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RESEARCH ARTICLE

Neuroprotective Effects and Amelioration of Ethyl Esters form of Fish Oil Supplementation in Neuroinflammation in a Mouse Model of Cuprizone-Induced Demyelination

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ABSTRACT

Multiple sclerosis (MS) is a disease that has inflammatory effects on the brain and spinal cord. Myelin sheath degradation is the prominent outcome of MS. This study is aimed to investigate protective effects of dietary fish oil in the form of ethyl esters (EE-FO) on demyelination in mice induced by cuprizone (CPZ). We found that EE-FO supplementation ameliorated CPZ-induced demyelination and improved learning and memory impairments of mice. It was observed that demyelination was alleviated in EE-FO-treated mice which was measured through luxol fast blue (LFB) staining and expression analyses of myelin basic protein (MBP). Additionally, it was also observed that activation of microglia in the corpus callosum was reduced in EE-FO treated mice. Furthermore, we demonstrated that EE-FO treatment down-regulated the expression of M1-markers, pro-inflammatory cytokine concentration i.e., TNF-α and IL-1β, and promoted M2-markers expression (CD206 and Arginase-1). Concomitantly, the EE-FO treatment showed an increased expression of SIRT1 and AKT but suppressed the expression of NF-κB p65 and NLRP3 inflammasomes in CPZ mice. Taken together, these results suggest that EE-FO supplementation exerts neuroprotective effects on demyelination in mice induced by CPZ by modulating the SIRT1/p-AKT and SIRT1/NF-κB/NLRP3 pathways.

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INTRODUCTION

Demyelination of the central nervous system (CNS) is a pathological state in case of multiple sclerosis (MS), which related to the reactive microglia and macrophages in brain (AlAmmar *et al.*, 2021). Recent studies have reported that fish oil supplementation improved clinical outcomes in MS patients (Zandi-Esfahan *et al.*, 2017). Naturally, docosahexaenoic acid (22: 6n-3) (DHA) exists as triglycerides and eicosapntemacnioc acid (EPA) (TG) as phospholipids, and formed to purified ethyl esters (EE) or free fatty acids (FFA) in fish oil (FO) products. Most of the studies focused on remyelination activity of TG-form FO in the MS along with anti-inflammatory effects in animal models. However, only a few studies are conducted on EE-form FO in the CPZ model. Cuprizone is a copper chelator that damages oligodendrocytes. It enhances the activation of microglia within the lesion, including demyelination in the mouse brain (C57BL/6). Therefore, the mouse model is useful to investigate demyelination and remyelination.

Microglia are important macrophage cells that are residents of the CNS. The functions of microglia in MS remained controversial. Depending on the environmental

milieu, two microglia phenotypes have been reported which include M1 and M2. M1 plays its role as pro-inflammation, whereas M2 is usually involved in immunoregulation through anti-inflammatory activity (Aryanpour *et al.*, 2017). It is reported that the n-3 PUFA supplementation reduced demyelination induced by CPZ accompanied by shift-in M2 phenotype microglial, both *in vitro* and *in vivo*. However, the effect of fish oil and its form (such as EE) on microglia responses and white matter pathology in the CPZ animal model of MS has not been investigated.

SIRT1 possesses neuroprotective effects, especially in neurodegenerative disease. SIRT1 is known to promote phosphorylation of Akt. Thereby for activation of the protein, SIRT1-dependent deacetylation of Akt is necessary (Peng *et al.*, 2017). It is also reported that oligodendrocyte maturation is enhanced by p-Akt in CNS. In addition, it has been reported that signaling pathway of nuclear factor kappa B (NF-κB) increases pro-inflammatory cytokines production i.e., TNF-α and IL-6 thus play a key role in promoting demyelination (Vega-Riquer *et al.*, 2019). Moreover, Nod-like receptor pyrin 3 (NLRP3) of the inflammasome is indicated to have a critical role in a prolonged inflammatory response and can further exacerbate myelin injury. Furthermore, previous research has shown that reduced expressions of NF-κB p65 and NLRP3 in the CPZ model can inhibit the pro-inflammation response and M1 type microglia, promoting the M2 type microglia, and attenuate the progression of the demyelination (Aryanpour *et al.*, 2017).

With respect to the mentioned findings, it was considered that FO acts as a therapeutic agent for mitigating symptoms and pathological features of MS. In this study, we demonstrated that EE-form FO treatment encouraged the expression of SIRT1/p-Ak and inhibited SIRT1/NF-κB/NLRP3 pathway (associated with M2 microglia polarization) and helps in the amelioration of inflammatory demyelination symptoms thus improves behavioral function. Consequently, EE-form FO treatment exhibits a strong potential strategy for alleviating demyelination disease.

MATERIALS AND METHODS

Animals: Seven-week-old male mice (C57BL/6) were procured from Animal Company of Jinan, China (Jinan Pengyue Experimental Animal Company). The study was approved by "Animal Ethics Committee" (permit # 20191018), and all protocols were performed as per directions of Shandong University, i.e., "Guidelines for Care and Use of Laboratory Animals".

