Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Docosahexaenoic acid, folic acid, and phosphatidylserine co-intervention alleviate cognitive impairment in *D*-galactose-induced aged mice

Yingying Lin[a,b], Yi Cui[b,c], Yi Xue[b], Xiaoya Lin[b], Yuning Zhang[a,b], Yiran Wang[b], Lining Chen[b], Zenghui Xia[b], Huiyuan Guo[a,b,c,*]

[a] *Key Laboratory of Functional Dairy, Department of Nutrition and Health, China Agricultural University, Beijing 100191, China*
[b] *College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China*
[c] *National Center of Technology Innovation for Dairy, Hohhot 010110, China*

ARTICLE INFO

Article history:
Received 30 April 2024
Received in revised form 7 August 2024
Accepted 10 October 2024

Keywords:
Cognitive impairment
Dietary nutrients
Behavioral experiments
Neuroinflammation
Synaptophysin

ABSTRACT

Following the aging of the population, cognitive impairment is becoming a major public health issue. Research in recent years has highlighted the potential of docosahexaenoic acid (DHA), folic acid (FA), and phosphatidylserine (PS) to improve synaptic plasticity and reduce inflammatory response. However, whether a combined intervention is more effective than using these supplements alone remains unclear. To investigate this, we conducted an experiment on *D*-galactose-induced aging mice and found that the combining DHA, FA, and PS (DFP) improved spatial learning memory. Moreover, the DFP treatment restored cognitive performance by regulating antioxidant levels, inhibiting microglia activation, and reducing inflammation. The treatment also increased the expression of neurotrophic factors and synaptic-related proteins in the hippocampal region of the brain. Our findings suggest that the supplementation of DPF could be more beneficial than using DHA alone. This combined intervention could improve neurotrophic factor expression and decrease neuroinflammation in the brain of mice, thus reducing neuron damage and enhancing synaptic plasticity. In summary, our results indicate that the combined supplementation of DFP may improve cognition, and may be more effective than supplement with DHA alone.

1. Introduction

With the rising trend in population aging, the cognitive decline accompanying aging has become a serious health problem. Cognitive decline is associated with the development of dementia, creating irreversible neurological damage. It is estimated that 15%–42% of older adults aged 65 years and older suffer from mild cognitive impairment (MCI), and approximately 5%–15% of those with MCI develop dementia[1]. Aging is the primary risk factor for cognitive decline[2], and changes in oxidative stress, neuroinflammation, and neurotrophic factor expression accompanying aging are important mechanisms affecting cognitive function in the brain[3-5].

Therefore, it is important to conduct early prevention to maintain the cognitive function of the elderly and promote healthy aging on a global scale. Epidemiological evidence indicates that dietary interventions, which are low-risk and cost-effective, an effective in ameliorating the detrimental effects associated with aging and neurodegenerative disease[6-7]. Long-chain *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), B vitamins, phosphatidylserine (PS), polyphenols,

* Corresponding author at: Department of Nutrition and Health, China Agricultural University, Beijing 100191, China.
E-mail address: guohuiyuan@cau.edu.cn (H.Y. Guo)
Peer review under responsibility of Beijing Academy of Food Sciences.

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2024.9250436

and other nutrients are reported to play a role in maintaining brain structure and function, induce age-associated cognitive impairment, and reduce the risk of developing dementia[8-10].

Docosahexaenoic acid (DHA), a key structural component of central nervous system membranes[11], shows down cognitive decline by improving anti-inflammatory and antioxidant activities, preserving membrane lipid flexibility, and ameliorating mitochondrial dysfunction and neuroinflammation[12]. Folic acid (FA), also recognized as vitamin B_9, is involved in homocysteine metabolism[13], thereby preserving the integrity of the blood-brain barrier and synaptic function. In older individuals, a dosage of over 400 μg of FA may alleviate cognitive impairment, while treatment with 3 mg or more of FA can remarkably improve cognitive performance and reduce peripheral inflammatory cytokine levels of the MCI subjects[14]. PS participates in the composition of neuron membranes and signaling, and is important for cognitive function[15-16]. In summary, dietary nutrition is closely related to brain health, and dietary nutritional interventions can effectively reduce the risk of cognitive impairment in middle-aged and elderly individuals. Thus it is important to develop functional foods with cognitive function improvement.

Several preclinical studies have demonstrated that specific combinations of nutrients, rather than a single component, can synergistically act to improve therapeutic outcomes. The administration of docosahexaenoic acid combine with phosphatidylserine (DHA-PS) has been shown to mitigate oxidative stress and yield significant benefits in addressing amyloid-beta pathology, mitochondrial damage, neuroinflammation and neurotrophic factor. The molecular mechanisms for these effects are closely linked to the phospholipid polar group[17]. Animal research has shown that a compound dietary supplement containing DHA, PS, and other micronutrients can improve cognitive ability in *D*-gal-induced aged rats, which proving that this compound dietary supplement has an ameliorative effect on cognitive impairment[18]. The concurrent supplementation of medium-chain triglycerides (MCTs) and DHA has the potential to enhance energy metabolism in APP/PS1 mice, leading to a reduction in nerve cell apoptosis and a suppression of the A expression[6]. Furthermore, numerous population-based studies have shown that multi-nutritional interventions are beneficial in reducing the risk of cognitive impairment[8,14,19]. The administration of daily oral FA, DHA and their combination over a 6-month period can significantly enhance cognitive function and attenuate plasma inflammatory cytokines in MCI individuals[9]. Moreover, the combination of FA and DHA is observed to yield greater benefits compared to either nutrient alone[9,19]. However, research on the combined intervention of DHA, FA, and PS is limited.

Therefore, utilizing a *D*-gal-induced aging mice model, this study examines whether the combined supplementation of DHA, FA and PS is more effective than DHA supplementation alone in ameliorating cognitive impairment. To assess the influence of the combination of DHA, FA, and PS (DFP) on cognitive impairment, Morris water maze (MWM), Y-maze and novel object recognition (NOR) tests were performed. The effect of DFP on neuroinflammatory responses and microglia activation was then investigated. Furthermore, synaptic dysfunction and the expression of brain-derived neurotrophic factor (BDNF) were evaluated. Based on the results, it is hypothesized that the combined intervention has a positive effect, and is more beneficial than that of DHA supplementation alone.

2. Material and methods

2.1 Chemicals

D-gal was purchased from Sigma-Aldrich Co., Ltd. (Saint Louis, MO, USA). DHA (algae oil origin, TG-DHA, 94%, and PL-DHA, 6%) was obtained from Inner Mongolia Yili Industrial Group, Co., Ltd. (Inner Mongolia, China), FA was obtained from Beijing Jinkangpu Food Science & Technology Co., Ltd. (Beijing, China) and PS (soybean origin) was purchased from Mihara Lihua Biotechnology Co., Ltd. (Xianyang, China). The rabbit monoclonal antibodies of anti-IBA-1, anti-BDNF, and anti-*β*-actin were obtained from Cell Signaling Technology, Inc. (Massachusetts, USA).

2.2 Animals administration

The experimental procedures involving animals and the corresponding protocols received approval from the Animal Care and Use Committee of China Agricultural University (Aw81104202-4-3). The 7-week-old C57BL/6 mice were purchased from GemPharmatech (Nanjing, China). The mice were maintained under controlled light (light/dark = 12:12, light off at 20:00) and constant conditions (22 ± 2) ℃, (45 ± 10)% humidity) in a pathoen-free animal facility. Following a 1-week acclimatization period, 48 mice were randomly allocated into 4 groups, each comprising 12 mice. 1) Control group (Con): mice without other treatments, 2) *D*-gal group (Dgal): mice administered with *D*-gal (300 mg/(kg·day), 3) DHA group (DHA): mice administered with *D*-gal (300 mg/(kg·day)) and simultaneously gavaged with DHA (300 mg/(kg·day)), 4) DHA + FA + PS group (DFP): mice treated with *D*-gal (300 mg/(kg·day)) and simultaneously were treated with DHA (300 mg/(kg·day)) + FA (10 mg/(kg·day)) + PS (200 mg/(kg·day)). The body weight was recorded weekly during an 8-week period. The flow chart of the animal study is depicted in Fig. 1A.

2.3 MWM test

The MWM test is a commonly used to assess rodent spatial learning and memory. We performed the test based on a previous study[20]. Signs of varying shapes and colors were placed on the walls and divided into 4 quadrants. Mice were familiarized with the maze and platform before the training trials. During the trials, the water was dyed white and the platform remained stationary. Mice were individually placed on the platform for 20 s and trained to find the platform within 1 min. If they failed, they were confined to the platform for 10 s. During the probe trial, mice explored the maze for 1 min in the absence of the platform. A video-tracking system and SuperMaze software (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China) recorded the escape latency platform crossings and the time in the target quadrant.

