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ORIGINAL ARTICLE

Eicosapentaenoic acid attenuates Aβ-induced neurotoxicity by decreasing neuroinflammation through regulation of microglial polarization

YiLong Dong[1], SiFan Long[1], YiLing Gu[1], WenJing Liu[1]

1 School of Medicine, Yunnan University, 2 Cuihu Bei Road, Kunming, Yunnan, 650091, People's Republic of China

Correspondence to: WenJing Liu, Ph.D
School of Medicine, Yunnan University, 2 Cuihu Bei Road, Kunming, Yunnan, 650091, People's Republic of China
TEL.: 86-871-65034358; E-MAIL: liuwenjing@ynu.edu.cn

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Abstract

OBJECTIVE: Although the cause of Alzheimer's disease (AD) is still controversial, it is generally accepted that neuroinflammation plays a key role in AD pathogenesis. Thus, regulating the polarization of microglia will help in recovering from AD since microglia can be polarized into classical M1 and alternative M2 phenotypes, M1 microglia leading to neuroinflammation and M2 microglia acting as anti-inflammatory effectors. Our previous study demonstrated that eicosapentaenoic acid (EPA), an essential n-3 polyunsaturated fatty acid, may modulate glial cell activity and functions, but it is not clear whether EPA plays a role in microglial polarization. Here, we aimed to test the hypothesis that EPA may regulate the polarization of microglia and subsequently alleviate neuroinflammation and neuronal damage.

METHODS: Male C57BL/6 mice were fed an EPA-supplemented diet or a palm oil-supplemented diet for 42 days. On day 28 of diet feeding, the mice received a single intracerebroventricular injection of β-peptide fragment 1-42($A\beta_{1-42}$) or saline. The polarization of M1 and M2 microglia was evaluated by western blot using the respective markers. Changes in inflammatory cytokine mRNA levels were examined using real-time PCR. Neurological deficits were analysed using the Morris water maze and TdT-mediated dUTP Nick-End Labeling (TUNEL) assays.

RESULTS: EPA supplementation effectively reversed the increasing trend of M1 microglial markers and the decreased expression of M2 microglial markers in the hippocampus mediated by $A\beta_{1-42}$ and normalized the Aβ-induced upregulation of proinflammatory cytokines and the downregulation of anti-inflammatory factors. Consistent with these findings, EPA significantly improved cognitive function and inhibited apoptotic neuronal death in the hippocampus.

CONCLUSION: These results demonstrated that EPA appears to have potential effects on regulating microglial polarization, which contributes to alleviating neuroinflammation and may have beneficial effects for preventing and treating AD.

Abbreviations:

Aβ	- Amyloid β
AD	- Alzheimer's disease
BDNF	- Brain-derived neurotrophic factor
CNS	- Central nervous system
EPA	- Eicosapentaenoic acid
IL-1β	- Interleukin-1β
NGF	- Nerve growth factor
PUFAs	- Polyunsaturated fatty acids
TUNEL	- TdT-mediated dUTP Nick-End Labeling
TNF-α	- Tumor necrosis factor-α

INTRODUCTION

Alzheimer's disease (AD) is a chronic neurodegenerative disease with a high incidence in the elderly population. It is characterized by the deposition of extracellular amyloid-beta (Aβ) in the brain and progressive cognitive decline. Although the pathogenesis of AD remains unclear, an increasing number of studies have shown that neuroinflammation plays a key role in AD (Kaur *et al.* 2019; Calsolaro *et al.* 2016). Therefore, microglia, the key effector cells of neuroinflammation, have received increasing attention.

Microglia are innate immune cells of the central nervous system (CNS). Similar to peripheral macrophages, macrophages can be polarized into proinflammatory M1 (classical activation) or anti-inflammatory M2 (alternative activation) phenotypes in response to microenvironmental disturbances (Walker *et al.* 2015). M1 microglia secrete proinflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), which initiate neuroinflammation and induce nerve injury, while M2 microglia release anti-inflammatory factors as well as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) to promote neuronal repair (Ghosh *et al.* 2016; Ju *et al.* 2015). Generally, M1 and M2 microglia maintain dynamic balances that benefit CNS homeostasis by releasing various cytokines under different conditions (Tang *et al.* 2016). However, it has been reported that the expression of proinflammatory cytokines is increased in the human AD brain accompanied by M1-skewed microglial activation (Hoozemans *et al.* 2006). These findings were reproduced by Jimenez and colleagues, who demonstrated that there was a distinctive shift from the M2 to M1 microglial phenotype in the hippocampus in AD mice (Jimenez *et al.* 2008). Obviously, excess M1 microglia ultimately result in the M1/M2 disequilibrium involved in AD pathology (McGeer *et al.* 2015). Therefore, repressing the overactivation of M1 microglia is beneficial for improving AD.