The demyelination induction and EE-FO treatment: The C57BL/6 male mice were assigned to one of the following three groups (n=10 mice/group): (i) control group (Ctrl) was fed with standard normal diet; (ii) CPZ+control group (CPZ+Ctrl) was fed with a standard diet with 0.2% CPZ for five weeks for induction of demyelination; (iii) CPZ+EE-FO group was fed with EE-(DHA+EPA, 12.6+8.4 g/kg) fish oil during the 0.2% CPZ feeding for five weeks. The body weight was recorded every two days.

Open field (OF) test: We performed OF test as described by Zhang *et al*. (2020). Each mouse was placed in the central area of OF to record its movement track through a video camera. Analysis of OF was performed through Panlab Harvard Apparatus (SMART 3.0, Spain).

Luxol fast blue (LFB) staining: The LFB staining was performed by the method described previously (Zhang *et al*., 2020). Myelin stained blue. Macroscopic images were obtained at 200× magnification using Image-Pro Plus (Mediacybernetics, Bethesda). Quantification of demyelinated areas were performed by the procedure described earlier by Zhong *et al.* (2021) by using a 3-point scale i.e., 0 = complete demyelination; 1 = 1-25%; 2 = 26-50%; 3 = 51-100% of myelin sheath.

Immunohistochemistry and Immunofluorescence labeling: We performed immunohistochemical staining as per method already described (Zhang *et al.*, 2020). The anti-MBP polyclonal antibody were used as primary antibodies for myelin basic protein (1:800 Proteintech); anti-CD68 polyclonal antibody (1:200, Abcam) were used for activated microglia; anti-Iba1 polyclonal antibody (1:1,000, Abcam). The $CD68^+$ cells and Iba-1^+ cells were counted with a clear and visible nucleus.

Immunofluorescence labeling was indirect. The primary antibodies used were: mouse anti-Iba1 (1:200, Servicebio), rabbit anti-CD68 (1:200, Abcam), rabbit anti-GST-π (1:100, Abcam). The secondary antibodies used were: FITC 488-conjugated secondary antibody (goat anti-mouse, 1:100)/Cy3-conjugated secondary antibody (goat anti-rabbit, 1:100) /Alexa Fluor® 488-conjugated secondary antibody (goat anti-rabbit, 1:200). Dako Fluorescence Mounting Medium was used to mount sections and sealed with coverslips.

Quantitative real-time PCR (qRT-PCR): Dissection of corpus callosum from whole brain was performed under light microscope. The qRT-PCR was performed by method described previously (Zhang *et al*., 2020). The primers were used: CD16 *forward* TTTGGACACCCAGATGTTTCAG; CD16 *reverse* GTCTTCCTTGAGCACCTGGATC; CD32 *forward* GTGTCACCGTGTCTTCCTTGAG; CD32 *reverse* GTGTCACCGTGTCTTCCTTGAG; CD206 *forward* CAAGGAAGGTTGGCATTTGT; CD206 *reverse* CCTTTCAGTCCTTTGCAAGC; Arginase1 *forward* TCACCTGAGCTTTGATGTCG; Arginase1 *reverse* CTGAAAGGAGCCCTGTCTTG; IL-1β *forward* TCCAGGATGAGGACATGAGCAC; IL-1β *reverse* GAACGTCACACACCAGCAGGTTA; TNF-a *forward* TTCCCAAATGGGCTC CCTCT; TNF-a *reverse* GTGGGCTACGGGCTTGTCAC; GADPH *forward* GAGGCCGGTGCTGAGATTGT; GADPH *reverse* GGTGGCAGTGATGGCATGGA. The cycle time values were normalized to GAPDH levels within the same sample.

Western blot analysis: Procedure of Western blot was performed as described by Liu *et al.* (2020). The primary antibodies used were: rabbit IgGs of NG2 (1:800; Proteintech), CNPase (1:10,000; Proteintech), CD16/32 (1:1,000; Abcam), Arginase1 (1:800; Proteintech), p-Akt

(Ser473) (1:125; Abcam), Akt (1:200; Abcam), Phospho NF-κB p65 (1:2,000; Abcam), NF-κB p65 (1:2,000; Abcam), NLRP3 (1:1,000; Abcam), β-actin (1:10,000; Proteintech), and SIRT1(moluse IgG, 1:2,000; Millipore Corporate). The secondary antibodies used were: conjugated goat anti-rabbit (1:10,000, Santa Cruz, US). Then, the signals were visualized by enhanced chemiluminescence (ECL) substrate with an Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

Statistical analysis: Data was statistically analyzed using one-way variance analysis (ANOVA), and significant differences between experimental groups were assessed. Data are expressed as means ± standard error (±SEM). The significant differences are indicated as *P<0.05, while highly significant difference is indicated as **P<0.01 and ***P<0.001.

RESULTS

Body weight: Animals fed on CPZ reduced body weight significantly (P<0.001) than control group, from day 3 to day 35 (Fig. 1). Interestingly, compared with CPZ+Ctrl group, body weight of CPZ+EE-FO group mice increased significantly (P<0.05) from day 33 till end of the experiment when compared with mice (Fig. 1).

