Neurobehavioral and Micro Structural Evaluation of the Anti-anxiety Potential of Flaxseed oil Following Rotenone-induced Parkinson' Disease in Mice

Philemon Dauda Shallie[1*], Bamidele Richard Babatunde[1],
Olugbenga Olawole Olayinka[1], Damilola Joseph Talabi[1], Helen Bassey Akpan[1],
Royhaan O. Folarin[1], Oluwole Ojo Alese[2] and Oluwadamilola Faith Shallie[1]

[1]Department of Anatomy, Olabisi Onabanjo University, Nigeria.
[2]Department of Anatomy, Ekiti State University, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author PDS designed the study and wrote the protocol and the final draft of the manuscript. Authors BRB and ROF managed the literature searches. Authors BRB and HBA wrote the first draft of the manuscript. Authors BRB, DJT and OOO managed the laboratory animals. Authors OFS, DJT, ROF, HBA and OOA performed the laboratory analyses. Authors OFS, OOA and ROF performed the statistical analysis. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: This study investigated the neurobehavioral and micro structural anti-anxiety potential of flaxseed oil following rotenone-induced Parkinson' disease [PD] in mice
Study Design: Fifty-six adult male and female mice (*Mus musculus)* weighing between 23.9-26.3 grams were used for this study. The mice were randomly placed into four groups of fourteen mice each: A (Control; mice pellets), B (Rotenone 3 mg/kg, IP), C (Rotenone + Flaxseed oil 0.3 ml orally), and D (0.3 ml Flaxseed + Rotenone).
Place and Duration of the Study: Department of Anatomy, Olabisi Onabanjo University. Between May and September, 2016.

_____

Corresponding author: E-mail: shallie.philemon@yahoo.com, ShallieP@ukzn.ac.za;

Methodology: The brains were excised, weighed and appropriate sections taken and processed histology and labelled with H&E, silver and Nissl stains and immuno-cytochemically with GFAP.
Results: The results showed significant (P<0.005) increase in anxiety related activities and neuronal structural derangement, and degeneration of astrocytes in the rotenone-induced Parkinson's mice, which were counter/ameliorated by flaxseed oil treatments.
Conclusion: In conclusion, flaxseed oil acts as a neuro-protective agent against the insult of rotenone model of Parkinson's disease, thus it should be further evaluated as a potential therapeutic candidate in the management/treatment of Parkinson's disease.

1. INTRODUCTION

Parkinson's disease [PD] is a chronic neurodegenerative disorder affecting over four million people worldwide [1]. It is classically characterized by the emergence of motor symptoms such as rigidity, tremor, postural unbalance, and bradykinesia/akinesia [2,3]. However, PD also involves non motor symptoms (NMS) [4,3] that appear in the early, often premotor, phase of the disease [5-7] and significantly contribute to the impairment of the quality of life of 50%–60% of PD patients [8].

Anxiety affects quality of life in those living with Parkinson's disease (PD) more so than overall cognitive status, motor deficits, apathy, and depression [9-11]. Although anxiety and depression are often related and coexist in PD patients [12], recent research suggests that anxiety rather than depression is the most prominent and prevalent mood disorder in PD [13,14]. Yet, our current understanding of anxiety and its impact on cognition in PD, as well as its neural basis and best treatment practices, remains meagre and lags far behind that of depression[15].

Two previous studies demonstrated that hippocampal atrophy is evident in PD with or without dementia [16,17]. Similar findings are observed in dementia with Lewy bodies (DLB), where diffuse Lewy bodies and, in many cases, the pathologic features of AD co-occur [18-20]. These changes overlap with those seen in PD with dementia (PDD) [21,22].

A growing body of evidence suggests that nutrition may play an important role in PD. Epidemiological and biochemical studies have recently identified promising components in certain food groups that may elicit neuroprotection in PD [23,24]. However, inclusion or exclusion of other food groups may trigger or exacerbate neurodegeneration.

Flaxseed oil comes from the seeds of the flax plant (*Linum usitatissimum, L.*). Flaxseed oil contains both omega-3 and omega-6 fatty acids, which are needed for health. Flaxseed oil contains the essential fatty acid alpha-linolenic acid (ALA), which the body converts into eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which is the omega-3 fatty acids found in fish oil. Some researchers think flaxseed oil might have some of the same benefits as fish oil. But the body is not very efficient at converting ALA into EPA and DHA. The benefits of ALA, EPA, and DHA are not necessarily the same [25].

Omega 3 and omega 6 fatty acids are essential fatty acids, that is, they cannot be synthesised and are essential components of the human diet for health. Omega 3s are found in large amounts in fish and seafood, particularly in oily fish, and in some plants, notably flaxseeds and purslane. While both omega 3 and 6 fatty acids have positive structural properties when incorporated into bodily cell membranes, in simple terms, omega 3s are essentially anti-inflammatory [26,27].

In this study, we investigated the relevance of flaxseed oil, as a potential neuro-therapeutic candidate targeting hippocampal neurones and astrocytes in line with the growing body of evidence that suggests nutrition may play an important role in PD.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Fifty-six adult male and female mice (*Mus musculus*) weighing between 23.9-26.3 g were used for this study. The animals were housed in clean plastic cages, well ventilated environment with temperature ranging between 24-28ºC in 12 hours light and 12 hours dark cycle. The animals were given standard mice pellets and water *ad*

libitum, and were allowed to acclimatize for two weeks before commencing the experimental protocols. Rotenone was bought from Abcam, while flaxseed oil was purchased from Organo Shoppe, Lagos, Nigeria. The institutional committee on Animal Care and Use in Research, Education and Testing (ACURET) approval was obtained and the animal experiments were conducted according to the NIH Guide on Laboratory Animals for Biomedical Research (NIH, 1978) and ethical guidelines for investigation of experimental pain in conscious animals [28].

