THE EFFECTS OF THE OMEGA THREE FATTY ACID, DHA, ON RAW 264.7 MACROPHAGE CELLS

By

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Abstract

Alzheimer's Disease (AD) is a progressive, degenerative disorder, which affects millions of people in the United States. Although it is currently unclear what exactly causes Alzheimer's disease, a link between AD and inflammation has been established. Our lab has previously shown that chronic peripheral inflammation in the body, simulated by treatment with LPS can contribute to or cause AD-like symptoms to develop within a mouse. Therefore, we believe that by reducing peripheral inflammation, we can potentially prevent AD-like symptoms, or at least slow the progression of the disease. One compound that has shown promise in reducing inflammation is omega three fatty acid, specifically docosahexaenoic acid (DHA). Previous studies have demonstrated that omega three fatty acids can have an anti-inflammatory effect on macrophage cells. Here, we test this claim further in order to establish a foundation for future studies. In cell culture, macrophage cells treated with omega three fatty acids and LPS produce less inflammation than cells treated with LPS alone. Although these *in vitro* results indicate a promising method for preventing AD, additional studies must be conducted in microglial cells and *in vivo* studies to access the use of omega three fatty acids in for AD prevention in humans. The purpose of this research was to replicate the observations made by other research groups in regards to macrophage treatment with DHA and to establish a reliable protocol for our lab to utilize in future studies further exploring the use of omega three fatty acids in the treatment of Alzheimer's disease

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Introduction

Alzheimer's disease (AD) is the sixth leading cause of death in the United States, affecting over five million individuals just within the U.S. It is estimated that one in three senior citizens die with AD or another form of dementia. Furthermore, AD is the only cause of death in the top 10 that currently cannot be prevented or slowed down. In addition, Alzheimer's disease is one of the most costly chronic diseases financially. In 2015, Americans paid an estimated two hundred and twenty-six billion dollars in care, with half of the cost carried by Medicare (1). Because of the great cost associated with AD, extensive research has been conducted to determine the exact cause of Alzheimer's and potential treatment options.

While the mechanism of AD is still unknown, numerous studies have shown that inflammation plays a critical role in the progression of the disease. Upon autopsy, AD is confirmed by the identification of amyloid beta plaques within the hippocampus. These plaques develop when precursor amyloid beta is transformed into amyloid beta. The amyloid beta then coagulates and disrupts signaling pathways, leading to the death of neurons within that area (11). Numerous studies have shown that this transformation from precursor amyloid beta to amyloid beta is closely correlated with inflammation within the brain. In addition, inflammation markers and amyloid beta create a feedback loop, which accelerates the progression of plaque formation and neuronal death (11). Currently, there are several hypotheses regarding how inflammation is initiated in sustained in the brain.

Our lab operates on the axiom that repeated bouts of peripheral inflammation, or inflammation within the body, leads to inflammation within the central nervous system and the formation of amyloid beta plaques. This model was developed after our lab showed that mice injected with lipopolysaccharide (LPS) repeatedly would not only form amyloid beta plaques, but will also present with symptoms of dementia such as poor cognitive function and memory deficits (9). LPS, a common molecule found in Gram-negative bacteria, mimics the effects of an infection by activating the immune system and

producing inflammatory markers in the process. Treatment with LPS serves to mimic the effects of chronic infection, and thus inflammation, over the course of a person's life.

Assuming that chronic peripheral inflammation can lead to the progression of AD, the natural next step is to look at anti-inflammatory medications as a possible preventative measure to AD. The most common anti-inflammatories, non-steroidal anti-inflammatory drugs (NSAIDs) are medications such as ibuprofen, naproxen, and aspirin. These drugs inhibit inflammation by targeting proteins such as Cox1 and 2. Unfortunately, the anti-inflammatory effects of ibuprofen, via the COX protein, also causes gastrointestinal ulceration with extended use (3). This limits the capabilities of NSAIDs like ibuprofen to potentially reduce chronic inflammation and in turn reduce inflammation within the brain.