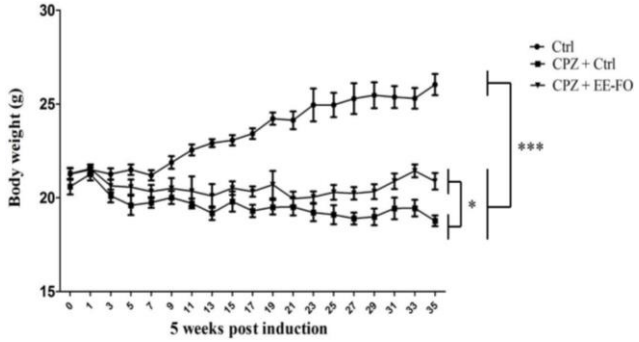

Fig. 1: Effect of EE-FO treatment on the body weight changes of mice and total food intake. The data thus are presented as the mean ± SEM. *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=10/group).

Clinical investigation

Effects of EE-FO on OF behavior of CPZ mice: Our results revealed behavioral abnormalities in mice treated with CPZ as reported by Zhang *et al*. (2020). In our study, mice treated with EE-FO for five weeks spent a significant (P<0.05) longer time in the central area and traveled longer distance (total distance and distance) than mice of CPZ+Ctrl group (Fig. 2) suggest that treatment of mice with EE-FO helped to decrease anxiety induced by CPZ.

Characterization and quantification of demyelination: When compared with CPZ+Ctrl group, mice of CPZ+EE-FO group showed a significantly (P<0.05) increase in myelin score (Figs. 3A, 3B, 3E and 3F). GST-π indicates the maturity of oligodendrocyte cells. Our results showed that a number of GST-π^+ cells significantly (P<0.05) increased in CPZ+EE-FO group when compared with CPZ+Ctrl group (Figs. 3C & G), white arrowhead indicate the high magnification of the GST-π-labeled cell. In addition, the CNPase protein expression significantly increased in EE-FO group as compared to CPZ+Ctrl group (P<0.05, Figs. 3D and 3I). Results of the present study revealed that EE-FO treatment decreased the expression of NG2 protein significantly (P<0.05) in mice fed on CPZ (Figs. 3D & H). Results of this study thus suggest that EE-FO treatment can promote the maturation of oligodendrocytes.

Effects of EE-FO on microglia in the CPZ-induced demyelination: Results of this study identified a significant (P<0.05) increase in $CD68^+$ and Iba-1^+cells of corpus callosum in mice of CPZ+Ctrl group as compared to Ctrl group (Fig. 4). Double positive cells $CD68^+$ and Iba-1^+ were shown in the picture (Fig. 4C); white arrowhead indicates co-localization of $CD68^+$ and Iba-1^+ (CD68, red; Iba1, green; yellow when co-localized). Furthermore, when compared to CPZ group, we found a significant decrease in $CD68^+$ and Iba-1^+cells in the CPZ+EE-FO group, (Figs. 4D & E). These results showed that EE-FO treatment is capable to decrease CPZ-induced activation of microglia. Therefore, we determined the effects of EE-FO treatment on microglial (phenotype of M1/M2) activation in the corpus callosum in the next step of our study.

Effects of EE-FO on the polarization of microglia: Expressions of M1 genes (CD16 & CD32) significantly increased due to CPZ feeding after five weeks (suggested in previous scientific findings (Zhang *et al.*, 2020). The EE-FO diet supplementation decreased the elevation of these M1 genes (Figs. 5A & B). CD16/32 protein expression also decreased in EE-FO treated group (P<0.01; Figs. 5G & H). Furthermore, the concentration of TNF-α and IL-1β decreased significantly in CPZ+EE-FO group as compared to CPZ+Ctrl group, (Figs. 5C & D). Moreover, EE-FO treatment enhanced M2 genes expressions (CD206 and Arginase-1, Figs. 5E & F) and Arginase-1 protein (Figs. 5G & I) in CPZ-treated mice. Interestingly, the expression of M2-markers was enhanced, while the M1-markers were down-regulated in the corpus callosum, suggesting that EE-FO treatment elevated microglial polarization towards M2 in comparison to the CPZ+Ctrl group.

Effect of EE-FO treatment on the SIRT1/Akt/NF-κB 65/NLRP3 protein expression in CPZ mice model: It is reported that activation of SIRT1 plays a neuroprotective role in any neurodegenerative diseases such as MS (Zhang *et al.*, 2020). The present study revealed that the CPZ+Ctrl group showed a decrease in SIRT1 protein expression, whereas this level was reverse in EE-FO treated group and a significant increase was observed (P<0.05; Figs. 6A & C). Furthermore, expression of NLRP3 protein increased significantly in CPZ+Ctrl group and showed highly significant (P<0.001) difference over Ctrl group (Figs. 6A & D). Interestingly, EE-FO treatment dramatically down-regulated the level of NLRP3 (P<0.05; Figs. 6A & D). In addition, CPZ treatment significantly increased phospho NF-κB p65)/ NF-κB p65 and phospho-AKT/total-AKT ratios. However, the ratios were decreased by EE-FO administration (Figs. 6E & F).