2.2 Experimental Design

Following the two weeks of acclimatization, the animals were randomly divided into four (4) groups of fourteen (14) animals each made up of equal number of male and female mice as follows:

- Group A: (Control Group) Mice were given dry food pellet and clean water *ad libitum.*
- Group B: (Negative Control Group) Mice were given 3 mg/kg/day of Rotenone per body weight subcutaneously for 5 consecutive days
- Group C: (Post-treated) Mice were given 3 mg/kg/day of Rotenone per body weight subcutaneously for 5 consecutive days followed by a fourteen (14) days oral treatment with (0.3 mil/mouse) flax seed oil.
- Group D: (Pre-treated) Mice were given (0.3mil/mouse) flax seed oil for fourteen (14) days consecutively followed by five (5) days administration of 3 mg/kg/day of Rotenone subcutaneously.

2.3 Elevated Plus Maze (EPM)

2.3.1 Introduction

This assay essentially determines a preference between a comparatively safe and comfortable environment (the closed arms) and a risky environment (elevated open spaces). This is often discussed in terms of avoidance or fear, but this is not strictly accurate (see the refs below). This is technically a preference test – one portion of the arm is avoided only in comparison to the other portion. The general principle is that the more "anxious" the subjects are, the less likely they will be to explore an uncomfortable, risky, or threatening environment. Thus, previous stress, presence of a predator odor, previous handling,

manipulation of stress hormones and peptides all effect behavior in the EPM. Unfortunately, not all these factors produce the same effects in each strain, sex, age species etc [29].

2.3.2 Procedures

Anxiety-related behavior is measured as preference for the closed arms. The percentage of open arm entries also indicates anxiety levels, especially in mice, which tend to be more impulsive and spend less time per entry in any arm. Controls include total arm entries, which is generally considered to indicate non-specific locomotor activity. The testing session consists of putting the animal in the apparatus and recording the following behaviors: Total time spent in the open arm, total time spent in the closed arm, total number of open arm entries, closed arm entries. The maze was cleaned with 70% ethanol after every trial and with 10% bleach at the end of every day [30].

2.3.3 Experimental design

The animals were tested in random order in a matched block design such that equal numbers of animals in each treatment group are represented in each testing block. The experimenter was blinded to the condition of the animals.

2.3.4 Scoring the EPM

An entry was defined when all 4 paws crossed the line into that arm. Time in the center was also be recorded.

2.4 Tissue Sample Preparation

At the end of the experimental period the mice were euthanized by administering 10 g/kg body weight of Pentobarbital. The mice brains were carefully dissected out, weighed and fixed in 10% formol-saline for routine histological and immunocytochemical procedures.

2.4.1 Haematoxylin and eosin routine staining

Tissue sections were rinsed in distilled water for 5 minutes, then stained in haematoxylin for 15 minutes, rinsed in running tap water and differentiated in 0.3% acetic acid and rinsed in tap water before staining with eosin for 2 minutes. Sections were then dehydrated in 70% for 1 minute, 95% alcohol for 1 minute, 100% alcohol for 1 minute (2 changes) respectively and

then taken to the oven overnight. Sections were subsequently cleared in xylene and then placed DPX mountant and cover slipped for light microscopy [31].

2.4.2 Bielschowsky's silver staining protocol

Deparaffinized sections to distilled water and washed three times, the slides were pre-warmed (40ºC) and stained in 10% silver nitrate solution for 15 minutes, the slides were then placed in distilled water and washed for 3 times; added to the silver nitrate solution, was concentrated ammonium hydroxide drop by drop until the precipitate formed was JUST clear. The slides were placed back in this ammonium silver solution and stained in 40ºC oven for 30 minutes or until sections become dark brown, slides were placed directly in developer working solution for about 1 minute, after this slides were dipped for 1 minute in 1% ammonium hydroxide solution to stop the silver reaction. Slides were then washed in distilled water in 3 changes. Slides were then placed in 5% sodium thiosulfate solution for 5 minutes, followed by yet another 3 changes of washing in distilled water. The sections were dehydrated and cleared through 95% ethyl alcohol, absolute alcohol and xylene and mounted with resinous medium [32].

2.4.3 Methods: Cresyl fast violet for nissl substance

Tissue sections were de-waxed in xylene (2 or 3 changes of 3 min each), dehydrated in alcohol (100% x2), 3 min each, followed by staining in 0.1% Cresyl Violet for 15 min.The slides were quickly rinsed in tap water to remove excess stain, then washed in 70% ethanol, followed by dehydration through 2x3min changes of absolute ethanol and finally cleared in xylene x2 and mounted in DPX [33].

2.5 Immunohistochemical Protocol

The paraffin embedded tissue was cut at 5 microns thick and allowed to heat on hot plate for 1 hour, then sections were taken to water, that is, through xylene, alcohols and finally water respectively. Antigen retrieval method was performed using citric acid solution pH 6.0 in a pressure cooker for 15 minutes. Sections were equilibrated by gently displacing hot citric acid with running tap water for 3 minutes. Blocking of peroxidises in tissue sections was done using peroxidise block for 15 minutes and then washed for 2 minutes with phosphate buffered saline

(PBS) with tween 20. Blocking of protein was then performed with Novocastra® protein block for 15 minutes. Tissue section was then washed for 2 minutes with PBS, then incubated with primary antibody e.g., Neurofilaments 1 in 100 dilution for 45 minutes, washed in PBS for 3 minutes and later added Secondary antibody for 15 minutes. Tissue section was then washed twice with PBS. Polymer was thereafter added and allowed for 15 minutes, washed twice with PBS and then added the diaminobenzidine (DAB) chromogen diluted 1 in 100 with the DAB substrate for 15 minutes, and then washed with water and counterstained for 2 minutes in Haematoxylin. Again the tissue section was washed, dehydrated, cleared and mounted in DPX mountant [34].

2.6 Photomicrography

Photomicrographs were taken using Omax led digital Microscope.

2.7 Statistical Analysis

Data were analysed using analysis of variance (ANOVA) by comparing values for different treatment groups with the values for individual controls. Results were expressed as mean ± SEM. The significant differences among values were analysed using Graph Pad version 7 at *P-value* = 0.05.