Eicosapentaenoic acid (EPA) is an essential n-3 polyunsaturated fatty acid (PUFA) that cannot be synthesized by the body itself but can only be supplied by food, especially cold-water fish. Growing evidence has demonstrated the links between diets high in EPA and a reduced risk of AD (Wiesmann *et al.* 2013; Wu *et al.* 2014; Wen *et al.* 2018). Importantly, many studies have shown that dietary supplementation with EPA reversed age-related increases in proinflammatory cytokine concentrations in the brain (Martin *et al.* 2002), and alleviating neuroinflammation is an important mechanism by which EPA protects against AD-like neuronal injury, confirming the role of EPA in the anti-inflammatory profile (Serini *et al.* 2012; Labrousse *et al.* 2012). Furthermore, our previously published data revealed that EPA may indeed ameliorate neuroinflammation by modulating microglial activity (Dong *et al.* 2018); however, which subtype of microglia was affected was not observed in this study. Thus, the objective of the present study was to test the hypothesis that EPA can improve AD, and these effects are mediated by abating M1 microglial polarization and attenuating neuroinflammation and neuronal damage. To further test these hypotheses, hippocampal samples were collected from mice fed a normal or EPA diet with or without Aβ administration, and the expression of M1/M2 microglial markers, inflammatory cytokines and neuronal death was measured. The learning and memory ability of the mice was also estimated.

MATERIALS AND METHODS

$A\beta_{1\text{-}42}$, HFIP, 3,3'-diaminobenzidine (DAB) substrate and palm oil were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Antibodies against Arg1, CCL2 and β-actin were purchased from Abcam (Abcam, England). Ethyl (E)-EPA (99% pure) was purchased from Amarin (Amarin Neuroscience Ltd., UK). The phosphatase inhibitor, protease inhibitor mixture, BCA protein assay kit and enhanced chemiluminescence substrate kit were purchased from Pierce Biotechnology (Rockford, IL, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The Ominiscript RT kit and SYBR Green Mix were purchased from Qiagen (Dusseldorf, Germany). The In Situ Cell Death Detection Kit was obtained from Roche (Basel, Switzerland). Guide cannulas were purchased from RWD Life Science (Shenzhen, China). All other chemicals used are the highest grade commercially available.

Preparation of $A\beta_{1\text{-}42}$ oligomers

The $A\beta_{1\text{-}42}$ peptide was dissolved in HFIP and then incubated at room temperature for 1 h. HFIP was slowly evaporated using nitrogen gas. The dried $A\beta_{1\text{-}42}$ pellet was dissolved in phosphate-buffered saline (PBS) to a final concentration of 1 μg/μl and then incubated for 96 h at 37°C before use.

Animals and treatments

Forty-eight male C57BL/6 mice (6-8 weeks, 20-25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Animals were housed 3 per cage at 22 ± 1°C with a 12-h light-dark cycle and free access to food and water at all times. After acclimation for 5 days, the animals were

anaesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg) and placed in a stereotaxic apparatus. Guide cannulas for PBS or $A\beta_{1-42}$ intracerebroventricular (i.c.v.) administration were located over the right lateral ventricle (L=1.1 mm, AP=0.48 mm, 2.2 mm depth). Following surgery, the mice were allowed to recover for 14 days. The animal protocol was approved by the animal care committee of Yunnan University and complied with the Yunnan University guidelines for the care and use of laboratory animals.

After recovery, the animals were randomly divided into 4 groups (n=12), including the control, EPA, Aβ and EPA+Aβ groups. Animals in the control and Aβ groups were fed a diet consisting of regular chow powder (Beijing Vital River) mixed with 0.8% palm oil, while animals in the EPA and EPA+Aβ groups were fed a diet mixed with 0.8% ethyl (E)-EPA as described previously (Dong *et al.* 2018). The diet was fed to the mice (8-10 g per mouse/day) before the light was turned off for the night and it was generally consumed over 12 h. The full feeding period was 42 days.

The animals received an i.c.v. injection of Aβ (Aβ and EPA+Aβ group) or PBS (control and EPA group) on day 28 of diet feeding. On the injection day, 2 μg Aβ or PBS (a total of 2 μl) was placed into a 4.2-mm internal needle connected to a PE50 polyethylene tube. The mice were gently restricted in a towel, and the cap of the guide cannulas was carefully unscrewed. The needle was then inserted into the guide cannulas, and Aβ or PBS was infused into the brain slowly over a period of 30 s. The injection needle was allowed to remain inside the guide cannulas for another 30 s and then slowly removed.