2.4 Y-maze test

The Y-maze test was performed following the protocol outlined in a previous study[20]. Mice were positioned centrally within a Y-maze and permitted to navigate the structure autonomously for a duration of 5 min. An alternation was characterized as the entries into each of the 3 arms without repetition (i.e., ABC, CAB, or BAC,

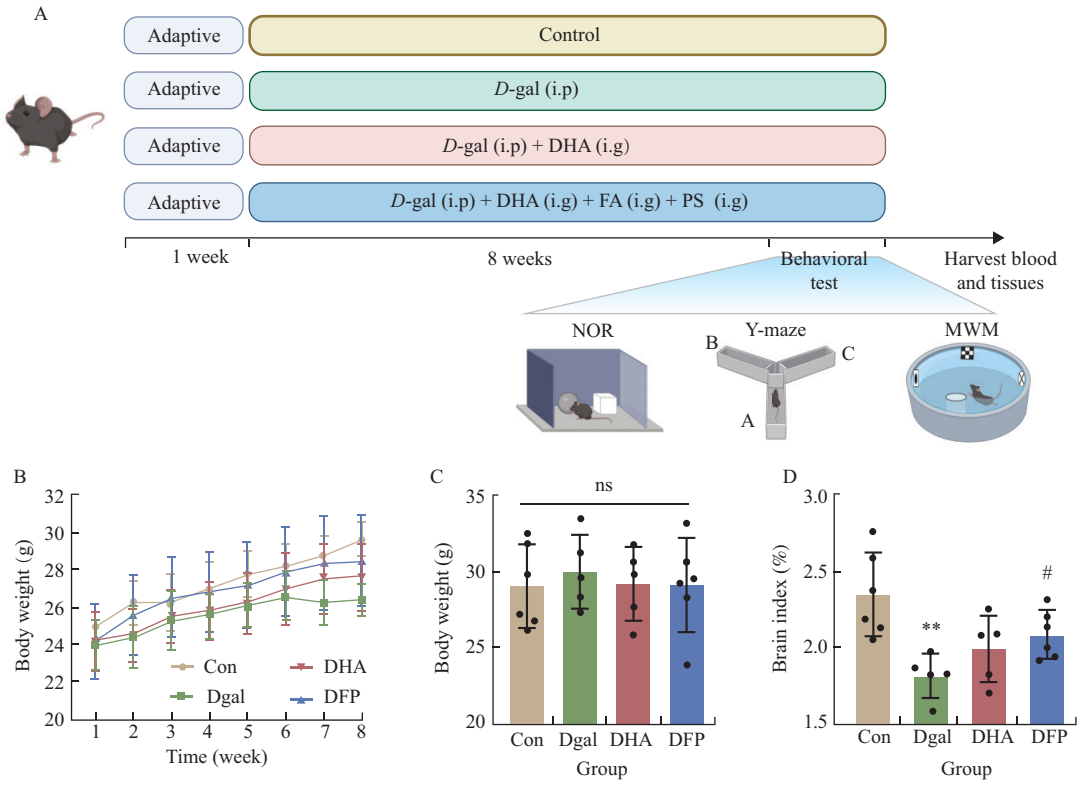

Fig. 1 Effect of the combined intervention on the body weight and brain. (A) Experimental schedule of animal treatments and behavior tests ($n = 12$). (B) Body weight changes of mice during the aging model establishment with *D*-gal ($n = 6$). (C) Body weight of mice in each group after 8 weeks. (D) Brain index of mice in each group. Data presented as the mean ± SD ($n = 6$), $^{**}P < 0.01$, compared with the Con group; $^{\#}P < 0.05$, compared with the Dgal group. ns, $P > 0.05$. i.g. intragastric, i.p. peritoneal injected.

excluding patterns such as ABA). The total number of arm entries and alternations were recorded. The percent alternation was then determined as:

$$\text{Alternation} = \frac{\text{Number of alternations}}{\text{Total arm entries} - 2} \quad (1)$$

2.5 NOR test

The NOR test[20] was conducted in an open field setting, comprising 3 distinct sessions: habituation, training, and testing. In the habituation session, mice were placed in an open field enclosure devoid of objects for a duration of 10 min. The following day, mice explored the same open field containing 2 identical objects for a 10-min training phase. After a 24-h interval, the test phase was conducted with 1 of the 2 identical objects was substituted with a new object. The apparatus was cleaned with 75% ethanol between each test interval. Object exploration was characterized by the mouse sniffing, licking, or touching the objects with its paws or head, maintaining a proximity of 2 cm, and excluding any climbing behavior. The NOR index was used to distinguish the novel object during the test phase. The duration of the mice's exploration of both novel and familiar objects during training and testing trials was recorded.

2.6 Brain index assay

After fasting overnight, the mice were given cardiac perfusion with saline. The brains were carefully isolated, rinsed with 4 °C saline and weighed by suction drying. The brain organ index was calculated as:

$$\text{Brain index (\%)} = \frac{\text{Brain weight (g)}}{\text{Body weight (g)}} \times 100 \quad (2)$$

2.7 Serum biochemical assay

Levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the serum were quantified in accordance with the protocols specified by the kits (Nanjing, China). Briefly, 200 μL of the indicator solution was added to 200 μL of the serum and reacted at 60 °C for 45 min. The absorbance was measured at 532 nm and compared to the MDA standard curve to quantify the MDA level in the serum. To measure ROS levels, 40 μL of physiological saline were added to 50 μL of the serum and reacted for 20 min at 37 °C. Fluorescence was measured at 450 nm to quantify the SOD activity in the serum.

2.8 Hematoxylin & eosin (H&E) and Nissl staining

The mice's brains were meticulously excised, rinsed with cold PBS, post-fixed in 4% paraformaldehyde, embedded with paraffin, and sectioned into 5-μm slices. The paraffin-embedded brain tissue sections were prepared through a routine procedure and stained with H&E and Nissl staining according to the standard protocol reported by previously references[21]. H&E (which stains nuclei blue and cytoplasm appears pink) was used to stain the brain sections and observed under light microscope. For Nissl's staining, which indicates

the number of neuronal cells loss, the slides were washed with 1% PBS followed by staining with a solution of Cresyl violet (0.5%) comprising a few drops of glacial acetic acids for 8–10 min. The stained tissues were examined with a confocal microscope (Nikon, Tokyo, Japan).

2.9 Immunohistochemical and immunofluorescence staining

Immunohistochemical staining and immunofluorescence staining were employed to detect the expression of IBA-1 and BDNF. The procedure was executed in accordance with a previously reported methodology[21]. For immunofluorescence staining, fixed tissue sections underwent overnight incubation with primary antibodies (anti-IBA-1) at 4 °C, and followed by three PBS washes, and treatment with the secondary antibody for 20 min. These slices were then examined using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

For immunofluorescence staining, following a similar overnight incubation with primary antibodies (anti-BDNF) at 4 °C, the slices were rinsed 3 times with PBS and underwent subsequent incubation with the respective secondary antibodies linked to horseradish peroxidase-streptavidin (Beijing, China) at 37 °C for 30 min. After post-nuclear staining with hematoxylin, the slices were mounted using neutral glue and analyzed under an optical microscope (Nikon, Tokyo, Japan).

2.10 Reverse transcription -quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from brain tissues using TRIzol® Reagent according to the manufacturer's (Tiangen Biotech, Beijing, China) instructions. Reverse transcription was performed with 1 μg of total RNA using the All-In-One RT MasterMix (Applied Biological Materials, Richmond, BC, Canada). The relative mRNA expression level of *Il6*, *Il1b*, *Tnfa*, *Jnk*, *Bdnf*, *TrκB*, *Snap25, Syn* and *Psd95* were quantified with SYBR® Premix Ex *Taq*™ (Thermo Scientific), with normalization to the *GAPDH* mRNA level. The thermal cycling conditions were performed in accordance with a previously reported methodology[18]. The primer sequences used for amplification are provided in Table 1.

Table 1
Primer sequences.