3. RESULTS AND DISCUSSION

Non-motor symptoms (NMS) in PD are common and often precede motor deficits. In the last years, affective symptoms of PD, such as anxiety and depression, or impaired sensory functions, such as olfactory disturbance attracted increased attention not only because they reduce the quality of life but also they may serve as early disease indicators [35]. This study compares anxiety in PD mice model with hippocampal structural integrity which is the seat of working memory. The findings showed that the mean number of entry and the time spent in the close arm of the elevated plus maze significantly increased in the PD mice [Figs. 1 & 2], while the number of entry and time spent in the open arm were decreased in the PD mice [Figs 3 & 4]; In elevated plus maze, anxiety-related behavior is measured as preference for the closed arms and dislike or avoidance of the open arms, hence our results showed that PD is positively associated with anxiety as shown by the significance preference for closed arm at the expense of

the open arm. Although the neurobiology of anxiety in PD remains unknown, many researchers have postulated that anxiety disorders are related to neurochemical changes that occur during the early premotor stages of PD-related degeneration [36,37] such as nigrostriatal dopamine depletion, as well as cell loss within serotonergic and noradrenergic brainstem nuclei (i.e., raphe nuclei and locus coeruleus, resp., which provide massive inputs to cortico-limbic regions).Overtime, chronic dysregulation of adrenocortical and catecholamine functions can lead to hippocampal damage as well as dysfunctional prefrontal neural circuitries [38, 39], which play a key role in memory and attention [40]. While both post and pre-treatment with flaxseed oil ameliorates and counters the heightened anxiety caused by rotenone induced PD in the experimental mice respectively [Figs. 1 and 2]. Our present study reported that intake omega 3 in the form of flaxseed oil significantly protected against H_2O_2-induced oxidative injury, suggesting that omega 3 might be an effective supplement for the prevention of neurodegenerative diseases which are associated with oxidative stress. DHA has been reported to scavenge the intracellular radical productions induced by hydrogen peroxide (H_2O_2), superoxide anion ($O_2 \cdot -$), and hydroxyl radical ($\cdot OH$) [41]. Many previous studies reported that DHA treatment could significantly reduce ROS production, which is a possible mechanism underlying DHA's protective effects [42].

We further investigated the role of flaxseed oil on the CA3 region of hippocampus of rotenone model of PD mice. Our results showed that rotenone induced neuronal degenerations in the mice model of PD [see Plates 1, B; Plate 2, B and Plate 3, B].One earlier study in PD using the same subfield segmentation technique found volume loss in CA2–3, CA4-DG, subiculum, and the whole hippocampus, and this correlated with verbal learning [43].

It has been shown in Parkinson's disease that mitochondrial function is disrupted, causing cells to become malnourished and die [44,45].The mechanism behind mitochondrial dysfunction in Parkinson's disease is hypothesized to be the PINK1 and Parkin complex which has been shown to drive autophagy of the mitochondria, also known as mitophagy [44-46]. PINK1 is a protein that is normally transported into the mitochondria, but can also accumulate on the surface of impaired mitochondria. Accumulated PINK1 then recruits Parkin which initiates the breakdown of dysfunctional mitochondria, a mechanism that acts as a "quality control" [44]. In Parkinson's disease, the genes coding PINK1 and Parkin are thought to be mutated, therefore preventing the breakdown of impaired mitochondria, causing abnormal function and morphology of mitochondria and eventually cell death [44,45]. Mitochondrial DNA (mtDNA) mutations have also been shown to accumulate with age [47] indicating that susceptibility to this mechanism of neuronal death increases with age.
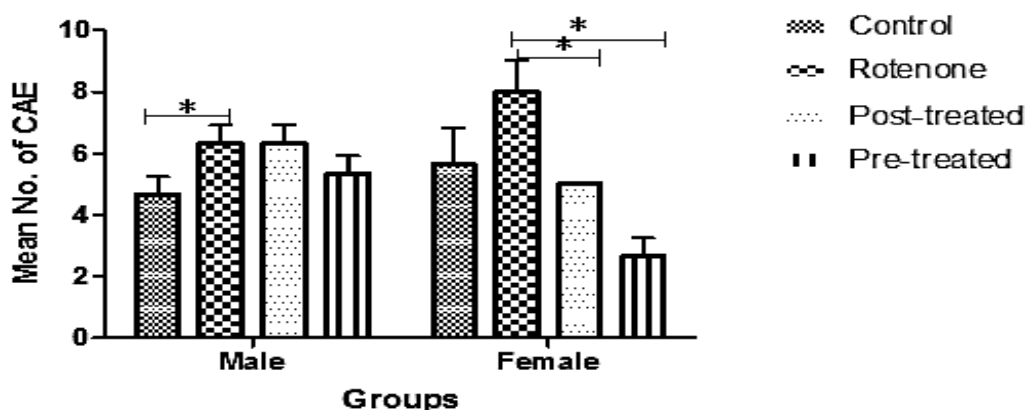

