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The Effect of Omega-3 Fatty Acids on Recovery from Traumatic Brain Injury

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Every year there can be up to 2.8 million traumatic brain injuries (TBIs) seen in emergency departments and hospitals in the United States (Taylor, Bell, Breiding, & Xu, 2017). About 75% of these injuries are mild traumatic brain injuries (mTBIs) which are also known as concussions (Taylor, et al., 2017; Creed, DiLeonardi, Fox, Tessler, & Raghupathi, 2011). These injuries not only restrict people from their everyday lives following an injury (Barrett, McBurney, & Ciappio, 2014; Creed, et al., 2011), but they can also cause major long-term neurological dysfunction, changes in cognition, sensorimotor function, and personality, and possibly even death (Begum, Yan, Li, Singh, Dixon, & Sun, 2014; Creed, et al., 2011; Johnson, Meaney, Cullen, & Smith, 2015; Taylor, et al., 2017). There is currently no therapy that has shown to be an effective treatment for this type of injury (Barret et al., 2014; Gupta, Summerville, & Senter, 2019; Petraglia, Winkler, & Bailes, 2011; Trojian, Wang, & Leddy, 2017). While there are ways to effectively treat symptoms, a treatment has not been discovered that actually helps the brain to recover. The physiological complexities of TBIs make it difficult to determine a treatment that effectively aids the brain through every aspect of recovery. Current research is looking at therapy that involves cognitive and physical rest as well as medicinal therapies (Barret, et al., 2014). One supplemental treatment strategy that has been a focus in this area of research is omega-3 fatty acids due to its structure and functional role within the brain (Barret, et al., 2014).

Various preclinical studies have found that omega-3 fatty acids are effective as both pre- and postinjury treatments (Barret et al., 2014). While many preclinical (animal) studies have shown significant improvement in recovery using omega-3s, this benefit has not been shown in clinical (human) trials (Gupta et al., 2019; Trojian et al., 2017). Although there are currently a

limited number of clinical trials that have been completed, this inconsistency suggests that the results found in animal studies looking at TBI are not applicable to human studies. There are numerous animal studies that use a variety of methodologies and techniques to examine the effectiveness of these supplements; therefore, it is difficult to determine the possible reasons that these results would not carry over to human trials. The goal of this investigation was to complete a meta-analysis and combine the results of individual studies to produce one common effect. Meta-analyses are conducted in order to improve estimates of size effects and to help resolve uncertainties created when results are conflicting (Russo, 2007). Therefore, this analysis will be helpful in better determining an effect estimate of omega-3 fatty acids and may help to answer the question of this analysis which is to determine reasons that results from preclinical trials have not carried to clinical trials.

Background

A TBI or more specifically a mTBI occurs when there is a direct or indirect mechanical force to the head which initiates a neurochemical cascade of events (Barret et al., 2014; Petraglia et al., 2011). The insult causes a stretching of axons which disrupt the membrane of neurons. This results in changes in the movement of ions within the brain (Barret et al., 2014; Petraglia et al., 2011). These ions help to maintain membrane potential; therefore, when the flow is disrupted, it can cause excitotoxicity which is increased firing of the neurons and can eventually lead to signaled cell death also known as apoptosis (Barret et al., 2014). To return ions to homeostasis, ATP-dependent pumps within the brain work at elevated levels leading to hypermetabolism. Hypermetabolism leads to a depletion of glucose which ultimately leads to hypometabolism in the brain. This can last for several weeks and makes the brain especially

vulnerable to a repeat injury (Barret, et al., 2014). Hypermetabolism can also cause oxidative stress which can again cause neuronal damage (Barret et al., 2014; Petraglia et al. 2011).

The membrane disruption allows for drastic increase in calcium influx. This change in movement of calcium ions has an effect on axonal structure and mitochondrial function (Barret et al., 2014). The influx of calcium inhibits polarization of the mitochondrial membrane resulting in its dysfunction. This dysfunction also leads to oxidative stress within the cell. When the axon is damaged and goes into oxidative stress, it sends signals to activate microglial cells (Barret, et al., 2014). These cells play a large role in the inflammatory response of neurons which can last up to 30 days after the initial injury. When repeated head injuries occur, the increased inflammation can be damaging to neurons and increase the risk of permanent damage (Barret, et al., 2014; Petraglia et a., 2011).

Neurofilaments and microtubules which work within the axon of a neuron can also be disrupted following a TBI (Petraglia et al., 2011). These filaments and tubules transport various proteins to and from the soma, therefore, they play a role in maintaining the structure of a neuron and continuing its normal function (Petraglia et al., 2011). In disrupting this activity, a cell is unable to function properly and goes into oxidative stress. The trauma to the brain can also cause proteins to be folded improperly or to unfold and inhibit their normal function (Yin, Sun, Li, Kiselyov, & Sun, 2017). The endoplasmic reticulum (ER) plays a role in synthesizing, modifying, and regulating the production of proteins; therefore, these misfolded proteins disrupt its normal function (Yin, et al., 2017). When this occurs, ER stress is induced and signals for stress gene activation (Yin, et al., 2017). This stress signals that the cell is damaged and needs to be removed, therefore, initiating apoptosis. This cell death is carried out by a mediated lysosome activity, autophagy, that eliminates damaged cells (Yin, et al., 2017). If autophagy is over

activated, which is common following signals of ER stress and oxidative stress, then it can further hinder cell function (Yin, et al., 2017). Overall, a TBI results in excitotoxicity, hypermetabolism, mitochondrial dysfunction, and inhibition of protein transport which all lead to oxidative stress, ER stress, inflammation, autophagy, and ultimately cell death.

There are several ways to measure these various effects of brain injury. Oxidative stress within the brain can be measured by looking at the production of amyloid precursor proteins (Barret et al., 2014). Damage due to the inflammatory response can be measured by looking at the production of lipid peroxidation products (Barret et al., 2014). Measuring the production of inositol requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6) can help to show ER stress because these proteins are activated due to ER stress (Yin, et al., 2017). Amount of apoptosis can be examined by measuring the amount of caspase 3 proteins produced due to its function of mediating apoptosis (Barret, et al., 2014). Various histology techniques can be used to measure the amount of these proteins produced in the brains of rodent subjects. Another way in which these physiological changes and impairments are revealed is through the physical and cognitive symptoms experienced by the people and animals that have been injured. Therefore, another way in which the damage can be measured is through behavioral tests. Various cognitive and physical abilities like memory, reaction time, and balance (Gupta, et al., 2019) as well physical symptoms like headache, fatigue, and sleep disturbances can be measured through behavioral tests (Barret, et al., 2014; Gupta, et al., 2019). The complex and numerous neurophysiological effects of TBI and mTBI make it difficult to determine a treatment that is beneficial to all consequences of the injury. The chemical makeup of omega 3 fatty acids and their function within the brain make them a promising treatment strategy for these injuries.

Omega-3 fatty acids are long chained polyunsaturated fats, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found in many fish, algae, and plants (Barret, et al., 2014; Petraglia et a., 2011; Vonder Haar, Peterson, Martens, & Hoane, 2016). They are essential for brain development and function due to their role in the structure of membranes as well as membrane fluidity, receptor affinity, and modulation of neurotransmission (Barret, et al., 2014; Petraglia et a., 2011; Vonder Haar, et al., 2016). DHA is the most prominent omega 3 in the brain by making up 97% of the omega 3 fatty acids (Barret, et al., 2014; Trojian, et al., 2017). Since it has such a high proportion in the brain many studies focused on this omega-3. There are multiple ways in which it is believed that omega-3s could be beneficial for recovery from TBI. One way is by regulating neuronal survival through inhibition of apoptosis following the injury (Vonder Haar, et al., 2016). This is accomplished through increasing BDNF levels, inhibiting synaptic degradation, and reducing oxidative stress. (Vonder Haar, et al., 2016). Omega 3 fatty acids are also anti-inflammatory agents because of their ability to inhibit pro-inflammatory cytokines like TNF-α, IL-6, and C-reactive protein (Vonder Haar, et al., 2016). While Omega 3 fatty acids appear to be a possible supplemental treatment for TBI, it is vital to test and observe its effectiveness in order to determine any ways in which they are not able to benefit the brain during recovery.

Methods

The purpose of a meta-analysis is to systematically search databases in order to obtain all studies related to the specific topic of interest, and to summarize the data from those studies through statistical methods (The University of Edinburg, 2017). A systematic literature search was conducted on EBSCOhost, PubMed, and PsycNet. Brain injuries and omega 3 were used for the first round of search terms with applying related words. The title and abstract were read for

each result from this search. All articles that were or appeared to be an experimental study looking at omega-3 fatty acid or DHA effects on TBI or mTBI recovery were then saved. Articles that looked at spinal cord injuries or treatment strategies other than use of omega-3s were not included. This was repeated using various other combinations of search terms including: brain injuries and fatty acids, omega-3 and recovery, head injury and Docosahexaenoic Acid (DHA), and finally Omega 3 fatty acids and brain injury or head injury or traumatic brain injury or acquired injury. From EBSCOhost 12 studies were found that fit the qualifications. The search on EBSCOhost was ended after searching the previously stated terms and not finding anymore related articles or finding articles that had already been saved. PubMed was searched second using the same search terms and qualifications. Sixteen articles were saved from PubMed. The search was completed when only unrelated articles or articles already saved from the previous database were appearing in the results. PsychNET was the final database to be searched and only one additional study was saved. The majority of the results from the searches were articles saved from the previous databases or articles that did not fit in the initial qualifications. Overall 29 articles were obtained to be read and examined more closely to insure they fit within the criteria for inclusion and were valid and unbiased studies.

All 29 articles were then obtained and read fully. After further inspection 9 articles were removed from the collection. Three of these studies looked at alternative head traumas to TBI, or they lacked sufficient explanation of methodologies and results to show the effect of omega-3s on TBI. The aim of this study was to determine a summarizing effect of animal studies; therefore, five human studies were also excluded from the group of articles. The final study was eliminated because it lacked the use of similar tests and analysis to compare to the results of the other studies. Study and subject characteristics, intervention strategies, and techniques to obtain

results were recorded for the remaining 20 articles. Seventeen of the studies used rats as their subjects and 3 used mice. In order to induce a TBI the studies used a variety of different techniques. Nine studies used a Controlled Cortical Impact (CCI). Five articles used a Fluid Percussion Injury (FPI). Three studies used Marmarou impact acceleration injury, and three others used the Feeney DM TBI model. The Feeney DM TBI model inflicts the injury by dropping a weight using an apparatus on the either the skull or brain of the rodent (Johnson, et al., 2015). Marmarou impact acceleration injury similarly drops a weight onto a plate that is fixed on the cranium (Johnson, et al., 2015). These two models differ because in the Feeney DM model the heads are secured and do not move with impact, however, in the impact acceleration model the heads remain loose allowing them to swing down following the impact (Johnson, et al., 2015). Because of the rapid acceleration that occurs after impact, it is thought that injury becomes a diffuse axonal injury. This means that instead of primarily causing damage directly at the point of impact the damage is spread to multiple other areas of the brain (Johnson, et al., 2015). The FPI model induces injury by injecting fluid into the epidural layer which causes a rapid wave of highly pressurized fluid in the closed cranial area (Johnson, et al., 2015). CCI uses an impactor to deform the brain at a specific velocity and depth. Recently many studies have been using this technique on closed skulls to lessen the impact, thus making the TBI milder (Johnson, et al., 2015). Each study looked at a variety of behavioral tests as well as various staining techniques in order to measure the benefits of omega-3 fatty acids during recovery from TBI.