Morris Water Maze

On day 39 of diet feeding, the Morris water maze task was used to evaluate spatial learning and memory. This process of the Morris water maze test consisted of 3 days of training and a testing trial on day 4. Animals were trained in a water tank (100 cm in diameter) located in a lit room with visual cues. A black escape platform (9.5 cm in diameter) was submerged 1.0 cm below the surface of water, located in the middle of the southwest quadrant of the tank. In the training trial, each mouse was immediately placed by the tail into the water and was allowed a maximum time of 60 s to find the platform. Mice that found the platform were allowed to stay on the platform for 10 s and subsequently placed into an empty and warm cage before the next trial began. If a mouse did not reach the platform within 60 s, it was gently guided to the platform by the experimenter, where it remained for 10 s. Two training trials were conducted each day for 3 consecutive days. In the test trial, the time required to escape onto the platform (escape latency) was recorded first, and then the platform was removed. The mice were allowed to swim freely for 60 s inside the tank. The time spent in the southwest quadrant (exploring time in the location where the platform was originally placed) and their swimming speed were recorded by a computerized video system (Beijing Sunny Instruments Co. Ltd., China).

Sample preparation

Twenty-four hours after the last behavioural test, 9 animals in each group were deeply anaesthetized by isoflurane inhalation and sacrificed by decapitation, and the brain was removed and dissected on ice. The hippocampus was removed and stored at -80°C until further analysis. The remaining 3 animals were perfused with PBS and 4% paraformaldehyde under deeply anaesthetized conditions. Their brains were removed and postfixed in 10% sucrose/4% paraformaldehyde for 8 h at 4°C and then placed in 20% sucrose/PBS at 4°C overnight. The 6 μm frozen sections were then cut for image analyses using a cryostat microtome (Leica, Germany).

Western blot

Hippocampal samples were homogenized in lysis buffer supplemented with protease inhibitor and then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected, and the protein concentration was estimated by a BCA Protein Assay Kit. Thirty micrograms of total protein from each sample was loaded onto a polyacrylamide gel for separation and then electrophoretically transferred to PVDF membranes. After incubation with 5% fat-free milk for 1 h at room temperature, the membranes were then incubated overnight at 4°C with the primary antibodies against CCL2 (1:1000), Arg1 (1:1000) and β-actin (1:5000). After washing, the membranes were incubated with goat radish peroxidase-conjugated secondary antibody (1:5000), followed by detection using enhanced chemiluminescence. Band intensities were quantified using ImagePro Express 4.0 software (Media Cybernetics Inc. Rockville, MD, USA).

Tab. 1. Real-time RT–PCR Primers. Sequences are shown for forward (F) and reverse (R) primers

Objective / control gene	primer sequence
BDNF	F: 5′- CAAAAGGCCAACTGAAGC -3′
	R: 5′- CGCCAGCCAATTCTCTTT -3′
NGF	F: 5′- AAACGGAGACTCCGTTCACC -3′
	R: 5′- GATTGTACCATGGGCCTGGA -3′
IL-1β	F: 5′- GGATGATGACGACCTGCTAGTG -3′
	R: 5′- ACATGGGTCAGACAGCACGA -3′
TNF-α	F: 5′- TCATTCCTGCTCGTGGCGGG -3′
	R: 5′- CGGCTGACGGTGGGGTGAG -3′
β-actin	F: 5′-GTCGTACCACTGGCATTGTG-3′
	R: 5′-CTCTCAGCTGTGGTGGTGAA-3′