Fig. 1. shows the mean number of close arm entry ± SE of Male and Female mice in control, rotenone, post-treated and pre-treated groups. The results of the two way ANOVA showed: 1. Significant interaction ($F_{(3, 16)}$ =12.22, P<0.0001; 2. Non-significant main effect of sex ($F_{(1, 16)}$ =1.33, P<0.2652); 3. Significant main effect of treatment ($F_{(3, 16)}$ =20.67, P<0.0001). The results showed that PD increased anxiety-like behaviors by increased preference for closed arms while flaxseed oil appears to ameliorate such behaviors
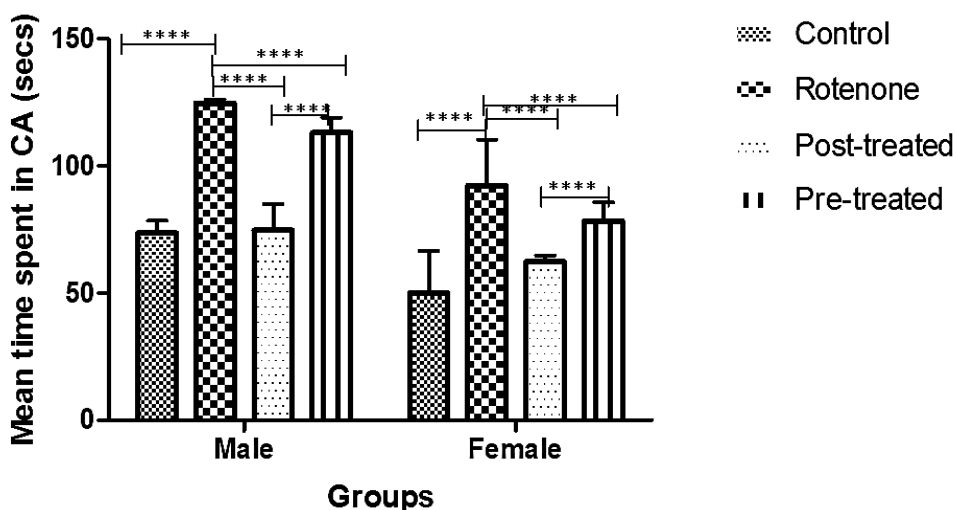
Fig. 2. Shows the mean time spent in close arm ± SE of Male and Female mice in control, rotenone, post-treated and pre-treated groups. The results of the two way ANOVA showed: 1. Non-significant interaction ($F_{(3, 16)}$ =1.489, P=0.2553); 2. Significant main effect of sex ($F_{(1, 16)}$ =28.46, P<0.0001); 3. Significant main effect of treatment ($F_{(3, 16)}$ =38.50, P<0.0001). The results just like in Fig. 1 above showed that PD increased anxiety-like behaviors by increased preference for closed arms while flaxseed oil appears to ameliorate such behaviors
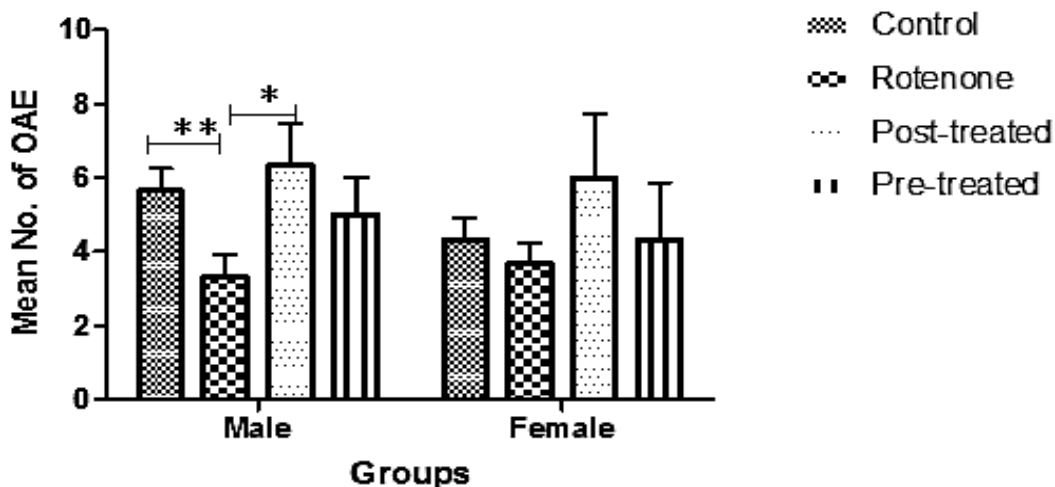

Fig. 3. shows the mean number of open arm entry ± SE of Male and Female mice in control, rotenone, post-treated and pre-treated groups. The results of the two way ANOVA showed: 1. Non-significant interaction ($F_{(3, 16)}$ =0.64, P<0.5991); 2. Non-significant main effect of sex ($F_{(1, 16)}$ =1.33, P=0.2652); 3. Significant main effect of treatment ($F_{(3, 16)}$ =6.42, P=0.0046). The results showed that PD increased anxiety-like behaviors by increased dislike for the open while flaxseed oil appears to ameliorate such behaviors