The aim of this paper was to statistically combine the results from these techniques. In an attempt to gather the statistical results from each test, it was discovered that most studies failed to report any numerical values of their results except for the level of statistical significance. The

lack of specific evidence reported by these studies prevented the running of a statistical meta-analysis. If a more extended period of time was given to work on this paper, the data would have been requested from the authors of each study. It would be a major breach of research ethics if they did not provide this information. The remainder of the paper, therefore, focuses on analyzing and summarizing the empirical data without statistical methods. It examines and compares the methodologies used within the different studies.

To determine the effect of omega 3 fatty acids on recovery it is important to look at the effects on both the behavioral aspects of recovery and neuroanatomical aspects of recovery. Each study performed a variety of tests and used various techniques to examine the animal's recovery. The behavioral tests that were most common throughout the studies were the beam balance and beam walk test, the neurological severity score, the novel object recognition test, the rotarod test, and the Morris Water Maze test. Other tests were used to look at emotional responses like fear, anxiety, and depression, or they were used as alternative ways to look at motor functions. These emotional tests will not be examined in this study because they were only examined in one study, and therefore, could not be compared to other studies, or they were only in two studies but one or both of those studies did not give sufficient explanation on that test to compare the results. The common histological techniques used to look at the neuroanatomical or molecular aspects of recovery were the western blot test, immunohistochemical staining, the ELISA test, immunofluorescent staining, TUNEL staining, Nissl staining, RT/PCR, and NeuN immunostaining. Like the behavioral tests, other techniques were used but not enough were studied or explained in order to compare and analyze. The following will look at each test individually to compare among the different studies that completed them. Details on the general methods of each study can be viewed in the Appendix.

Beam Balance Test and the Beam Walk Test

The beam balance test and the beam walk test help to measure motor deficits in fine motor skills, balance, and coordination (Hamm, Pike, O'Dell, Lyeth, & Jenkins, 2009). Typically, these tests are comprised of a long beam that can have varying lengths, widths, and heights depending on the study (Hamm, et al., 2009). In the beam balance test the subjects were placed on a suspended wooden stick, and the time the animal is able to stay balanced on the stick is recorded (Begum, et al., 2014; Lin, Chao, Li, Xu, Liu, Bao, Hou, Liu, Wang, You, Liu, & Ji, 2017). In the beam walk test the animals are given an incentive to walk across the beam, and the number of hind foot slips or falls are counted (Hamm, et al., 2009). Two of the studies that were examined used the beam balance test to measure motor function and three of the studies used the beam walk test.

Begum, et al. (2014) assessed the beam balance task the day before the injury and days 1-5 following the injury. Three 60 second trials were performed per animal per day. The amount of time before the animal fell off the beam or completed a spin, was recorded. The results of this study showed that the sham injury control group did not have significant fluctuations of scores for this test. They also showed that both the TBI control group and the TBI and DHA group had a significant decrease in their scores between the pre-injury test and the test 1 day after injury. On day five the TBI control had shown some improvement but it was not a significant increase. The TBI and DHA group showed a significant increase in score on day 4 and 5 following injury. The study concluded from these results that DHA can significantly help rat sensorimotor skills following TBI.

Lin, et al. (2017) used a similar way of assessing the subjects as well as tested on the same days surrounding TBI. Additionally, this study looked at another variable, the presence of

GW1100. GW1100 is an antagonist for G- protein coupled receptor 40 (GPR40), a suspected receptor involved in anti-inflammation (Lin, et al., 2017). This study hypothesized that the omega-3s work through this pathway to reduce inflammation caused by trauma. Therefore, by inhibiting this pathway, the researchers hypothesized that the omega-3s would not be as beneficial (Lin, et al., 2017). To test this a group was created in which some of the rats that had gotten a TBI and who were on the omega-3 rich diet, were injected with GW1100 30 minutes before injury and continuously every day after injury (Lin, et al., 2017). The results of this study showed that rats on the omega-3 rich diet performed significantly better on the beam balance test than the rats on the standard diet. Results also showed that this improvement in motor skills by the omega-3 rich diet group could be reversed when given the GW1100 injection (Lin, et al., 2017). They concluded from these results that omega-3s improved motor deficits created by TBI through the GPCR40 pathway.

These two studies showed that overall omega-3 treatment helped improve motor function. The first study showed that DHA started to show significant improvement in motor function on day 4 and 5 after injury whereas the second study found the DHA had done significantly better overall. This difference may be explained due to the fact that the second study had its subjects on a DHA diet throughout their entire life instead of simply injecting DHA after the fact (see Appendix). This may mean that it is more beneficial to take omega-3 supplements or to eat food containing omega-3s throughout life instead of just taking them after an injury. Although both tests showed significant differences, they had low sample sizes averaging to be about 10 each (see Appendix). These low sample sizes can inflate differences found, therefore, reducing the likelihood that these are true significant effects (Button, Ioannidis, Mokrysz, Nosek, Flint,

Robinson, & Munafò, 2013). If these results found are not real effects than this may explain the lack of similar results in human trials.

Desai, et al. (2014) used a beam walk test with a beam that was 50 cm long and 7 mm wide. The subjects were trained for two days and then given a baseline test the day before the surgery (Desai, et al., 2014). After the surgery each subject completed three trials of the test for the next 7 days. There was a statistical difference in number of foot slips between the omega-3 deficient and adequate groups from day 2 through day 7 with the adequate diet group performing better. There was no significant difference in performance before injury (Desai, et al., 2014). This led the researchers to conclude that the inadequate amounts of omega-3 does not affect performance before an incidence, but following an injury, adequate amounts of omega-3s is beneficial to recovery in motor functions.

Tang, et al. (2018) used a beam that was 1390 mm long, 21 mm wide, and 430 mm high. Two days before injury the rats were trained to walk across the beam by placing a box at the other end and slowly increasing the distances needed to walk on the beam to get to the box. The tests were administered on day 1, 3, and 5 after injury with 3 trials completed each day (Tang, et al., 2018). Performance was scored on a scale of 0-6, which ranged from the rat falling off the beam to no foot slips while crossing the entire beam. The results from this study showed that there was a significant impairment one day after surgery for animals that received TBI. It also showed that there was a significant difference in scores with rats that were treated with DHA (Tang, et al., 2018). The study concluded from this that DHA treatment improves motor function.

Begum, et al. (2014) looked at the beam walk test, and it trained the subjects to escape a loud noise and bright light by traversing a beam and entering a box. They were then assessed on

the day before surgery and the five days following surgery with three trials completed each day (Begum, et al., 2014). The beam was 2.5 cm wide and 100 cm long. The dependent variables examined were average latency to cross the beam or 60 seconds if they did not cross it completely, and a score of up to five points where 5 points were allotted if they successfully crossed and less points given depending on how far they reached on the beam (Begum, et al., 2014). The results showed that the DHA group had significantly better latency times on the fifth day after injury, and significantly better beam walk scores on day 4 and day 5 after the injury. In general, the DHA group had significant improvement on scores and latency between day 1 and day 5, however, the TBI group without DHA did not show significant improvement (Begum, et al., 2014). The study concluded from this that DHA given immediately after injury can help to improve motor function.

Overall, the studies showed improvement of motor function with administration of omega-3s. More specifically, two of the studies showed improved early on in recovery, day 1 and day 2, and the third saw significant improvement on day 4 and 5. One study had examined mice that had been treated with omega-3s for generations (Desai, et al., 2014) while the other two studies examined rats that were injected with DHA fairly soon after injury (Lin, et al.,, 2017; Begum, et al., 2014) (see Appendix). The two studies that showed improvement were one of the mice studies and one of the rat studies. There are no apparent differences in methods that would explain the lack of improvement in the Begum, et al. study. This difference may be accounted for by the small sample sizes. Each of the groups in these studies had 10 subjects or less which means that the results may not be as reliable as portrayed (Button, et al., 2013). This may indicate that the improvement may not happen as early as 1-2 days after injury. Additional studies need to be completed to compare these results.

Neurological Severity Score (NSS)

As indicated by its name, the NSS generally looks at neurological functions of the subjects. Two of the studies completed the subtests for the common NSS assessments. These tests assess coordination, alertness, and motor function (Tang, et al., 2018; Zhu, Ding, Kong, Li, & Chen, 2018). A modified score (mNSS) was completed by three of the studies. This encompasses a variety of tests that measure motor, sensory, and reflex systems within the rodents (Chen, Wu, Chen, Xie, Fang, Hu, Chen, Fu, & He, 2017; Chen, Chen, Fan, Wu, Yang, Fang, Fu, & Li, 2018; Chen, Pan, Fang, Lin, Wu, Yang, Li, Fu, Gao, & Li, 2018).

Tang, et al. (2018) used the normal NSS assessments. The scores for this test were ranged from 0-10 with 0 being normal activity and 10 being maximum neurological damage. The subjects were assessed from days 1-5 after injury (Tang, et al., 2018). The results showed that all groups showed some improvement in score throughout the five days, however, the DHA group showed significantly greater improvement in scores compared to the TBI control group earlier on during the recovery time (Tang, et al., 2018). The study concluded from these results that DHA did help to aid in the recovery of neurological functioning after FPI (Tang, et al., 2018).

Zhu, et al. (2018) gave the NSS evaluation one hour after the injection and 1, 4, 7, 14, and 21 days after injury. Each subject completed 10 different tasks and were given one point if they failed to successfully complete the task. Therefore, a ten was the highest a subject could score representing maximum injury and 0 was the lowest they could score representing normal function (Zhu, et al., 2018). The results showed that rats treated with DHA performed much better starting on day 4 than the rats that were treated with saline or not treated at all. The group given 555 mg of DHA performed the best of the three groups given DHA, however, there was not a significant difference between the groups given DHA (Zhu, et al., 2018). They concluded

that DHA had an effect on neurological recovery, however, more research is necessary to determine the dose that is most beneficial.

Chen, et al. (2017) The next study that will be discussed used a modified NSS to determine the neurological effect of omega-3 on TBI recovery. The subjects were tested on day 1, 3, and 7 after injury. This test was modified because it was on a scale from 0-18 instead of 0-10 like the other two previous studies. This means that 8 additional assessments were given to determine the score (Chen, et al., 2017). The meaning of the ranges was similar to the first two with 18 being the maximum amount of dysfunction and 0 being normal function (Chen, et al., 2017). The results from this study showed significantly better neurological function of the omega-3 treated animals than the TBI control group on day seven after injury (Chen, et al., 2017). In other words, they concluded that DHA was beneficial to recovery.

