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Research Article

Pharmacology for drug discovery

Fish Oil Ameliorates Doxorubicin Induced Memory Impairment in Wistar Rats

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Abstract: According to the World Health Organization, cancer is the leading cause of death in 112 countries under the age of 70 in 2019. Cognitive impairment is a common chemotherapeutic drug side effect that affects 15-80 % of cancer patients. These cognitive changes persist for one or two years following chemotherapy and sometimes persist for life time. Due to impaired cognitive function, survivors find utmost difficulty to perform day to day activities and lose their independency which has a negative impact on standard of living (SOL). The Antineoplastic drug doxorubicin has been associated with severe neurotoxicity, which manifests as a loss of cognitive abilities, most likely due to oxidative stress in the brain. Naturally occurring Omega-3 fatty acids have potential health benefits. They are abundant in fish oil. Fish oil is used as dietary supplement and is well known for anti-inflammatory, anti-hyperlipidaemia, cardio protective, and antioxidant and neuro protective functions. The current study explored to investigate Fish oil potential neuro protection and memory-improving advantages against Doxorubicin-induced cognitive and neurobiological impairments. The preventive effect of Fish oil against doxorubicin-induced memory impairments in rats was evaluated by using a novel object recognition task and the Morris water maze test. Doxorubicin-induced memory impairment was considerably prevented by fish oil treatment (50 mg/kg and 100 mg/kg). The levels of tumour necrosis factor (TNF-α) and acetyl cholinesterase activity were dramatically reduced with both dosages of Fish oil. Furthermore, Fish oil protected the frontal cortex and hippocampus parts of the brain from doxorubicin-induced oxidative and inflammatory damages. These findings suggest that Fish oil may be a promising adjuvant therapeutic option for decreasing doxorubicin-related side effects

Keywords: Fish oil, Chemo brain, Doxorubicin, Novel object recognition test, Morris water maze, oxidative stress markers.

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1. INTRODUCTION

Cancer is a primary cause of death and a major impediment to extending life expectancy in the world. According to the WHO in 2019 cancer is the primary cause for death in 112 countries below the age of 70 years. The International Agency for Research estimated 19.3 million cancer case and 10 million cancer deaths were documented in the year 2020. Among all type of cancers female breast cancer is the common diagnosed cancer in the world. The worldwide cancer burden will be increasing and it is expected to be 28.4 million cancer cases in 2040. [1] The overall cancer death rate dropped due to advancements in cancer treatment strategies and breast cancer survivor are increasing as compared to other cancer pateints.[2] Although survival rates have grown dramatically a significant fraction of cancer survivors (35-72%) are struggling with post chemotherapy complications.[3] One such negative effect was cognitive dysfunction also known as Chemo brain or chemo fog or mental fog. The clinical manifestations of chemo brain are deficits in episodic, visual, verbal, language, spatial, and working memory, as well as a reduced processing speed of information. [4] This complication frequently remain for a period of two to three years, and in some situations, they can last for the rest of one's life. Hence, chemo-brain is a substantial source of concern for cancer survivors and has a high impact on standard of life (SOL).[5] hence there is a need for novel drugs to prevent chemo-brain in cancer survivors. Chemotherapeutic drugs are commonly used to treat cancer are cyclophosphamide (Cytoxan), daunorubicin (Cerubidine, DaunoXome), epirubicin (Ellence), idarubicin (Idamycin), mitoxantrone (Novantrone), paclitaxel (Abraxane, Onxol), trastuzumab. Among all anti-cancer medications Doxorubicin is an anti-tumour antibiotic most commonly used and the oldest chemotherapy drug in the treatment of different types of cancers. Despite its therapeutic benefits, doxorubicin causes a number of adverse reactions in cancer patients.[6] Chemotherapy-induced cognitive dysfunction is assumed to be caused by myelosuppression, decreased blood flow and metabolism, increased oxidative stress, and chronic neuro inflammatory, according to several hypotheses.[7] Even though doxorubicin will not reach the brain like other anti-cancer drugs it produces chemobrain by indirect mechanisms such as production of inflammatory and oxidative mediators and induces neuro inflammation in the brain [8] Fatty acid is often a necessary component for some bodily functions and a critical building block for the body and brain. Good fats, such as omega-3 fatty acids, play an important role in brain development and function. There are also studies that show their ability to help improve neurodegenerative mechanisms in the brain, as well as their role in improving mood.[9] Long-chain n-3 polyunsaturated fatty acids (PUFAs), such as eicosahexaenoic acid (EPA) and docosahexaenoic acid (DHA), have been linked to improved brain health. DHA is abundant in synaptic membranes and changes fluidity and neurotransmitter levels in the brain whereas EPA is an eicosanoid precursor.The fatty acids DHA and EPA play a role in improving cognitive function and reducing depression.