Another mitochondrial-related mechanism for cell death in Parkinson's disease is the generation of Reactive Oxygen Species (ROS) [47,48]. ROS are highly reactive molecules that contain oxygen and can disrupt functions within the mitochondria and the rest of the cell. With increasing age, mitochondria lose their ability to remove ROS yet still maintain their production of ROS, causing an increase in net production of ROS and eventually cell death [47,48].
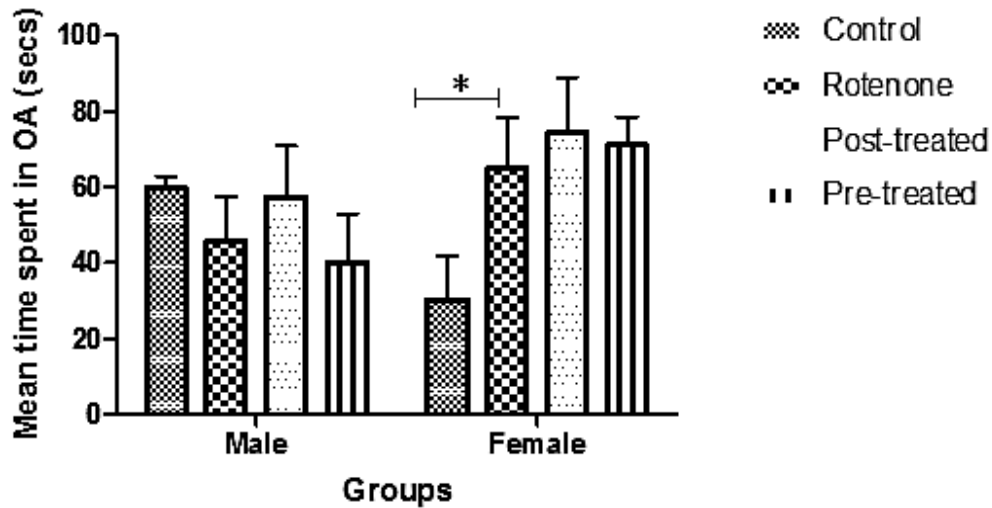
Fig. 4. Shows the mean time spent in open arm ± SE of Male and Female mice in control, rotenone, post-treated and pre-treated groups. The results of the two way ANOVA showed: 1. Significant interaction ($F_{(3, 16)}$ =7.98, P<0.0018); 2. Non-significant main effect of sex ($F_{(1, 16)}$ =4.069, P=0.0608); 3. Significant main effect of treatment ($F_{(3, 16)}$ =6.42, P=0.0046).The results just like in Fig. 3 above showed that PD increased anxiety-like behaviors by increased dislike for the open while flaxseed oil appears to ameliorate such behaviors.
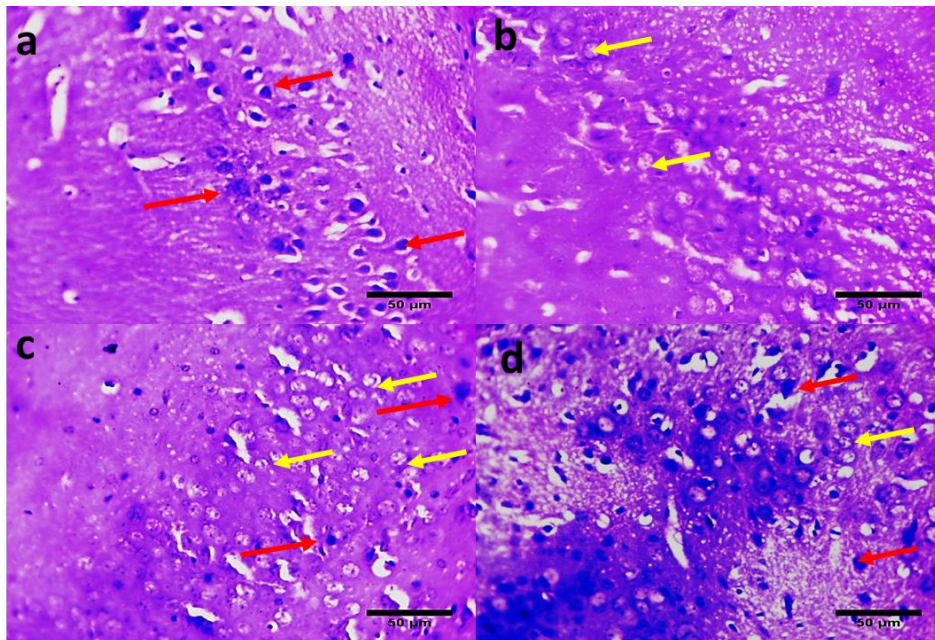

Plate 1. Showing the photomicrographs of mice Cornu Armonis 3 (CA3) region of the hippocampus stained with H&E X1000: (a) CA3 region of the hippocampus of control group showing normal neuronal nuclei (red arrows); (b). CA3 region of the hippocampus of Rotenone treated mice showing degenerated neurons (yellow arrow); (c)CA3region of the hippocampus of Rotenone exposed mice and post-treated with flaxseed oil showing some normal neurons (red arrows) and degenerating neurons (yellow arrow); (d) CA3 region of the hippocampus of Rotenone exposed mice and pre-treated with flaxseed oil showing some normal neurons (red arrow) and degenerating neurons (yellow arrow)
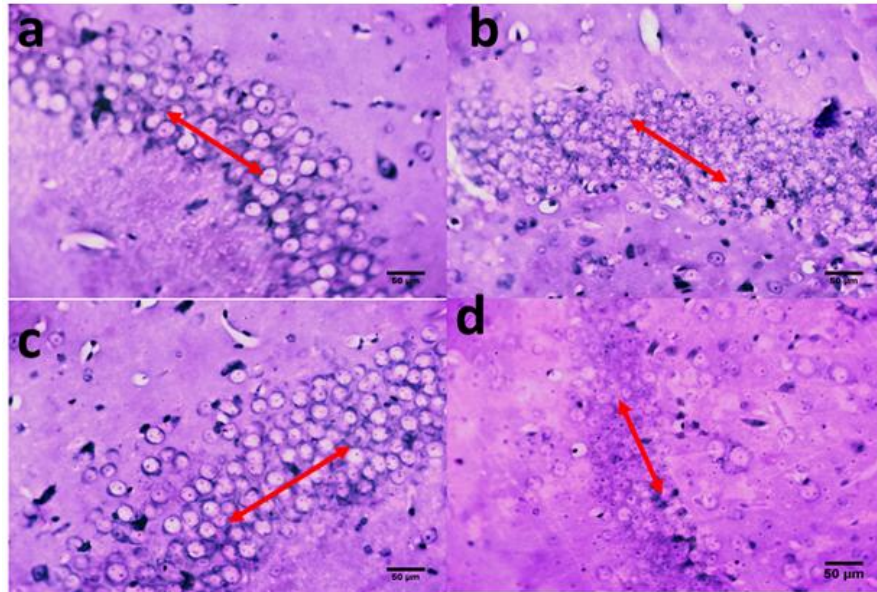
Plate 2. Showing the photomicrographs of mice Cornu Armonis 3 (CA3) region of the hippocampus stained with Nissl X1000: (a) CA3 region of the hippocampus of control group showing some nissl substance around the normal neuronal nuclei (red double-arrows); (b)CA3 region of the hippocampus of Rotenone treated mice showing degenerated chromotolytic neurons (red double-arrow); (c)CA3 region of the hippocampus of Rotenone exposed mice and post-treated with flaxseed oil showing some nissl substance around normal neuronal nuclei (red double-arrows); (d) CA3 region of the hippocampus of Rotenone exposed mice and pre-treated with flaxseed oil showing degenerated chromotolytic neurons (red double-arrow).
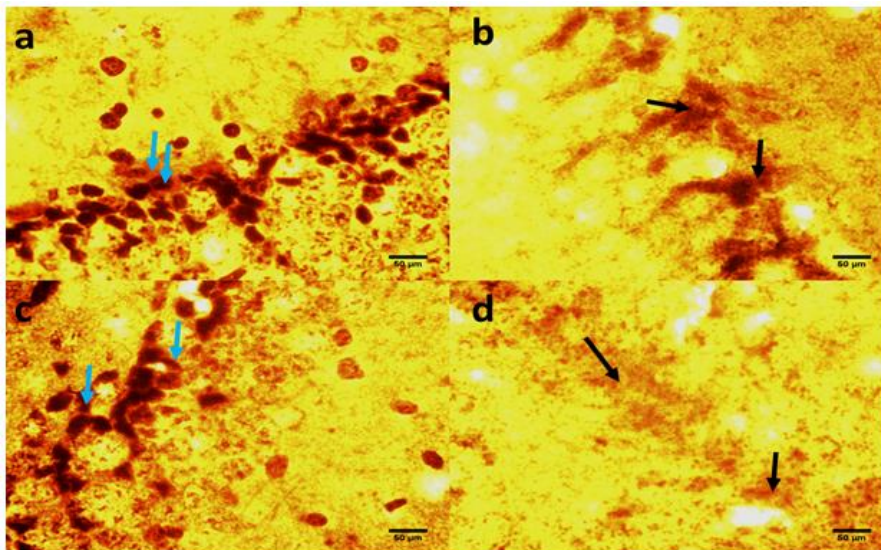