Gene		Primer sequences (5′ to 3′)
Gapdh	Forward	ACAGCAACAGGGTGGTGGAC
	Reverse	TTTGAGGGTGCAGCGAACTT
Il6	Forward	CAACGATGATGCACTTGCAGA
	Reverse	GTGACTCCAGCTTATCTCTTGGT
Il1b	Forward	AATGAAAGACGGCACACCCA
	Reverse	CTCTGCTTGTGAGGTGCTGA
Tnfa	Forward	CCCTCACACTCACAAACCAC
	Reverse	ATAGCAAATCGGCTGACGGT
Jnk	Forward	CAGCCGTCTCCTTTAGCACA
	Reverse	TGTATCCGAGGCCAAAGTCG
Bdnf	Forward	CCGGAGAGCAGAGTCCATTC
	Reverse	TTGGCTTGACAGCGAGGAAA
TrκB	Forward	CACTTCGCCAGCAGTAGCA
	Reverse	GGTCGCAGAACCGCTAAACC
Snap25	Forward	GACATGAAGGAGGCCGAGAA
	Reverse	CATCTGCTCCCGTTCATCCA
Syn	Forward	TTTGCCATCTTCGCCTTTGC
	Reverse	TTTAACGCAGGAGGGTGCAT
Psd95	Forward	CCCAGGATATGTGAACGGAAC
	Reverse	ATGCTGTCGTTGACCCTGAG

2.11 Western blotting

The brain tissues of mice underwent Western blotting analysis following previous protocols. In brief, total proteins were isolated using lysis buffer, and protein concentration was measured with the BCA method (Vazyme, Nanjing, China). Equal amounts of protein were separated by SDS-polyacrylamide gels, and then transferred onto PVDF membranes (Millipore, Burlington, USA). The membranes were subsequently blocked using 5% non-fat dry milk in Tris-buffered saline for 1 h at room temperature. Following the blockade, proteins were identified *via* incubation with primary antibodies specific to IBA-1 (1:100; Abcam) overnight at 4 °C, and then incubated with secondary antibodies for 2 h at room temperature. The detection of protein blots was performed with an electrochemiluminescence detection kit (Millipore, Burlington, USA).

2.12 Statistical analysis

Results are presented as the means ± standard deviation (SD). Prism 9.2 (GraphPad Software, San Diego, CA) was used for the statistical analysis. The Shapiro–Wilks normality test was performed on the data. Data that passed the normality test was subjected to the two-independent sample *t*-test and Mann–Whitney *U* test for intergroup comparisons (for normal and abnormal data distribution, respectively). One-way analysis of variance (ANOVA) with LSD's post-hoc test was applied to determine group differences.

3. Results

3.1 Effect of DFP on the body weight and brain index of mice

The *D*-gal-induced oxidative stress mice model was established to study the effects of DHA and DFP. Eight-week-old male C57BL/6J mice were peritoneal injected with a *D*-gal solution to reach an aging state. Then, DHA and DFP were then administered to mice. The experimental design is illustrated in Fig. 1A. The results indicated that there were no significant changes in body weight among all experimental groups throughout the whole experiment (Figs. 1B, C). As shown in Fig. 1D, there was a significant decrease in the brain index in the Dgal group compared to the Con group. The brain index of the DFP groups was significantly elevated compared with those in the Dgal group. Thus DFP effectively attenuated brain index changes.

3.2 DFP improved spatial learning and memory

Behavioral experiments (MWM, Y-maze, and NOR tests) were conducted to explore the learning and memory abilities of each group of mice. In the MWM test, mice in the DFP group exhibited a significantly lower latent escape period during positioning navigation. Moreover, during spatial exploration, mice in the DFP group demonstrated a significantly higher number of crossing escape platforms and a significantly higher number and duration of stays in the target quadrant (Fig. 2A). In the Y-maze experiment, the mice in the DFP group showed significant exploratory behavior, with a significant increase in the total number of entries into each arm and the number of entries into the new opposite arm, and a significant increase in the number of spontaneous alternations and the rate

of spontaneous alternations (Fig. 2B). Following the combined intervention, the number of new objects explored and the exploration time of mice in the NOR experiment increased, and the relative discrimination index between new and familiar objects was significantly improved (Fig. 2C). The behavioral experiments reveal the ability of the DFP intervention in improving the learning and memory abilities of mice.

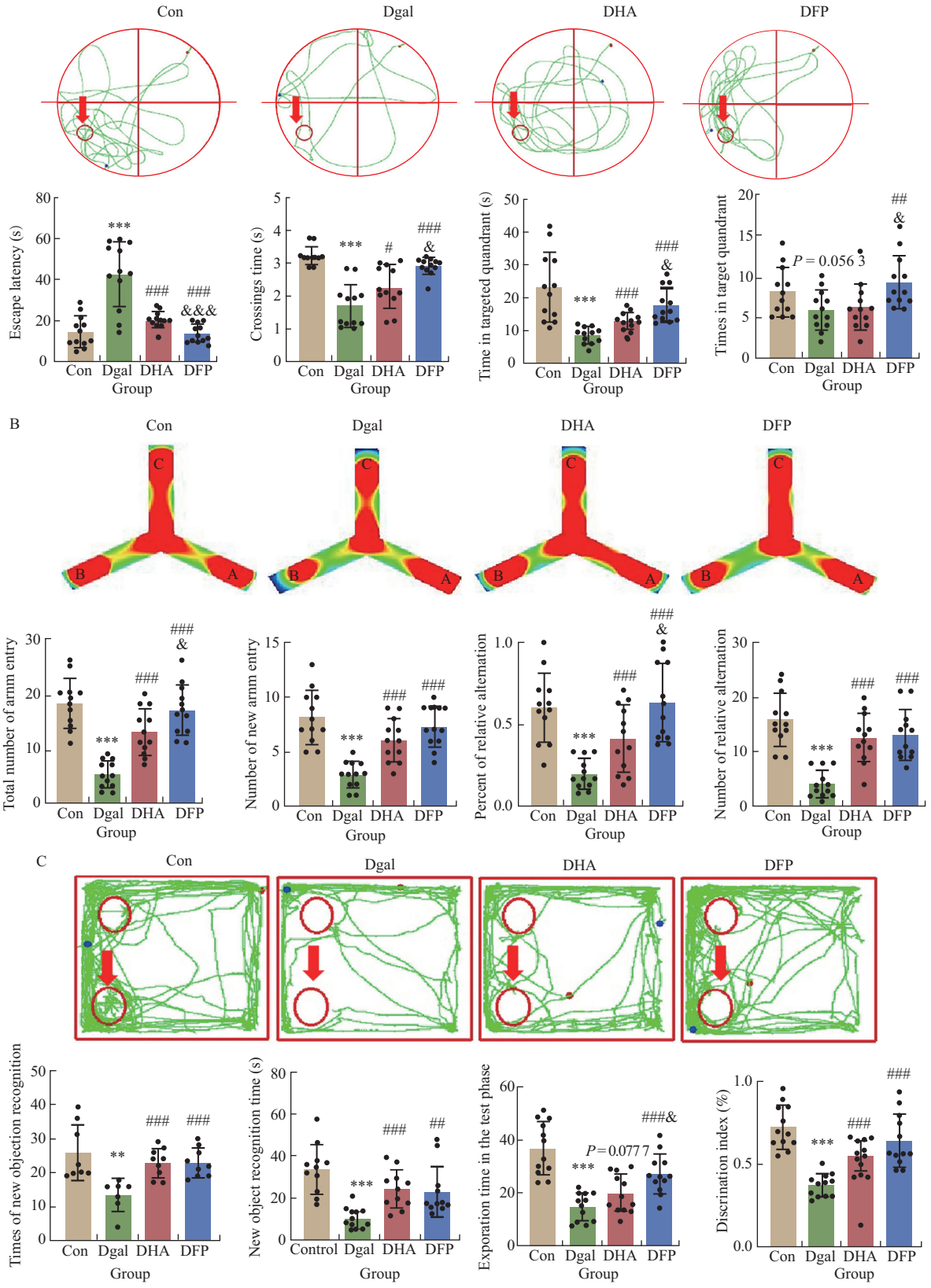

Fig. 2 Effect of the combined intervention on behavioral disorders in aging mice. (A) MWM test results (n = 12). (B) Y-maze test results (n = 12). (C) NOR test results (n = 12). The schematic drawing of the behavioral test was created in www.biorender.com. All data are presented as the mean ± SD (n = 6). ***P < 0.001, compared with the Con group. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 compared with the Dgal group. $^{\&}P$ < 0.05 compared with the DHA group.