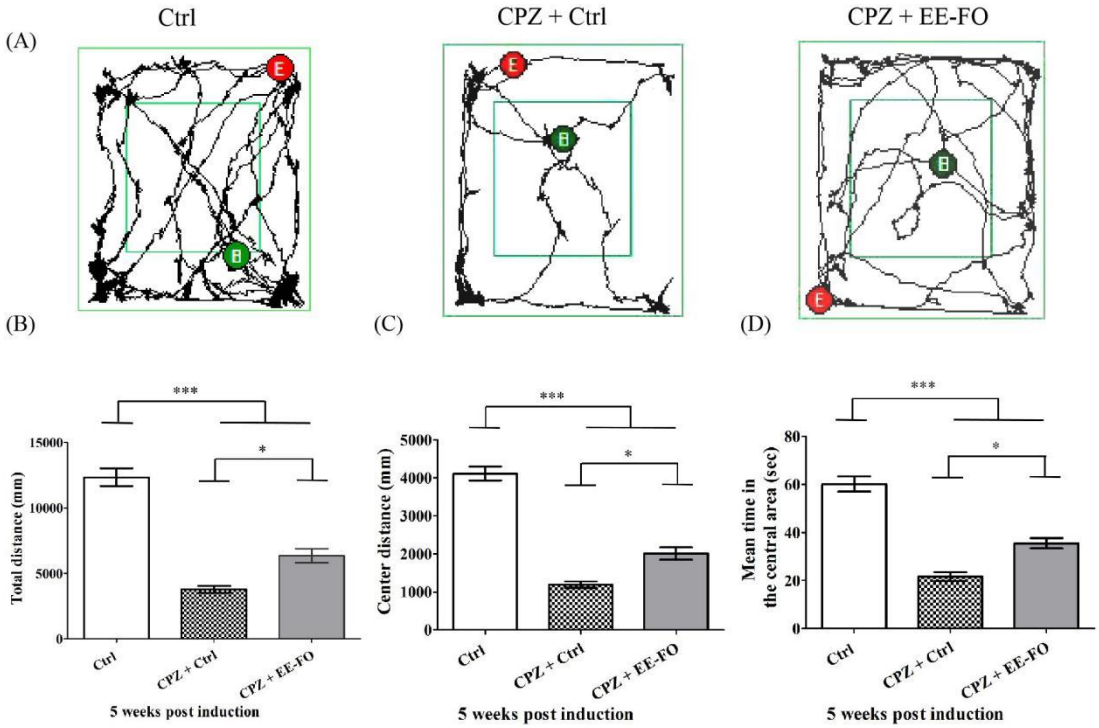

Fig. 2: EE-FO treatment rescued the exploratory activity of mice. (A) Images show exploratory behavior of mice in each group. (B) Total distance traveled. (C) Distance traveled in central area. (D) Time spent in the central area. The data thus are presented as the mean ± SEM (n = 10/group). *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=10/group).