Plate 3. Showing the photomicrographs of mice Cornu Armonis 3 (CA3) region of the hippocampus stained with Silver X1000: (a) CA3 region of the hippocampus of control group showing normal neuronal nuclei (blue arrows); (b). CA3 region of the hippocampus of Rotenone treated mice showing degenerated neurons (black arrows); (c)CA3 region of the hippocampus of Rotenone exposed mice and post-treated with flaxseed oil showing some normal neurons (blue arrows); (d) CA3 region of the hippocampus of Rotenone exposed mice and pre-treated with flaxseed oil showing degenerated neurons (black arrows)
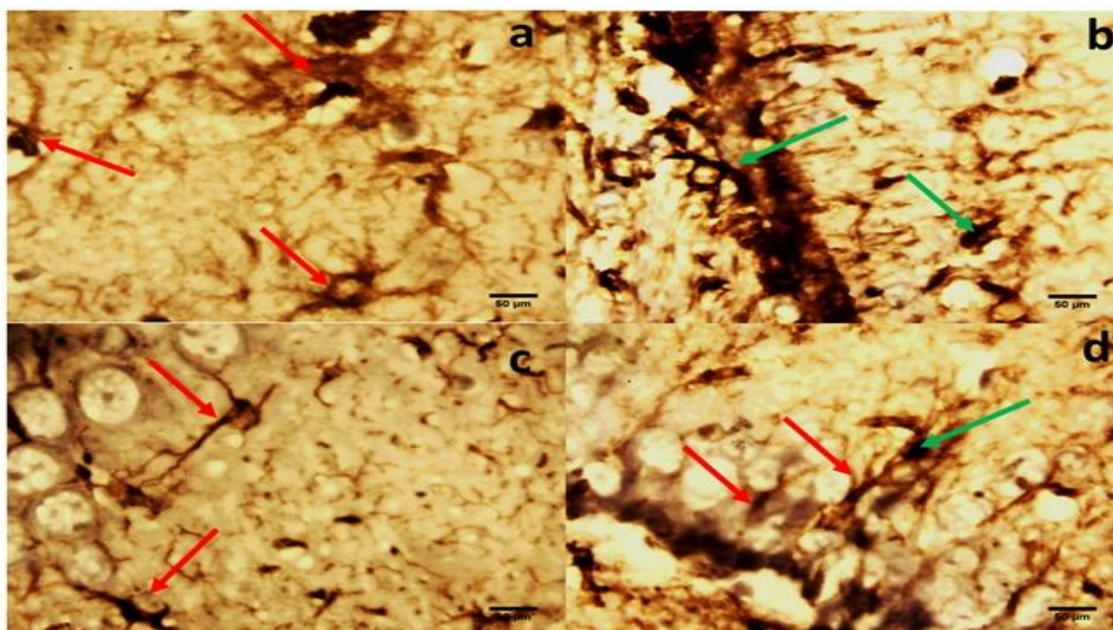
Plate 4. Showing the photomicrographs of mice Cornu Armonis 3 (CA3) region of the hippocampus stained with GFAP X1000: (a) CA3 region of the hippocampus of control group showing normal astrocytes (red arrows); (b). CA3 region of the hippocampus of Rotenone treated mice showing clumped astrocytic processes (green arrows); (c)CA3 region of the hippocampus of Rotenone exposed mice and post-treated with flaxseed oil showing some normal astrocytes (blue arrows); (d) CA3 region of the hippocampus of Rotenone exposed mice and pre-treated with flaxseed oil showing few apparently normal astrocytes (red arrow and some clumped astrocytic processes (green arrows).

Finally we looked at the role of astrocytes in rotenone induced Parkinson's disease and on the intervention with flaxseed oil. Our finding showed complete loss of astrocytes in the rotenone group [Plate 3, B]; During brain damage (including diseases, brain injury and oxidative stress), these astrocytic functions become transiently or permanently impaired, and the subsequent impact on neuronal cells may lead to pathological conditions and neurodegenerative diseases [49,50]. Neurons are more susceptible to injury than astrocytes, as they have limited antioxidant capacity, and rely heavily on their metabolic coupling with astrocytes to combat oxidative stress [49]. However, severe brain damage also results in astrocyte dysfunction, leading to increased neuronal death [51]. Our results also revealed that intake of flaxseed oil was able to preserve some astrocytes [Plate 3, C & D]. A large amount of evidence, in cellular and animal models under neurotoxic stimuli, has suggested that DHA can prevent inflammation by modulating glial cell activity [52-55]. DHA modulation of astrocytes also demonstrates fine tuning of neuronal activity through inhibition of pro-inflammatory mediators and an important regulation of astrocytic activity.