3.3 DFP recovered neuron damage

The histopathological alterations within the regions of the brain were further examined utilizing H&E (Fig. 3A) and Nissl staining (Fig. 3B). Neurons in the Con group displayed normal morphology characterized by a well-preserved cytoplasm, prominent nucleus, and nucleolus, In contrast, the neurons from the Dgal group displayed signs of degeneration, including neuronal shrinkage and nucleus condensation. Notably, the neuron damage was alleviated in the DHA and DFP groups (Fig. 3A). The Nissl body in the cell plasma of neurons stained with Nissl stain is dark blue, and u is typically employed to reveal the basic neural structures. The DHA and DFP group mice showed a higher Nissl body expression in the hippocampal and cortical regions, compared with the mice in the Dgal group (Fig. 3B). Furthermore, the Nissl body expression in the hippocampus was more pronounced in the DFP-treated mice than in those treated with DHA.

3.4 DFP increased SOD activity and decreased MDA concentration

Serum SOD activity and MDA concentrations were quantified to evaluate the impact of DFP on oxidative capacity. The *D*-gal treatment markedly reduced the SOD capacity and elevated the MDA concentration (Fig. 4). Conversely, the DHA and DFP treatments attenuated the serum MDA level and enhanced the production of SOD (Fig. 4).

3.5 DFP suppressed microglia activation and inflammation in the brain

The activation of microglia is associated with the pathogenesis of neuroinflammatory and neurodegenerative diseases. Therefore, we examined the ionized calcium-binding adaptor molecule 1 (IBA-1) as the activation marker of microglia. Immunohistochemical staining analysis showed the IBA-1 expression in mice hippocampus (Figs. 5A, C) and cortex (Figs. 5B, D) was significantly downregulated in the DHA and DFP group when compared with that of the Dgal group. Similarly, compared with the Dgal group, the DHA and DFP treatments significantly downregulated the protein expression levels of IBA-1 significantly in the hippocampus (Fig. 5E) and cortex (Fig. 5F). DFP treatments were observed to exert a more significant effect. Then, we analyzed the expression of neuroinflammation (*Il6*, *Il1b*, *Tnfa*, and *Jnk*) on mRNA. The results showed that the expression of pro-inflammatory cytokines *Il6*, *Il1b*, *Tnfa* and *Jnk* were decreased significantly after the DFP treatment (Fig. 5G). Thus, DFP prevented *D*-gal-induced microglia activation and neuroinflammation.

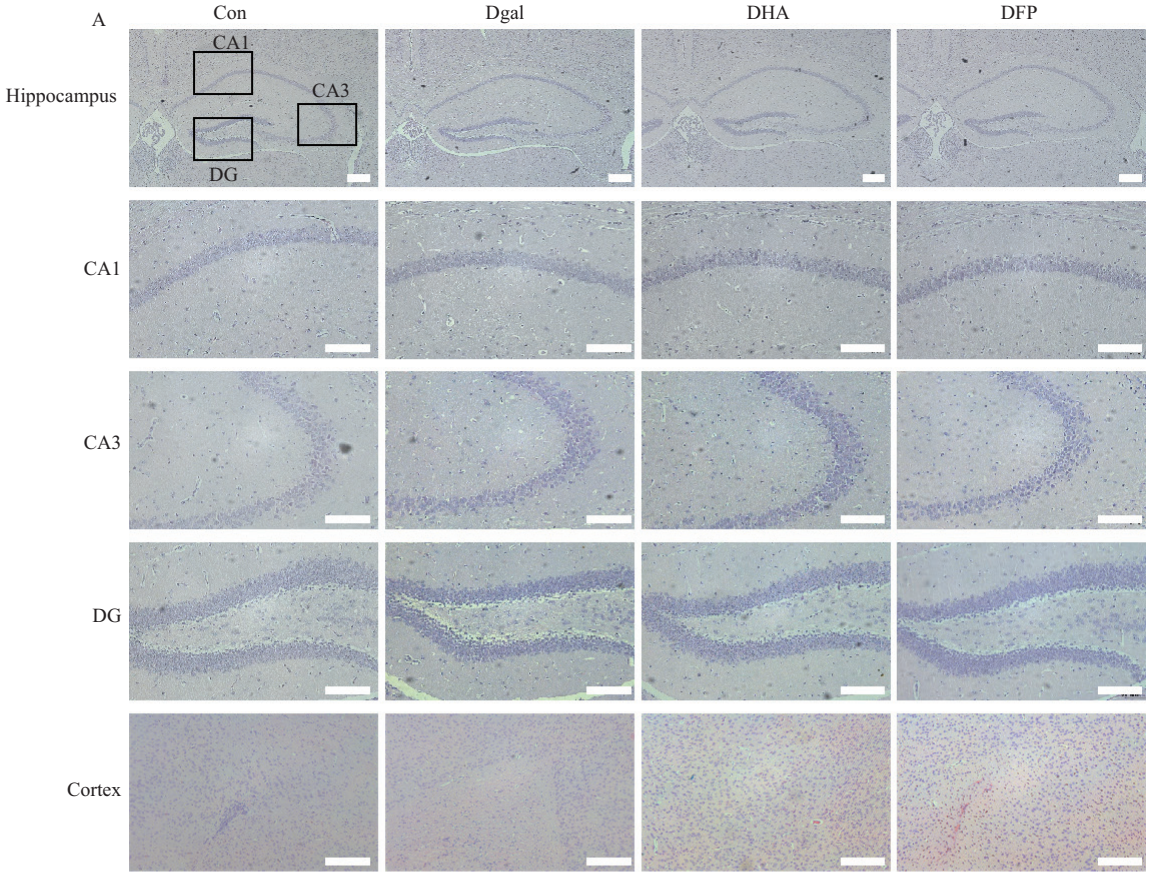

Fig. 3 Combined intervention recovered neuron damage in *D*-gal-induced mice. (A) Representative images of H&E staining in different regions of the brain ($n = 3$, scale bar, 100 μm), Haematoxylin is a blue-purple basic dye, which stains the nucleus. Eosin is a pink acid dye that dyes the cytoplasm of most cells red. (B) Representative images of Nissl staining in the hippocampus and cortex ($n = 3$, scale bar, 100 μm). (C) Quantification of the Nissl area based on Nissl-stained sections in the hippocampus by ImageJ (Version 1.52a, NIH, Bethesda, MD, USA) ($n = 6$). Data are presented as the mean ± SD ($n = 6$). $^{***}P < 0.001$, compared with the Con group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the Dgal group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared with the DHA group.