Chen, Chen, et al. (2018) completed the mNSS evaluation and assessed its subjects on days 1, 3, 7, and 14. Six subjects total were evaluated with the mNSS. The mNSS was completed the same way as the previously discussed study. The results of this study found that rats in the omega-3 group performed significantly better on their assessments starting on day 3 after TBI (Chen, Chen, et al., 2018). They concluded from this that the rats benefited from the omega-3s during recovery. Chen, Pan, et al. (2018) completed the mNSS test very similarly to the two previous studies. It used the same kind of subjects, technique to induce TBI, and timing to induce TBI. The mNSS was also determined in the same way which was on a scale from 0-18. The results for this study found a significant difference between the omega-3 TBI group and the TBI control group on days 3, 7, and 14 after injury (Chen, Pan, et al., 2018).

Overall, the studies all showed significant improvement in NSS score with the use of omega-3s. They all used rats and gave injections about 30 minutes after injury was induced (see

Appendix). They also showed similar improvements on the same days after injury. Each study had low sample sizes that ranged from 10-12 subjects per group. This means that the level of statistical significance could have been overestimated (Button, et al., 2013). Since multiple studies seemed to show very similar results, this may suggest that the effect size was in fact representative of the population.

Novel Object Recognition Test (NOR)

The NOR test consists of a habituation, familiarization, and testing phase (Desai, et al., 2014; Schober, et al., 2016). In this test the subject is placed into an open field that is enclosed at varying distances depending on the specific research. For the habituation phase the subject was placed in the field and allowed to explore. In the familiarization field two identical objects are placed in the field and the animal is allowed to investigate and "familiarize" themselves with the object. In the test phase one of the objects is the familiar object and the other is an unfamiliar or novel object (Desai, et al., 2014; Schober, et al., 2016). The amount of time spent investigating the novel object is recorded. This test helps analyze cognition by looking at how memory may be affected by a brain injury (Desai, et al., 2014; Schober, et al., 2016). A higher time spent exploring the unknown object is considered a better memory because it shows that the animal remembers seeing the familiar object and realizes that there is a new object within the room.

Desai, et al. (2014) compared mice on an omega-3 deficient or adequate diet. On the fifth, sixth, and seventh days after receiving a TBI, the mice were given five minutes to explore an open field (Desai, et al., 2014). On the seventh day two identical objects were placed in the area, and the mice were allowed to explore the arena until they examined the objects for 30 seconds total or it had been 15 minutes. It was considered examining when the mouse was within 2 cm of the object (Desai, et al., 2014). Then the animal was placed in the arena with a familiar

and novel object for three minutes, and the amount of time they spent at each object was measured. The study found that there was significantly less exploration of the novel object done by the DHA deficient mice than the DHA adequate mice (Desai, et al., 2014). It also found that the sham groups, one with a DHA deficient diet and one with a DHA adequate diet, did not have a difference in exploration of the novel object. This shows that the difference in diet did not affect memory when no injury has occurred (Desai, et al. 2014).

Schober, et al. (2016) used the NOR test allowed the animals to explore in the arena for 15 minutes for two days in order to be habituated to the environment. On the third day, the subject was placed in the arena for five minutes, but during this day the arena had two identical objects within it. For the testing phase a day later, the rat was allowed 5 minutes to explore (Schober, et al., 2016). This exploration time was recorded on video and the total time spent within 2 cm of the object was recorded (Schober, et al., 2016). There was no statistically significant difference in time spent at the novel object. This means that the CCI did not have an effect on memory, therefore, they were unable to determine any effect DHA may have on memory recovery (Schober, et al., 2016).

These two studies had conflicting results. It is difficult to draw conclusions because the second study didn't show a difference in the sham injury group and TBI injury groups. As stated above, this suggests that the mechanism of injury did not have an effect on memory, however, as both studies used CCI as a mechanism of injury the lack of difference is puzzling (Button, et al., 2013). Overall, more studies are needed in order to interpret and compare the results and determine the effect of omega-3s on memory recovery after TBI.

Rotarod Test

The rotarod test looks at motor deficits like motor coordination and balance (Hamm, et al., 2009). In this test animals are placed on a cylinder or rod that is continuously spinning. The speed of the rod slowly increases to a speed that is chosen by the experimenters (Hamm, et al., 2009). The animals are given three practice trials before completing the test trial. The trial is finished after 5 minutes or once the subject falls off the cylinder (Hamm, et al., 2009). The time taken to fall of the rod is recorded as the dependent variable in the experiment. This test measures similar functions as the beam walk test and the beam balance test mentioned above, however, it is a more sensitive in testing motor impairments (Hamm, et al., 2009). Five of the studies used this test as a way to measure any motor deficits that may appear after injury, as well as any motor improvements that may occur after taking omega 3s.

Desai, et al. (2014) used the beam walk and NOR test. In this study the rotarod slowly increased from 4 to 400 rotations per minute (rpm) for five minutes, and the animals were tested for the six days following TBI (Desai, et al., 2014). The results showed delayed recovery from day 1-7, and the DHA adequate group had significantly higher scores on days 2 and 4. This group was higher on the other days, but it was not a significant amount (Desai, et al., 2014). The study concluded that deficiencies in DHA hindered recovery. Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) had very similar methodologies. In these studies, the rotarods were increased slowly from 5 rotations per minute (rpm)-45 rpm over the course of 5 minutes. Chen, Pan, et al. (2018) tested subjects on days 1, 3, 7, and 14 days after injury, and Chen, Chen, et al. (2018) was the same except it did not have animals tested one day after injury. Both studies showed significant improvement in the TBI and omega-3 group on day 7 and day 14.

Tang, et al. (2018) used three training trials at 16 rpm for 1 minute, and one baseline test for 2 minutes given an hour before TBI. Three test trials were given on days 1, 3, and 5, and they were averaged and recorded to be compared. This study found that DHA treated groups had significantly better scores than the TBI control group (Tang, et al., 2018). Schober, et al. (2016) a test trial that slowly increased the rotarod speed from 4-45 rpm for 5 minutes on days 12 and 35 after injury, and this study showed no significant differences in time spent on the rotarod between any of the groups. They concluded that their CCI model did not produce significant motor impairments according to the rotarod test (Schober et al., 2016).

The results from these studies all showed improvement and an effect of omega 3s, however, they all showed effects on different days. All but the first article started DHA treatment around the time of injury (see Appendix); therefore, one would posit that these studies would have similar days of improvement. The study that had third generation mice with adequate or deficient amounts of omega-3s (see Appendix) showed significant differences on day 2 and 4. Although the other days showed a difference in scores, it seems unlikely that having an omega-3 adequate brain would have such an effect on one day but not the next. This inconsistency leads to questions regarding the stability of the effect. The final study did not see an effect of injury on any of the subjects' motor performances. The small sample sizes of all the studies may have resulted in effect sizes that were not actually representative of the true effect which would explain the variations of results (Button, et al., 2013).

Morris Water Maze Test

In general, the Morris water maze test looks at change in cognition through the measurement of spatial memory and learning (Wang, Jiang, Pu, Zhang, An, Hu, Liou, Leak, Gao, & Chen, 2013). The test consists of a water tank divided into four quadrants in which the

rat or mice is released from one of the four quadrants and timed to see how long it takes for them to discover an escape platform (Wang, et al., 2013). Four consecutive trials are run in a row. These consecutive trials measure learning because by the fourth trial the animal should have learned through visual cues the quadrant that contains the platform. The time or latency to get to the platform should, therefore, decrease with every trial (Wang, et al., 2013). After four trials, a fifth trial is run, and the platform is removed. In this trial the subject is released into the tank and forced to swim. Then the amount of time spent in each quadrant is recorded. This test measures spatial memory in the animal (Wang, et al., 2013). If the animal remembers which quadrant had the platform, they would spend more time in that quadrant. Ten of the studies used this test to measure the effectiveness of omega-3s.

Pu, et al. (2017) examined that looked at this test assessed C57BL/6J mice that had been given a TBI through the CCI model. The animals were pretrained for the test three days before injury with three trials each day. The actual tests were completed on days 29-34 after injury with four trials on each day. The animals were given 10 seconds on the platform once they found it, or once they were directed there if they were unable to find it within 60 seconds (Pu, et al., 2017). On the last day of tests, the animals performed a sixth trial with the platform removed to test the memory of the animal. In this trial, the researchers also tested swim speed (Pu, et al., 2017). The results from this study indicated that the FO group or the FO+N3 group had significantly better latency times to the platform when compared to the control group (Pu, et al., 2017). The FO+N3 group was also beneficial for memory retention because this group had significantly better scores than the control group and the other two experimental groups in the fifth trial test (Pu, et al., 2017). All the groups had similar swim speeds, therefore, any differences seen in the other tests are not due to motor dysfunction (Pu, et al., 2017).

Wang, et al. (2013) gave the MWM test on days 10-14 after injury, in which the rats were given 120 seconds to find and climb on the escape platform. If they did not find the platform they were guided there. After finding the platform each subject was given 30 seconds on top of the platform and then given a 4-minute break between trials. Four trials were completed each day with the rats starting in a different quadrant each trial (Wang, et al., 2013). A fifth trial was conducted each day as well in which the platform was removed, and the animals were timed to see how long they spent in each quadrant (Wang, et al., 2013). This study additionally tested for visual acuity by placing a visible platform in a different quadrant. This was a control performed to ensure that a subject's eyesight did not affect their ability to perform the test (Wang, et al, 2013). The results from this study found that on day 14 the fish oil group had significantly shorter times to find the platform than the control diet group. In the trial that took away the platform, the fish oil group also performed better because they spent more time in the quadrant that originally contained the platform (Wang, et al., 2013). There was no difference in time to find the visible platform which indicated that vision did not have an effect on the subjects (Wang, et al., 2013). The researchers concluded from this study that fish oils aided in the recovery of memory and learning following TBI (Wang, et al., 2013).

Zhu, et al. (2018) conducted this test on days 5-8 after injury, and the same general five trials were given on each day. The results showed that there were significant memory and learning deficits in all of the TBI groups. They also showed that the rats in the TBI with DHA groups had significantly reduced times from the TBI group and the TBI with saline group (Zhu, et al., 2018). Both the sham injury group and the TBI groups with DHA swam significantly longer in the target quadrant than the other groups. The researchers concluded from these results

that DHA helped to improve memory retention and learning when recovery from TBI (Zhu, et al., 2018).