Omega three fatty acids have been shown to reduce inflammation. Omega three fatty acids are polyunsaturated fatty acids that are characterized by a double bond on carbon number three, a carboxyl group on one end, and a methyl group on the other. The location of the double bond in comparison to the methyl group determined the type of omega three fatty acid (6). Omega three fatty acids are capable of crossing the blood brain barrier and entering cells within the body (7). They have previously been shown to reduce inflammation by inhibiting key steps within the inflammatory pathway. Specifically, DHA has been shown to inhibit the migration of NF-κB into the nucleus, which prevents it from acting as a transcription factor for numerous inflammatory cytokines (5). Currently, there are three types of omega three fatty acids utilized by the body– α-linolenic acid (ALA), docasohexaenoic acid (DHA), and eicosapentaenoic acid (EPA). In our study, we have chosen to utilize DHA due to the availability of the omega three fatty acid and the amount of previous research conducted with DHA. While our bodies ingest small amounts of omega three fatty acids regularly from sources such as nuts and fish, higher dosages of omega three are needed to see anti-inflammatory effects.

Previous studies on macrophages, an immune cell of the peripheral immune system, have shown that macrophages treated with omega three fatty acids and LPS showed a reduction in the amount of inflammatory cytokines produced (5). These studies have been carried out in both cell cultures and in

animal models such as mice. In cell culture, Raw 264.7 murine macrophages, a common cell line model for studying the processes associated with macrophages, are known to produce numerous inflammatory cytokines when exposed to molecules such as LPS (13). They have also been shown to be differentially affected by DHA. Specifically, the studies show that NF-κB, a major inflammatory pathway protein, was reduced by thirty-two percent. In addition, two other inflammatory markers, TNF-α and IL-6, were shown to decrease significantly (8). Because omega three fatty acids are already commercially available and relatively inexpensive, they have great potential as a supplementary drug to reduce inflammation. In addition, no known side effects are currently associated with taking omega three supplements regularly.

Several studies have investigated the beneficial effects of omega three fatty acids in patients with Alzheimer's disease. In pilot trials using thirty-nine patients and monitoring them over the course of twelve months, patients taking omega three fatty acids showed less decline in their mental state examinations in comparison to placebo patients (12). These preliminary results suggest that omega-three fatty acids can slow the cognitive and functional decline of patients already diagnosed with AD. However, because the sample size is small, further studies must be conducted to definitively prove that omega-three fatty acids can slow the progression of AD. It is also important to note that this study looked at patients diagnosed with AD and their disease progression. In practice, omega three fatty acids could also potentially serve as a preventative measure for high-risk individuals for AD.

While this study looked at cognitive function levels within patients, to our knowledge, there have been no studies showing the effects of omega three fatty acids on inflammatory markers within the brain. By generating quantifiable data regarding the effects of omega three fatty acids on neural immune cells, we hope to establish a proof of concept for future studies regarding animal models. In the brain, microglial cells act similarly to macrophages. They phagocytose debris and monitor the brain for foreign substances. Previous studies have shown that like macrophages, microglial cells also respond to LPS stimulation and produce inflammatory cytokines.

Because of the similarities between macrophages and microglial cells, we hypothesize that microglial cells treated with an omega three fatty acid, such as DHA, and LPS, will produce lower levels of inflammation than microglial cells treated with LPS alone. This would provide us with the data necessary to continue studying the effects of omega three fatty acids within a mouse model. Furthermore, if inflammation is in fact reduced, it would corroborate the slowing progression of AD patients who were given omega three fatty acids.

If in fact, omega three fatty acids are capable of reducing inflammation, the significance would be several fold. Our lab has shown that peripheral inflammation can contribute to and cause AD-like pathology to develop in a mouse model. This means that the amount of inflammation present and available to cross the blood brain barrier would be reduced. In addition, if microglial cells operate similarly to the macrophages, as is suspected, inflammation would be reduced in the central nervous system as well. If this is the case, then omega three fatty acids could potentially reduce inflammation on both ends of the spectrum and reduce the amount of inflammation which enters the amyloid beta loop, which in theory, would slow the progression of the disease.