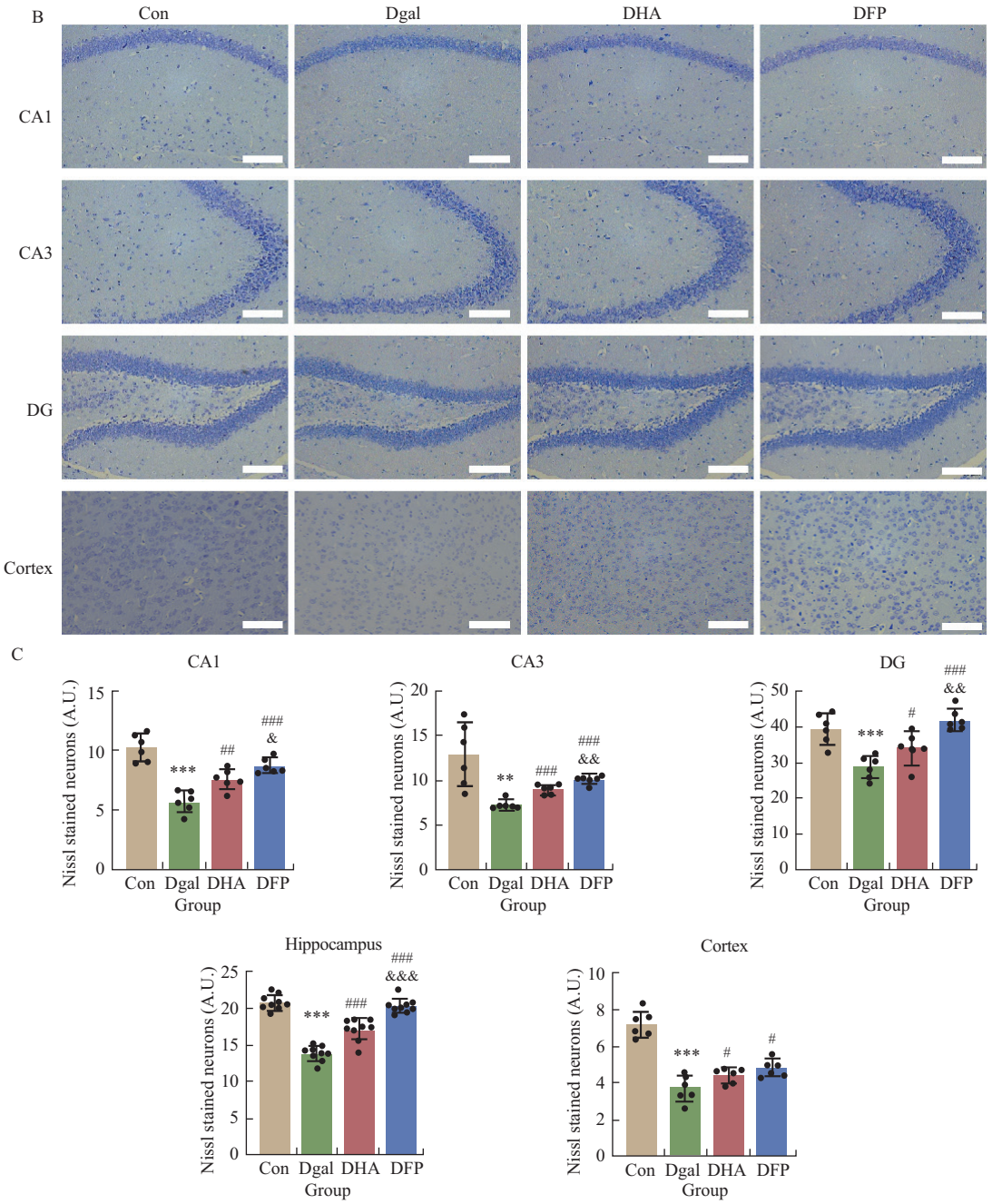

Fig. 3 (*Continued*)

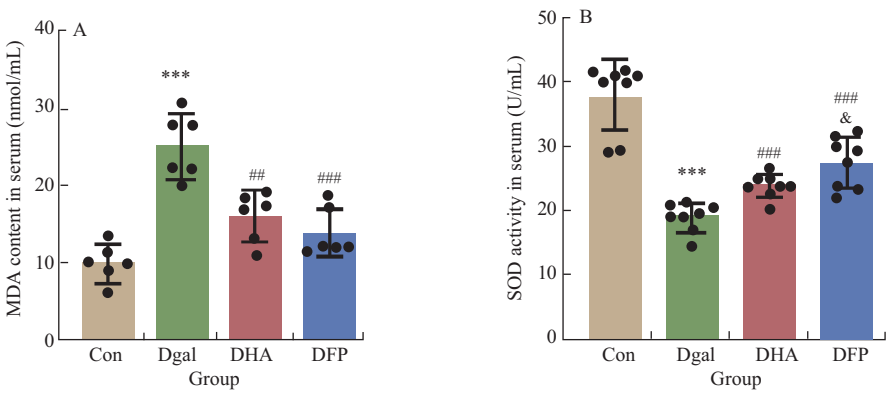

Fig. 4 Combined intervention reversed *D*-gal induced oxidative stress in mice serum. (A) MDA content in serum. (B) SOD activity in serum. ***$P < 0.001$, compared with the Con group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the Dgal group. $^{\&}P < 0.05$ compared with the DHA group.

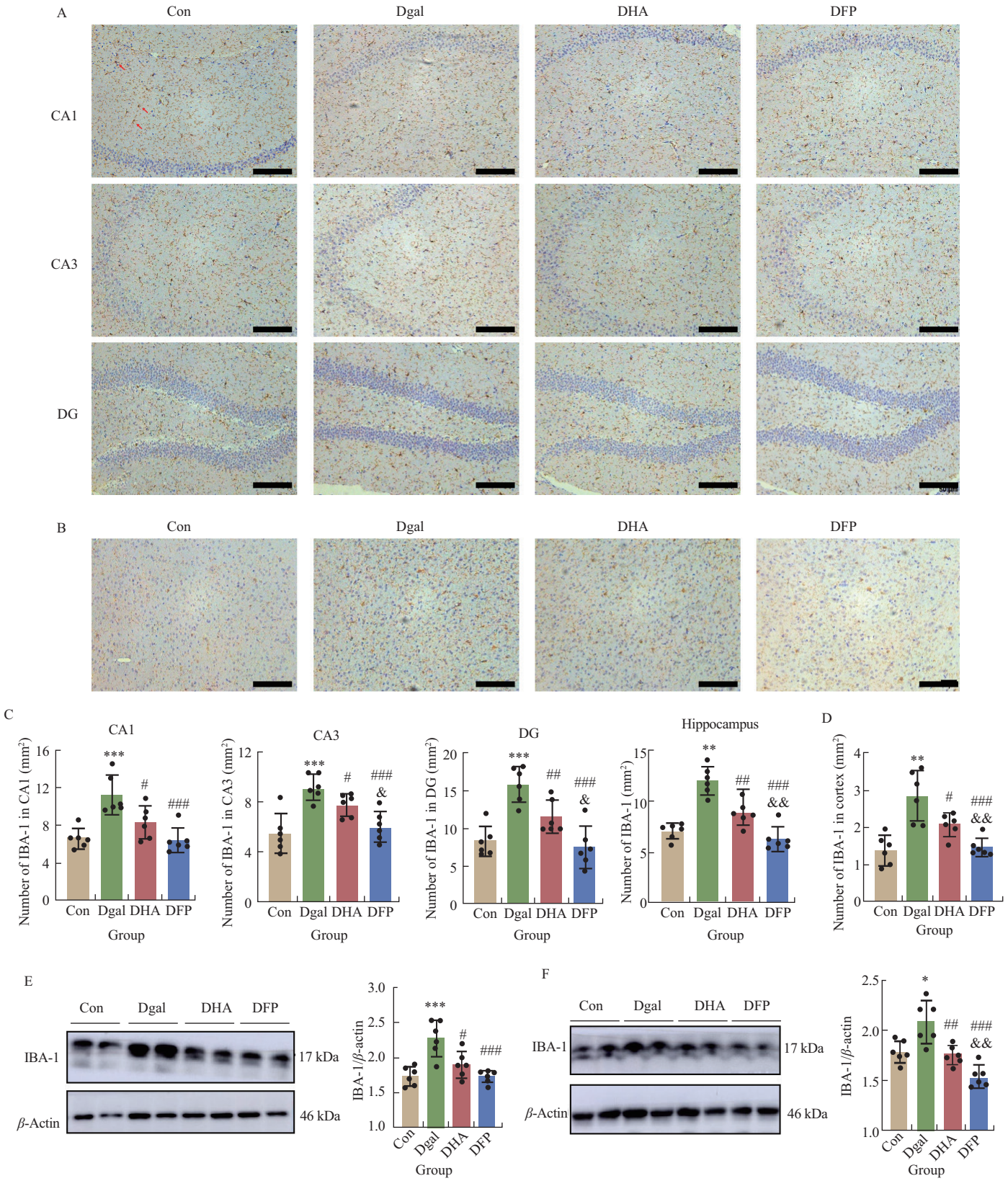

Fig. 5 Combined intervention suppressed *D*-gal-induced microglia activation and inflammation. Representative images of immunohistochemical staining of IBA-1 in the hippocampus (A) and cortex (B) ($n = 3$, scale bar, 100 μm). Quantification of the IBA-1 positive area based on immunohistochemically stained sections in the hippocampus by ImageJ (C) and cortex (D). Representative images were captured from 9 slices of 3 mice per group and data are presented using means ± SD ($n = 6$). (E, F) Representative Western blots of IBA-1 and the relative levels of IBA-1/*β*-actin in the hippocampus (E) and cortex (F). (G) RT-qPCR analysis of the transcript levels of inflammatory cytokines in the hippocampus. Data presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the Con group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the Dgal group. $^{\&\&}P < 0.01$ compared with the DHA group.