In Yin, et al. (2018) the animals were assessed with the MWM days 14-20 after injury. On days 14-18 the tests that involve the invisible escape platform were given. On these days they completed four trials that lasted until they found the platform with 4-minute intervals between each trail. They were given 30 seconds on the platform once they found it or were guided to it (Yin, et al., 2018). On days 19-20 the test that involves the visible platform in the pool was given (Yin, et al., 2018). On days 18 and 20 the group treated with DHA performed better than the TBI control group. The researchers concluded from this that treatment of DHA following CCI on mice improved memory retention and spatial learning (Yin, et al., 2017).

Schober, et al. (2016) completed this test using Sprague Dawley rats and the CCI TBI model. The animals were tested on days 41-45 after injury with four trials each day, a maximum swim time of 120 seconds, and 14 minutes of rest between each trial (Schober, et al., 2016). On day 46 and 47 they were tested with the visible platform to test swim speed. On day 48 the platform was removed to analyze time spent out of 60 seconds in the target quadrant (Schober, et al., 2016). The DHA group performed similarly to the sham group, and they performed significantly better than the regular diet group on day 43 and 44. There were no differences found in the trials when the platform was visible and when there was no platform (Schober, et al., 2016). Therefore, the study concluded that DHA improved cognition during recovery (Schober, et al., 2016).

One study that used the MWM test used C57BL/6J mice and the CCI model for injury (Pu, Guo, Zhang, Huang, Wang, Liou, Zhang, Zhang, Leak, Wang, Chen, & Gao, 2013). The tests were given on days 22-26 after injury. This study had five trials a day. The first four trials

had the platform and the animals were started from a different quadrant each time while in the fifth trial the platform was removed (Pu, et al., 2013). The specifics of time given to swim and rest in between trials were not stated in the study. This study showed a statistically significant difference between the group that was treated with DHA and the group that was deficient with DHA on day 25 and 26 after injury (Pu, et al., 2013). There was also a significant difference found between these two groups in the trial without the platform (Pu, et al., 2013). These results indicate that DHA helps to improve memory and spatial learning in rat's recovery from TBI.

Lin. et al. (2017) used the MWM test and examined Sprague Dawley rats after they received TBI by CCI. The test with the hidden platform was performed on days 11-15 after injury with 30 seconds between each of the four trials performed on that day. The animal was allowed 100 seconds to look for the platform until they were eventually led to the platform (Lin, et al., 2017). On day 16 and 17 the visible platform test was given. The results showed that the TBI and DHA group performed significantly better than the group on the regular diet. They also showed that there was no difference in swim speed between the groups suggesting that any difference in time on the hidden platform test was likely due to the TBI (Lin, et al., 2017). Some animals were also given GW1100, an antagonist to an anti-inflammatory receptor, to see if it would inhibit the benefits of the omega-3 rich diet (Lin, et al., 2017). The results showed a significant difference between the TBI and DHA group with GW1100 and the TBI and DHA group without it. Suggesting that omega-3s are beneficial to TBI recovery through the receptor pathway that GW1100 inhibits (Lin, et al., 2017).

Wu, et al. (2011) looked at the MWM test and completed the tests on days 1-5 following injury. Two trials were completed every day, and each trial the animal was given one minute to find the hidden platform (Wu, et al., 2011). If the animal did not find the platform, they were

placed on it. Each subject stayed on the platform for 1 minute (Wu, et al., 2011). The animals with the enriched omega-3 diet performed better than the regular diet group on days 4 and 5. Therefore, the study concluded that DHA improved any learning disability that was caused by the TBI (Wu, et al., 2011).

Mills, Hadley, et al. (2011) administered the test on day 14 after injury. On this day ten trials were performed with up to 120 seconds a trial. Three pretraining trials were also completed the day before injury. The test analyzed the time or latency to get to the platform as well as the number of errors made (times they crossed the center of the pool). The results from this study showed that animals not supplemented took longer to find the platform and made more errors than the supplemented group (Mills, Hadley, et al., 2011). The results also showed that the group that received 40 mg of DHA took significantly longer to find the platform and made more errors (Mills, Hadley, et al., 2011). While the 4 mg DHA group and the 12 mg DHA group showed improvement, they were not statistically significant changes. The study suggested that DHA does improve learning some but not a significant amount, and it showed that too much DHA can also hinder learning as well (Mills, Hadley, et al., 2011).

Wu, et al. (2004) gave the tests on days 5, 6, and 7 after injury with ten trials on each day and 120 seconds per trial. If the animal did not find the platform within 2 minutes, it was placed on the platform. After each trial the animal was allowed to rest on the platform for one minute (Wu, et al., 2004). The subjects in the regular diet and TBI group performed significantly worse on the test than the sham group. The TBI group given the DHA supplement showed improvement but there was no statistically significant difference between the TBI group with the regular diet (Wu, et al., 2004). The study concluded the DHA supplementation could aid in improving learning impairments caused by TBI (Wu, et al., 2004).

Overall, 8 of the 10 studies showed significant improvements in learning and memory in the omega-3 treated groups. Five of those studies showed improvements on the later days of testing, and 3 showed better scores on the majority, if not all, of the days. Overall, there were too many differences between these groups to explain why some only showed improvement on the last two days of testing and some showed improvements on every day of testing. The two studies that did not show significant improvements both ran 10 trials in one day. This larger number of trials may have allowed even the TBI control group to improve in memory. This could have given them an average of a slightly quicker time, therefore, closer to the omega-3 treated groups. Similar to the other behavioral tests, these studies all have extremely low sample sizes (see Appendix); therefore, any significant effect size that is seen is unreliable (Button, et al., 2013). Since an effect was seen in the majority of the studies, however, this may indicate that omega-3s do help improve recovery, but it may not be a practically significant amount.

Western blot Test

A Western blot test is performed to identify specific proteins (Mahmood, & Yang, 2012). This is accomplished by separating the proteins by molecular weight by gel electrophoresis, transferring them to a solid support such as a membrane, and finally marking the proteins with a primary or secondary antibody to help visualize it (Mahmood, & Yang, 2012). Any unbound antibodies are washed off. Since antibodies only bind to a specific protein, only one band will appear, and the thickness of the band corresponds to the amount of protein (Mahmood, & Yang, 2012). Therefore, this technique can be used to identify a variety of proteins that represent various physiological problems that can result after a TBI. Fourteen of the studies completed this test.

Desai, et al. (2014) used Western blot testing to look at the amount of α-spectrin breakdown products (SBDP) 24 hours after injury and to look at synapsin 1 protein expression 7 days after injury. SBDPs have been shown to be good indicators of brain injury and to be helpful in determining the severity of a TBI (Desai, et al., 2014). Synapsin 1 is a phosphoprotein that implicates synaptogenesis, therefore, less synapsin 1 means more loss of synapses (Desai, et al., 2014). The results from the Western blot showed that the omega-3 deficient group had significantly more SBDP produced. Since SBDPs are produced during injury, these results indicate that the omega-3 deficient subjects had a more damage caused by the TBI (Desai, et al., 2014). A significantly less amount of synapsin 1 in the brain was also found in both the TBI injury groups when compared to the sham group. This shows that TBI induces synaptic loss. The DHA sufficient group had more synapsin 1 than the deficient group which implies that DHA plays a role in maintaining synapses following TBI (Desai, et al., 2014).

Wu, et al. (2007) used the Western blot test and looked at Sprague Dawley rats that were given an TBI by FPI. Hippocampal tissue samples were used to extract the proteins and complete the analysis (Wu, et al., 2007). The study tested for Sir2α expression, oxidized proteins, AMPK and p-AMPK levels, and uMtCK levels. Sir2α, silent information regulator 2, plays a role in cellular metabolism and homeostasis (Wu, et al., 2007). It has also been shown that oxidative stress which is a product of metabolic dysfunction can reduce the expression of Sir2α. Therefore, since it is known that TBI causes oxidative stress it was hypothesized that Sir2α would be affected by TBI (Wu, et al., 2007). The results from this study showed that rats that were on a normal diet had significantly lower expression of Sir2α in the hippocampus on days 7 and 14 after injury (Wu, et al., 2007). There was no decrease on day 1. The results also showed that the animals in the fish oil diet had restored some of the Sir2α lost from TBI (Wu, et al., 2007). To

visualize the amount of oxidative stress that occurred, the western blot test was also used to show the amount of oxidative proteins within the hippocampus. The results showed an increased amount of oxidative proteins in the TBI group and a reduced number was seen in the fish oil group (Wu, et al., 2007). AMPK and phosphorylated AMPK (p-AMPK) also play a role in maintaining energy balance within the brain. The result of this study supports this statement by showing a decrease in these proteins in the TBI group. The benefit of omega-3s was also shown because the levels of these proteins had been restored back to an almost normal level in the fish oil group (Wu, et al., 2007). The final protein they looked at was the ubiquitous mitochondrial creatine kinase (uMtCK). This is an enzyme that has been known to maintain Ca2+ homeostasis (Wu, et al., 2007). The TBI group showed decreased levels of this protein while the fish oil group showed a reversal of this effect and returned the levels to normal (Wu, et al., 2007).

Chen, et al. (2017) looked at this form of analysis by extracting proteins through a RIPA lysis buffer then running it through gel electrophoresis. The study used antibodies for caspase 3, Bax, SIRT1, Iba-1, (p)-IκB, TLR4, and NF-κB p65 (Chen, et al., 2017). The results showed that caspase 3 and Bax, which regulate apoptosis, significantly increased in the TBI control group and were significantly reduced in the omega-3 group 3 days following TBI (Chen, et al., 2017). SIRT1 levels, which play a role in the regulation of autophagy, were similarly upregulated in the omega-3 group. The other factors that were tested are all inflammatory factors that contribute to the inflammatory response (Chen, et al., 2017). The study found that the omega-3 group had significantly reduced the expression of these factors when compared to the TBI group (Chen, et al., 2017).

Both Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) completed the Western blot test 7 days post-injury by extracting the proteins with a radioimmunoprecipitation assay lysis

buffer and then running them through gel electrophoresis. Chen, Chen, et al. (2018) examined (Bcl)-2, Bcl-2-associated X factor (Bax), CD16, CD206, HMGB1, Iba-1, and NF-κB p65 with their own specific antibody. Bcl-2 and Bax are both involved in the apoptosis. Bax encourages apoptosis while Bcl-2 works against it. The results showed that omega-3s counteract apoptosis by reducing the amount of Bax and increasing the amount of Bcl-2 (Chen, Chen, et al., 2018). The results showed that CD16, Iba-1, and HMGB1, which are all microglial activation factors, were reduced in the brains of the omega-3 groups. This shows the protectiveness that omega-3s have on the brain. CD206 inhibits the microglial activation and had an increase in production in the omega-3 group (Chen, Chen, et al., 2018). NF-κB p65 is involved in the inflammatory process and the omega-3 group similarly showed inhibition of its activation (Chen, Chen, et al., 2018). Chen, Pan, et al. (2018) examined P62, HO-1, NQO1, UGT1A1, and cleaved caspase-3, Beclin-1, ATG-3, and ATG-7, each with their own individual antibodies. The results showed that Beclin-1, ATG-3, and ATG-7, regulatory autophagic markers, all significantly increased in the omega-3 group compared to the TBI control group (Chen, Pan, et al., 2018). The cleaved caspase-3 factor which helps initiate apoptosis was reduced in the omega-3 group. The rest of the factors that were tested are all helpful in reducing oxidative stress. The presence of omega-3 in the omega-3 group helped to significantly increase the production of these factors, therefore, reducing oxidative stress (Chen, Pan, et al., 2018).