In this study, we replicated the procedures carried out on Raw 264.7 cells using LPS and DHA in order to establish a reliable protocol model for future studies to be based on. In addition, we looked at the levels of inflammatory cytokine, TNF-α , present in cells exposed to LPS and compared that to cells exposed to LPS and DHA. The potential for omega three fatty acids is significant because it could not only aid in slowing the progress of AD and patients suffering from dementia, but could also serve as a preventative measure for individuals. This study will allow us to replicate the previous research done on macrophages and DHA treatment, establish our own protocol, which we can apply to future studies further exploring the potential ability for omega three fatty acids to act as a preventive/treatment method for Alzheimer's disease.

Materials and Methods

Cell Culture

The macrophage cell line, Raw 264.7, was obtained in our lab after being frozen down by a previous undergraduate. Cell cultures were kept in standard petri dishes of 100 mm X 15 mm. The cells were incubated at 37.0ºC and 5% CO_2 and were monitored daily for confluency. The cells were submerged in Dulbecco's Modified Eagle's Medium (DMEM) which contained 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine. Cells were passaged upon reaching 75-80% confluency.

Lipopolysaccharide Treatment

Raw 264.7 cells were plated in 12-well plates at a concentration of 250,000 cells/well. The cells were then mixed with a specified amount of LPS depending on the experiment. In our first experiment, we established a standard curve of LPS by using 10 ng/mL, 100 ng/mL, and 1000 ng/mL of LPS in each respective well in triplicate. In addition to the cells and LPS, each well had 1 mL of growth media. Plates were run with varied amounts of cells, concentrations of LPS, and incubation periods. From the first experiment, we determined that a concentration of 100 ng/mL and an incubation period of 90 minutes was ideal. After 90 minutes, the supernatant in each well was collected and stored at - 80 ˚C until it could be analyzed with an ELISA. All experimental variables were run in triplicate. This procedure allowed us to determine the ideal levels of LPS necessary to elicit a significant inflammatory response (2).

Omega Three Fatty Acid Treatment

To begin our research on omega three fatty acids and inflammation, we first needed to determine the ideal levels of omega three fatty acids necessary to elicit a significant response. In order to do this, we pre-treated cells with varying levels of DHA-using concentrations of 400 µM, 200µM, and 100µM for 24 hours. We then exposed the cells to a constant amount of 100 ng/mL LPS and an incubation period of 90 minutes. Plates were run according to the same protocol as the LPS treatment, with 250,000 cells/well and 1 mL of total media. The supernatant was then collected and stored it at - 80 °C until it could be analyzed with an ELISA (8).

Analysis of TNF-α and IL-1β Levels Using ELISA

ELISAs testing for the levels of TNF-α were run on 96 well plates and read using a plate reader. The wells were first blocked using a coating buffer and antibody overnight. After washing, we added a series of reagents and washes in accordance with the BioLegend ELISA protocol. Each sample was run in duplicate and at concentrations of 1:1, 1:2, and 1:4. The samples were compared to a standard curve generated in the protocol with TNF-α standards of 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL, and 0 pg/mL. The LPS treatment plate incubated for 90 minutes was read using samples with a 1:1 dilution. The DHA treatment plate was read using samples with a 1:2 dilution. Plates were read at 570 nm and 450 nm and applied to a four-parameter fit as set forth in the BioLegend protocol.