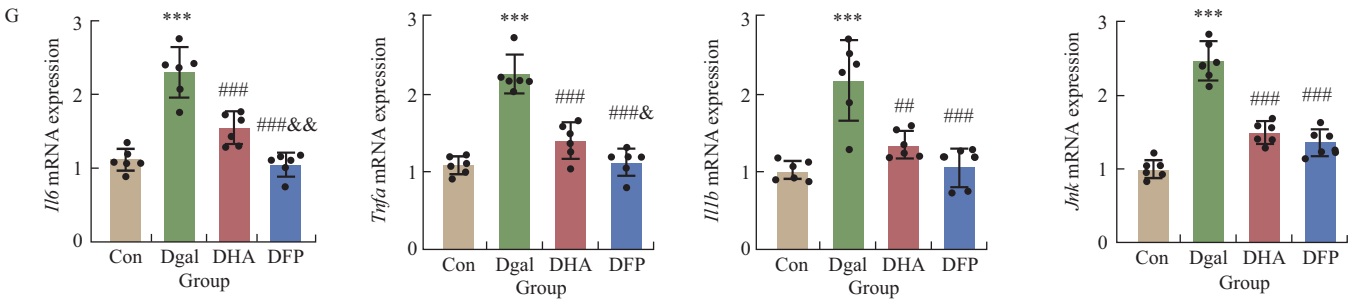

Fig. 5 (*Continued*)

3.6 DFP upregulated the expression of brain-derived neurotrophic factors

BDNF was employed to assess the synaptic function. Immunofluorescent staining analysis demonstrated that the hippocampal BDNF expression levels were significantly reduced in the Dgal group, compared to the Con group, while the DFP group exhibited a marked increase in BDNF expression compared to that of the Dgal group (Figs. 6A-D). We further analyzed the expression of neurotrophic factors and the receptor (*TrκB*) and synaptic proteins (Snap25, Syn, and Psd95) proteins on mRNA by RT-qPCR. Both *Bdnf* and its receptor *TrκB* were upregulated by the combined intervention (Figs. 6E, F). Similarly, the expression levels of the *Snap25*, *Syn*, and *Psd95* genes in the hippocampus were upregulated by the combined intervention. The above results reveal that the DFP did significantly increased synaptic plasticity and improved cognitive impairment in the *D*-gal-induced aging mice.

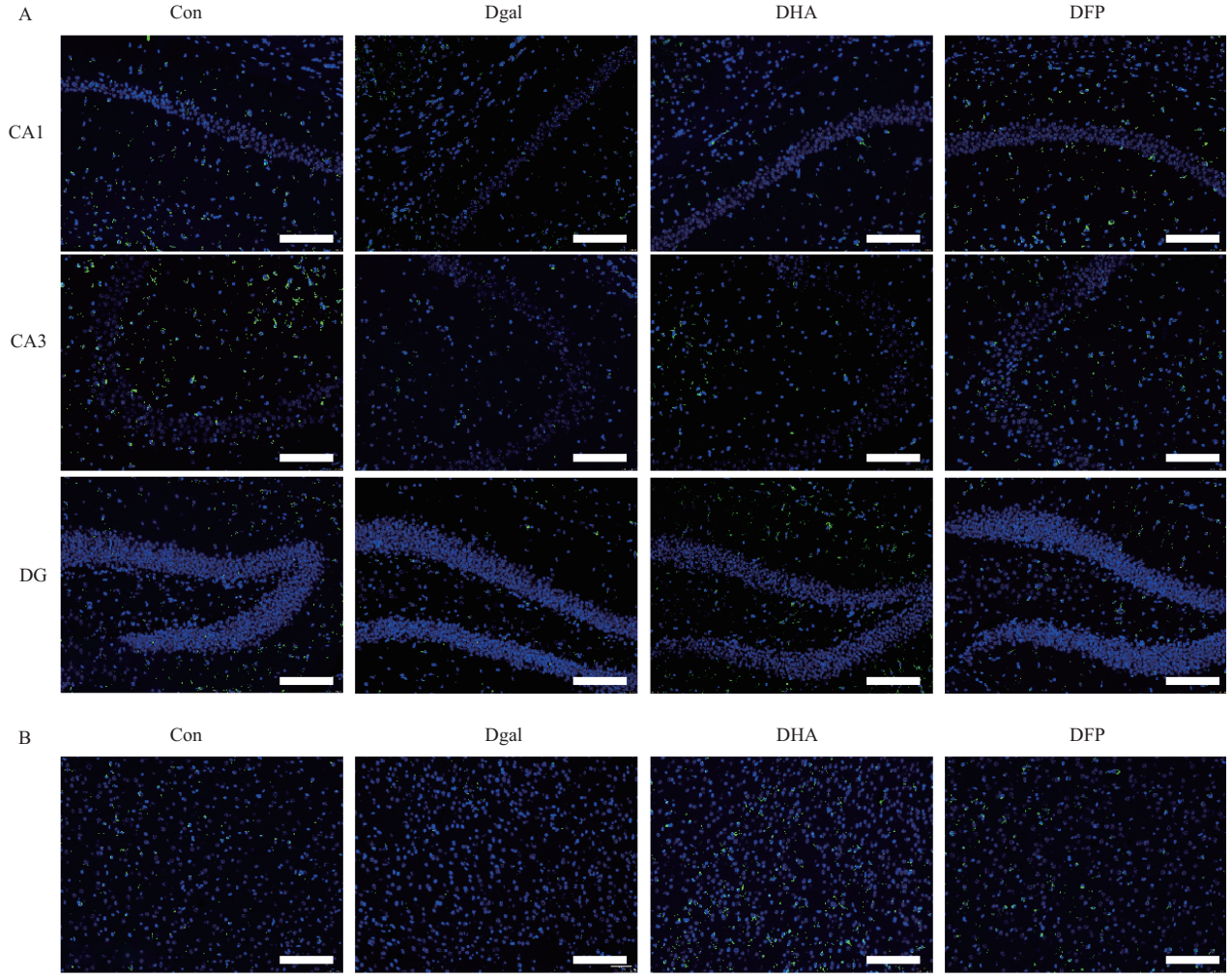

Fig. 6 Effects of combined intervention on the level of neurotrophic factor in the brain of aging mice. Representative images of immunofluorescence of BDNF ($n = 3$, scale bar, 100 μm) in the hippocampus (A) and cortex (B). Quantification of the BDNF area based on immunofluorescence staining sections in the hippocampus by ImageJ (C) and cortex (D) ($n = 6$). Effect of the combined intervention on the expression of *Bdnf* (E), *TrκB* (F), *Snap*25, *Syn*, and *Psd95* (G) in the hippocampus. Data presented as the mean ± SD ($n = 6$), $^{**}P < 0.01$,$^{***}P < 0.001$, compared with the Con group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the Dgal group. $^{\&\&}P < 0.01$ compared with the DHA group.

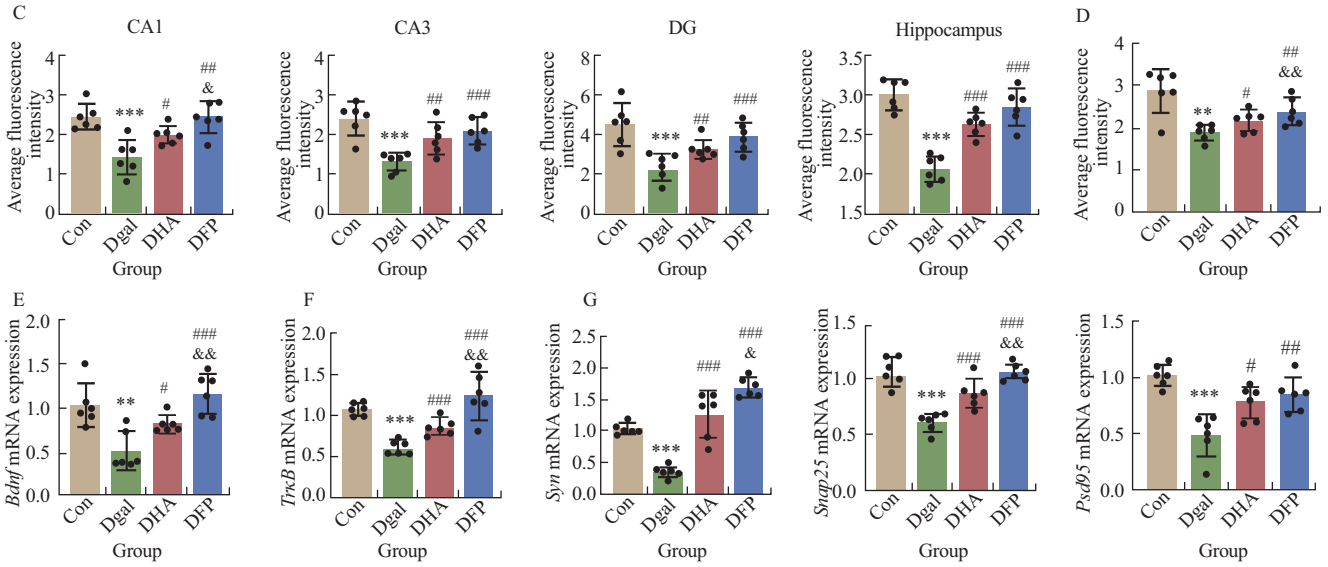

Fig. 6 (*Continued*)

4. Discussion

In recent years, the role of dietary nutrients in the prevention of cognitive impairment has been the focus of much research[18,22]. Nutrients play a significant role in brain functional integrity and metabolism, and a rational intake of dietary nutrients in healthy older adults may reduce the risk of cognitive decline. Previous studies have found that nutrients such as DHA[11,17,22-23], FA[14,24] and PS[18,23] have a wide range of roles in the maintenance of brain structure and function. However, research on the multi-nutrient complexes of DHA, FA, and PS and their effects on cognitive disorders is limited. This study investigated the cognitive impact of the DFP supplement on age-related cognitive decline utilizing a *D*-gal-induced aging mice model.