4. CONCLUSION

In conclusion, flaxseed oil acts as a neuro-protective agent against the insult of rotenone model of Parkinson's disease, thus it should be further evaluated as a potential therapeutic candidate in the management/treatment of Parkinson's disease.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this paper and accompanying images.

ETHICAL APPROVAL

As per international standard or university standard, written approval of ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, Marshall FJ, Ravina BM, Schifitto G, Siderowf A, et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. Neurology. 2007;68:384–386.

2. Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 2003;24:197–211.

3. Jankovic J. Parkinson's disease: Clinical features and diagnosis. J Neurol Neurosurg Psychiatry. 2008;79:368–376.

4. Chaudhuri KR, Healy DG, Schapira AH. Non-motor symptoms of Parkinson's disease: Diagnosis and management. Lancet Neurol. 2006;5:235–245.

5. Abbott A.. Neuroscience: The molecular wake-up call. Nature. 2007;447:368–370.

6. Abbott RD, Ross GW, White LR, Tanner CM, Masaki KH, Nelson JS, Curb JD, Petrovitch H. Excessive daytime sleepiness and subsequent development of Parkinson disease. Neurology. 2005;65:1442–1446.

7. Claassen DO, Josephs KA, Ahlskog JE, Silber MH, Tippmann-Peikert M, Boeve BF. REM sleep behavior disorder preceding other aspects of synucleinopathies by up to half a century. Neurology. 2010;75:494–499.

8. Aarsland D, Ballard C, Rongve A, Broadstock M, Svenningsson P. Clinical trials of dementia with Lewy bodies and Parkinson's disease dementia. Curr Neurol Neurosci Rep. 2012;12:492–501.

9. Hanna KK, Cronin-Golomb A. Impact of anxiety on quality of life in Parkinson's disease. Parkinson's Disease. 2012;8. (ArticleID640707)

10. Jones JD, Butterfield LC, Songetal W. Anxiety and depression are better correlates of Parkinson's disease quality of life than apathy. Journal of Neuropsychiatry and Clinical Neurosciences. 2014;27(3):213–218.

11. Deane KHO, Flaherty H, Daley DJ, et al. Priority setting partnership to identify the top 10 research priorities for the management of Parkinson's disease. BMJ Open. 2014;4(12).

12. Marsh L, Neuropsychiatric aspects of Parkinson's disease. Psychosomatics. 2000;41(1):15–23.

13. Landau S, Harris V, Burn DJ, et al. Anxiety and anxious depression in Parkinson's disease over a 4-year period: A latent transition analysis. Psychological Medicine. 2016;46(3):657–667.

14. Brown RG, Landau S, Hindle JV, et al. Depression and anxiety related subtypes in Parkinson's disease. Journal of Neurology, Neurosurgery and Psychiatry. 2011;82(7):803–809.

15. Seppi K, Weintraub D, Coelho M, et al. The movement disorder society evidence-based medicine review update: Treatments for the non-motor symptoms of Parkinson's disease. Movement Disorders. 2011;26 (supplement3):S42–S80.

16. Camicioli R, Moore MM, Kerr D, Kaye J. Dementia in Parkinson's disease is associated with hippocampal atrophy. Neurology. 1999;52:A486.

17. Laakso MP, Partanen K, Riekkinen P, et al. Hippocampal volumes in Alzheimer's disease, Parkinson's disease with and without dementia and in vascular dementia: An MRI study. Neurology. 1996; 46:678–681.

18. Hashimoto M, Kitagaki H, Imamura T, et al. Medial temporal and whole-brain atrophy in dementia with Lewy bodies: A volumetric MRI study. Neurology 1998;51:357–362.

19. Barber R, Gholkar A, Scheltens P, Ballard C, McKeithI G, O'Brien JT. Medial temporal lobe atrophy on MRI in dementia with Lewy bodies. Neurology. 1999; 52:1153–1158.

20. Barber R, Ballard C, McKeith IG, Gholkar A, O'Brien JT. MRI volumetric study of dementia with Lewy bodies: A comparison with AD and vascular dementia. Neurology. 2000;54:1304–1309.

21. Hurtig HI, Trojanowski JQ, Galvin J, et al. Alpha-synuclein cortical Lewy bodies correlate with dementia in Parkinson's disease. Neurology. 2000;54:1916–1921.

22. Harding AJ, Halliday GM. Cortical Lewy body pathology in the diagnosis of dementia. Acta Neuropathol (Berl). 2001; 102:355–363.

23. Searles Nielsen S, Franklin GM, Longstreth WT, Swanson PD, Checkoway H. Nicotine from edible *Solanaceae* and risk of Parkinson disease. Ann. Neurol. 2013;74:472–477.

    DOI: 10.1002/ana.23884

24. Shaltiel-Karyo R, Frenkel-Pinter M, Rockenstein E, Patrick C, Levy-Sakin M, Schiller A, et al. A blood-brain barrier (BBB) disrupter is also a potent alpha-synuclein (alpha-syn) aggregation inhibitor: a novel dual mechanism of mannitol for the treatment of Parkinson disease (PD). J. Biol. Chem. 2013;288:17579–17588.

    DOI: 10.1074/jbc.M112.434787

25. Steven D. Ehrlich, NMD, Solutions Acupuncture, a private practice specializing in complementary and alternative medicine, Phoenix AZ. Review provided by VeriMed Healthcare Network. Also Reviewed by the A.D.A.M Editorial Team; 2015.