Summary of Conditions

LPS Standard Curve At 90 Minutes, 4 Hours, 6 Hours, and 24 Hours

Macrophage Only

Macrophage + 10 ng/mL

Macrophage + 100 ng/mL

Macrophage + 1000 ng/mL

DHA Standard Curve 24 Hour Pretreatment At 90 Minutes With 100 ng/mL LPS

Macrophage + LPS

Macrophage + LPS + 400μM DHA

Macrophage + LPS + 200μM DHA

Macrophage + LPS + 100μM DHA

Results

LPS Treatment

12 well plates, with 250,000 cells in each well, were treated with LPS concentrations of 0 ng/ mL, 10 ng/mL, 100 ng/mL, and 1000 ng/mL. The plate was then incubated for 90 minutes and analyzed with an ELISA in order to determine the amount of TNF-α produced. The ELISA was run at a 1:1 sample concentration. As hypothesized, inflammation was produced in a concentration dependent manner based on the amount of LPS used. It is important to note that the control also produced low levels of inflammation. This is due to a mutation in the Raw 264.7 macrophage cell line that causes low levels of inflammatory cytokines to constantly be produced. LPS treatment in the control resulted in 6.8 pg/mL of TNF-α. The LPS concentrations in increasing order resulted in 33.1 pg/mL, 42.6 pg/mL, and 52.6 pg/mL of TNF-α production respectively.

LPS and DHA Treatment

In our second experiment, we pretreated our cells with DHA concentrations of 0 μM, 100 μM, 200 μM, and 400 μM for 24 hours. The cells were then exposed to 100 ng/mL of LPS and incubated for 90 minutes. The plate was then analyzed for TNF-α levels using an ELISA. The ELISA was run at a 1:2 sample concentration. Similarly to LPS, DHA's ability to reduce inflammation was concentration dependent. The baseline for inflammation reduction, DHA concentration 0 μM, produced 569.2 pg/mL of TNF-α. In increasing concentration, the DHA samples produced 507.5 pg/mL, 390.9 pg/mL, and 24.4 pg/mL of TNF-α production respectively.

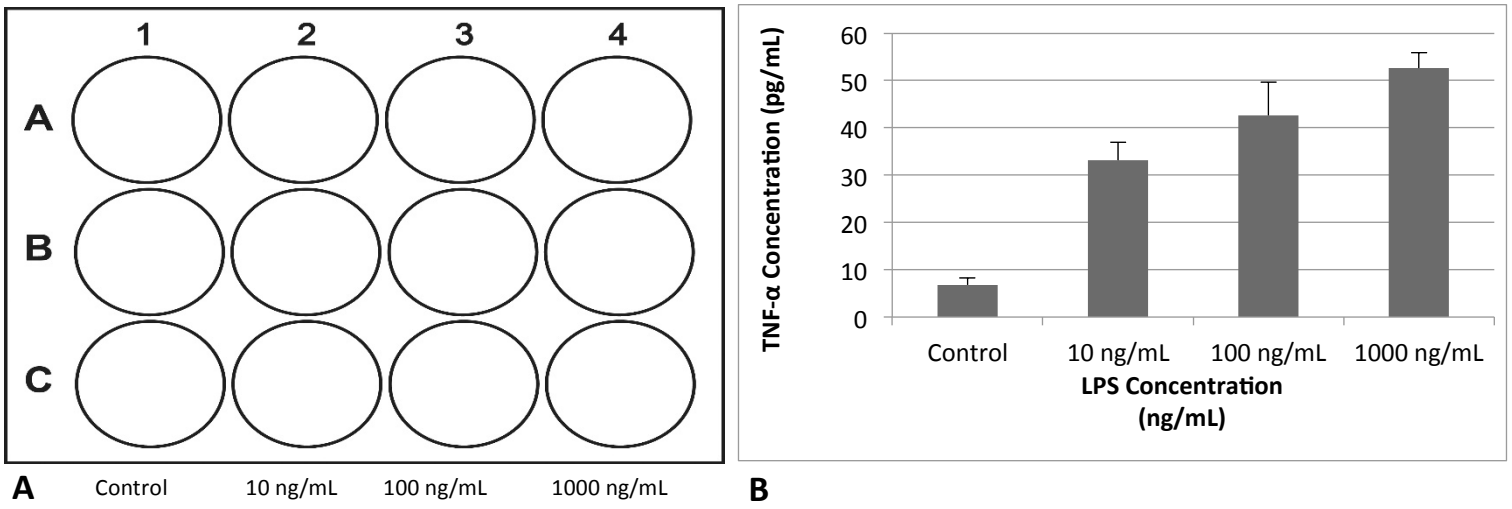

Figure 1: A) 12 well plate map used for LPS treatment. Wells contained 250,000 cells and 1 mL of media. B) Concentration of TNF-α present following 90 minutes of exposure to varied concentrations of LPS. Each concentration was run in triplicate and analyzed in duplicate for increased accuracy. Error bars represent variation from the mean.