Furthermore, according to a recent meta-analysis, the ratio of supplemented EPA to DHA is important because only supplements containing more than 60% EPA of total n-3 PUFAs effectively reduced depressive symptoms.[10] Fish is consumed as food all over the world and is thought to be a good source of essential nutrients for a healthy lifestyle. Fish oil supplementation has been adopted as a solution to prevent or cure many pathophysiological states and diseases by both professionals and the general public. Fish oil that is extracted from certain cold-water fish (e.g. sardines, salmon, anchovies) is the richest source of n-3 polyunsaturated fatty acids (PUFAs), mainly EPA and DHA. Recent studies reported that Fish oil which contain polyunsaturated fatty acids has anti-inflammatory,[11] anti-hyperlipidaemia, cardio protective,[12] antioxidant and neuroprotective functions. [13] Several clinical and preclinical studies on Resveratrol [14], Astaxanthin[15], Rutin[16] and Sodium valproate [17] to alleviate cognitive complication were reported in a recent review, but they were not completely effective in treating chemobrain. There is also a pressing need to investigate novel therapeutic interventions to prevent cognitive complications, as there is a significant gap in this area. Hence the present study was aimed to assess the effect of fish oil in doxorubicin produced cognitive impairments in rats. Furthermore, the current study promotes additional clinical studies into the neuroprotective benefits of fish oil use in cancer patients receiving doxorubicin treatment.

2. MATERIALS AND METHODS

2.1 Animals

Adult female rats having weight about 180-200g were used for the study and the animals were procured from Mahveer Enterprises Hyderabad. The study protocol was authorised by IAEC (Institutional animal ethical committee: 1921/PO/Re/S/16/CPCSEA) of QIS College of Pharmacy, Ongole, Andhra Pradesh, India. The animals were placed in clean PVC cages and housed in the animal house of QIS College of pharmacy and provided 12 hours daylight and dark cycles at room temperature. The animals were allowed for free access to diet and water.

2.2 Study design

The animals were divided into four groups and assigned nine animals per group. The group I rats received normal saline as a vehicle. For groups II, III, and IV, doxorubicin was given to the rats at a dose of 2.5 mg/kg through an i.p (intraperitoneal) injection. The doxorubicin injection was given every fifth day from the starting day for a period of 50 days. Along with doxorubicin, fish oil (FO) was given to group III and IV rats with a daily dose of 50 mg/kg (p.o) as a lower dose and 100 mg/kg as a higher dose. The fish oil therapy started one week before the doxorubicin treatment and continued throughout the study period. The study design is given in Figure no. 1.

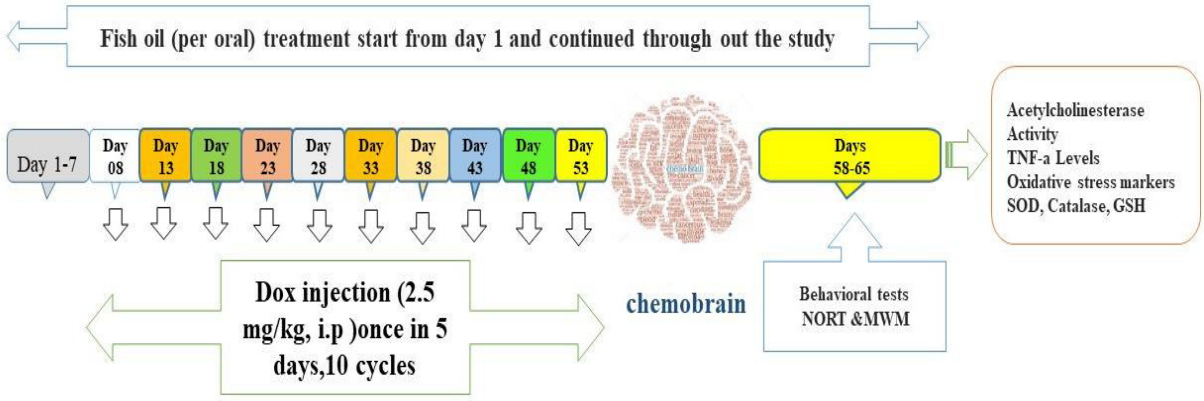

Fig 1.Study protocol of doxorubicin treatment and fish oil treatment.