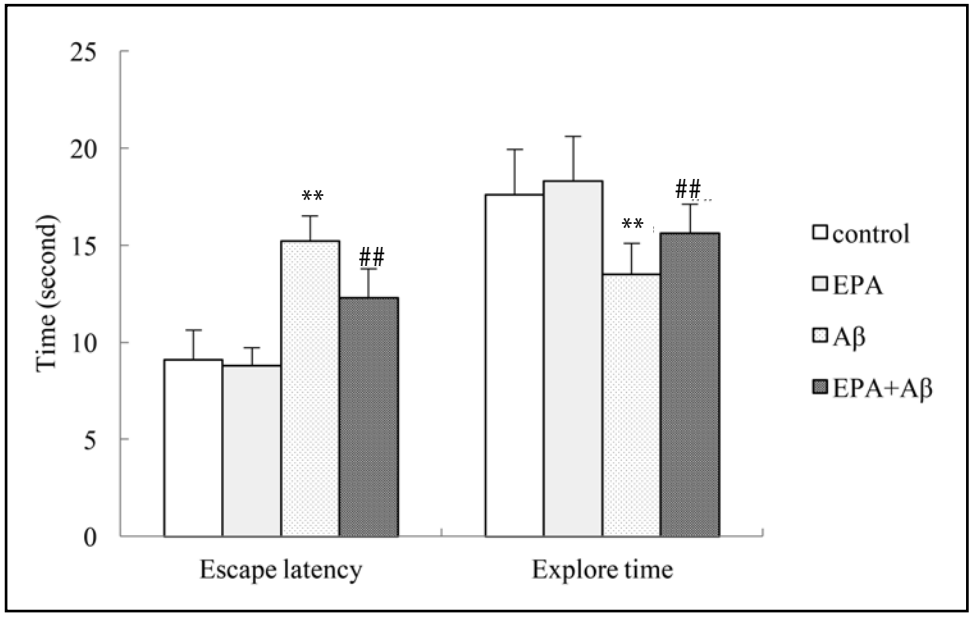

Fig. 1. EPA supplementation improved deficits in spatial learning and memory mediated by Aβ. Morris water maze training began on the 39th day of feeding the EPA diet, 4 days in a row. The escape latency and exploration time were analysed on day four. **p <0.01 vs. control group; ##p <0.01 vs. Aβ group.

Real-time PCR

Total RNA was extracted from the hippocampus using TRIzol, and first-strand cDNA was synthesized from 1 μg of total RNA using the Ominiscript RT kit according to the manufacturer's instructions. The PCR sequence was run on a Corbett Life Science (Sydney, Australia) Rotor-Gene 6000 system with SYBR Green fluorescence. The PCRs were carried out in a reaction mixture consisting of 7.5 μl SYBR Green Mix, 1 μl cDNA, 0.5 μl of each primer (10 μM) and 5.5 μl RNase-free water. Amplification was performed using an initial 15 min step at 95°C, followed by 45 cycles of 94°C for 15 s, 59°C for 30 s and 72°C for 30 s. Each sample was tested in triplicate. Gene expression levels were quantified by normalizing Ct values of the target genes to the Ct values of the reference gene (beta actin) with the ΔΔCt method. The following primers (Sengong, Shanghai, China) were used to measure the target gene expression (Table 1).

TdT-mediated dUTP Nick-End Labelling Assay (TUNEL)

The TUNEL assays were performed using the in situ Cell Death Detection kit according to the manufacturer's

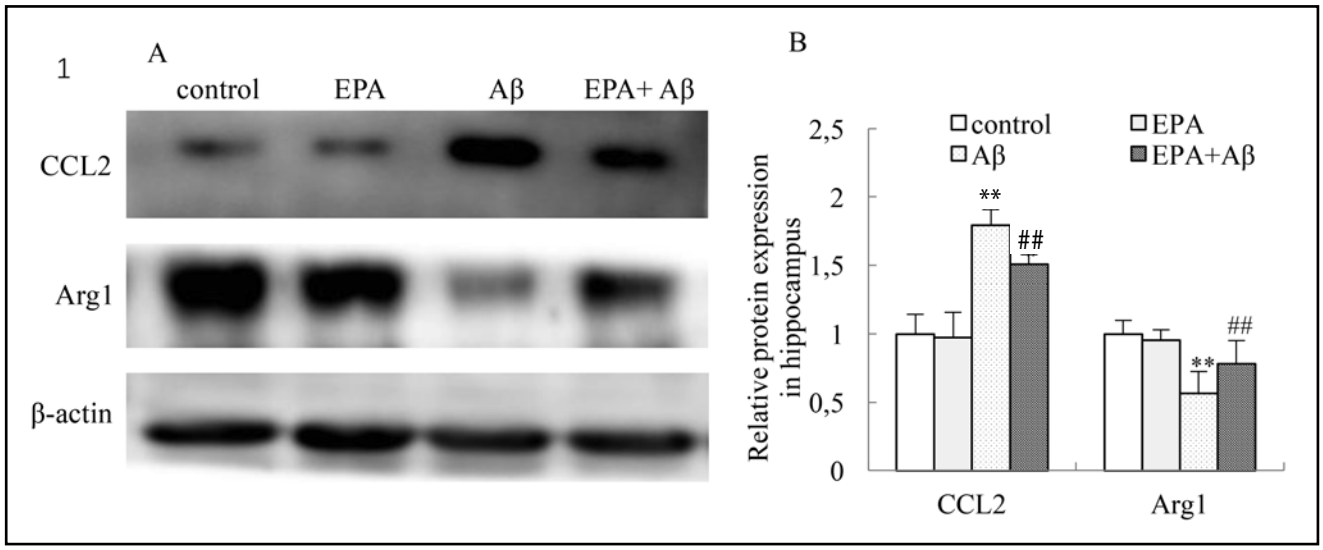

Fig. 2. EPA promotes microglia polarization into the M2 phenotype. Different microglial marker proteins were assessed by western blotting (A) and quantified by densitometry (B). Data from the western blots were normalized by taking the value of the control group as 1. **p <0.01 vs. the control group; #p <0.05, ##p <0.01 vs. the Aβ group.

