SEROTONIN IN THE MEDIATION OF BEHAVIORAL EFFECTS OF OMEGA-3 FISH OIL SUPPLEMENTS: A STUDY IN RATS.

F. Khan[1,2*], K. Jalal[1,2], A. Gohar[2], R. Afroz[1], S. Nawaz[1], T. Salman[1], I. A. Khan[1] and D. J. Haleem[1]

[1] Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, 75270, Pakistan.
[2] H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, 75270 Karachi, Pakistan.
Corresponding Author e-mail: Faisal Khan (faisalkhanzarrar@gmail.com)

ABSTRACT: Omega-3 polyunsaturated fatty acids (omg-3 PUFAs) are widely prescribed for improving therapeutic strategies in many diseases. There are reports that the use of these supplements can reduce disease-related depression and anxiety in patients taking these supplements. However, pre-clinical research shows mixed results. In view of the role of serotonin (5-hydroxytryptamine, 5-HT) in anxiety and depression, the present study concern effects of omg-3 on anxiety-like behavior and on serotonin metabolism in brain regions known to have a role in anxiety. It was found that administration of omg-3 for 20 days did not alter food intake and body weight. The treatment reduced locomotor activity in an open field arena and enhanced anxiety-like behavior in an elevated plus-maze test. 5-HT concentration increased in the hippocampus (HPC) and midbrain (MB) but decreased in the prefrontal cortex (PFC). Levels of 5-hydroxyindoleacetic acid (5-HIAA) were decreased in these regions. The tryptophan levels were not affected in the PFC but increased in the HPC and MB at a low dose; suggesting that decreases of 5-HT and 5-HIAA were not due to a decrease in the availability of tryptophan. The present study implied that unnecessary use of omg-3 supplements can reduce serotonin neurotransmission via the PFC to predispose to anxiety.

Key words: n-3 PUFA, DHA/EPA, Locomotor, Anxiety, 5-HT, Prefrontal Cortex.

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INTRODUCTION

An important global health concern is the rise in the incidences of anxiety and depression. (Al Omari *et al*., 2020). Conventional drugs for treating anxiety and depression are not very effective and relapse is common (Bandelow *et al*., 2017; Bandelow *et al*., 2015). Moreover, these treatments are associated with extensive side effects, which includes addiction, headaches, seizures, and suicidal attempts (Fajemiroye, da Silva, de Oliveira, Costa, & pharmacology, 2016). Therefore, there has been a significant rise in treatment of anxiety and depression with natural remedies. Together with this, there is an escalating rise in research interest on the positive health effects of omega-3 poly unsaturated fatty acids (omg-3 PUFAs).

Early epidemiological studies reveal that diets rich in fish oil can reduce the occurrence of cardio-cerebrovascular diseases (CCVDs) (Shahidi & Miraliakbari, 2004). Several observational and experimental studies have now shown that these beneficial effects are due to omg-3 PUFAs (Calzolari, Fumagalli, Marchionni, & Di Bari, 2009; Shahidi, Ambigaipalan, & technology, 2018). The complex of omg-3 PUFAs includes stearidonic acid (SDA), eicosa-pentaenoic acid (EPA), docosa-pentaenoic acid (DPA), alpha-linolenic acid (ALA) and docosa-hexaenoic acid (DHA) (Belayev *et al*., 2011; Chang *et al*., 2019; Mehta, Dworkin, & Schwid, 2009; Rapoport, Rao, Igarashi, & Acids, 2007; Yang, Emma-Okon, Remaley, & disease, 2016). These secondary metabolites cannot be synthesized directly in the body yet are necessary for normal physiological functions. Thus, ALA is an essential fatty acids from which other omg-3 PUFAs including EPA and DHA are synthesized in the humans (Rapoport *et al*., 2007). In a recent time, a study shows that EPA is metabolized into DHA and this could be advantageous in the treatment of psychiatric illnesses counting as anxiety and depression (Palak, Virginia, & Sana, 2021).

Numerous clinical research and pre-clinical studies have been carried out to examine the potential beneficial health impacts of omg-3 supplements in anxiety and depression (Hallahan *et al*., 2016; Lin *et al*., 2017). A meta-analysis shows that omg-3 supplements can improve the treatment efficacy of conventional anti-depressant drugs (Lin *et al*., 2017), while another meta-analysis shows that omg-3 supplementation produces a very little or even no effects in preventing the symptoms of anxiety and depression (Mocking *et al*., 2016). Based on these reports, the potential anxiolytic and anti-depressant effects of omg-3 components such as EPA and

DHA are not yet established. The importance of serotonin, also called 5-hydroxytryptamine (5-HT) in the pathophysiology, and treatment of anxiety as well as in depression is well established (Udenfriend, Weissbach, & Medicine, 1958). In the raphe nuclei of midbrain (MB) the long and extended cell bodies of serotonergic neurons are present. Axons of these cell bodies are extended to almost every brain region. Raphe-hippocampal and raphe pre-frontal cortical serotonergic systems are highly implicated in anxiety and depression (Brady, Siegel, Albers, & Price, 2005).