Cognitive assessments were conducted through behavioral tests (MWM, Y-maze, and NOR), which can reflect the changes in hippocampal and synaptic functions to evaluate the learning and memory capabilities of mice. Behavioral experiments provide an important basis for our understanding of the ability of DFP to enhance learning and memory functions. The improvement of cognitive functions by DFP may be potentially attributed to their anti-inflammatory properties and requires further exploration at the molecular level.

The chronic administration of *D*-gal can disturb the oxidative and antioxidative equilibrium, induce the production of reactive oxygen species (ROS), and consequently result in impaired learning and memory functions. Elevated ROS not only significantly inhibits neurogenesis but also exacerbates neuroinflammation and neurodegeneration[25]. MDA and SOD are commonly used indicators of oxidative stress and have important roles in the study of cognitive decline. MDA is a marker of free radical damage to cell membrane lipids, and the increase in its content reflects the degree of lipid oxidation during oxidative stress. Muka et al.[26] evaluated the relationship between oxidative stress and cognitive decline by using a meta-analysis system, with MDA as a key indicator of oxidative stress. Previous research employed a mouse model to explore the effects of chronic low-dose dichloro-diphenyltrichloroe-thane (DDT) exposure on cognitive function, revealing that decreased SOD activity in the treated group was strongly associated with cognitive impairment[27]. Our results showed that the DHA and DFP treatment protected the hippocampus against oxidative stress by elevating the SOD activity and decreasing the MDA level, resulting in a decline in ROS during aging.

As individuals age, microglia progressively enter a pro-inflammatory state, a transition attributed to the diminished quiescent signaling from neurons and astrocytes[26]. Moreover, neurons positive for IBA-1, which is indicative of microglial activation, have been identified as a significant source of inflammatory cytokines originating within the brain. In our study, the number of IBA-1 positive cells was significantly lower in the DHA and DFP treatment groups than those in the Dgal group, possibly due to the anti-inflammatory activities of the DHA and DFP supplements. Additionally, the over-release and aberrant activation of inflammatory factors can have negative effects during the aging process. The binding of IL-6 binding to its receptor can activate the JAK–STAT signaling pathway, leading to alterations in inflammation-related gene expression and cellular function, as well as directly affecting synaptic plasticity and synaptic connectivity and interfering with neuronal information transmission. The binding of TNF-α to its receptor triggers a signaling cascade that activates downstream transcription factors such as nuclear factor-κB (NF-κB), which induces activation of cysteine proteases, enzymes associated with apoptosis and neurodegenerative changes, resulting in increased apoptosis and impaired synaptic function in hippocampal neurons[28]. JNK is involved in biological processes such as apoptosis and inflammation, and the over-activation of the JNK signaling pathway triggers inflammatory responses and neuronal apoptosis, leading to cognitive decline[29]. Our results showed that *D*-gal induction upregulated the expression of pro-inflammatory cytokines *Il6*, *Il1b*, *Tnfa*, and *Jnk* genes in the hippocampal tissues of mice, while DHA and DFP downregulated the expression of inflammatory factors compared to *D*-gal-induced mice, suggesting that the DHA and DFP improves cognitive function by decreasing the occurrence of neuroinflammation. Our study shows that DFP provided greater benefits than DHA in ameliorating neuroinflammation in aging mice. Neurotrophins are involved in neuronal survival, differentiation, and synaptic plasticity, and are important for maintaining the structural

and functional integrity of the brain[30]. The specific binding of BDNF and its receptor TrκB can activate tyrosine kinases, which in turn activate MAPK and other pathways involved in the regulation of cell survival, proliferation, and synaptic connectivity, affecting the nervous system function. Lian et al.[31] showed that BDNF and TrκB play significant roles in the cognitive function of the brain, and the decline in BDNF and TrκB expression during aging is an important cause of cognitive impairment. Our results indicate that the DHA and DFP treatment can significantly up-regulate the expression of *Bdnf* and *TrκB*, thus playing an improved role in cognitive function.

SNAP25, SYN and PSD95 are important proteins that are closely related to synaptic function and neurotransmission and have key roles in cognitive function. SNAP25 is present in the presynaptic membrane and can interact with other proteins to promote the fusion of neurotransmitter vesicles with the synaptic membrane. Decreased expression levels of SNAP25 are associated with synaptic dysfunction and impaired cognitive function[32-33]. SYN is able to influence neurotransmitter release and synaptic plasticity by regulating the release and distribution of synaptic vesicles. It can also influence cognitive function by regulating glutamate receptors and synaptic plasticity expression, and plays a critical role in spatial learning and memory[34]. In addition, SYN has been found to interact with calcium-regulated proteins and calcium-regulation-dependent kinases, thus influencing cognitive function[35]. PSD95, located in the postsynaptic density can promote neurotransmitter receptor aggregation and signaling through interactions with a variety of proteins involved in interneuronal connectivity. Studies have demonstrated that PSD95 promotes stable synaptic connectivity and plays an important role in the development and maintenance of cognitive function[36]. The abnormal expression or impaired function of SNAP25, SYN, and PSD95 may lead to synaptic transmission disorders, which affect learning, memory, and other cognitive functions, and is closely related to brain injury and the development of neurodegenerative diseases. Our results showed that during *D*-gal-induced aging in mice, the hippocampal tissues showed a cognitive decline with reduced expression of synapse-related proteins Snap25, Syn, and *Psd95* gene. In addition, the expression of synapse-related protein genes was significantly upregulated by the DFP treatment, which had an ameliorative effect on the cognitive decline following aging.

Based on the previous literature, the present study explores the effects of the multi-nutrient complexes of DHA, FA, and PS on the improvement of cognitive impairments. It provides a theoretical basis for evaluating the improvement of cognitive function by the joint intervention of multi-nutrient complexes. However, this study just compared the effect of DFP and DHA on cognition, did not compare the DFP with FA or PS, or a pairwise combination of the three, follow-up experiments can be conducted to further explore it. Moreover, there are three common molecular forms of DHA including DHA-enriched ethyl esters (DHA-EE), DHA-enriched triglyceride (DHA-TG), and DHA-enriched phospholipids (DHA-PLs), and the efficacy of DHA is closely related to its molecular form. Interestingly, previous studies suggested that dietary DHA-PLs was more effective in alleviating neurodegenerative diseases such as AD and Parkinson's disease in rodent model than DHA-EE and DHA-TG[37]. Similarly, the efficacy of PS follows the same pattern, with DHA-containing PS (DHA-PS) being superior to soy-based PS without DHA[23]. The DHA used in this study was mainly DHA-EE, and the PS was soy origin PS without DHA, both of which are commonly used ingredients in the dairy industry. The follow-up research should explore other sources of DHA and PS to find more effective combinations. DFP improved cognitive impairment *via* multiple pathways, such as oxidative stress, neuroinflammation, and synaptic plasticity. Further in-depth animal and *in vitro* cellular experiments are required to determine the interactions between the pathways and the signaling processes. At present, research on the cognitive functions of dietary nutrients mainly focuses on animal experiments and cellular models, while studies on combined interventions are limited, and the results of population studies are insufficient. Thus long-term follow-up and intervention studies are required to verify the effects of combined interventions.

The combination of multi-nutrient efficacy factors is conducive to achieving the development and industrialized production of functional dairy products to improve the memory of the middle-aged and elderly population. This is of great significance in maintaining the cognitive function of the middle-aged and elderly individuals, and actively promoting healthy aging.

5. Conclusion

The results of this study reveal that the combined intervention of DHA, FA, and PS influences the cognitive function of mice brains in aging models. Its observed effects include regulating the antioxidant level, and improving the expression of inflammatory responses, neurotrophic factors, and synaptic-related proteins. The combination of interventions improves the ability of mice in NOR and spatial learning memory, and holds potential as a promising candidate for the neuroenhancement of age-related cognitive impairments. This study provides a theoretical basis for the combined intervention of DHA, FA, and PS to improve cognition, and achieve product development and industrial production of functional dairy products.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (32301994) and the National Center of Technology Innovation for Dairy (2021-National Center of Technology Innovation for Dairy-5).