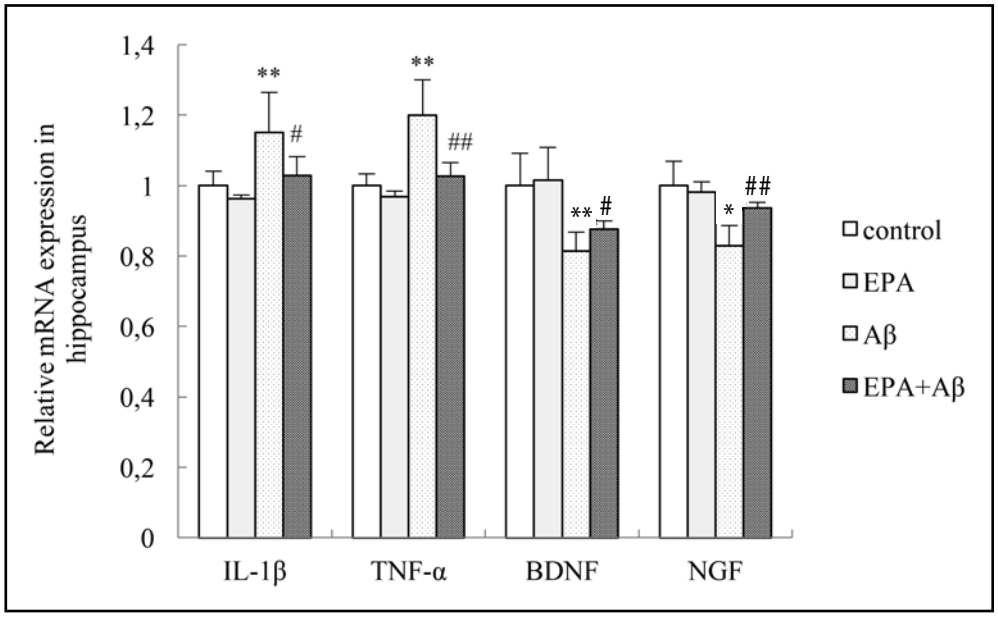

Fig. 3. EPA modulated Aβ mediated neuroinflammation. Proinflammatory cytokines were significantly increased, but anti-inflammatory cytokines were decreased by Aβ, whereas EPA attenuated this effect. Data from the PCRs were normalized by taking the value of the control group as 1. **p* <0.05, ***p* <0.01 vs. the control group; #*p* <0.05, ##*p* <0.01 vs. the Aβ group.

instructions. Briefly, tissue sections on slides were treated with 0.3% hydrogen peroxide at room temperature for 10 min. After washing with PBS, the slides were quenched in proteinase K at room temperature for 15 min. The sections were then rinsed and incubated in TUNEL reaction mixture containing digoxigenin antibody with TdT enzyme at 37°C for 1 h, blocked with stop/washing buffer, and incubated with peroxidase-conjugated anti-digoxigenin antibody at room temperature for 15 min. Finally, the reaction product was visualized by incubation with DAB substrate. To quantify the TUNEL-positive cells, 12 slides were selected randomly from each group, and the positive cells were counted in the hippocampal CA region using an optical microscope (Leica, Germany) at 40× magnification. Apoptotic cells were stained brown.

Statistical analysis of data

Data are expressed as the mean ± SEM. Statistical significance was analysed using ANOVA, followed by Dunnett's test (SPSS 18.0). $p< 0.05$ was considered statistically significant.

RESULTS

1 EPA improves learning and memory impairment induced by Aβ

Spatial learning and memory were evaluated by the Morris water maze. As shown in Fig. 1, Aβ-treated animals took a longer time to find the platform and spent less time in the target quadrant in the test trial than animals in the control group (both $p<0.01$). However, EPA-treated animals showed significantly shorter escape times but longer exploration times than the Aβ animals (both $p<0.05$). EPA alone caused no changes in healthy animal behaviour in the Morris water maze tests.

2 EPA attenuates the overactivation of M1 microglia induced by Aβ

The expression of the M1 marker CCL2 and the M2 marker Arg1 was detected by western blot. As shown in Fig. 2, the expression of CCL2 was increased, but Arg1 expression was decreased in the Aβ group compared to the control group, while these changes were partially reversed by EPA treatment. Although EPA alone repressed CCL2 expression compared with the control group, the changes were not significantly different.

3 EPA alleviated neuroinflammation induced by Aβ

The expression of inflammatory cytokines was examined by real-time PCR. As shown in Fig. 3, Aβ significantly increased IL-1β and TNF-α expression but decreased BDNF and NGF expression compared to the control group, while these changes were alleviated by EPA. Additionally, EPA alone downregulated the expression of proinflammatory cytokines compared with the control group, but there was no significant difference between the two groups.