The goal of the current study was two-fold. Firstly, potential role of commercially marketed omg-3 supplements was monitored on the anxiety like behavior in rats. Secondly, the effects of treatment with omg-3 were determined on the levels of serotonin, 5-hydroxy indole-acetic acids (5-HIAA), and tryptophan in the areas of brain specifically involved in the regulation of anxiety, i.e., pre-frontal cortex (PFC), hippocampus (HPC), and midbrain.

To achieve the goal, fish oil supplements containing 500 mg/kg (EPA and DHA contents in a ratio of 3:2) were used. The omg-3 was given orally to rats in doses of 500 mg/kg and 1000 mg/kg (or 0.5 g/kg and 1 g/kg) daily for a period of 20 days in a row. Anxiety like behavior and locomotor activity were observed in an elevated plus maze (EPM) and open field (OF), respectively, after the single and repeated administration of omg-3 supplements. Animals were killed after repeated treatment with omg-3 to collect brain regions. Serotonin and its metabolite (5-HIAA) and tryptophan levels were determined in the PFC, HPC and MB of rats by HPLC-ECD.

METHODS AND MATERIAL

Animals, Omg-3 Supplements and Treatment: Albino Wistar rats (male) of weight 200 ± 20 grams and age of 5 to 6 weeks were received from the animal resource facility of "Dr. Panjwani Center for Molecular Medicine and drug research (PCMD), International Center for Chemical and Biological Center (ICCBS), University of Karachi-Pakistan". Two animals per cage (2 rats/cage) were kept in cages made up of opaque polyacrylic, and they had free access to tap water and the regular rat chow diet. The light and dark cycles (12:12 hrs) were maintained (from 6:00 a.m. to 6:00 p.m.) with humidity (40 ± 5%) and a controlled room temperature (25±3°C), and. Rats were kept in this novel environment for 5 days for acclimatization to reduce handling stress. Experimental protocol was approved by the committee of "Institutional Animal Ethical Care" (IAEC) and conducted according to the NIH guide for care and use of lab animals (IAEC; Animal Study Protocol No. 2020-016).

Locally purchased softgel (The Vitamin company, USA) omega-3 (omg-3) fish oil capsules containing 500 mg omg-3 (EPA, and DHA in a ratio of 3:2) were used. Oral gavage connected to 1ml syringe was used to administer doses.

Experimental Protocol: After acclimatization, 24 rats randomized into three equal groups (8 rats/in each group) and were orally treated with (i) tap water 1ml/kg (control group), (ii) 500 mg/kg omg-3 (low dose treatment group) and (iii) 1000 mg/kg omg-3 (high dose treatment group). On experimental day 1, body weights and food placed in the hopper were measured at 9:00 a.m. to 10:00 a.m. Water or omg-3 were also given during this time via oral gavage. The treatment continued daily at the same time from day 1 to 20. Activities in an EPM and OF were monitored respectively after 60 and 70 min of the treatment in between of 11:00 a.m.-01:00 p.m. These activities were monitored on day 1, day 10, day 16 and day 20. Body weight gain and the changes in food intake were monitored on day 20 after the repeated treatment of omg-3. On 20th day of the experiment, after monitoring EPM and OF activities, decapitation of rats was carried out from 01:00 p.m. to 02:00 p.m.

Monitoring Body Weight and Food Intake: Body weights of animals were monitored on first day (day 1), and on last day (day 20) of experiment, in between 9:00 a.m. to 10 a.m. Percentage changes in the body weight during the treatment were used to calculated as:

"%Age changes in body weight = {(Body weight after the treatment) / (body weight before starting the treatment)} x 100"

Cumulative food intake during the treatment was measured by subtracting the food intake given on starting day from the leftover food after the treatment on day 20.

Elevated Plus Maze Activity: EPM activity was monitored to determine the effects of treatment on anxiety like behavior. EPM apparatus was comprised of four equal arms made up of opaque polyacrylic plastic sheet, extending from a central open area of 10cm x 10cm. Each arm had a length of 50 cm and a width of 10 cm. Two of these arms were open called "open arms" but the other two arms were surrounded by three-sided walls with a height of 15 cm while one side was open called "close arms". The apparatus was elevated from the ground at a height of 60cm. A camera was placed above the apparatus to record the behavior of rats in the apparatus.