2.3 Fish oil dosing

Commercially available fish oil capsules were purchased from a health kart manufactured by bright life care, India. Each capsule consists of Omega 3- fatty acids, Eicosapentanoic acid (EPA) and Docosahexaenoic acid (DHA). Doses of fish oil was calculated by converting HED (human equivalent dose) to AED (animals equivalent dose) by using conversion factor formula that is

Animal dose (mg/kg) = Human dose (mg/Kg) × conversion factor.[18]

From dosing calculation two doses 50mg/kg and 100mg/kg were used for the experiment.

2.4 Object recognition test

The rats were given a NORT test after completing the Doxorubicin 10 cycle chemo brain induction phase. The current investigation followed a procedure described by Ramalingyya et al.[16] The experiment was carried out in square boxes constructed of acrylic sheet with a volume of 40 cm ×40× cm ×40 cm. The test was conducted in three phases: habituation, familiarisation, and a recognition trial. During the habituation phase, the animals were acclimated to the experimental room's surroundings, placed in square arenas, and encouraged to explore the experimental set up for 20 minutes. Two identical objects were placed in square arenas on day 2 (familiarisation trail), and the animals were allowed to explore the objects for around 3 minutes. Before the subsequent trial, the arena was cleansed with 25% ethanol to remove any scent indicators. Recognition trails were done after a 2-hour inter-trial interval (ITI). One of the identical objects was changed with a novel object, and each animal's time spent analysing the familiar and novel objects during a three-minute period was recorded in recognition trails. The entire experiment was captured by a mounting camera on top of the arenas. Recordings were analysed by using the ANY-maze video tracking system trail software (6.35 version, 1999–2021 Stoelting Co.). The recognition index (RI) and discriminative index (DI) of the animals were calculated by using the below formula.[19, 20] ORT protocol was depicted in Figure no2.

Recognition index (RI) = b/ (a + b)
Discriminative index (DI) = (b-a)

a= Animals spent time (sec) near the familiar object during recognition trails
b= Animals spent time (sec) near the novel object in recognition trails