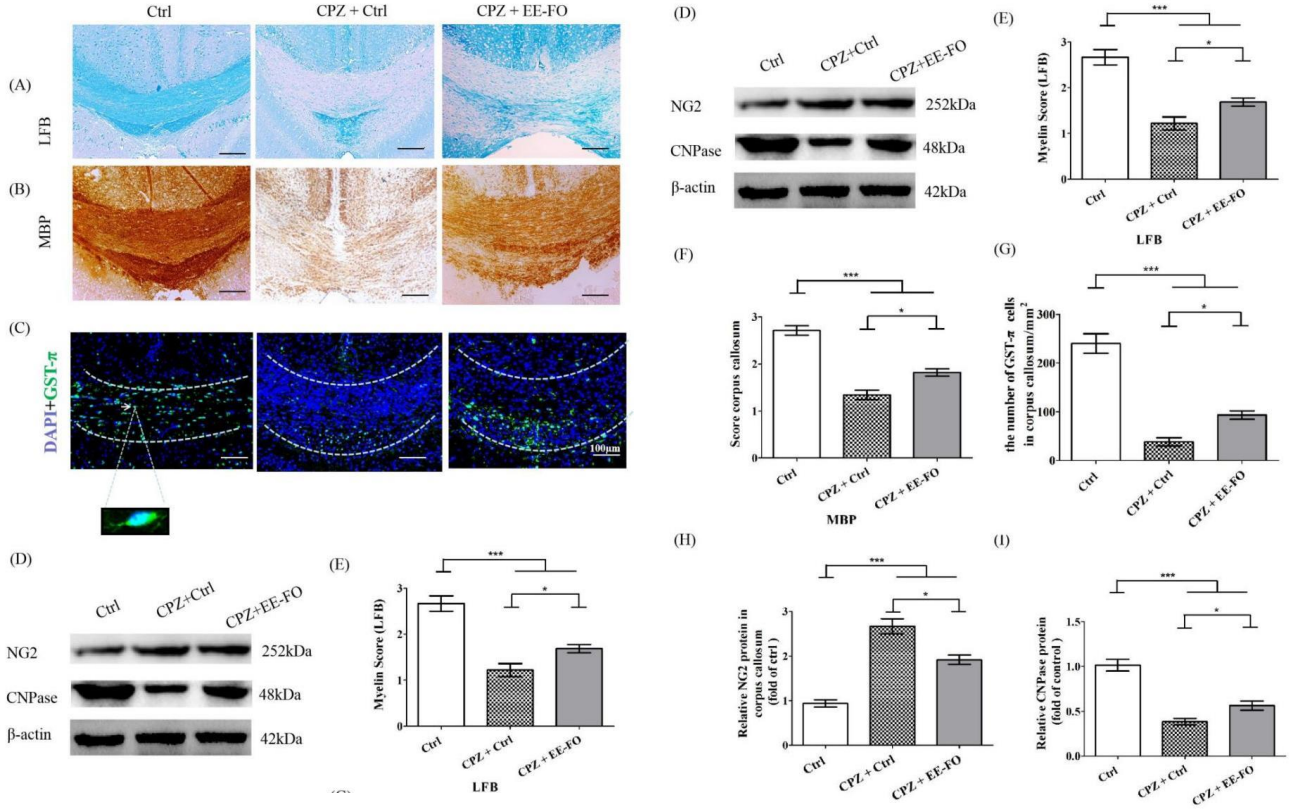

Fig. 3: Effect of EE-FO treatment on the myelination in CPZ-model of MS. (A-B). LFB staining (A) and MBP staining (B) were used to evaluated demyelinating lesions in the corpus callosum. (C) Representative microphotographs of immunofluorescence staining showing localization of GST-π (green) and blue DAPI nuclear staining in corpus callosum from mice of different groups. The small box in the Ctrl group presents high magnification of GST-π-labeled cell at 100 μm of scale bars. (D) Protein levels of CNPase and NG2 in corpus callosum by Western blotting were detected, and β-actin was used for normalization of internal control. (E-F) Predicts the score of myelinations obtained from LFB and MBP-stained images among different groups. (G) Quantitative analysis of corpus callosum GST-π$^+$ cells in each group. (H-I) The quantitative analysis of NG2 (H) and CNPase (I) levels was performed by Western blotting in each group. Data thus obtained are presented as mean ± SEM. *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=5/group).