To measure the activity in EPM, animal was placed in the central spaced area, and head of the animal was facing towards one of the closed arms. The animal was left free for five minutes to explore open and closed arms. During this time, total no. of entries and time passed in open arm were recorded. After monitoring the behavior in EPM, the rat was kept back in its respective

home cage and the EPM apparatus was cleaned for the next rat.

Open Field Test: Activity in OF was monitored to determine effects of the treatment on the exploratory behavior. The OF apparatus was comprised of a square area of 76 cm x 76 cm, surrounded by four-sided boundary walls. The boundary walls were made up of white opaque polyacrylic plastic with a height of 42 cm. The square area was separated by black coloured lines into equal 25 (5x5) squares. A camera was installed above the apparatus to record the exploration of rats in the apparatus.

To measure locomotor behavior, an animal was placed in central arena of an OF. The idea was to monitor the no. of square crossings with all four paws which were calculated for 5 minutes. Afterward, the rat was picked from the OF apparatus and housed back in its cage. The OF apparatus was cleaned for exposure to the next rat.

Sample Collection for Neurochemical Analysis: The animals were decapitated on day 20 with a sharp conventional guillotine. The chopped heads were washed with a cold 0.9% saline solution. The cranium was opened at the atlanto-occipital joint with sharp scissors and brains were taken out and dipped in cold 0.9% saline solution. A brain slicer was used to make slices of 1mm thickness from which PFC, HPC, and MB were dissected. The regions were kept in HPLC-ECD at temperature of -80°C for the analysis of serotonin, 5-HIAA, and tryptophan.

Extraction of Serotonin, 5-HIAA, and Tryptophan: For extraction of serotonin, 5-HIAA, and tryptophan, brain regions were homogenized in cold extraction medium using an electric homogenizer (DLAB D-160). The extraction medium was 0.4 M per-chloric acid ($HClO_4$), containing 0.1 % of sodium meta-bisulphite (Na2S2O5), 0.01% of EDTA, and 0.01% of cysteine. The samples were homogenized in the 5 volumes of the extraction medium. The homogenates then centrifuged at 12000 rpm at 4°C for 15 mins in a temperature-controlled centrifuge (ThermoScientific Heraeus Megafuge 8-R; Rotor Cat #75005719). The supernatants collected were again centrifuged for 5 mins and clear supernatants were used for the quantification of serotonin, 5-HIAA, and tryptophan.

Quantification of serotonin, 5-HIAA, And Tryptophan: The separation of serotonin, and 5-HIAA, and tryptophan was done by reverse phase high-performance liquid chromatography (HPLC: waters® e2695). A Shim-Pack-ODS-column with a diameter (4.0 mm), length (15 cm), and particle size (5µ) was used. Separation was achieved by a mobile phase containing 0.1 M of phosphate buffer solution (PBS: pH 2.9), 0.023% of octyl sodium sulfate, 14 % of methanol, and of 0.0035% EDTA, at an operating pressure (1500–2500 psi) with a flow rate (1ml/min) using Shimadzu HPLC pump. An electrochemical detector (Shimadzu LEC 6A) was used to quantify 5-HT and 5-HIAA at an operating potential (0.8mV), and the level of tryptophan was detected by using UV/Vis detector (waters® 2489) at an absorbance of 273nm wavelength. The detection signal was quantified by computer attached-HPLC software (Empower™3 software).

Statistical analysis: SPSS-IBM (21.0) software was used to analyze the data. Data are represented as means ± SD. Effects of omg-3 on body weight changes, cumulative food intake, and data on serotonin, 5-HIAA and tryptophan concentration were tested statistically by using 1-way ANOVA. Effect of omg-3 on OF activity and EPM test; scored on days 1, 10, 16, and 20 were evaluated by 2-way ANOVA (repeated measure) design, factor 1 (dose), and factor 2 (repeated measure) on days 1, 10, 16, and 20. Posthoc analysis was performed via Tukey's test and a p-value of <0.05 was considered significant.

RESULTS

Effect of Omg-3 On Bodyweight and Food Intake: Figure 1 reveals the effect of daily oral administration of omg-3 for 20 days on (0.5 g/kg and 1 g/kg) doses on percentage (%) changes in body weight (Fig. A), and total food consumption (Fig. B). Data analysed by 1-way ANOVA revealed non-significant effects on percentage changes in body weight ($F=0.057$ df2,21 $p>0.05$) and cumulative food intake ($F=0.109$ df2,21 $p>0.05$).