4 EPA attenuates apoptotic neuronal death induced by Aβ

Apoptotic neuronal death in the hippocampal CA1 subregion was evaluated using TUNEL staining. As shown in Fig. 4, abundant brown nuclei cells were found in the Aβ animals, and the number of apoptotic

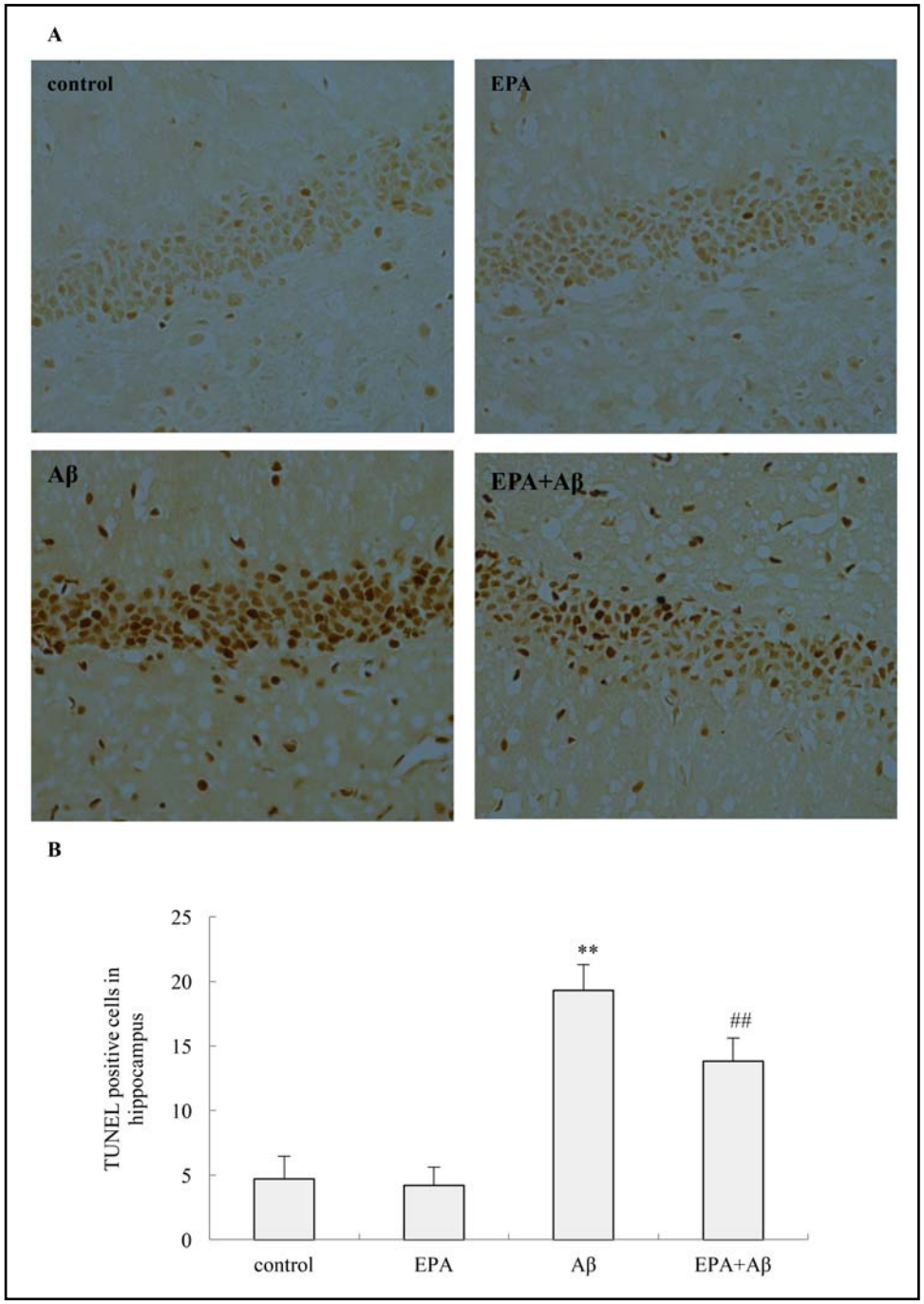

Fig. 4. EPA normalized apoptotic cell death induced by Aβ. A. TUNEL staining in hippocampal CA regions (×400). B. Quantitative analysis of TUNEL positive cells. ***p* <0.01 vs. control group; ##*p* <0.01 vs. Aβ group.

cells was increased significantly compared to control animals. However, the number of TUNEL-positive neurons was markedly reduced in EPA-treated animals.

DISCUSSION

In this study, we demonstrated that regulation of microglial polarization resulted in attenuation of neuroinflammation to inhibit apoptotic cell death as one of the neuroprotective effects of EPA.