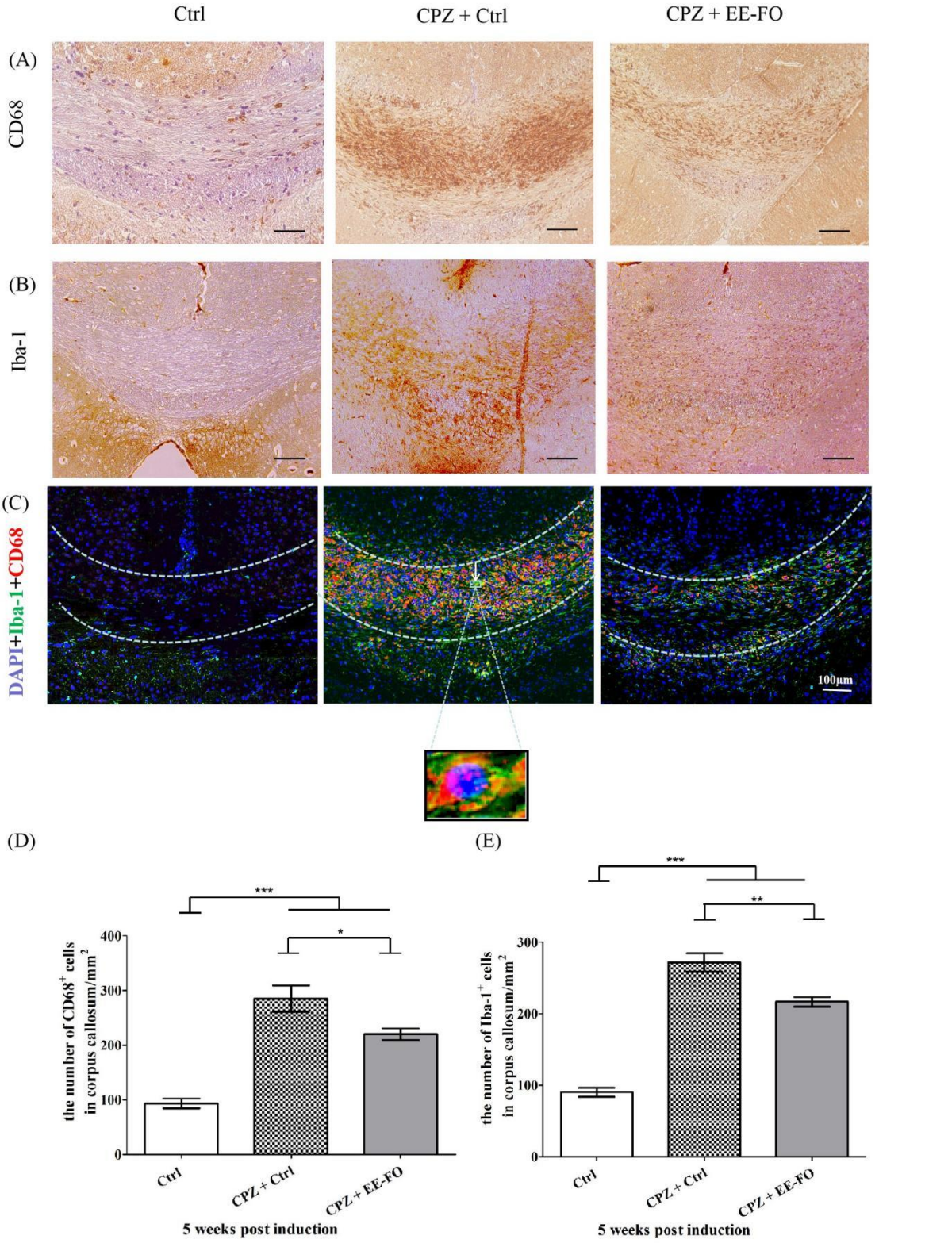

Fig. 4: The EE-FO treatment decreased microglia activation in the CPZ-induced demyelination model. (A-B) The images of the immunohistochemistry staining for CD68 (A) and Iba-1(B) in the corpus callosum of mice in Ctrl, CPZ+Ctrl and CPZ+EE-FO groups, and the nuclei were stained in blue. (C) Merged image of Iba-1(green), CD68 (red) and blue DAPI nuclear staining in corpus callosum from mice of different groups. The small box in the CPZ+Ctrl group present the high magnification of the Iba-1 and CD68 double-labeled cells. Scale bars, 100 μm. (D-E) Analysis of numbers of CD68- and Iba-1 positive cells in the corpus callosum in different groups. The data are presented as mean ± SEM. *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=5/group).

DISCUSSION

Previous clinical studies have suggested that FO enriched supplements are more effective in preventing and treating of MS (Torres-Sanchez *et al.*, 2018). The present study explored the capacity of EE-formed FO on neuro-protection of the myelin pathology in the mice model of CPZ. In the present study, we found that 0.27g/mouse/day oral EE-FO treatment obviously ameliorated the course and severity of the disease compared to untreated animals.

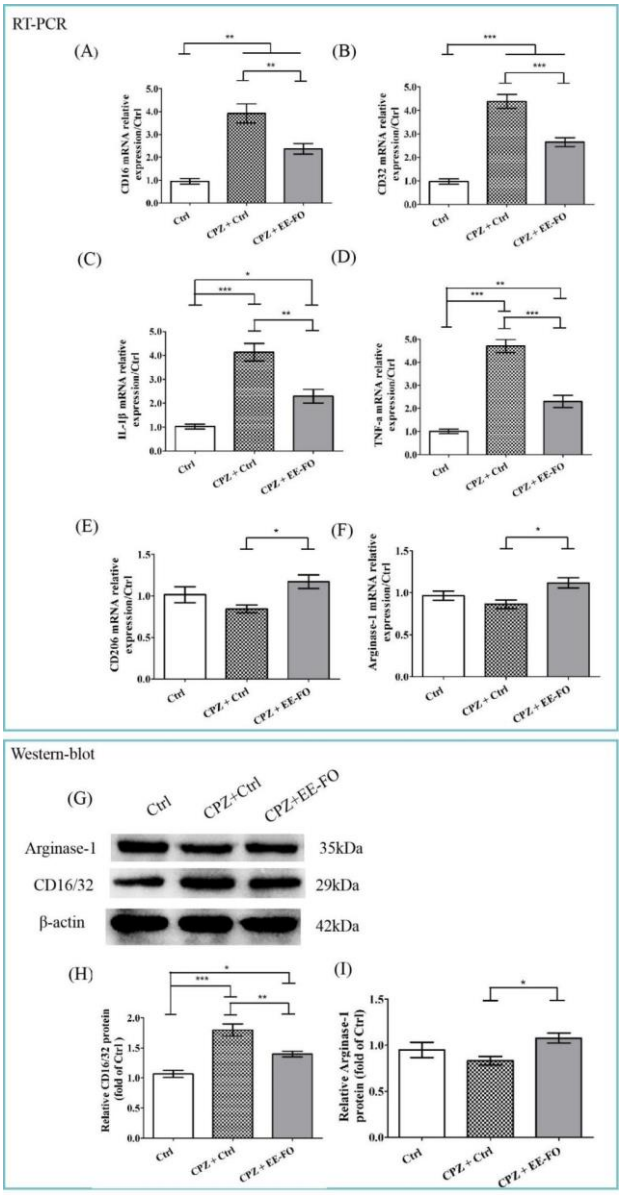

Fig. 5: Effect of EE-FO treatments on M1- and M2-microglia associated markers in CPZ-model. (A-F) The total RNA extracted from corpus callosum through Real time-PCR for M1-markers (A & B), pro-inflammatory cytokines and M2-markers (C & D and E & F, respectively). The CD16 quantification (A), CD32 (B), IL-1β (C), TNF-α (D), CD206 (E) and Arginase-1 (F) corpus callosum gene expression. (G) In the detection of Arginase-1 in the corpus callosum and CD16/32 protein levels, the β-actin was used to normalize internal control. (H-I) In each group, the quantitative analysis was performed through western blot; (H) CD16/32 and (I) Arginase-1 levels. The data are presented as mean ± SEM. *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=5/group).