Effects of Omg-3 on Elevated Plus-Maze behaviour: Figure 2 show the effects of omg-3 in (0.5 g/kg and 1 g/kg) doses on anxiety-like behaviour in the EPM test, monitored as total time passed in open arm (Fig. 2A), and total no. of entries in the open arm (Fig. 2B). The effects studied on days 1, 10, 16, and 20, after 60 min of omg-3 administration, analysed by the two-way ANOVA (repeated measure) design exhibited a significant effects of omg-3 ($F=225.5$ df2, 21 $p<0.01$), repeated measure, i.e., days ($F=184$ df3, 63 $p<0.01$). A significant interaction was exhibited by repeated measure x days x omg-3 ($F=45.2$ df6, 63 $p<0.01$). Post-hoc analysis showed that administration of omg-3 in both doses (500 mg/kg and 1000 mg/kg) lowered the time passed in the open arms. Moreover, values in water treated control, as well as omg-3 treated groups, were smaller on day 10, day 16, and day 20 than the respective values on day 1, suggesting habituation effect.

Two-way ANOVA repeated measure design was again utilized to assess the data of total no. of entrances in EPM's open arm, which exhibited a significant effect of omg-3 ($F=135.1$ df2,21 $p<0.01$), repeated measure, i.e., days ($F=200.4$ df3,63 $p<0.01$) and significant interaction between repeated measure; days x omg-3 ($F=$

27.1 df6, 63 p<0.01). Post-hoc analysis showed that a single and repeated administration of both doses (0.5 g/kg and 1 g/kg) of omg-3 decreased the total number of entries in the open arm on day 1, day 10 and day 16 and day 20 than the water-treated rats. Further, number of entries in water treated controls as

well as in drug treated group were smaller on day 10, day 16, and day 20 compared to respective values on day 1 showing that rats were habituated to the maze on repeated exposure.

Effects of Omg-3 on behavior in Open Field: Figure 3 show the effects of single and repeated treatment with omg-3 (0.5 and 1 g/kg) on the OF activity monitored on day 1, day 10, day 16, and day 20, 70 min after the administration of omg-3. Data on the total numbers of squares crossed were analysed via 2-way ANOVA (repeated measure) exhibited a significant effect of omg-3 (F=10.586 df2, 21 p<0.01), repeated measure (days) (F=183.066 df3, 63 p<0.01) and significant interaction between repeated measure; days x omg-3 (F=6.345 df6, 63 p<0.01). Analysis by post-hoc showed that administration of omg-3 whether single and repeated in doses of 0.5 and 1 g/kg significantly decreased the total no. of square crossed on day 1, day 10, and day 16 suggesting that omg-3 had motor depressant effects. The motor depressant of omg-3 effects did not occur day 2. Like habituation effects seen in EPM activity habituation to OF also occurred and motor activity in controls as well as omg-3 treated groups were smaller on day 10 and day 16, and day 20 than their respective values on day 1.