Zhu, et al. (2018) performed the Western blot test 24 hours after injury was induced and 5 rats from each group were used to complete this analysis. They used anti-Nrf2, anti-HO-1, anti-NQO-1, anti-Bcl-2, anti-cleaved caspase-3, and rabbit anti-Bax to stain their respective factors. The results showed that the apoptosis mediators, cleaved caspase 3, and bax were all reduced in the TBI and DHA group (Zhu, et al., 2018). Bcl-2 which works against apoptosis was increased.

Nrf2 is involved with the induction of autophagy while HO-1 and NQO-1 are involved in the regulation of the Nrf2 pathway (Zhu, et al., 2018). DHA administration following TBI helped to reduce the Nrf2 factors and increase the expression of the HO-1 and NQO-1 factor, therefore, acting as neuroprotection to prevent cell death (Zhu, et al., 2018). Tang, et al. (2018) performed the Western blot test to examine protein expression of TLR4, p65 polyclonal, TNF-α polyclonal, and IL-1β polyclonal by treating them with their specific antibody. All are mediators to inflammation through the TLR4/NF-Kappa B signaling pathway (Tang, et al., 2018). All were upregulated in the TBI group but reduced by the administration of DHA. This supported the researchers' hypothesis of omega-3s role in inflammation reduction (Tang, et al., 2018).

Yin, et al. (2018) used the RIPA lysis buffer to extract the proteins from the sample. The appropriate antibodies were placed on the LC3B, Lamp-1, Lamp-2, CTSD, and p62 (Yin, et al., 2018). The Lamp-1 and Lamp-2 factors, which play a role in autophagy response, showed a significant decrease in expression in the DHA group than the TBI group. LC3B, CTSD, and p62 are all involved in the regulation of autophagy. CTSD also showed a significant decrease following treatment of omega-3s (Yin, et al., 2018). LC3B had a lack of build-up in the TBI groups for unclear reasons (Yin, et al., 2018). The factor p62 was explained as having increased expression in both TBI groups initially following TBI, but the levels decreased in the DHA group around day 3 (Yin, et al., 2018).

Lin, et al. (2017) analyzed IL-1β, caspase -1, NLRP3, ARRB2, and GPR40 by treating them with their corresponding antibody. Caspase-1 and IL-1β play a role in inducing inflammation. The results showed that the expression of these proteins was inhibited significantly in the omega-3 treatment group (Lin, et al., 2017). They also showed that GPR40 expression was silenced by the omega-3 treatment group. Since this plays a role in induction of

inflammation, these results show that omega-3s can inhibit inflammation following injury (Lin, et al., 2017). It was also found that both NLRP3 and ARRB2, which play a role in inflammation, were significantly reduced by the presence of omega-3 (Lin, et al., 2017). Overall, the results indicate that omega-3s reduced inflammation responses following TBI (Lin, et al., 2017).

The primary protein tested in Harvey, Yin, Attarwala, Begum, Deng, Yan, Dixon, & Sun, (2015) was the presence of NF-kB p65. The results showed that the DHA group had decreased amounts of NF-kB p65 expressed, however, difference from the TBI control group was not statistically significant (Harvey, et al., 2015). Since this factor plays a role in the inflammatory response, the results show that DHA plays a role in inflammation inhibition (Harvey, et al., 2015). Begum, et al. (2014) used Western blot testing to examine the expression of p-eIF2α, eIF2, ATF-4, IRE1α, and CHOP. P-eIF2α is a marker for ER stress, and it was found to be expressed significantly less in the DHA treatment group. There was no effect on the eIF2 factor. IRE1α and ATF-4 also play a role in ER stress and were similarly produced less in the DHA treatment group (Begum, et al., 2014). CHOP places a role in linking ER stress and apoptosis. The results showed that DHA significantly reduced the expression of these molecules which showed the significance of DHA on inhibition of ER stress and apoptosis (Begum, et al., 2014).

Wu, et al. (2011) completed the Western blot test to examine the expression of BDNF levels, Syn-I, CREB, CaMKII, SOD, Sir2, 4-hydroxynonenal (4-HNE), iPLA2, and STX-3 by treated them with their corresponding antibodies. Both BDNF levels and CaMKII play a role learning and memory in the hippocampus. DHA affected them similarly by increasing their expression that had been reduced from the TBI (Wu, et al., 2011). Syn-I and CREB play a role in synaptic plasticity and were reduced following TBI, however, the DHA supplement group showed significant increases in their production (Wu, et al., 2011). 4-HNE plays a role in

neuronal signaling of lipid peroxidation. DHA significantly reduced the production of this factor. SOD and Sir2 both play a role in controlling oxidative stress within the brain, and the addition of DHA caused a drastic increase in their expression. This shows the importance of DHA on reducing oxidative stress (Wu, et al., 2011). iPLA2 and STX-3 play a role in membrane homeostasis and membrane expansion respectively. Both are increased in expression when they are treated with DHA which shows DHA's role in normal membrane function (Wu, et al., 2011).

Wu, et al. (2004) completed a Western blot test to analyze BDNF levels, synapsin-1, and CREB by treating them with their respective antibody during the staining process. BDNF helps to maintain synaptic plasticity. When the fish oil group showed BDNF levels return to normal, this showed that DHA plays a protective role in the brain following a TBI (Wu, et al., 2004). Synapsin-1 and CREB similarly play a role in synaptic plasticity. Expression of both returned to normal for the fish oil supplement group (Wu, et al., 2004).

Immunohistochemical Staining

Immunohistochemical staining is similar to Western blot staining, however, one important difference is that Western blot is done by extracting and separating out the specific protein being analyzed while in this analysis the actual brain tissue is stained and analyzed (De Matos, Trufelli, De Matos, & Da Silva Pinhal, 2010). A slice is made in the section of the brain that is of interest, and then it is processed and prepared so that the cellular components within the tissue are not damaged (De Matos, et al., 2010). The tissue then goes through a complex process in which various enzymes and antibodies are stained onto the sample to reveal the protein or other cellular structure that is being analyzed (Matos, et al., 2010). The specific type of antibodies added depends on the protein that is of interest to the researchers. This staining allows

the researcher to not only identify the proteins but specifically shows their location and distribution throughout is original source (Matos, et al., 2010). 10 studies used this method.

Wu, et al. (2007) that used this type of staining sacrificed rats on days 1, 7, and 14 after injury to obtain the brain samples. To do this immunostaining, coronal slices were made 25μm apart and then mounted on slides before being coated with anti-Sir2 (Wu, et al., 2007). A control was also done by excluding the addition of the antibody from the staining process. The results showed that the Sir2 was distributed in the mossy fiber system and granule layer of the dentate gyrus in the hippocampus. Rats in the TBI group showed reduced Sir2α expression in this region, and the TBI with fish oil diet group showed that the omega-3s counteracted this reduction (Wu, et al., 2007).

Bailes & Mills (2010) used immunohistochemistry staining to analyze APP positive axons and Caspase-3 positive axons. Serum levels for DHA, EPA and overall omega-3s were obtained by blood samples to ensure that there were significant differences in the amounts of these fatty acids based on the treatment methods (Bailes & Mills, 2010). The results showed that the group with no supplement had decreased amounts of fatty acids following injury. To complete this staining, sagittal slices were made 50μm thick and mounted on a plate. They were then diluted with the APP (β-amyloid precursor protein) antibody (Bailes & Mills, 2010). These proteins travel from the cell body to the axon periphery by fast axonal transportation (Reichard, Smith, & Graham, 2005). Therefore, when the neuron and axon are disrupted like in a TBI, APP can start to build up and be detected within 2 hours after injury (Reichard, et al., 2005). Because of this, they can be used to indicate when neurons have been damaged from TBI. The results from this study showed that there were scarce amounts of APP shown in the sham injury rats. They also showed that the TBI control group had high expressions of APPs in the area of injury

while both groups given the supplements had reduced this expression and had similar amounts to the sham injury group (Bailes & Mills, 2010). While the APP counts differed between the TBI groups they both, unlike the sham group, had similar morphological characteristics like swelling and disconnections that were observed by the researchers (Bailes & Mills, 2010). This experiment also looked at caspase-3-positive axons. As stated earlier, caspase-3 proteins help to mediate apoptosis, therefore, they are helpful indicators of a brain injury (Barret, et al., 2014). This study found that the TBI control group had a significant number of caspase-3 proteins being expression compared to the sham injury group, but the supplemented groups showed a significant decrease in that expression which indicates that DHA can help protect the brain from neuronal damage following TBI (Bailes & Mills, 2010).

To study microglia activation C57BL/6J mice had to be sacrificed 24 hours after receiving a CCI in the Pu, et al. (2013) study. Brain slices were made through the cortex and striatum, stained with the lba-1 antibody, and mounted on slides for further examination (Pu, et al., 2013). The results showed that the number of microglia, or lba-1 positive neurons were significantly raised from the sham injury group. The omega-3 rich diet group showed a decrease in microglia activation from the other TBI group which supports the idea that omega-3s may play a role in diminishing the inflammatory response (Pu, et al., 2013). The study also used histochemical staining to analyze the presence of SMI32 a non-phosphorylated neurofilament, and MBP, a myelin sheath protein (Pu, et al., 2013). The presence of this neurofilament and/or loss of the protein indicates myelin sheath damage. Therefore, when the corpus callosum, cortex, and striatum of TBI subjects were stained with anti-SMI32 and anti MBP 35 days post-injury, the researchers saw elevated levels of SMI32 and low levels of MBP (Pu, et al., 2013). The

results also showed that animals that were given omega-3 high diets had reversed these changes seen in the low omega-3 diet group (Pu, et al., 2013).

An immunohistochemical staining was completed by Chen, et al. (2017) by creating 4 µm slices in the cortical lesion area. Anti- HMGB1, Iba-1, and GFAP antibodies were stained onto the slices 3 days after TBI was induced. These three factors all indicate the inflammatory responses either by microglia cells or astrocytes (Chen, et al., 2017). The results showed an increase in expression for all 3 factors in the TBI control group. The omega-3 group had a decrease in the expression for Iba-1 and HMGB1 the factors that activate microglia, but there was no difference in the expression of GFAP, the astrocyte factor. This implies that omega-3s protect the brain by inhibiting microglia activation but not astrocyte activation (Chen, et al., 2017). Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) completed this analysis with 4µm slices in the cortical lesion area. These two studies stained with a SIRT1 antibodies. SIRT1 is a factor that regulates autophagy which is a way to eliminate damaged cells. Both of the studies had results that showed an increase in expression of the SIRT1 factor in the omega-3 supplement group (Chen, Chen, et al., 2018; Chen, Pan, et al., 2018). This supported their hypothesis that omega-3s are neuroprotective through suppression of autophagy (Chen, Chen, et al., 2018; Chen Pan, et al., 2018).