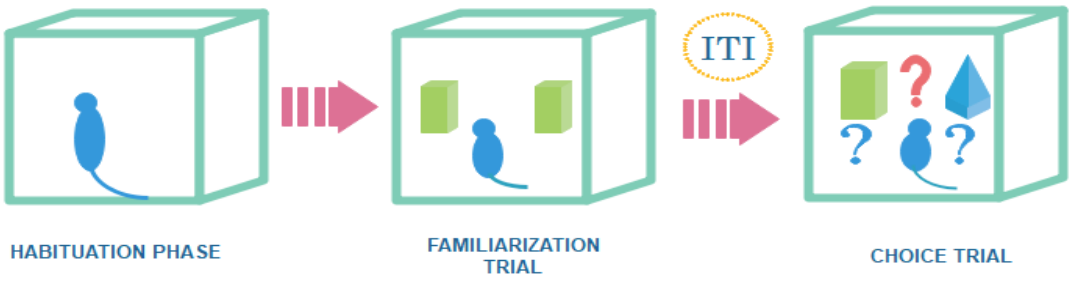

Fig 2.Illustration of ORT Protocol

2.5 Morris water maze task

The investigation was conducted in a black circular tank with a diameter of 120cm and a height of 50cm. During the training days, four distal landmarks of various shapes (circular, square, triangle, and diamond) and colour visual cues were provided to the animals around the tank, and these cues served as spatial cues for the animals. In one of the four quadrants, a circular plexiglass platform with a diameter of 10cm and a height of 24cm was positioned. Throughout the trails, the platform was retained in the same location and position. The water maze task was completed in two stages. The first phase was the Acquisition phase, in this phase each rat was subjected to four consecutive trials per day for four days, with a 15-minute time gap between them. The rats were released into the water and allowed them to identify the hidden platform using visual cues. Each day, the sequence of starting points was changed to aid the rat in gaining spatial memory of the space. The trail was completed when the rat reached the hidden platform and remained there for at least 10 seconds. The rats were placed on the platform for 10 seconds if the rats couldn't find it in 60 seconds. To boost visibility, a flag was hoisted on the platform. Throughout the acquisition trails, the platform location remained consistent. On day 5, the probe test was carried out by removing the platform from the tank and releasing the animals into the pool, allowing them to reach the target quadrant where the platform had previously been positioned. ANY-maze video tracking system trail software (6.35 version, 1999–2021 Stoelting Co.) was used to record the duration spent and frequency of entry into the target quadrant. After completing the assignment, the rats were dried and examined for normothermia before being returned to their home cage. [21, 22]

2.6 Estimation of TNF-α levels

By using an ELISA kit TNF- α concentrations were measured in frontal cortex region homogenates and hippocampal homogenates. Assay was done according to the instruction given by manufacturer.[23]

2.7 Acetylcholinesterase activity

Hippocampal and frontal cortex supernatants were used for the estimation of Ach esterase activity. Ellman et.al [24] protocol was used for the estimation of Ach esterase.

2.8 Oxidative stress indicators

To estimate the concentration of cellular oxidative defensive enzymes such as Glutathione (GSH) [25] superoxide dismutase (SOD) [26], catalase [27] and total Thiols [28] the rats were euthanized and the brains were rapidly removed and isolated the hippocampus region and frontal cortex regions. A homogenizer (REMI) was used to homogenise the samples and these homogenates were stored at 20°C and used to measure brain antioxidant levels.

3. STATISTICAL ANALYSIS

Statistical analysis performed by Graph pad statistical software trial version (9.2.0). Exploration time between the objects and within the groups was calculated by using Student's paired t-test. Kruskal–Wallis test and Dunn's post hoc test were used to compare groups in the discriminative index and recognition index in the ORT behavioural study. Students' unpaired t-test was used to compare the groups in Morris water maze data, which included target latency, Q4 latency and Q4 time. One-way ANOVA, followed by Dunnett's post hoc test was used to analyse Acetylcholinesterase, TNF-α, and oxidative indicators. $P < 0.05$ was chosen as a statistically significant value.

4. RESULTS AND DISCUSSIONS

4.1 ORT Results

In the familiarisation trails, all groups of rats spent the same amount of time near the two identical objects, indicating that rats did not exhibit any discriminating between the two identical objects. Normal control rats were able to identify familiar objects during recognition trials and spent much more time with novel objects than familiar ones, demonstrating discrimination following a 2-hour ITI. However, the Doxorubicin (DOX) treated rats spent almost as much time investigating familiar objects as they did novel objects, indicating that these animals had forgotten about the familiar object. This indicates that long-term Doxorubicin (DOX) treatment led to episodic memory impairments for object identification, which may be considered an additional complication of chemotherapy-induced neurocognitive dysfunction (chemo brain). When the chronic Doxorubicin group was compared to the normal control group, both the discriminative and recognition indices were considerably ($p < 0.001$) lower in the chronic DOX group. Pre-treatment with Fish oil (FO) at doses of 50 mg/kg and 100 mg/kg, on the other hand, significantly repaired episodic memory deficits in a dose-dependent manner, as demonstrated by a significant difference in exploration time. When the groups treated with Fish oil (FO) were compared to the groups treated with Doxorubicin, it was revealed that the discriminative and recognition indices were significantly improved in the Fish oil (FO) groups (Figure no 3). This indicates that combining Fish oil (FO) with doxorubicin provides considerable protection against DOX-induced chemobrain in terms of episodic memory and cognitive functioning.