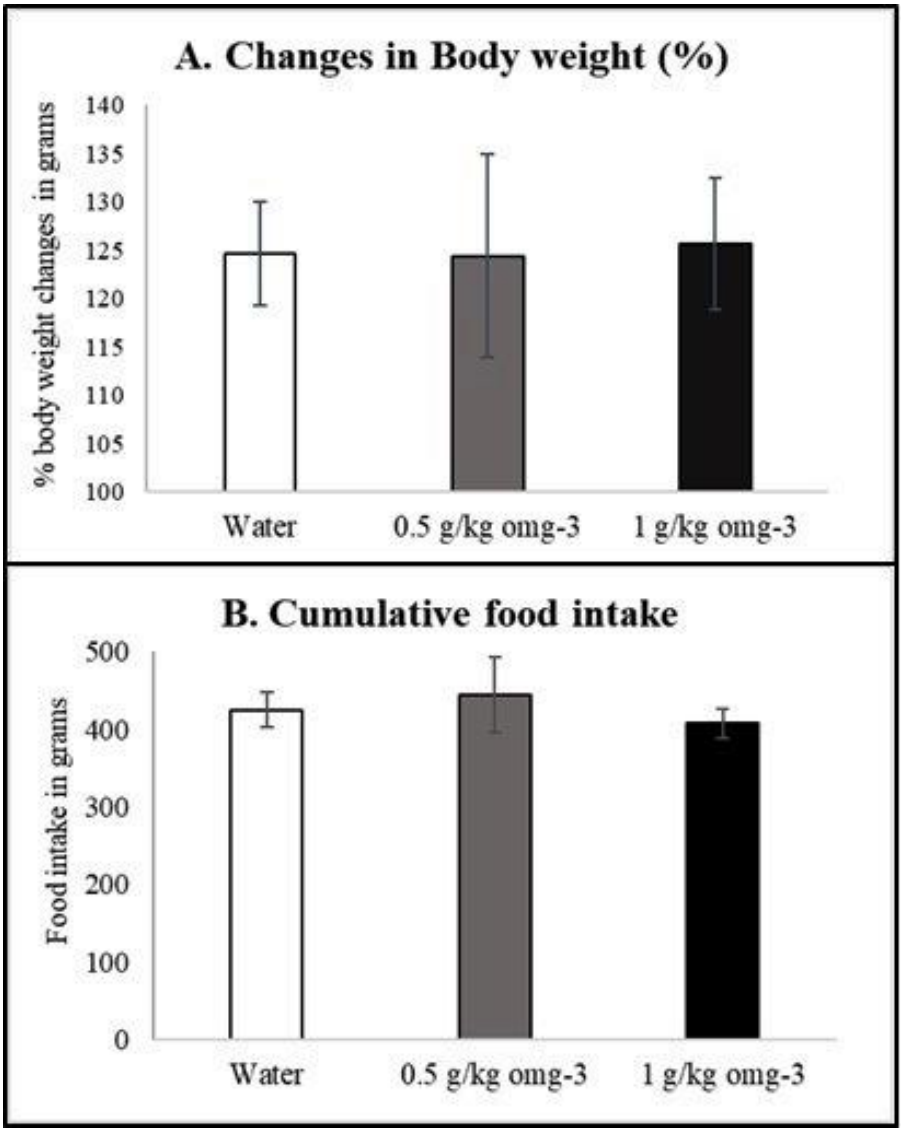

Fig. 1. Dose-dependent effects of omg-3 on percentage (%) changes in body weight (A) and total food consumption (B). Data are presented as mean ± S.D. (n=8). Difference by 1-way ANOVA was not significant (P>0.05).

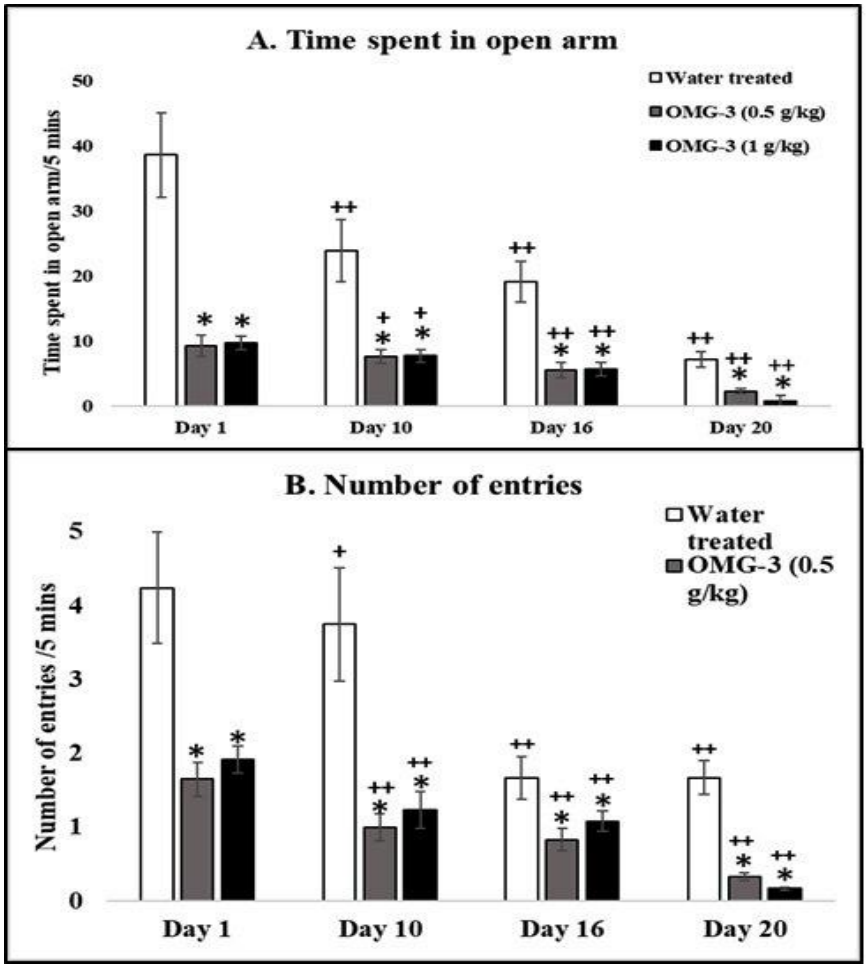

Fig.2. Effects on the single and repeated administration of omg-3 (0.5 and 1 g/kg) on the EPM behavior; time passed in the open-arms (A) and a no. of entries in the open-arm (B). Data are presented as mean ± S.D. (n=8). Tukey's test showed significant differences: *p<0.01 from the respective day value of water-treated control animals, +p<0.05 and ++P<0.01 from day 1 value of similarly treated group.