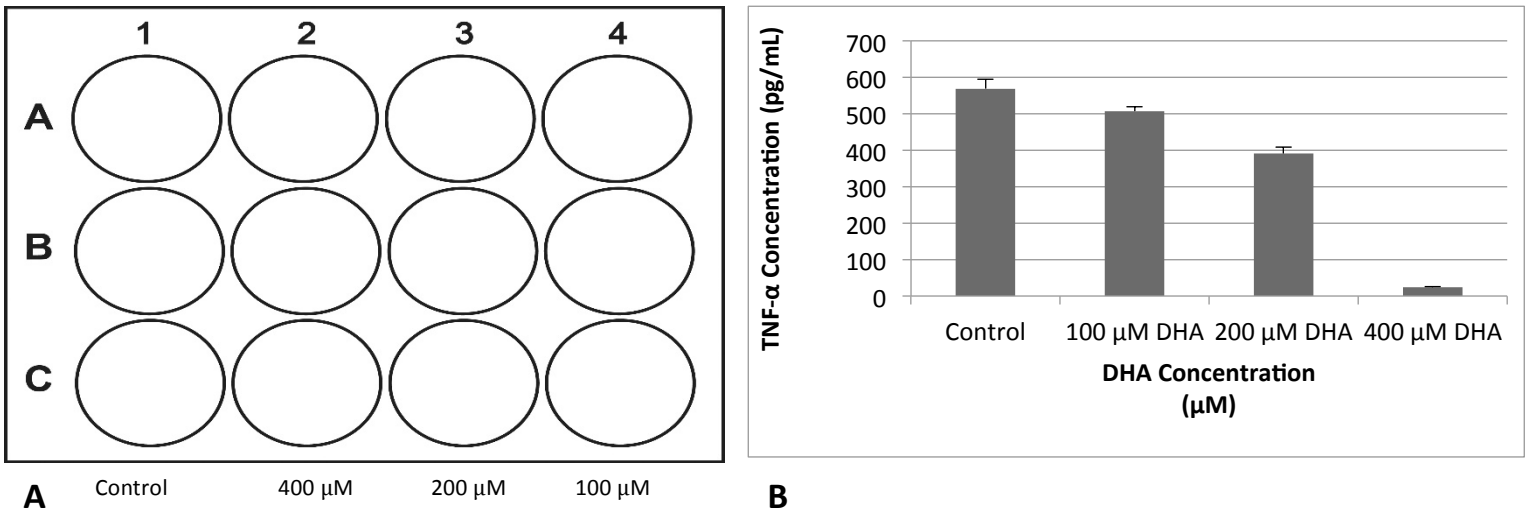

Figure 2: A) 12 well plate map used for DHA treatment. Wells contained 250,000 cells and 1 mL of media. Wells were treated with 100 ng/mL of LPS after DHA pretreatment. B) The reduction of TNF-α after 24 hours of pretreatment to varied concentrations of DHA. All samples were incubated for 90 minutes. Each concentration was run in triplicate and analyzed in duplicate for increased accuracy. Error bars represent variation from the mean.

Discussion

Our original hypothesis was that if we exposed cells to DHA prior to LPS treatment, we would observe a reduction in the amount of inflammation, via TNF-α levels, present. In addition, our goal was to develop a repeatable and reliable procedure for this process for future studies within our lab. This would allow us to conduct future experiments utilizing DHA and LPS, with the intention of gaining a better understanding of the anti-inflammatory mechanism of DHA. Initially, when we ran our experiment, we encountered several issues in generating significant data. However, after manipulating variables and adjusting several key steps in the experimental procedure, we were able to generate promising results.