Another immunohistochemical staining that was completed looked at the presence of CD11b and GFAP by treating them with their antibodies (Tang, et al., 2018). This test was completed 24 hours after injury and showed that DHA reduced the number of these cells present within the hippocampal region (Tang, et al., 2018). Both of these play a role in immune reactivity; therefore, they play a role in causing various responses when the brain is in distress (Tang, et al., 2018). The reduction of these proteins from DHA administration shown in the

results, suggest that DHA can aid as protection to the brain following TBI (Tang, et al., 2018). This staining is also performed in Mills, Hadley, et al. (2011) that examines beta APP expression. In this study animals received 4, 12, or 40 mg of DHA supplements, and only the 40 mg DHA group showed a decrease in the amount of APP expression. This suggests that a larger dosage of DHA is needed to have an effect on injured axons within the brain (Mills, Hadley, et al., 2011). Immunohistochemical labeling was also done for CD68, which marks macrophages presence, and Caspase3 proteins, which mark apoptosis. Similar to APP expression, DHA supplementation of 40 mg was the only group that showed significant reduction of CD68 and caspase 3 positive neurons, however, the other treatment groups showed some reduction (Mills, Hadley, et al., 2011).

Mills, Bailes, Sedney, Hutchins, & Sears (2011) that also completed this staining cut the brain slices sagittally and 50μm thick. This study analyzed expression of APP positive neurons and caspase 3 proteins. APP positive staining represents the number of injured neurons within that specific area of the brain and caspase 3 is an indicator of neuronal apoptosis (Mills, Bailes, et al., 2011). Both were significantly reduced in the omega-3 treatment group which shows omega-3 role in reducing neuron degradation and apoptosis (Mills, Bailes, et al., 2011). Wu, et al. (2004) looked at immunohistochemical staining and only observed BDNF expression in the CA3 hippocampal region. Expression of BDNF levels shows synaptic plasticity in the brain. The results from this study showed a decrease in their expression in the TBI control group and a return to normal expression in the fish oil supplement group (Wu, et al., 2004).

ELISA Test

An ELISA, or an enzyme-linked immunosorbent assay, is used to detect different antigens produced in the body through the use of antibodies (Milner, Mack, Coates, Hill, Gill, &

Sheldrick 1990). In that sense it is similar to Western blot analysis, however, instead of transferring the extracted cell matter onto a solid membrane surface, ELISAs are conducted on a microtiter plate (Milner, et al., 1990). In ELISA an enzyme is bound to the antibody that binds to the antigen of interest. After this binding occurs a substrate, usually a chromogen, is added, and when it binds to the enzyme, it becomes colorful (Parker, Schneegurt, Tu, Forster, & Lister, 2016). This reaction illuminates the location of an antigen and allows the researchers to observe and quantify them (Parker, et al., 2016). Three studies used this test.

Chen, et al. (2017) completed this analysis to look at inflammatory factors like TNF-α, IL-1β, IL-6, IFN-γ, and HMGB1. The study found that there were higher expression levels for these factors in the TBI group compared to the omega-3 group. This indicates the neuroprotective role that omega-3s may play by inhibiting the inflammatory response in the brain (Chen, Wu, et al., 2017). An ELISA test was also used to analyze TNF-a and IL-1b in the Harvey, et al. (2015) study. These are cytokines involved in activating inflammation. Three days after TBI, there was no change seen in the expression of these cytokines (Harvey, et al., 2015). This is consistent with another study's results which suggested the optimal time of cytokines expression was 1-3 hours after injury (Harvey, et al., 2015). Wu, et al. (2004) used ELISA testing to analyze the expression of BDNF protein expression in the CA3 hippocampal region. The results showed a decrease in these proteins in the TBI control group, and they also showed that the levels were returned to normal in the fish oil supplementation group (Wu, et al., 2004).

Immunofluorescent staining

Immunofluorescent staining is similar to the ELISA test due to its identification and quantification of antigens present in the brain (Parker, et al., 2016). It differs from these other staining techniques because it uses a fluorogen instead of a chromogen and an enzyme to identify

the antigen. A fluorogen is a fluorescent marker that directly binds to the antigen region and enables researchers to quickly and easily recognize and measure the amounts of the antigen (Parker, et al., 2016). The fluorescent antibodies are placed on a microscope slide and then observed through a fluorescent microscope (Parker, et al., 2016). Seven studies used this staining.

Wang, et al. (2013) used this staining only as a preliminary study to examine the neuronal degeneration that would occur 24 hours after FPI was administered. Four Sprague Dawley rats were examined, and they were on a standard laboratory rat diet. In this study tissues sections were mounted on plates and then stained with FJ-B staining solution to detected FJ-B positive neurons (Wang, et al., 2013). In the results the experimenter found the majority of these neurons were present within the hippocampus and parietal cortex (Wang, et al., 2013). These neurons have been shown to indicate neuronal degeneration following TBI, therefore, they found that 24 hours following FPI in rats can cause neuronal degeneration of hippocampal and parietal cells (Wang, et al., 2013).

Chen, et al. (2017) that used immunofluorescent staining tested for expression of NeuN, Iba-1 and HMGB1 factors. These stainings were completed 3 days following injury. The study found that expression of these factors, and therefore, microglia activation increased following TBI (Chen, et al., 2017). The study also found that supplementation of omega-3s helped to significantly reduce this expression. This supports the study's hypothesis that omega-3s have neuroprotective effects on the brain by inhibiting expression of HMGB1, Iba-1, and NeuN inflammatory factors (Chen, et al., 2017).

Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) performed the immunofluorescent staining with 4 μm thick slices. Chen, Chen, et al. (2018) tested for CD16, CD206, Iba-1, and

HMGB1. CD16, Iba-1, and HMGB1 are microglial factors that activate microglial activation while CD206 is a factor that inhibits microglial release (Chen, Chen, et al., 2018). When the omega-3 group inhibited the expression of CD16, Iba-1, and HMGB1 and increased the expression of CD206, this supported the hypothesis that omega-3s inhibit the inflammation response (Chen, Chen, et al., 2018). Chen, Pan, et al. (2018) stained with antibodies for LC3, NeuN, and Beclin-1. LC3 and Beclin-1 are markers for autophagy. The results showed that the omega-3 group had significantly reduced the suppression of autophagy by increasing the expression of these factors (Chen, Pan, et al., 2018).

Yin, et al. (2018) performed immunofluorescence to look at the presence of Lamp-1, Lamp-2, and CTSD expression in the hippocampal CA 1, CA 2, and CA 3 regions. These factors all play a role in autophagy responses (Yin, et al., 2018). The results showed a statistically significant decrease in CTSD production in the DHA treated group. They also found a significant decrease in LAMP-1 expression which shows DHA's aid in inhibition of autophagy in the brain (Yin, et al., 2018). There was a slight decrease in the Lamp-2 factor, however, it was not a significant amount.

Harvey, et al. (2015) used this technique to examine the expression of CD206, CD16/32, Iba-1, Lamp-1, and CHOP by treating each with their corresponding antibody. All of these were tested in the frontal lobe of the brain. The results showed that there was no effect of DHA on Iba-1 and a very small decrease in the inflammatory response factor CD16/32. CD206, which inhibits inflammation, was found to be increased in the DHA treated groups which suggested that DHA had an effect on this aspect of the inflammatory response (Harvey, et al., 2015). CHOP is a marker for ER stress within the brain, and it was shown that DHA administration reduced the production of CHOP as well as reduced its association with the microglial neurons. This means

that DHA lowers ER stress within the brain by reduction of CHOP, and it may play a role in microglial reduction due to microglial interactions with CHOP (Harvey, et al., 2015). DHA also lowered the expression of Lamp-1 which plays a role in lysosomal activation (Harvey, et al., 2015). Harvey, et al. (2014) also used a variation of this test called Fluor-Jade C staining. This staining measures neuronal degeneration in the brain by determining the number of F-J C stained positive cells present (Harvey, et al., 2014). The results showed that DHA reduced degeneration on days 3, 7 and 21 following injury because it increased the amount of F-J C positive cells present in the brain (Harvey, et al., 2014).

Immunofluorescence was also examined by Begum, et al. (2014) looked at in a study that cut coronal slices 35 μm thick. This study examined p-Tau, CHOP, ATF4, MAP-2, Aβ-APP, and ubiquitin (Begum, et al., 2014). CHOP, ATF4, MAP-2, p- Tau and Aβ-APP all play roles in ER stress which can lead to apoptosis. Similarly, they were all reduced in the DHA treatment group, which shows DHA reduces ER stress following TBI (Begum, et al., 2014). Ubiquitin is a good signal for protein misfoldings which follows ER stress. Therefore, when it was shown that the DHA group had significantly reduced amounts of ubiquitin proteins, it showed DHAs role in ER stress reduction (Begum, et al., 2014).

TUNEL Assay

TUNEL assay is used to label programmed cell death within the brain (Gavrieli, Sherman, & Ben-Sasson, 1992). It stands for Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (Gavrieli, et al., 1992). The name itself describes the important specifics of the assay. A terminal deoxynucleotidyl transferase is an enzyme used to label the end of a broken DNA strand with dUTP (Gavrieli, et al., 1992). The labeled portion is amplified by a peroxidase which allows the labels to be seen though light microscopes (Gavrieli, et al., 1992). A DNA

break indicates the areas when programmed cell death, apoptosis, has occurred; therefore, by using this assay to label DNA breaks, the test can help researchers estimate the amount of apoptosis that has occurred in that area (Gavrieli, et al., 1992). Since apoptosis is a common effect of TBI, this assay can help compare the impact that omega-3s may have on TBI protection. Six studies used this form of analysis.

TUNEL assay was completed by Chen, et al. (2017) three days post-injury to observe TUNEL positive neurons. As stated above these neurons help to depict the amount of apoptosis that has a occurred within the lesioned area (Chen, et al., 2017). The results showed that there were significantly more TUNEL positive neurons in the brains of the TBI control group, and that those neurons decreased in the omega-3 group. Supporting the hypothesis that omega-3s can play a neuroprotective role within the body (Chen, et al., 2017). Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) completed this assay to account for the number of TUNEL positive cells in a lesion slice. They both had results that showed that the number of TUNEL positive neurons decreased in the omega-3 group (Chen, Chen, et al., 2018; Chen, Pan, et al., 2018).