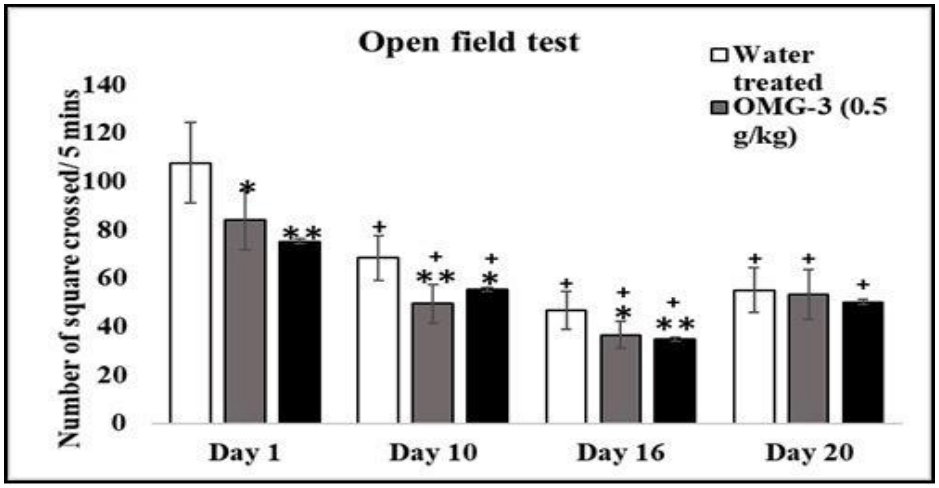

Fig.3. Effects on the single and repeated administration of omg-3 (500 and 1000 mg/kg) on locomotor behavior in open field apparatus. Values are presented as mean ± S.D. (n=8). Tukey's test showed significant differences: *p<0.05 and **p<0.01 from respective day water-treated animals. +p<0.01 from day 1 value of control and omg-3 in both doses.

Effects of Omg-3 on 5-HT metabolism in PFC, HPC and MB: Figure 4 shows the effect of administration of omg-3 (0.5 and 1 g/kg) on the levels of serotonin, 5-HIAA, and also the levels of tryptophan in different regions of brain (PFC, HPC, and MB). Data of the PFC (Fig 4A) showed significant effects of omg-3 on levels of serotonin ($F=61.637$ df2, 21 $p<0.01$) and 5-HIAA ($F=24.807$ df2, 21 $p<0.01$); however, effect on tryptophan levels ($F=0.831$ df2, 15 $P>0.05$) was not significant. One-way ANOVA showed significant effects of omg-3 on 5-HT levels ($F=170.457$ df2, 21 $p<0.01$), 5-HIAA ($F=109.035$ df2, 21 $p<0.01$) and tryptophan ($F=22.158$ df2, 15 $p<0.01$) in the HPC (Fig 4B). The data on the MB (Fig 4C), also revealed significant effects on 5-HT levels ($F=47.860$ df2, 21 $p<0.01$), 5-HIAA ($F=102.533$ df2, 21 $p<0.01$) as well as tryptophan levels ($F=5.204$ df2, 15 $p<0.05$).

Post-hoc analysis revealed that administration of omg-3 at both doses (0.5 and 1g/kg) reduced serotonin and 5-HIAA levels however had no effects on tryptophan in the PFC. Both doses of omg-3 increased 5-HT levels but only low dose increased tryptophan levels in the HPC. However, both doses of omg-3 decreased 5-HIAA levels in the HPC. The treatment with low dose omg-3 increased 5-HT as well as tryptophan but decreased 5-HIAA, and treatment with high dose of omg-3 decreased 5-HIAA but had no effects on 5-HT and tryptophan in the MB. Results showed that the repeated (20 days) administration of omg-3 increased the levels of 5-HT largely by decreasing its degradation to 5-HIAA in the HPC as well as MB. Effects of omg-3 induced increase tryptophan in enhancing 5-HT levels were evident only at low dose of omg-3.