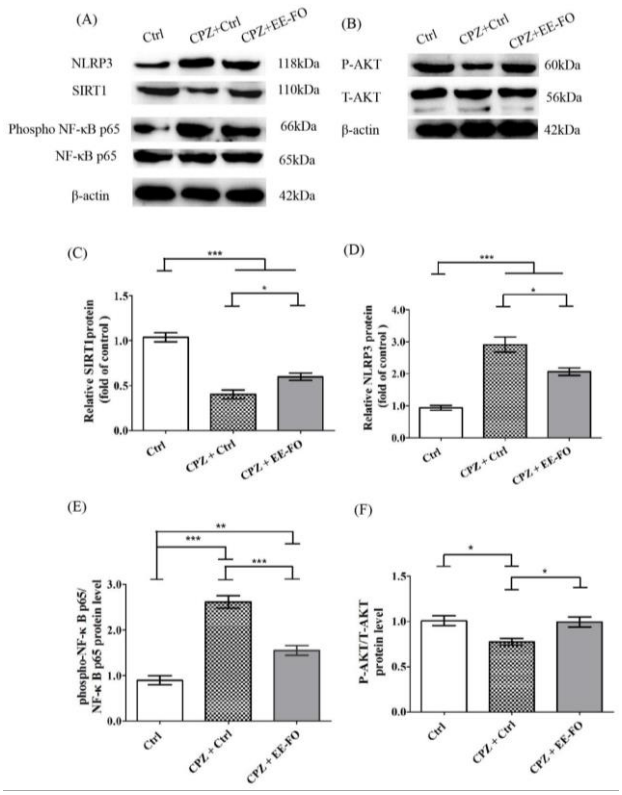

Fig. 6: The effect of the EE-FO treatment on CPZ-induced alterations in the SIRT1, p-AKT, NF-κB p65, NLRP3and Phospho NF-κB p65, and protein expression levels. (A) Detection of SIRT1, NF-κB p65, and NLRP3 corpus callosum protein levels, β-actin was used for normalization of internal control. (B) The detection of phospho-AKT and total-AKT corpus callosum protein levels, β-actin, was used for normalization of internal control. (C-D) The SIRT1quantitative analysis (C) and NLRP3 (D) corpus callosum protein expressions at day 35. (E) Predicts the ratio of (phospho NF-κB p65) / (NF-κB p65). (F) Quantification of immunoblots of (phospho-AKT)/(total-AKT). The data are presented as mean ±SEM. *P<0.05 is considered for significant differences between groups, **P<0.01 and ***P<0.001 were considered for highly significant differences (n=5/group).

CPZ induced the behavioral like-anxiety, as demonstrated by the OP, which agrees with previous reports (Zhang *et al.*, 2020). Way and Popko (2016) reported that these disorders of behavior are associated with demyelination due to impairment in the regenerative ability of OLs themselves and their precursors. In addition, previous studies have shown the final body weight decrease in mice that received CPZ, which was associated with the demyelination of CPZ intoxication (Liu *et al.*, 2020). This study revealed that EE-FO treatment remarkably improved the final body weight and spontaneous exploration in the mice that received CPZ. It is suggested that EE-FO treatment ameliorated the CPZ-induced demyelination.

It is established that OPCs are always located next to demyelinated lesions hence contribute to myelination repair. In CNS of normal mammalian, NG2 proteoglycan is expressed by OPCs (Sakry and Trotter, 2016). In this study, after CPZ ingestion, upregulation of NG2 protein expression was observed, which is in line with the results of the study conducted by Zhang *et al*. (2019). However, the EE-FO treatment can decrease the NG2 protein expression, as indicated by its normalization. These results suggest that EE-FO treatment promote the OPCs differentiate into OLs, and in keeping up with the CNPase western blot results and MBP immunohistochemistry staining.

Microglia plays a major role in the progression of MS. It is reported that neurodegenerative disease is developed by phenotypic change of M1 microglia to M2 microglia, including MS (Chu *et al.*, 2018). The capability of M1-polarized microglia to produce reactive oxygen species (ROS) along with various pro-inflammatory cytokines result in dysfunction of CNS, while M2-polarized microglia can express cytokines and receptors that have anti-neuroinflammation activity and restoration of homeostasis. It has been reported that the n-3 PUFAs