It is well accepted that neuroinflammation is a major risk factor for AD (Kaur *et al.* 2019). As resident macrophages in the brain, microglia are the main effectors of neuroinflammation. They can be activated by extracellular Aβ. Their activation states can be categorized into classically activated (M1) and alternatively activated (M2) subtypes (Walker *et al.* 2015). Although the two activated cells are both derived from resting microglia, they show different or even opposite functions. M1 microglia are the main source

of proinflammatory cytokines, including IL-1β and TNF-α, which trigger inflammatory effects. Conversely, M2 microglia function as anti-inflammatory cells by releasing neuroprotective factors such as BDNF and NGF. With Aβ deposition, microglia tend to polarize into the M1 phenotype in AD brains (Zhang *et al.* 2017).

In the present study, Aβ was applied to imitate AD pathology and the microglial polarization process. Similar to Cheng *et al.*, who reported that Aβ contributes to microglial M1-like activation (Cheng *et al.* 2019), our results clearly showed that M1 markers were elevated, whereas M2 markers were reduced in the hippocampal tissue of Aβ-treated mice, suggesting that Aβ may stimulate M1-like microglia. Importantly, the present findings showed that EPA could attenuate microglial polarization into the M1 phenotype and promote M2 activation, as evidenced by decreased CCL2 expression and increased Arg1 expression. Specifically, Arg1 can metabolize arginine into hydroxyproline, proline and polyamine, and products such as hydroxyproline and proline are important sources of collagen synthesis that help strengthen tissues and are used for cell repair (Wu *et al.* 2011). Additionally, polyamines such as agmatine could help neurons counteract injury from proinflammatory cytokines (Xu *et al.* 2018). Thus, the present findings, on the one hand, showed that EPA regulates microglial M1/M2 polarization; on the other hand, the increase in Arg1 expression suggested increasing neuroprotection from EPA.

In parallel with our previous studies implicating the anti-inflammatory role of EPA (Dong *et al.* 2018), the present work demonstrated the upregulation of proinflammatory cytokines and the downregulation of anti-inflammatory cytokine expression induced by Aβ, which could be partially reversed by EPA. As mentioned earlier, M1 microglia are the main source of proinflammatory cytokines, while anti-inflammatory cytokines are the main source of M2 microglia (Ghosh *et al.* 2016; Ju *et al.* 2015). These findings not only support EPA normalizing AD-like neuroinflammation but also confirm that EPA regulates microglial polarization. Obviously, the effect of EPA on polarizing microglia from the M1 phenotype to the M2 phenotype is beneficial for attenuating neuroinflammation, which overall contributes to alleviating apoptotic cell death and improving cognitive impairment induced by Aβ.

Notably, when following the EPA diet without Aβ injections, although EPA alone downregulated the expression of proinflammatory cytokines and M1 microglial markers, these changes were not statistically significant. These results seem to support the hypothesis EPA is not a direct anti-inflammatory precursor and that the regulation of microglial polarization is a key mechanism by which EPA improves neuroinflammation.

Although the signalling involved in EPA modulating microglial polarization was not explored in this study, it was reported that M1/M2 microglial polarization is mainly regulated by JAK/STAT signalling (Choi *et al.* 2016), based on the findings that EPA may regulate Fyn, an important upstream regulator of JAK/STAT (Kurahara *et al.* 2020; Jiang *et al.* 2008). We speculate that the effect of EPA on microglial polarization may be mediated by the Fyn-STAT pathway, which needs to be verified by further experiments.

We know there are some limitations to this study, such as: 1) the signalling related to EPA modulating microglia polarization should be investigated; 2) additional EPA concentrations should be studied to compare the efficiency of different EPA levels on microglia polarization; and 3) more M1/M2 microglia markers should be analysed. Our present results supported the hypothesis that EPA can effectively improve cognitive impairment and neuronal death in AD animals and that the neuroprotective effect of EPA, at least in part, is attributable to its ability to repress microglia polarization into the M1 phenotype but promote cell conversion into the M2 phenotype and subsequently alleviate excessive neuroinflammation. Therefore, the benefits of EPA on microglial polarization suggest it has potential future uses for treating neuroinflammatory conditions associated with AD.

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Competing Interests

The authors declare that they have no competing interests.