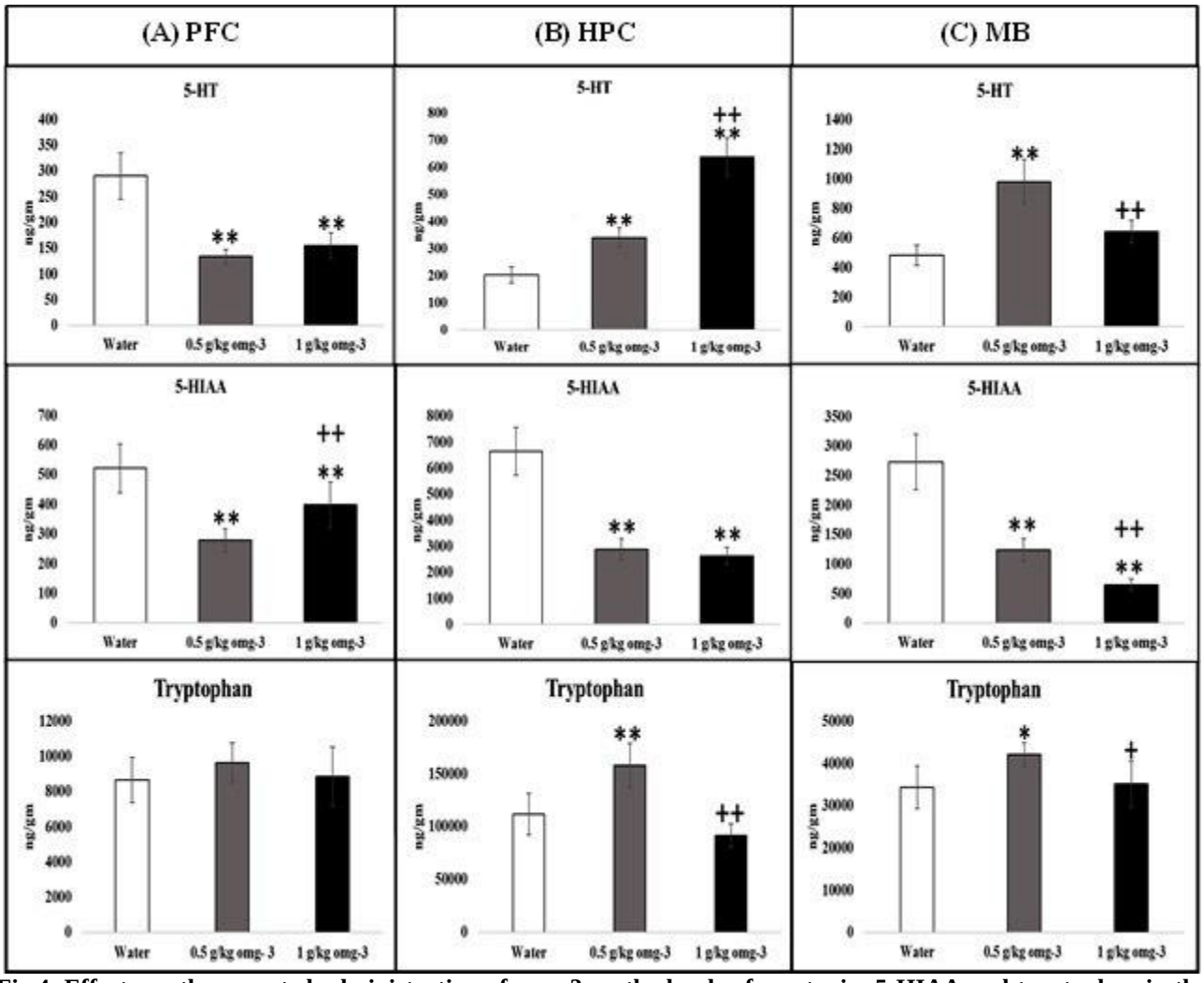

Fig.4. Effects on the repeated administration of omg-3 on the levels of serotonin, 5-HIAA and tryptophan in the PFC *(A)*, HPC *(B)*, MB *(C)*. Values are presented as means ± S.D. (n = 8). Significant differences by Tukey's test: *p < 0.05 and **p < 0.01 from water administered animals; +p < 0.01 and ++p < 0.05 from low dose (0.5 g/kg) omg-3 treated animals using one-way ANOVA.

DISCUSSION

The novelty in the current study is the behavioral expression of greater anxiety in rats treated with omg-3. Together with this, there is a reduction in 5-HIAA levels of the PFC, HPC, and MB suggesting a decrease in the metabolism of 5-HT. This study does not support that the use of omg-3 supplements for reducing anxiety in normal conditions. Conversely, it suggests that unnecessary use of these supplements can decrease the metabolism of 5-HT in all the regions studied and also levels of 5-HT in the PFC to produce anxiogenic like effects and predispose to depression.