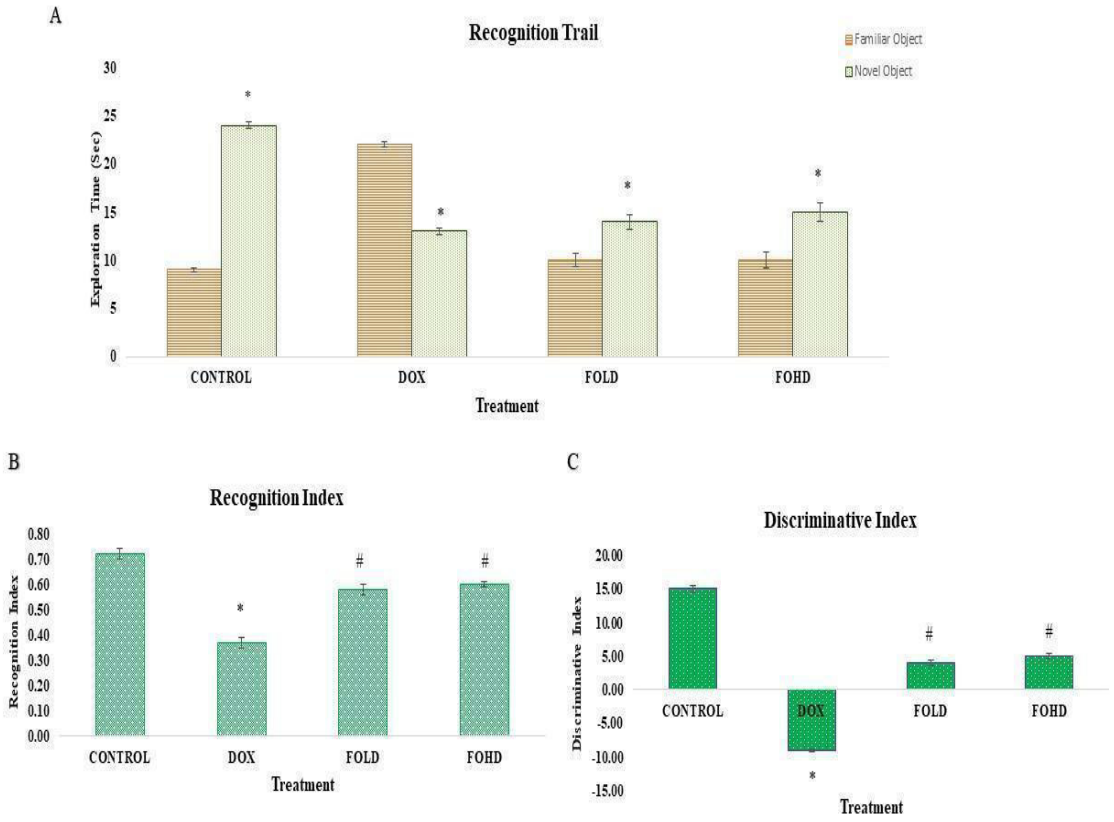

Fig 3. Effect of Fish oil on Doxorubicin Induced Memory Impairment in Rats

Note: Data were given as mean ± SEM. A. Recognition trails time spent at novel object and familiar object (*p<0.001 vs familiar object), B. Recognition index and Discriminative index (*p<0.001 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

4.2 MWM task

There were no significant differences in escape latency among the six groups in the MWM task for measuring spatial memory during four training days. When compared to a normal control, chronic doxorubicin therapy dramatically shortened Q4 duration and increased Q4 delay. This suggests that persistent Doxorubicin treatment has an effect on spatial memory. After four days of spatial learning training, the animals were placed in a water maze without a platform in probing trails. The animal's performance was evaluated using Q4 time and Q4 latency. When compared to the doxorubicin-treated rats, the animals treated with Fish oil doses of 50mg/kg and 100mg/kg significantly increased time spent in the target quadrant (Q4 time) and decreased time to reach the target quadrant (Q4 latency) (Figure no 4). This study revealed that rats given Fish oil were protected from the learning and memory loss caused by doxorubicin. These results support the previous studies on krill oil where krill oil improves the spatial memory by increasing the concentrations of polyunsaturated fatty acids in the brain. [29]