Our first experiment was meant to determine the amount of time needed for LPS exposure and the concentration of LPS needed to generate significant amounts of inflammation. Based on previous studies, we believed that the ideal concentration would fall somewhere between 100 ng/mL and 1000 ng/mL. We also believed that the amount of time for LPS incubation would be incredibly variable, with research showing significant inflammation as early as 30 minutes and as late as 24 hours. Because of this, we ran experiments at time points: 90 minutes, 4 hours, 6 hours, and 24 hours. Initially, we had planned to measure the inflammatory cytokines TNF-α and IL-β. We arbitrarily chose to proceed with IL-β measurements first to determine if the experimental procedures were working. Unexpectedly, our first three experiments did not produce detectable amounts of the inflammatory cytokine IL-β. This caused us to begin trouble-shooting and manipulating variables based on other LPS protocols. First, we tested to see if the LPS we were using was working correctly. Next, we thought that we could be running the experiment for too long, causing our inflammatory molecules to degrade. Finally, we hypothesized that we could be using too few cells and be diluting out our end product due to the

large volume of growth media. We tested these variables and still could not generate significant results. Eventually, we elected to utilize a procedure previously used in our lab and adjusted for the plate type we were using. This resulted in us using 250,000 cells per well, instead of the initially intended 100,000 cells. In addition, we utilized a LPS incubation period of 90 minutes versus our initial experiments of 4 hours and beyond. We also decided to perform an ELISA looking for TNF-α instead of IL-β because some previous research indicated that RAW 264.7 cells produce more TNF-α than IL-1β within our experimental parameters (14). Expectedly, the results showed that LPS produces inflammation in a concentration dependent manner, meaning that as the amount of LPS used increases, so does the amount of TNF-α present. In addition, the results indicated that a concentration of 100 ng/mL and a time period of 90 minutes was ideal for future experiments. Furthermore, the concentration of 100 ng/mL gave us data well within our observable standard curve of TNF-α. After determining our ideal concentration of LPS and incubation time, we were able to proceed to the next step of determining the effects of varied concentrations of DHA on LPS treated cells.

We next exposed the macrophage cells to varied concentrations of DHA for 24 hours of pretreatment and subsequently incubated the cells in cells in 100 ng/mL of LPS for 90 minutes, we ran an ELISA to determine the amount of TNF-α produced in comparison to a control, which received no omega three fatty acid treatment. The samples in this ELISA were run at a 1:2 ratio. In our experiment, we utilized DHA concentrations of 400 μM, 200 μM, and 100 μM. These concentrations were determined based on previous studies and adapted to our protocol based on the number of cells and amount of LPS used in comparison (8). Differences in TNF-α production between the LPS treatment alone and the DHA plus LPS treatment are due to the pretreatment period with DHA. As seen in the LPS control, unstimulated Raw 264.7 macrophages express low

levels of TNF-α and this accumulates over the 24 hours. Additionally, the replication time for Raw 264.7 macrophages is between 12-18 hours, meaning that the original 250,000 cells has likely doubled by the time LPS treatment is initiated. Similarly to the LPS treatment ELISA, DHA reduces inflammation in a concentration dependent manner. Initially, the lower concentrations of DHA do reduce the amount of TNF-α being produced, but the reduction is minimal. This is likely due to the feedback loop that NF-κB exhibits, in which it stimulates increased transcription of inflammatory molecules. At a concentration of 400 μM, the DHA is able to overcome this feedback loop and significantly reduce the amount of inflammation being produced by the cells. However, it is important to note that there are saturation limits in which increased concentrations of DHA will no longer reduce inflammation further. In addition, at extremely low levels of DHA, it is unable to inhibit inflammation significantly (4). This observation made in cell culture would also apply in an animal model and ultimately in the human system as well.