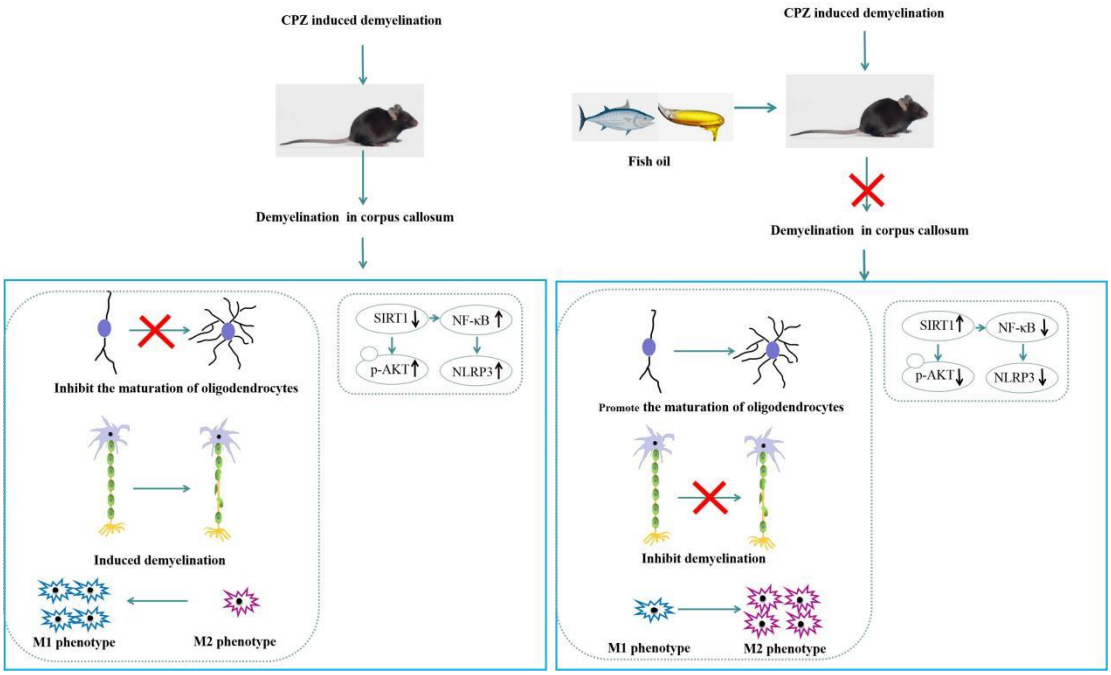

Fig. 7: Possible neuroprotective mechanism of EE-FO in mice that received CPZ.

treatment in vivo significantly elevated M2 microglial quantity in the brain, thus ameliorating myelin sheet loss in CPZ mice, suggesting that M2 phagocytes might be a promising therapy for MS due to the protective M2 features. In line with this report, mice of EE-FO treated group showed a substantial increase in M2 microglial number whereas a significant ($P<0.05$) decrease in M1 marker at day 35 of CPZ demyelination model indicating a protective role of EE-FO.

Previous studies described the role of SIRT1 in inhibiting demyelination (Zhang *et al.*, 2020). In addition, earlier studies have demonstrated that the histone deacetylase enzyme of SIRT1 deacetylates Akt thereby promoting the activation of Akt. The p-Akt activation enhances the oligodendrocyte maturation present in the CNS. It has been confirmed that activating the SIRT1/Akt pathway can protect rats from early brain damage. The current results indicate that the EE-FO treatment significantly increased SIRT1 expression and p-Akt in CPZ mice. These results, therefore, suggest that EE-FO treatment activates SIRT1/p-AKT, which plays a protective role against CPZ-induced demyelination.

The NF-κB activation plays an essential role in regulating the pro-inflammation gene expression (Zhou *et al.*, 2020). Furthermore, the M1 microglia polarization increased the pro-inflammatory molecules production, which explains the activation of NF-κB signaling pathway, was reported (Huang *et al.*, 2019). The present result shows that NF-κB p65 expression was lower in mice treated with EE-FO than in the CPZ+Ctrl groups. This demonstrates that the EE-FO treatment has the potential to decrease the polarization of M1 type microglia, which could relate to the downregulation of NF-κB p65. Importantly, NF-κB, which is a key activator of inflammation, upregulated the NLRP3 inflammasome response (Huang *et al.*, 2019). Previously, Aryanpour *et al*. (2017) reported that NLRP3 has a significant role in neuroinflammation, accelerated loss of mature oligodendrocytes and enhanced the demyelination in response to the CPZ-induced model. This study showed that NLRP3 expression decreased significantly in mice with EE-FO treatment, suggesting EE-FO suppressed signaling pathway of NLRP3 inflammasome thereby inhibiting immunoinflammatory responses activation consequently enhancing remyelination levels.

Conclusion: It is, therefore, concluded that treatment with EE-FO had a beneficial effect on the clinical course in the CPZ mouse model and attenuated the demyelination. The beneficial and protective effects of EE-FO were mediated, at least in part, through the induction of the M1 to M2 switch in microglia phenotype within the CNS. The data also demonstrates the role of the SIRT1/p-AKT and SIRT1/NF-κB/NLRP3 signaling pathways in the protective effect of EE-FO. These findings indicate that fish oil with the EE forms may be a promising nutritional agent to improve the symptoms of neurodegenerative disorders like MS.

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Authors contribution: WS, BL, JY, MW, GL and SW helped in the study execution and investigation methodology. MAK, MAA and MAZ contributed to data analysis and interpretation. NZ prepared the manuscript, and MAZ revised the manuscript. All authors gave final approval of the manuscript.

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