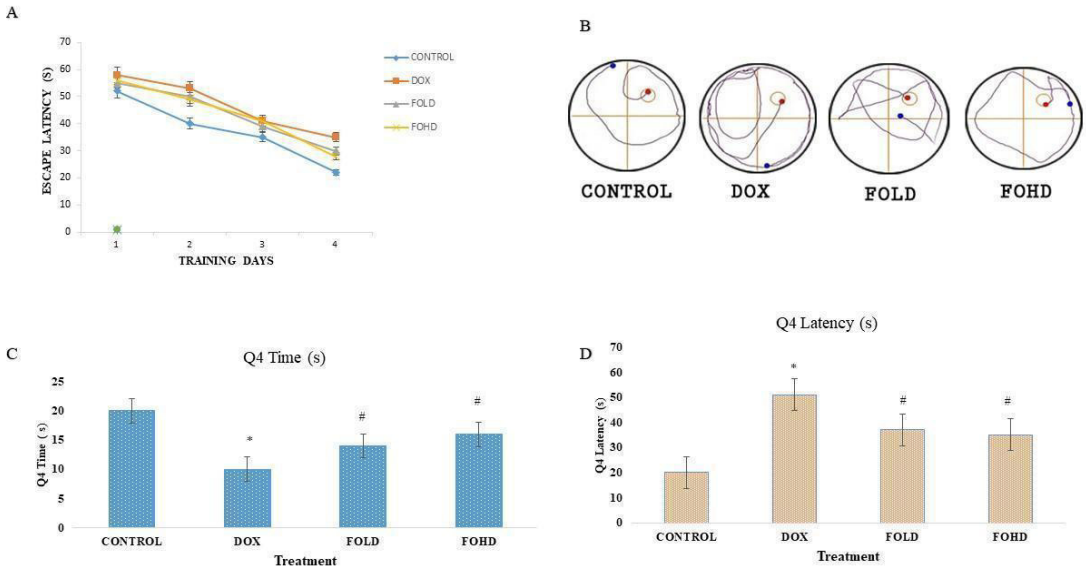

Fig 4. Effect of Fish oil on Doxorubicin Induced Spatial Acquisition Learning and Memory Deficits in MWM Test

Note: Data were given as mean ± SEM. A. Escape latency during trail days B. Track plots C. Q4 (Target) time D. Q4 (Target latency in probe trails (*p<0.01 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

4.3 TNF-α Levels

Doxorubicin-treated rats exhibited considerably higher levels of TNF- α in hippocampus and frontal cortical tissues than vehicle-treated animals. These findings back up previous research that found an increased concentration of TNF- α in the brains of cancer patients who received doxorubicin treatment. [30] Chronic fish oil administration decreased doxorubicin-induced TNF- α concentrations in the hippocampus and frontal cortical regions of the brain significantly (Figureno5).

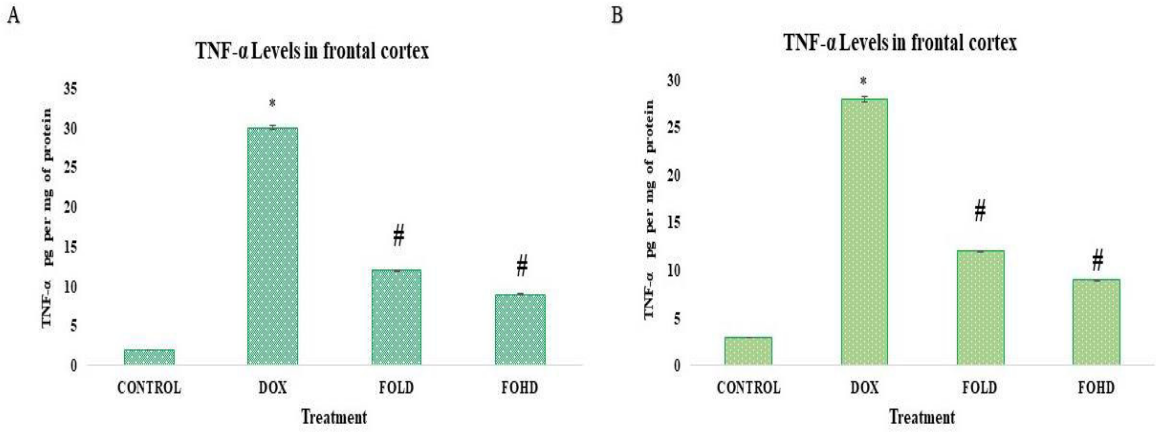

Fig 5. Effect of Fish oil Treatment on Doxorubicin Induced Inflammatory Marker TNF-α in Hippocampus and Frontal Cortex

Note: Data were given as mean ± SEM. A. TNF-α levels in frontal cortex B. TNF-α levels in hippocampus (*p<0.01 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

4.4 Acetylcholinesterase levels

According to the earlier studies Acetylcholine concentration is important for learning and memory function in the brain.[31] Doxorubicin treatment increased acetylcholinesterase (AChE) activity in compared to the vehicle-treated group. The co-administration of fish oil considerably reduced the alterations in enzyme activity (Figure no 6).