Although these results are relevant and show what we hypothesized, there are several limitations in our study that prevented us from gathering significant data. While each treatment condition was run in triplicate, additional samples and plates would need to be run to generate prove a significant difference. In addition, it is important to note that these samples can only be compared within each plate run due to differences in amount of time that cells were kept in the wells. We also had hoped to demonstrate the effects of pretreating cells with DHA alone, but due to time constraints were not able to do so. It is also unclear how long the anti-inflammatory effects of DHA can be observed in LPS treatment. Our research provided constant omega three fatty acid exposure for 24 hours, followed by a brief exposure to LPS. Additional studies need to be conducted to measure how long this effect is observable and if it is viable for chronic

treatment. It is possible that the cells would stop responding to DHA after repeated exposure, similarly to the endotoxin resistance observed in repeated LPS treatments (9). Unfortunately, these results are not novel in terms of the literature–numerous studies have shown the effects of DHA on LPS treated cells. Our hope was to push this research forward by looking at microglial cells as well, but unforeseen issues prevented this. However, the data we generated is applicable because it enabled us to establish a protocol that works reliably and expectedly within our lab. These experimental parameters will be applied to future studies with macrophages and microglial cells.

While there are numerous directions that this data could take us in regards to future studies, two areas of great promise are determining the effects of omega three fatty acids on amyloid beta production and the application of omega three fatty acid treatment in an animal model. Some previous research indicates that omega three fatty acids, such as DHA, may be able to reduce the production of amyloid beta and inhibit the conversion of pre-cursor amyloid beta protein into amyloid beta (10). This is significant because if this hypothesis was true, omega three fatty acids could potentially reduce all steps within the Alzheimer's disease process that we currently study. We have already shown that DHA can reduce peripheral inflammation within the body, and it is likely that this phenomenon also applies to microglial cells inhibiting inflammation in the brain. Therefore, if DHA could also prevent the deposition of amyloid beta in the brain, the feedback loop, which leads to Alzheimer's disease could be greatly reduced. This would potentially slow the progression of the disease greatly and possibly prevent it from initially occurring. While our results and others show great promise in cell culture, to our knowledge, this has not been applied to an animal model looking at the anti-inflammatory effects *in vivo* as well as the cognitive improvements due to the potential slowing of disease progression.

Our lab has previously shown that exercise can decrease the amount of amyloid beta present in a mouse brain (17). Further researching this observation in conjunction with omega three fatty acid treatment could also be promising. Human trials have correlated a slowed progression of AD with omega three fatty acid treatment, but have not been able to look at the actual levels of inflammation present within the body and how they change over time. These two areas of research are promising for the future studies of omega three fatty acids and AD. Our research was intended to establish the protocol, which these future studies could utilize.

Although there is still much to uncover regarding omega three fatty acid treatment and its viability within Alzheimer's disease, the initial results are promising. At the very least, it has been demonstrated that DHA can reduce the amount of inflammation produced by macrophages within the body. Our research lab was previously shown that this inflammation is the first step that can lead to Alzheimer's disease-like symptoms and pathology. Thus, by reducing the amount of inflammation present within the body, we also reduce the amount of inflammation available to cross the blood brain barrier and contribute to the inflammation-amyloid beta feedback loop. There is great promise that omega three fatty acids can also reduce the amount of inflammation produced by microglial cells, the immune cell within the brain. Finally, some previous research indicates that omega three fatty acids could also reduce amyloid beta production. These possibilities make omega three fatty acids an exciting field of research for Alzheimer's disease treatment. The commercial availability, cost, and absence of known chronic side effects make omega three fatty acids a potentially viable treatment option for Alzheimer's disease. Our research established a reliable procedure for us to base future studies regarding DHA on. We were able to determine that we could produce significant inflammation at an LPS concentration of 100 ng/mL and an incubation time of 90 minutes. In addition, we demonstrated

that DHA reduces inflammation in a concentration dependent manner. Using this data, we will be able to effectively research omega three fatty acid treatment methods, while also maximizing our efficiency in terms of time, reagents, and cost. The purpose of this research was to replicate previous results demonstrating the anti-inflammatory effects of DHA on LPS treated macrophages and to establish a reliable protocol for use in our lab and we accomplished both of these goals.

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