Zhu, et al. (2018) used TUNEL assay to identify TUNEL positive neurons, and therefore, examine the amount of apoptosis that occurred. This study, similar to the previous ones, found a decrease in TUNEL positive cells in the DHA group compared to the control TBI group (Zhu, et al., 2018). Tang, et al. (2018) used TUNEL assay to analyze the brains of rats that were given DHA 30 minutes after injury and the brains of rats that were not given any treatment after injury. The results showed that there were significantly less TUNEL positive cells in the DHA group (Tang, et al., 2018). TUNEL assay was completed in Lin, et al. (2017) by creating 7μm slices and then continuing the normal TUNEL staining process. The results showed a reduction in TUNEL positive cells in the omega-3 treated group (Lin, et al., 2017).

Nissl/ Cresyl Violet Staining

Nissl staining is used to help understand the pathology and morphology of neurons (Kádár, Wittmann, Liposits, & Fekete, 2009). Certain stains especially Cresyl violet interact with the nucleic acids (Kádár, et al., 2009). Therefore, when these stains are added to a brain section it binds to the DNA content of the cell's nuclei (Kádár, et al., 2009). Due to the Cresyl violets ability to bind to the nuclei of each cell separately, researchers are able to distinguish and quantify the number of neurons within a specific area of the brain (Kádár, et al., 2009). Six studies used this form of staining.

Cresyl violet staining was used in Wang, et al. (2013) that examined Sprague Dawley rats after getting a TBI from FPI. Two weeks following injury the rats were sacrificed. Forty-five μm coronal slices of the hippocampus were created, processed, and then stained with Cresyl violet (Wang, et al., 2013). The stains were looking at pyramidal neuron counts of the CA2-3 hippocampal regions (Wang, et al., 2013). Results from this staining showed that the fish oil supplement group did have a larger number of these neurons present than the standard diet group, however, this difference was not statistically significant (Wang, et al., 2013).

Pu, et al. (2013) used Nissl staining and examined C57BL//6J mice following CCI. Similar to the previous study, this study was interested in looking at the viable neuron count of the CA3 hippocampal region as well as the lesion volume of the brains (Pu, et al., 2013). Rats were sacrificed 35 days post-injury to obtain brain tissue that was used to observe the hippocampal viable neuron count while the brains used to examine the lesion volume were evaluated 5 weeks following injury (Pu, et al., 2013). The results from this study showed a significant increase in the number of surviving neurons within the CA3 hippocampal region in

the omega-3 supplementation group. There was no statistically significant difference in tissue loss between the groups (Pu, et al., 2013).

Desai, et al. (2014) used Cresyl violet staining to examine the lesion volume of TBI mice brains. To ensure a true difference in DHA within these mice brains a cerebellar lipid composition was done. It showed that the deficient group had significantly less DHA within their brain (Desai, et al., 2014). At 3-4 months old the mice were given TBIs through the CCI model. Seven brain sections 400μm apart were placed on glass slides and stained with Cresyl violet. A polygon tool was used to determine the lesion area, and then the volume was determined by multiplying the sum of the lesion areas found in each section by the distance between those areas (Desai, et al., 2014). The results from this analysis showed that the deficient mice did have larger volumes, however, they were not statistically significantly different from the omega-3 adequate group (Desai, et al., 2014). The researchers stated that while their technique to determine the lesion volume may not have been sensitive enough to detect small differences in the lesions, their results did support results found in other studies (Desai, et al., 2014).

Chen, et al. (2017) completed a Nissl staining by cutting 4μm slices into the cortical area of the site of the lesion in the subject's brain. These sections were processed, stained for 5 minutes in the Nissl staining solution, and finally, observed for positively stained cells (Chen, et al., 2017). Small amounts of stained neurons would indicate more apoptosis has occurred. The results showed a significantly higher amounts of apoptotic cells in the TBI group compared to the sham injury group (Chen, et al., 2017). They also showed that the omega-3 group had a much lower percentage of apoptosis occur in their brains indicating that omega-3s had a protective effect following TBI (Chen, et al., 2017). Chen, Chen, et al. (2018) and Chen, Pan, et al. (2018) used Nissl staining and cut 4μm slices in the cortical area of the lesion site. They each stained

their slices with Nissl staining solution for 5 minutes and then examined the cell counts. Both found in their results that the apoptotic cell counts were greatly reduced in the omega-3 supplementation group (Chen, Chen, et al., 2018; Chen, Pan, et al., 2018).

RT/PCR

Reverse Transcriptase polymerase chain reaction (RT/PCR) is a test used to identify specific nucleic acids that are present within the body and, in this case, the brain (Parker, et al., 2016). This process begins with the conversion of mRNA to cDNA by a reverse transcriptase enzyme (Parker, et al., 2016). The cDNA is then used as the template to complete normal PCR which is a way to amplify or make copies of a specific strand of DNA (Parker, et al., 2016). While this test can be used to identify DNA that is present, it is unable to quantify the amount of that DNA present. This is because the results are produced by duplicating the DNA making it impossible to identify the original amount present (Parker, et al., 2016). Some studies instead used quantitative RT/PCR (qRT/PCR). This variation of the test adds a fluorescent that allows observation of the DNA as it goes through the PCR process and allows quantification of the original target strand (Parker, et al., 2016). Four studies used this technique.

Pu, et al. (2013) used this method of analysis to measure the amount of mRNA cytokines like IL1-a, IL1-b, and TNFa, as well as measure the presence of COX-2 and iNOS, 24 hours after TBI was induced. These various cytokines and other proteins play a role in inflammation. When it was discovered that the low omega-3 diet group had high expressions of these DNA strands and the high omega-3 diet had a significantly less amount, this supported the hypothesis that omega-3 could help protect the brain from TBI by inhibiting the inflammatory response (Pu, et al., 2013). It was stated that the study also completed a Western analysis to determine the

amount of COX-2 proteins were present. They found a positive correlation between the gene expression and protein presence (Pu, et al., 2013).

Zhu, et al. (2018) which used RT/PCR examined the primer expression of HO-1 and NQO-1. The results showed that the DHA group had significantly more expression of these factors which are involved in the regulation of autophagy through the Nrf2 pathway (Zhu, et al., 2018). This shows that omega-3s can help protect the brain by increasing inhibition of the Nrf2 pathway (Zhu, et al., 2018). Tang, et al. (2018) used qRT/PCR to analyze the expression of TLR4, a factor that indicates neuronal injury. Following a TBI this factor is overexpressed, however, when DHA is administered is suppresses the up regulation, therefore, depicting a reduction in neuronal injury (Tang, et al., 2018).

A qRT/PCR test was completed by Yin, et al. (2018) to analyze and identify the amount Actb, Cstd, Lamp-1, and Lamp-2 three days after TBI. All of these are involved in autophagy related factors. The DHA group showed significant reduction in these factors that were all almost down to the same amounts as the sham injury group had shown (Yin, et al., 2018). This shows the role DHA can play in reducing immune responses like autophagy (Yin, et al, 2018).

NeuN Immunostaining

NeuN immunostaining is a version of immunohistochemical staining that looks specifically at the presence of NeuN proteins (Gusel'nikova & Korzhevskiy, 2015). This means that to identify the NeuN protein an anti-NeuN antibody is used to stick to the protein to identify it (Gusel'nikova & Korzhevskiy, 2015). NeuN is a protein found only in neurons that binds to DNA. They are found in a large number of the neurons throughout the brain except for a few areas that have been discovered that do not contain them (Gusel'nikova & Korzhevskiy, 2015). Since they are found in neurons, they are a good indicator of the neuronal population, meaning

the number of neurons in a specific site (Gusel'nikova & Korzhevskiy, 2015). This technique is therefore used to look at the amount of neuronal loss that may have occurred by examining the amount of NeuN proteins expressed in animals following TBI. Two studies used this technique.

Desai, et al. (2014) used specifically NeuN immunostaining and took five brain slices used per mice near the center of the injury. After completing the staining process, the NeuN positive cells were counted using a metamorph software (Desai, et al., 2014). The results showed that there was significantly less NeuN-positive cells in the injured areas of the brain. They also showed that as the sections got farther from the injury site, there was an increase in the number of NeuN-positive cells (Desai, et al., 2014). The results showed that the omega-3 deficient mice had significantly less NeuN-positive cells than the omega-3 adequate group (Desai, et al., 2014). Pu, et al. (2017) similarly used the NeuN immunostaining technique. The results showed that following TBI there was significant loss of neurons in the CA-1 and CA-3 hippocampal areas. The results also showed that there was no change seen in the omega-3 treated groups suggesting that omega-3s do not protect the hippocampal area from neuronal loss (Pu, et al., 2017).

All of these analyses are slightly varied procedures with the same goal of determining the presence or amount of expression of a particular protein. These proteins are all associated with a specific physiological process that follows a TBI. One common occurrence is an inflammatory response. Four commonly looked at protein factors that effect inflammation are NF-kB p65, TNF-α, IL-1β, and TLR4. They were tested by multiple studies via the western blot test, ELISA, and RT/PCR test, and each of these studies similarly found a significant reduction of these factors with the administration of omega-3s. Although each of these studies had low sample sizes (see Appendix), the common result found in each of them suggests that these results do have a high probability of being representative of the true effect in the population. Another group of

factors are associated with the inflammation process; however, they specifically play a role in microglial activation. The factors that were looked at most in the studies are CD16, CD206, Iba-1, and HMGB1. CD206 plays a role in inhibition of microglial activation while the other three promote it. By using the Western blot test, immunohistochemical staining, ELISA, and immunofluorescence staining, the studies found a common result that CD16, Iba-1, and HMGB1 were all reduced with the use of omega-3s. One study did not find a significant effect on these factors between any of the groups. This study, however, looked at slices that were in the frontal lobe. This result is not surprising because most of the other studies found results in the hippocampal region. Similar results were found between the techniques and studies that looked at CD206. They found significant increases in the expression of this factor which inhibits microglial activation. The supporting results of all of these studies suggest that omega-3s are protective to the brain following TBI specifically through inhibition of microglial cells.

Another response in the brain that is activated following a TBI is the activation of autophagy. Autophagy can be portrayed by the presence of multiple factors including SIRT1, Beclin-1, LC3, Lamp-1, Lamp-2, CTSD, HO-1, NQO-1, and p62. Multiple studies examined these proteins by western blot and immunofluorescence. SIRT1, Beclin-1, HO-1, NQO-1 and p62 factors all regulate autophagy either directly or by regulating oxidative stress which can promote autophagy. The role of omega-3 in regulating oxidative stress and autophagy was supported when an increase in production of these factors was observed following omega-3 supplementation. All of these studies found significant increases in the production; therefore, it is likely that this function of omega-3s does occur in the brain. The extent of that function, however, may not be as significant as the studies found due to the fact that they all had low sample sizes (see Appendix). Lamp-1, Lamp-2, and CTSD, unlike the previous ones, promote

autophagy activation. They all decreased in their amount of expression when omega-3s were given as a treatment. One of the times Lamp-2 was tested this reduction was not significant, however, all of the other effects found were. These results have the same problem as the other proteins because they had low sample sizes (see Appendix). LC3 was examined by two studies, one using western blot and the other immunofluorescence, and the western blot showed a lack of expression of LC3 in the TBI group making the results inconclusive. A mistake could have been made on the western blot test due to its multistep process which is unlike immunofluorescence that simply involves adding a fluorescent dye. Excluding these results, the rest of findings for the autophagy factors portrayed that omega-3s significantly inhibit autophagy cell death.