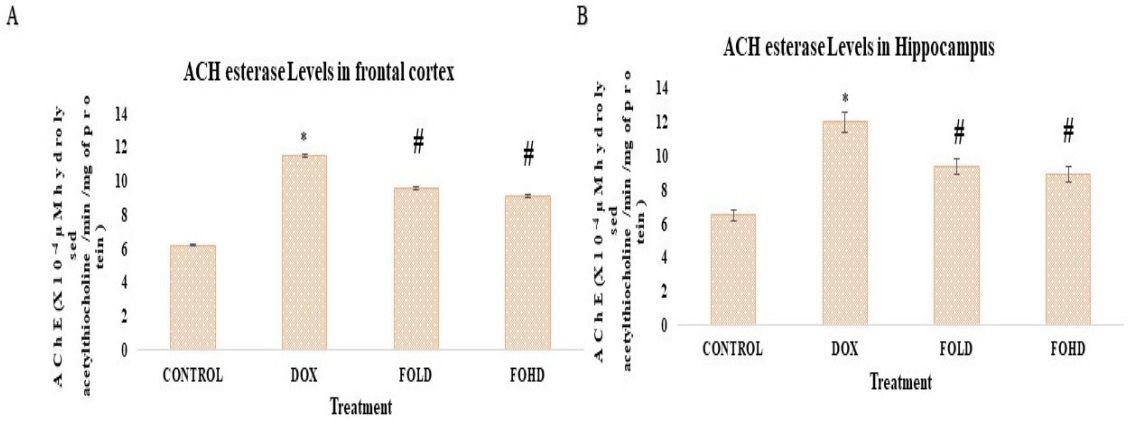

Fig 6. Effect of Fish oil Treatment on Hippocampal and Frontal Cortex Acetylcholinesterase Activity in Doxorubicin Treated Rats

Note: Data were given as mean ± SEM of A. Ach esterase activity in frontal cortex B. Ach esterase activity in hippocampus (*p<0.01 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

4.5 Oxidative stress markers

We measured the quantities of Glutathione, superoxide dismutase, thiols, and catalase to see how fish oil protects Doxorubicin produced oxidative stress in the frontal cortex and hippocampus. Doxorubicin administration shows a strong pro-oxidant effect as compared to the vehicle-treated group, as demonstrated by significant reductions in GSH, SOD, thiols, and catalase levels. Co-treatment of fish oil with Doxorubicin, on the other hand, considerably restored normal GSH, SOD, thiol levels, and catalase activity. The results are shown in tables 1 and 2.

Table 1. Effect of Fish oil Treatment on Oxidative Stress Markers in Frontal Cortex of Doxorubicin Treated Rats

Groups	GSH (µg/Mg Of Protein)	Thiols (µg/Mg Of Protein)	SOD (Units/Mg Of Protein)	Catalase (Units/Mg Of Protein)
Control	5.61 ± 0.71	11.62 ± 0.87	13.42 ± 1.24	3.69 ± 0.42
Dox	1.28 ± 0.16*	3.80 ± 0.44*	4.56 ± 0.75*	1.15 ± 0.41*
Fold	2.89 ± 0.12#	8.12 ± 1.12#	9.27 ± 1.45#	2.55 ± 0.16 #
Fohd	3.54 ± 0.14#	8.54 ± 1.34#	9.76 ± 1.63#	2.78 ± 0.42#

Note: Data were given as mean ± SEM (*p<0.01 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

Table 2. Effect of Fish oil Treatment on Oxidative Stress Markers in Hippocampus of Doxorubicin Treated Rats

Groups	GSH (µg/Mg Of Protein)	Thiols (µg/Mg Of Protein)	SOD (Units/Mg Of Protein)	Catalase (Units/Mg Of Protein)
Control	6.81 ± 0.87	12.41 ± 0.91	14.61 ± 2.14	2.70 ± 0.22
Dox	1.91 ± 0.26*	4.50 ± 0.63*	6.43 ± 0.85*	1.01 ± 0.33*
Fold	3.42 ± 0.82#	9.22 ± 1.26#	10.38 ± 1.36#	1.80 ± 0.16 #
Fohd	3.96 ± 0.34#	9.82 ± 1.42#	11.28 ± 2.04#	1.98 ± 0.32#

