Differentiated SH-SY5Y Cells*

![Figure 4: Fatty Acid content of control cells (n=3) and ALA cells (n=3). Bar values are means with standard error bars. * Statistical significance determined via a student’s T-test with a p value < 0.05.](image)
From the gas chromatography chromatogram for ALA supplemented SH-SY5Y cells a pronounced ALA peak can be seen in relation to the other fatty acids detected in the cells (Figure 5). Additionally, three other fatty acids were detected in significant amounts that did not map to a corresponding fatty acid in comparison to the Supelco-37 standard. These unknown fatty acid peaks were consistently not present in control cell chromatograms. The Supelco-37 standard does not contain standards for fatty acids that are intermediates in the elongation and desaturation of ALA to EPA and then subsequently to DHA. These include 18:4n-3, the product of Δ6-desaturase, and 20:4n-3, the subsequent product of an elongase. Considering the typical pattern of separation and elution of fatty acid methyl esters from the column under these conditions, both of the fatty acids would most likely elute between arachidonic acid (20:4n-6) and EPA (20:5n-3). Two of the unknown peaks elute between AA & EPA. The third unknown peak elutes after EPA but before DHA which could correspond to docosapentaenoic acid (DPA, 22:5n-3) which would be formed via two-carbon elongation of EPA. The identity of these peaks remains to be validated with authentic standards, but the higher content of EPA together with the presence of these unknown peaks suggest that the elongase and desaturase pathway is at least partially active in these cells. Further, the results would suggest that longer incubation with ALA may lead to greater proportions of EPA and DHA.
Evidence for Elongation and Desaturation in ALA Treated Cells

 Attempts at Evidence for Functional Impact: Attempts were made to examine the effect of ALA supplemented SH-SY5Y cells in comparison to control SH-SY5Y cells on dopamine release as a measure of functional impact. This neurotransmitter was chosen for these studies because RA-differentiated SH-SY5Y cells produce dopamine predominantly rather than norepinephrine which is the main catecholamine produced in undifferentiated cells. A successful standard curve was achieved using authentic dopamine with electrochemical detection; however the test samples had a dopamine content that was below detection limits (Figure 6).
Figure 6: HPLC chromatogram of the response and retention times of a dopamine standard (5µmol), ALA supplemented cells, and control cells.

Additionally, an alternative approach was undertaken to evaluate the impact of fatty acid enrichment on functionality by evaluation the expression of tyrosine hydroxylase via Western blot as a marker for dopamine biosynthetic potential. Once again, sensitivity did not seem to be adequate as there was a lack of visible bands. Thus, there is a need for more sensitive techniques such as an enzyme-linked immunosorbent assay (ELISA), or a way to stimulate dopamine release such as through addition of KCL, to measure the content of dopamine in the SH-SY5Y cell line.
Discussion and Conclusions

The method of supplementing SH-SY5Y cells with ALA conjugated with BSA demonstrated an increase in the proportion of ALA in cells. Additionally, through gas chromatography we have discovered that the unenriched SH-SY5Y cells have no detectable ALA and EPA present and very little DHA, suggesting poor omega-3 fatty status. Supplementing SH-SY5Y cells with ALA also increased the amount of EPA, but not DHA, present in the cell. This data suggest that although components of the elongation and desaturation pathway are present in these cells, conversion of EPA to DHA may be limited or sluggish in this cell line. As discussed previously, the three unknown peaks observed in all samples from ALA-treated cells, but in none of the control cell samples, may correspond to 18:4n-3, 20:4n-3, and 22:5n-3, which are intermediates in the biosynthetic conversion of ALA to DHA.1 Further studies in the lab will verify the identity of these peaks.

The results of this experiment are similar with the results of Alessandri et al. who also showed an increase in EPA, when undifferentiated SH-SY5Y cells were treated with ALA enriched media via adding the sodium salt in ethanol.7 In this experiment however, differentiated SH-SY5Y cells were used as these cells exhibit a phenotype more closely resembling the appearance and functionality of neurons in the brain.4 Additionally, we enriched the media with ALA in the free acid form prior to incubation with the cells. Alessandri et al. also found DPA significantly increased, but DHA was only affected minutely.7 Thus, it is likely that the last unknown omega-3 peak that we observed was DPA. Arterburn et al. indicated that there is a block in the conversion of ALA to DHA after the DPA step in humans. This suggests that the
same phenomenon of the block in the conversion of ALA to DHA after the DPA step is present in differentiated SH-SY5Y cells.

Acknowledging the uptake of the free ALA into the cell, and the conversion of ALA to EPA, it can be concluded that these cells exhibit the ability to generate and utilize acyl-CoA synthases. These enzymes function to convert fatty acids to acyl-CoAs, and are thus critical in the cellular uptake, activation, and metabolism of fatty acids. The acyl-CoAs can then go on to be used in processes such as complex lipid formation, lipid remodeling, and effects on signal transduction. My results would suggest that the differentiated SH-SY5Y cells is an appropriate model to study the role of acyl-CoA metabolism in long-chain fatty acid associated pathways as the appropriate machinery is present in these cells.

The work of Geraldine et al. documented that enrichment of cell membrane DHA by over 7-fold by supplementing the media with 70 µM concentration of this fatty acid led to an increase in the release of neurotransmitter by 25% in comparison to cells treated with media containing AA. This functional change with omega-3 fatty acid enrichment was only observed with agonist-stimulated release of the neurotransmitter and not the total amount in cells. We attempted to quantify the amount of neurotransmitter extracted from cells enriched with ALA but the methods utilized lacked sensitivity. Future studies will address the sensitivity issue and should provide insight into the question of whether or not omega-3 fatty acid driven improvements in agonist-stimulated release are DHA-dependent in these cells.

In conclusion, a method has been successfully developed to enrich SH-SY5Y cells with omega-3 fatty acids using an ALA treatment media. The parent fatty acid and the elongation and desaturation product, EPA, were found to be enriched in the isolated and washed cells. Additionally, the presence of relatively large unknown peaks eluting at positions between AA
and EPA, and EPA and DHA, suggests that these cells can partially convert the precursor ALA to additional omega-3 polyunsaturated fatty acids products. Thus research to investigate whether supplementation with the omega-3 fatty acid ALA on neuronal cells has an effect on functional outcomes, such as playing a part in neurotransmitter content and release, is now closer to fruition.

References