Another major way to examine the result of a TBI is by determine the amount of cell death that has occurred as a result. This can be done by examining factors that promote apoptosis, factors that promote synaptogenesis, or simply just the presence of healthy and injured neurons. Some of the factors that were examined most frequently in the studies were Bax, caspase-3, APP, CHOP, BDNF, and CREB. The first four are involved in the activation of apoptosis. They were all shown to be significantly reduced in the omega-3 treatment groups. TUNEL assay similarly tests for cell death that has occurred in the brain, and the results for these tests all showed significant decreases in the number of damaged neurons within the brain. The combination of all of these results suggests that omega-3s are helpful in reduction of neuronal loss following TBI. This function of omega-3s can also be done by testing for factors that indicate synaptic functions are working like BDNF and CREB, and by testing the presence of functioning neurons through NeuN staining. All of these studies found a reduction in BDNF, CRER, and NeuN positive cells following TBI, and reversal of this reduction in groups that were treated with omega-3s. Considering these multiple factors that all represent the loss of neurons

after TBI and then an inhibition of this loss with the treatment of omega-3s, it is likely that this function does in fact exist. However, the true effect of that function may not be as significant as is depicted in these studies due to the fact that they all have small sizes (Button et al., 2013).

Conclusion

Overall, the majority of the studies did come to similar conclusions that omega-3s do provide a neuroprotective effect for the brain following a TBI. In the majority of the results, the methodology showed a reverse effect in the omega-3 or DHA groups from the TBI control groups. This includes studies that had subjects on supplementations for their entire life and studies that provided supplementations about 30 minutes – one hour after injury was induced. Since these results were so similar in their findings it is intriguing that they have not carried over to clinical trials.

One reason these conflicting results could have occurred is due to the small sample sizes used in many of these studies (see Appendix). As stated earlier a small sample size can make an effect seem larger than it actually is in reality (Button, et al., 2013). By statistically combining the results of multiple studies it can improve the probability of the size effect (Russo, 2007); therefore, giving a better estimate of how omega-3s may help humans. A limitation found during this analysis was that not enough statistical evidence was reported by these studies to combine their results. Many of the studies reported finding statistical differences, however, did not report the actual statistics that allowed them to come to that conclusion. While some studies did provide the statistical values, not enough information was given for related tests to combine for estimation of overall effect sizes. This made the completion of statistical meta-analysis impossible to complete. It is important for this information to be included so other studies can complete meta-analyses, replicate the studies and compare results, and reference these articles

for future research. The lack of effective sample sizes and sufficient explanation of results contribute to the replication crisis that is prevalent within the psychology field (Button, et al., 2013). Another limitation of this paper was that more attention could have been given to the exact dosages provided in each study. The few studies that compared dosages within their experiments showed differences in the recovery depending on the amount given and even the way it was given. Analyzing this more closely may have given more insight into any differences that were seen.

Future studies should use larger sample sizes to see if they produce the same results as the ones described above. This could help improve the likelihood of an estimated effect size similar to the real effect size. It is also important to remember in future studies to fully report the statistical findings so that this information can be used by other research. It is also important to complete more clinical trials to better determine the effect that omega-3s may have on humans. Although omega-3s may be effective and helpful to rodents following a TBI, they may not have the same effect in people simply because they are a different species. Although the general presence of omega-3s is similar in rats and humans, the dosage provided to them may have substantially different effect in the body making it not as helpful as a treatment for humans. That being said, it is also important to test and analyze the different dosages provided to help determine what is best for aiding in recovery. Another future study could look at beginning omega-3 treatment at varying time periods around the injury to determine when treatment is most effective as well as when it is no longer effective.

It may also be interesting to know how far the damage spreads to the rest of the brain, and if after various impacts is it more likely for surrounding areas of the brain to be injured as well. A few of these studies tested regions other than the primary region where the impact

occurred and found little to no effects on dysfunction in those regions. It would be helpful to see if similar studies that tested for this found the same results. Many studies also gave the impact in a similar area on the head; therefore, future studies could look at whether impacts from different sides and angles are more likely to cause different behavioral difficulties depending on the region of the brain closest to the hit. These various other applications may be helpful in applying to human TBIs and mTBIs which often occur in different ways. Few emotional tests were given following injury; therefore, future studies could look at the effects of TBIs on different mental and emotional side effects like depression and anxiety. Since these are common symptoms seen in many people with these injuries, it would be helpful to see if omega-3s would be helpful in resolving those symptoms.

The large number of TBIs that occur throughout the United States every year and around the world shows the importance of determining a treatment that can help individuals that suffer from their symptoms. In general, these studies found similar results which shows an effect of omega-3s on recovery from TBI, however, as of now this has not shown to be as effective in human trials. These conflicting results may be due to the small sample sizes used by the majority of the studies that completed this research. It is important for future studies to use more substantial sample sizes and to completely share all results that they find in order to determine if this treatment should still be considered for human use. While the functions of omega-3s appear to be a good treatment for the complexities of traumatic brain injuries, it is still unclear if they will be effective enough to help recover from this debilitating injury.

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Appendix: General Methods Description of Each Study

Study	Subjects	Mechanism of Injury	Administration of Omega-3
Bailes & Mills, 2010	Sprague Dawley rats	Marmarou impact acceleration injury	Sham injury group, TBI group with no supplement, TBI group with 10 mg DHA supplement, TBI group with 40 mg DHA supplement. Supplements started 1-day post-injury and for 30 days following. (n=10/group)
Desai, et al., 2014	E14 C57BL/6J mice	CCI model	Three generations of mice on omega-3 deficient or adequate diet. When third generation was 3-4 months old induced TBI. Also had sham injury group (n= 7-8/group)
Pu, et al., 2013	C57BL/6J mice	CCI model	Low omega-3 diet or high omega-3 diet, started 2 months before injury (n=10/group)
Wang, et al., 2013	Sprague Dawley rats	FPI model	TBI group given standard diet throughout the experiment. TBI group with fish oil diet four weeks before injury and 2 weeks post-injury. (n=16/group)
Wu, et al., 2007	Sprague Dawley rats	FPI model	Standard diet for one week then exposed to TBI. Sacrificed either 1,7, or 14 days after injury. Two other groups put on either standard diet or fish oil diet 4 weeks before injury and continued on that diet for 7 days following injury. Also had sham injury group. (n=5-6/group)
Chen, Wu, et al., 2017	Sprague Dawley rats	Feeney DM TBI	Sham injury group, TBI and saline injection group, and TBI and omega-3 fatty acid injection group, first injection 30 minutes post-injury then given once a day for seven days following (n=12/group)
Chen, Chen, et al., 2018	Sprague Dawley rats	Feeney DM TBI	Sham injury group with saline injection, sham group with omega-3 injection, TBI group with saline injection, TBI group with omega-3 injection. First injection given 30 minutes after injury then given once a day for 7 days. (n=12/ group)
Chen, Pan, et al., 2018	Sprague Dawley rats	Feeney DM TBI	Sham injury group with saline injection, sham group with omega-3 injection, TBI group with saline injection, TBI group with omega-3 injection. First injection 30 minutes after injury then given once a day for 7 days (n=12/group)

Zhu, et al., 2018	Wistar rats	FPI model	Sham injury group, TBI group, TBI group no injection, TBI group given saline, TBI group given 370 mg of DHA, TBI group given 555 mg of DHA, and TBI group given 740 mg of DHA. Injections given 30 minutes after injury (n=10/group)
Tang, et al., 2018	Sprague Dawley rats	CCI model	Sham injury group, TBI group with saline injection, TBI group with DMSO injection, TBI group with DHA injection. Injections given 30 minutes after injury and 1, 3, and 5 days after injury. (n=10/group)
Yin, et al., 2017	Sprague Dawley rats	CCI odel	Sham group with DMSO injection, TBI group with DMSO injection, and TBI group with DHA injection. Injections given 10 minutes after TBI and days 3 and 7 after injury (n=20 total)
Lin, et al., 2017	Sprague Dawley rats	CCI model	Pregnant mice started on a standard diet with limited DHA and EPA or omega-3 rich diet. After birth rats were continued omage-3 rich or limited diet. 8 weeks old when injury was induced and had sham injury group. GW1100, GPR40 inhibitor, given 30 minutes before injury in an additional group (n=8-12/group)
Pu, et al., 2017	C57BL/6J mice	CCI model	Sham injury group, a TBI group with a saline injection, a TBI group with an omega-3 injection, a TBI group given an omega-3 supplement, and a TBI group given both the injection and supplement. Injections given 2 hours after TBI and 14 days following. Supplements started day of injury and 35 days following (n=6-8/group)
Harvey, et al., 2015	Sprague Dawley rats	CCI model	Sham injury group with DMSO, TBI group with DMSO injection, and TBI group with DHA injection. Injections given 5 minutes following injury and given on days 3, 7, and 21 after injury (n=72total)
Schober, et al., 2016	Sprague Dawley rats	CCI model	Sham injury group, TBI group with regular diet, and TBI group with DHA diet. DHA diet started one day before induced TBI. (n=7/group)
Begum, et al., 2014	Sprague Dawley rats	CCI model	Sham injury group and DMSO injection, TBI and DMSO injection, TBI and DHA and DMSO combination injection. Injections given 5 minutes following injury

			as well as 3, 7, and 21 days following injury (n=10/group)
Wu, et al., 2011	Sprague Dawley rats	FPI model	Sham injury group on regular diet, TBI with regular diet, TBI group with DHA diet. DHA diet started day of injury and continued 12 days following injury. (n=12/group)
Mills, Hadley, et al., 2011	Sprague Dawley rats	Marmarou impact acceleration injury	Sham injury group, TBI group with no supplement, TBI group with 4 mg supplement of DHA, TBU group with 12 mg supplement of DHA, TBI group with 40 mg supplement of DHA. Supplements given 30 days before injury and stopped on the day before injury. (n=10/group)
Mills, Bailes, et al., 2011	Sprague Dawley rats	Marmarou impact acceleration injury	Sha injury group, TBI control group, TBI group with a 10 mg omega-3 supplement, and TBI group with a 40 mg omega-3 supplement. Supplement given 1 day post-injury and once a day for the next 40 days after that (n=10/group)
Wu, et al., 2004	Sprague Dawley rats	FPI model	Sham injury group, TBI group on standard diet, TBI group on DHA diet. Diets started 4 weeks before injury and continued to 1-week post-injury. (n=6-8/group)