REFERENCES

1 Calsolaro V, Edison P (2016). Neuroinflammation in Alzheimer's disease: Current evidence and future directions. Alzheimers Dement. **12**: 719–732.
2 Cheng YW, Chang CC, Chang TS, Li HH, Hung HC, Liu GY, ET AL (2019). Aβ stimulates microglial activation through antizyme-dependent downregulation of ornithine decarboxylase. J Cell Physiol. **234**: 9733–9745.
3 Choi JW, Kwon MJ, Kim IH, Kim YM, Lee M, Nam TJ (2016). Pyropia yezoensis glycoprotein promotes the M1 to M2 macrophage phenotypic switch via the STAT3 and STAT6 transcription factors. Int J Mol Med. **38**: 666–674.
4 Dong Y, Xu M, Kalueff AV, Song C (2018). Dietary eicosapentaenoic acid normalizes hippocampal omega-3 and 6 polyunsaturated fatty acid profile, attenuates glial activation and regulates BDNF function in a rodent model of neuroinflammation induced by central interleukin-1beta administration. Eur J Nutr. **57**: 1781–1791.
5 Ghosh M, Xu Y, Pearse DD (2016). Cyclic AMP is a key regulator of M1 to M2a phenotypic conversion of microglia in the presence of Th2 cytokines. J Neuroinflammation. **13**: 9.
6 Hoozemans JJ, Veerhuis R, Rozemuller JM, Eikelenboom P (2006). Neuroinflammation and regeneration in the early stages of Alzheimer's disease pathology. Int J Dev Neurosci. **24**: 157–165.

7 Jiang L, Li ZQ, Rui LY (2008). Leptin stimulates both JAK2-dependent and JAK2-independent signaling pathways.J Biol Chem. **283**: 28066–28073.
8 Jimenez S, Baglietto-Vargas D, Caballero C, Moreno-Gonzalez I, Torres M, Sanchez-Varo R, et al (2008). Inflammatory response in the hippocampus of PS1M146L/APP751SL mouse model of Alzheimer's disease: age-dependent switch in the microglial phenotype from alternative to classic. J Neurosci. **28**: 11650–11661.
9 Ju L, Zeng H, Chen Y, Wu Y, Wang B, Xu Q (2015). Dual polarization of microglia isolated from mixed glial cell cultures. J Neurosci Res. **93**: 1345–1352.
10 Kaur D, Sharma V, Deshmukh R (2019). Activation of microglia and astrocytes: a roadway to neuroinflammation and Alzheimer's disease. Inflammopharmacology. **27**: 663–677.
11 Kurahara LH, Hiraishi K, Yamamura A, Zhang Y, Abe K, Yahiro E, et al (2020). Eicosapentaenoic acid ameliorates pulmonary hypertension via inhibition of tyrosine kinase Fyn. J Mol Cell Cardiol. **148**: 50–62.
12 Labrousse VF, Nadjar A, Joffre C, Costes L, Aubert A, Grégoire S, et al (2012). Short-term long chain omega3 diet protects from neuroinflammatory processes and memory impairment in aged mice. PLoS One. **7**: e36861.
13 Martin DS, Lonergan PE, Boland B, Fogarty MP, Brady M, Horrobin DF, et al (2002). Apoptotic changes in the aged brain are triggered by interleukin-IL-1β-induced activation of p38 and reversed by treatment with eicosapentaenoic acid. J Biol Chem. **277**: 34239–34246
14 McGeer PL, McGeer EG (2015). Targeting microglia for the treatment of Alzheimer's disease. Expert Opin Ther Targets. **19**: 497–506.
15 Serini S, Bizzarro A, Piccioni E, Fasano E, Rossi C, Lauria A, et al (2012). EPA and DHA differentially affect in vitro inflammatory cytokine release by peripheral blood mononuclear cells from Alzheimer's patients. Curr Alzheimer Res. **9**: 913–923.
16 Tang Y, Le W (2016). Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. Mol Neurobiol. **53**: 1181–1194.
17 Walker DG, Lue LF (2015). Immune phenotypes of microglia in human neurodegenerative disease: challenges to detecting microglial polarization in human brains. Alzheimers Res Ther. **7**: 56.
18 Wen M, Ding L, Zhang LY, Cong PX, Zhang TT, Xu J, et al (2018). A comparative study of eicosapentaenoic acid enriched phosphatidylcholine and ethyl ester in improving cognitive deficiency in Alzheimer's disease model rats. Food Funct. **9**: 2184–2192.
19 Wiesmann M, Jansen D, Zerbi V, Broersen LM, Garthe A, Kiliaan AJ (2013). Improved spatial learning strategy and memory in aged Alzheimer AβPPswe/PS1dE9 mice on a multi-nutrient diet. J Alzheimers Dis. **37**: 233–245.
20 Wu FJ, Xue Y, Liu XF, Xue CH, Wang JF, Du L, et al (2014). The protective effect of eicosapentaenoic acid-enriched phospholipids from sea cucumber Cucumaria frondosa on oxidative stress in PC12 cells and SAMP8 mice. Neurochem Int. **64**: 9–17.
21 Wu GY, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA,et al (2011). Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids. **40**:1053–1063.
22 Xu WL, Gao LS, Li T, Shao AW, Zhang JM (2018). Neuroprotective Role of Agmatine in Neurological Diseases. Curr Neuropharmacol. **16**: 1296–1305.
23 Zhang Y, He ML (2017). Deferoxamine enhances alternative activation of microglia and inhibits amyloid beta deposits in APP/PS1 mice. Brain Res. **1677**: 86–92.