Note: Data were given as mean ± SEM (*p<0.01 vs control, #p<0.001 vs Doxorubicin control); DOX-Doxorubicin, FOLD-Fish oil lower dose (50mg/kg), FOHD-Fish oil higher dose (100mg/kg)

5. DISCUSSION

A key, life-changing discovery would be the development of a drug that may shield cancer patients from cognitive damage caused by chemotherapy. Omega-3 fatty acids, found in fish oil, are essential for brain function and development. Fish oil may also help people with cognitive problems, such as Alzheimer's disease or other forms of cognitive impairment and improve their brain function. Using fish oil supplements has been shown in several studies to aid people with milder forms of dementia, such as moderate cognitive impairment or age-related cognitive decline, enhance their brain function.[32, 33] Doxorubicin is a drug that is often used to treat cancer.[34] Doxorubicin produces multiple adverse reactions like cardio toxicity, nephrotoxicity, and hepatotoxicity. In addition to these side effects, doxorubicin-induced cognitive impairment is quite common in doxorubicin-treated patients.[15] Doxorubicin toxicity is caused by oxidative stress,

apoptosis, and inflammation, which are all potential mechanisms for Doxorubicin toxicity.[35] In our behavioural study Doxorubicin produced episodic memory impairment in ORT task and spatial memory impairment in Morris water maze task, corroborating previous studies of Philpot et.al.[36] Fish oil treated rats protected from memory impairment caused by doxorubicin indicating the fish oil potential in protecting the memory impairment caused by doxorubicin. Reduced levels of SOD, GSH, thiols, and catalase activity were observed in doxorubicin treated rats. These observations were consistent like earlier studies of Kuzu M et.al.[37] Fish oil protected the rats from reduction of SOD, GSH, thiols, and catalase activity represents fish oil antioxidant ability. Increased tumour necrosis factor levels after doxorubicin therapy were reported in earlier studies.[38] This represents TNF-α mediated neuro inflammation and neuro degeneration are typical characteristics of doxorubicin-induced memory impairment.[39-41] TNF-α levels in the hippocampus and frontal cortical sections of rats brains were drastically raised after doxorubicin treatment, indicating enhanced neuro inflammation. TNF-α levels were dramatically lowered in rats that received fish oil, showing that it has an anti-inflammatory and inhibition of neurodegeneration impact. Earlier Pharmacological data clearly indicated that both muscarinic and nicotinic acetylcholine receptors have a role in the encoding of new memories.[42] Acetylcholinesterase hydrolyses the neurotransmitter acetylcholine to choline and acetate in the synaptic cleft. Mounting evidence has shown reduced activity of AChE in several brain disorders.[43] Hippocampal acetylcholinesterase activity was reduced in cognitive impairment and early Alzheimer's disease and so the value of in vivo acetylcholinesterase measurements in detecting the early Alzheimer process is vital.[44] Using cholinesterase inhibitors are efficacious for mild to moderate Alzheimer's disease (AD). [45] In this current study fish oil decreased the acetylcholine esterase activity in the brain and protected memory impairment produced by the doxorubicin. All results in this study represent fish oil effectively preventing the doxorubicin produced memory impairment.

6. CONCLUSION

According to our findings, chronic Doxorubicin treatment resulted in episodic memory deficits in wistar rats. The wistar rats were given Fish oil and then subjected to behavioural tests such as the Novel object recognition test and the Morris water maze. The findings from behavioural tests revealed that fish oil had a positive effect on cognitive function by reducing oxidative damage in the brain. Hence, fish oil may be a promising adjuvant therapeutic intervention to alleviate the cognitive deficits associated with DOX-induced chemobrain.

7. AUTHORS CONTRIBUTION STATEMENT

Mr Jalaiah Marri conceptualized the study and obtained the data upon experimentation. Dr Jaya Sharma and Dr.Pankaj Sharma supported in designing and analysing the data and also provided the necessary guidance for the interpretation of the result. All the authors discussed the methods and reviewed the result and approved the final version of the manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none

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