Limited preclinical data is available on the therapeutic effects of omg-3 PUFAs on the anxiety like behavior. These studies show that omg-3 supplementation during the critical phase of brain development can induce restraint stress-induced corticosterone increases to predispose to anxiety like behavior (Pérez, Terreros, Dagnino-Subiabre, & Functions, 2013). Restraint stress-induced decreases in body weight gain are also attenuated. That supplementation of fish oil during the prenatal and postnatal periods can decrease olfactory bulbectomy-induced anxiety and depression like behavior has been also shown (Pudell *et al*., 2014). The present investigation concerns anxiolytic like effects, if any, of omg-3 supplementation in control rats not exposed to any stress challenge. We find that single administration or repeated daily administration omg-3 fish oil (0.5 or 1 g/kg doses) enhanced anxiety like behavior in an EPM test but no effects occur on food intake and gain of body weight.

This study shows that the single as well as repeated administration of omg-3 decreases locomotor activity in an OF. Previous literature on the effects of omg-3 supplementation and deficiency on motor behavior is not consistent, As, the study conducted by Hauser and colleagues *et al*., shows that diet enriched with omg-3 PUFAs given to rat for six weeks reduced locomotor activity (Hauser *et al*., 2014). An another study, supplementation of omg-3 PUFAs (1000 mg/day) for two weeks before mating to two-month-old female rats decreased motor activity (Chalon *et al*., 1998).

It is tempting to note that in the previously described study of (Pérez *et al*., 2013) *et al*., omg-3 supplementation during the critical period of brain development reduced stress induced anxiety but in unstressed animals it produced anxiety. We found that not only during the critical period of the brain development but also in adult rats omg-3 supplementation produced anxiogenic effects. Conversely, rats fed on omg-3 PUFAs diet exhibited a non-significant increase in time passed in the open arms of EPM but the total number of entries in open arms were significantly increased (Mathieu, Denis, Lavialle, Vancassel, & acids, 2008) suggesting that the deficiency of omg-3 tends to produce anxiety like effects in control animals. Clinical studies show that omg-3 supplements can ease anxiety in people with a range of mental health problems (Su *et al*., 2018). On the other hand, it has been also shown that the supplementation produces no effects in healthy, normally developing and impairment free population (Jackson, Reay, Scholey, & Kennedy, 2012). The present study also suggests that unnecessary use of omg-3 PUFAs can enhance normal level of anxiety.

To further understand the neurochemical basis of omg-3 PUFAs induced anxiety, the level of serotonin, its metabolite, i.e., 5-HIAA, as well as tryptophan are determined. A reduction in 5-HIAA levels in all brain regions is consistent with the view (Veenema *et al*., 2005) that 5-HT metabolism is decreased. In addition, 5-HT levels were also reduced in the PFC. Data therefore support notion that omg-3 decreases 5-HT metabolism to produce anxiety.

Tryptophan is a well-known essential amino acid and reported to be the sole precursor of serotonin (Venero, Herrera, Machado, & Cano, 1992). The aminoacid is converted into 5-HIAA by tryptophan hydroxylase (TPH), a rate limiting enzyme. In normal physiological conditions ,this enzyme is unsaturated with its substrate (Esteban *et al*., 2004). The levels of tryptophan in brain are therefore determined to understand that if a decrease or low levels in the availability of tryptophan is the cause of smaller 5-HT metabolism in omg-3 treated animals. Our findings, that levels of tryptophan are not reduced in any brain regions tend to suggest that a reduction in 5-HIAA levels may be in part due to the decrease in degradation of 5-HT to 5-HIAA. An activity of enzyme monoamine oxidase (MAO) that converts 5-HT to 5-HIAA (Peters *et al*., 2021) seems to have been affected by omg-3. In vitro or in vivo studies, on the activity of omg-3 can help to understand the mechanism involved in omg-3 enhanced modulation of 5-HT metabolism.

Overall the conclusion of the current investigation is that single or repeated administration of omg-3 PUFA (DHA and EPA) enhances the anxiety like behavior. A reduction in the metabolism of serotonin and decreases in the levels of serotonin particularly in the PFC observed after repeated administration of omg-3 seems important in the expression of anxiety like behavior because a decrease in 5-HT increase the risk for depression and anxiety, while anti-anxiety and anti-depressant drugs tend to enhance 5-HT neurotransmission. Although, the present study suggest that unnecessary intake of omg-3 supplements can enhance anxiety like behavior, studies on the effects of omg-3 on anxiety and depression like behavior are needed to understand the need of omg-3 in relieving stress-induced anxiety and improving therapies in mental illnesses.

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Contributors:

Conceptualization and Methodology: Faisal Khan, Darakhshan J. Haleem

Investigations: Khurshid Jalal, Atia Gohar, Rushda Afroz

Formal analysis: Faisal Khan, Shazia Nawaz

Writing original draft preparation: Faisal Khan, Tabinda Salman

Supervision, Writing/reviewing & editing: Ishtiaq Ahmad Khan, Darakhshan J. Haleem

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