26. Erasmus U. Fats that heal, fats that kill: the complete guide to fats, oils, cholesterol and human health. 3rd ed. Summertown, TN: Alive Books; 2010.

27. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr. 2002;21(6):495–505.

28. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain. 1983;16:109–110.

29. Pellow S, Chopin P, File SE, Briley M. Validation of open: Closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods. 1985;14:149–167.

30. Rodgers RJ, Johnson NJ, Factor analysis of spatiotemporal and ethological measures in the murine elevated plus - maze test of anxiety. Pharmacol Biochem Behav. 1995;52(2):297-303.

31. Drury RAB, Wallington EA. Carleton's histological technique, 5th ed. Oxford, UK: Oxford University Press, Oxford. Wenk modification is substitution of acetaldehyde for paraldehyde. Wenk PA. J. Histotechnology. 1996;19(4):353-355.

32. NISSL Caceci T. Histology: Lecture 29: Introduction to special stains techniques: Nerve Tissue Staining. 1 – 29.

    Available:www.histology.com,

    (Accessed 2nd March, 2014)

33. Luna LG: Histopathologic methods and color atlas of special stains and tissue artifacts; Am Erican Histolabs, Inc. Silver. 1992;483-485.

34. O'Callaghan J, Sairam K. Glial fibrillary acidic protein and related glial proteins as biomakers of neurotoxicity. Expert Opinion on Drug Safety. 2005;4:433–442.

35. Gustafsson H, Nordstrom A, Nordstrom P. Depression and subsequent risk of Parkinson disease: A nationwide cohort study. Neurology. 2015;84:2422–2429.

36. Braak H, Ghebremedhin E, R¨ub U, Bratzke H, Del Tredici K. Stages in the development of Parkinson's diseaserelated pathology. Cell and Tissue Research. 2004;318(1):121–134.

37. Prediger RDS, Matheus FC, Schwarzbold ML, Lima MMS, Vital MABF, Anxiety in Parkinson's disease: A critical review of experimental and clinical studies. Neuropharmacology. 2012;62(1):115–124.

38. Sullivan GM, Coplan JD, Kent JM, Gorman JM. The noradrenergic system in pathological anxiety: A focus on panic with relevance to generalized anxiety and phobias. Biological Psychiatry. 1999;46(9): 1205–1218.

39. Espejo EF. Selective dopamine depletion within the medial prefrontal cortex induces anxiogenic-like effects in rats placed on the elevated plus maze. Brain Research. 1997;762(1-2):281–284.

40. Ryder KA, Gontkovsky ST, Mcswan KL, et al. Cognitive function in Parkinson's disease: Association with anxiety but not depression. Aging, Neuropsychology, and Cognition. 2002;9(2):77–84.

41. Shimazawa M, Nakajima Y, Mashima Y, Hara H. Docosahexaenoic acid (DHA) has neuroprotective effects against oxidative stress in retinal ganglion cells. Brain Res. 2009;1251:269–275.

42. Kim EJ, Park YG, Baik EJ, Jung SJ, Won R, Nahm TS, Lee BH. Dehydroascorbic acid prevents oxidative cell death through a glutathione pathway in primary astrocytes. J. Neurosci. Res. 2005;79: 670–679.

43. Pereira JB. et al. Regional vulnerability of hippocampal subfields and memory deficits in Parkinson's disease. Hippocampus. 2013;23:720–728.
44. Chen H, Chan DC. Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. Human Molecular Genetics. 2009;18(R2).
   DOI: 10.1093/hmg/ddp326
45. Pickrell A, Youle R. The Roles of PINK1, Parkin. Mitochondrial fidelity in Parkinson's disease. Neuron. 2015:85(2):257-273.
   DOI: 10.1016/j.neuron.2014.12.007
46. Narendra D, Tanaka A, Suen D, Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. The Journal of Cell Biology. 2008;183(5):795-803.
   DOI: 10.1083/jcb.200809125
47. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006;443(7113).
48. Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. Molecular and Cellular Biochemistry Mol Cell Biochem. 2010;345(1-2):91-104.
   DOI: 10.1007/s11010-010-0563-x
49. Zahler S, Kupatt C, Becker BF. Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-alpha. FASEB J. 2000;14:555–564.
50. Zhao Z, Wen H, Fefelova N, Allen C, Guillaume N, Xiao D, Huang C, Zang W, Gwathmey JK, Xie LH. Docosahexaenoic Acid reduces the incidence of early after depolarizations caused by oxidative stress in rabbit ventricular myocytes. Front. Physiol. 2012;3.
   DOI: 10.3389/fphys.2012.00252
51. Nanou A, Higginbottom A, Valori CF, Wyles M, Ning K, Shaw P, Azzouz M. Viral delivery of antioxidant genes as a therapeutic strategy in experimental models of amyotrophic lateral sclerosis. Mol. Ther. 2013;21:1486–1496.
52. Li L, Wu Y, Wang Y, Wu J, Song L, Xian W, et al. Resolvin D1 promotes the interleukin-4-induced alternative activation in BV-2 microglial cells. J Neuroinflammation. 2014;11:72.
53. Xu J, Storer PD, Chavis JA, Racke MK, Drew PD. Agonists for the peroxisome proliferator-activated receptor-alpha and the retinoid X receptor inhibit inflammatory responses of microglia. J Neurosci Res. 2005;81:403–11.
54. Manzhulo IV, Ogurtsova OS, Lamash NE, Latyshev NA, Kasyanov SP, Dyuizen IV. Analgetic effect of docosahexaenoic acid is mediated by modulating the microglia activity in the dorsal root ganglia in a rat model of neuropathic pain. Acta Histochem. 2015;17:659–66.
55. Lu Y, Zhao LX, Cao DL, Gao YJ. Spinal injection of docosahexaenoic acid attenuates carrageenan-induced inflammatory pain through inhibition of microglia-mediated neuroinflammation in the spinal cord. Neuroscience. 2013; 241:22–31.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/20201