References

[1] D. Wang, L. Liu, J. Yan, et al., Cardiotrophin-1 (CT-1) improves high fat diet-induced cognitive deficits in mice, Neurochem. Res. 40 (2015)843-853. http://doi.org/10.1007/s11064-015-1535-z.

[2] M.P. Mattson, T.V. Arumugam, Hallmarks of brain aging: adaptive and pathological modification by metabolic states, Cell Metab. 27 (2018) 1176-1199. http://doi.org/10.1016/j.cmet.2018.05.011.

[3] M.J. Yousefzadeh, R.R. Flores, Y. Zhu, et al., An aged immune system drives senescence and ageing of solid organs, Nature 594 (2021) 100-105. http://doi.org/10.1038/s41586-021-03547-7.

[4] A. Fernandes, M. Tábuas-Pereira, D. Duro, et al., C-reactive protein as a predictor of mild cognitive impairment conversion into Alzheimer's disease dementia, Exp. Gerontol. 138 (2020) 111004. http://doi.org/10.1016/j.exger.2020.111004.

[5] D.F. Xu, D. Lian, J. Wu, et al., Brain-derived neurotrophic factor reduces inflammation and hippocampal apoptosis in experimental *Streptococcus*

pneumoniae meningitis, J. Neuroinflammation 14 (2017) 156. http://doi.org/10.1186/s12974-017-0930-6.

[6] Z. Wang, D. Zhang, C. Cheng, et al., Supplementation of medium-chain triglycerides combined with docosahexaenoic acid inhibits amyloid beta protein deposition by improving brain glucose metabolism in APP/PS1 mice, Nutrients 15 (2023) 4244. http://doi.org/10.3390/nu15194244.

[7] K. Fischer, D.M. Van Lent, S. Wolfsgruber, et al., Prospective associations between single foods, Alzheimer's dementia and memory decline in the elderly, Nutrients 10 (2018) 852. http://doi.org/10.3390/nu10070852.

[8] R. Power, J.M. Nolan, A. Prado-Cabrero, et al., Omega-3 fatty acid, carotenoid and vitamin E supplementation improves working memory in older adults: a randomised clinical trial, Clin. Nutr. 41 (2022) 405-414. http://doi.org/10.1016/j.clnu.2021.12.004.

[9] M. Li, W. Li, Y. Gao, et al., Effect of folic acid combined with docosahexaenoic acid intervention on mild cognitive impairment in elderly: a randomized double-blind, placebo-controlled trial, Eur. J. Nutr. 60 (2020) 1795-1808. http://doi.org/10.1007/s00394-020-02373-3.

[10] Y.P. Zhang, Y.Y. Lou, J. Hu, et al., DHA supplementation improves cognitive function via enhancing Aβ-mediated autophagy in Chinese elderly with mild cognitive impairment: a randomised placebo-controlled trial, J. Neurol. Neurosurg. Psychiatry 89 (2018) 382-388. http://doi.org/10.1136/jnnp-2017-316176.

[11] L. Lauritzen, P. Brambilla, A. Mazzocchi, et al., DHA effects in brain development and function. Nutrients 8 (2016). http://doi.org/10.3390/nu8010006.

[12] A.J. Sinclair, Docosahexaenoic acid and the brain - what is its role? Asia Pacific J. of Clin. Nutr. 28 (2019) 675-688. http://doi.org/10.6133/apjcn.201912_28(4).0002.

[13] R. Singh, S.S. Kanwar, P.K. Sood, et al., Beneficial effects of folic acid on enhancement of memory and antioxidant status in aged rat brain, Cell. Mol. Neurobiol. 31 (2011) 83-91. http://doi.org/10.1007/s10571-010-9557-1.

[14] M. Xu, Y. Zhu, J. Chen, et al., Effects of folic acid supplementation on cognitive impairment: a meta-analysis of randomized controlled trials, J. Evid. Based Med. 17 (2024) 134-144. http://doi.org/10.1111/jebm.12588.

[15] W. Xiong, B. Fang, X. Wang, et al., Dietary lipid intervention in the prevention of brain aging, Engineering 37 (2023) 128-137. http://doi.org/10.1016/j.eng.2023.04.012.

[16] B. Liu, N. Zhang, J. Yang, et al., Preparation, characterization, evaluation of neuroprotective effect, and related mechanisms of phosphatidylserine emulsion in 5- and 12-week old mice, J. Agric. Food Chem. 70 (2022) 1852-1864. http://doi.org/10.1021/acs.jafc.1c07403.

[17] M.M. Zhou, L. Ding, M. Wen, et al., Mechanisms of DHA-enriched phospholipids in improving cognitive deficits in aged SAMP8 mice with high-fat diet, J. Nutr. Biochem. 59 (2018) 64-75. http://doi.org/10.1016/j.jnutbio.2018.05.009.

[18] Q. Ren, J. Sun, D. Xu, et al., A dietary supplement containing micronutrients, phosphatidylserine, and docosahexaenoic acid counteracts cognitive impairment in *D*-galactose-induced aged rats, Front. Nutr. 9 (2022) 931734. http://doi.org/10.3389/fnut.2022.931734.

[19] S.L. Dawson, S.J. Bowe, T.C. Crowe, A combination of omega-3 fatty acids, folic acid and B-group vitamins is superior at lowering homocysteine than omega-3 alone: a meta-analysis, Nutr. Res. 36 (2016) 499-508. http://doi.org/10.1016/j.nutres.2016.03.010.

[20] M. Riva, S. Moriceau, A. Morabito, et al., Aberrant survival of hippocampal Cajal-Retzius cells leads to memory deficits, gamma rhythmopathies and susceptibility to seizures in adult mice, Nat. Commun. 14 (2023) 1531. http://doi.org/10.1038/s41467-023-37249-7.

[21] H. Zhang, L. Mo, X. Chen, et al., The effect of exogenous free N^{ε}-(carboxymethyl) lysine on diabetes-associated cognitive dysfunction: neuroinflammation, and metabolic disorders, Food Sci. Hum. Wellness 13 (2024) 2970-2981. http://doi.org/10.26599/fshw.2022.9250240.

[22] H. Che, H. Li, L. Song, et al., Orally administered DHA-enriched phospholipids and DHA-enriched triglyceride relieve oxidative stress, improve intestinal barrier, modulate inflammatory cytokine and gut microbiota, and meliorate inflammatory responses in the brain in dextran sodium sulfate induced colitis in mice, Mol. Nutri. Food Res. 65 (2021) e2000986. http://doi.org/10.1002/mnfr.202000986.

[23] Z.J. Xu, Q. Li, L. Ding, et al., A comparative study of the effects of phosphatidylserine rich in DHA and EPA on Aβ-induced Alzheimer's disease using cell models, Food Funct. 12 (2021) 4411-4423. http://doi.org/10.1039/d1fo00286d.

[24] F. Ma, Q. Li, X. Zhou, et al., Effects of folic acid supplementation on cognitive function and Aβ-related biomarkers in mild cognitive impairment: a randomized controlled trial, Eur. J. Nutr. 58 (2019) 345-356. http://doi.org/10.1007/s00394-017-1598-5.

[25] T.F. Yuan, S. Gu, C. Shan, et al., Oxidative stress and adult neurogenesis, Stem Cell Rev. Rep. 11 (2015) 706-709. http://doi.org/10.1007/s12015-015-9603-y.

[26] T. Muka, M. Glisic, J. Milic, et al., A 24-step guide on how to design, conduct, and successfully publish a systematic review and meta-analysis in medical research, Eur. J. Epidemiol. 35 (2019) 49-60. http://doi.org/10.1007/s10654-019-00576-5.

[27] H. Sies, Oxidative stress: a concept in redox biology and medicine, Redox Biol. 4 (2015) 180-183. http://doi.org/10.1016/j.redox.2015.01.002.

[28] T. Muhammad, M. Ikram, R. Ullah, et al., Hesperetin, a citrus flavonoid, attenuates LPS-induced neuroinflammation, apoptosis and memory impairments by modulating TLR4/NF-κB signaling, Nutrients 11 (2019) 648. http://doi.org/10.3390/nu11030648.

[29] Y. Zhao, K. Kuca, W. Wu, et al., Hypothesis: JNK signaling is a therapeutic target of neurodegenerative diseases, Alzheimers Dement. 18 (2022) 152-158. http://doi.org/10.1002/alz.12370.

[30] M. Zhang, L. Xu, H. Yang, *Schisandra chinensis* fructus and its active ingredients as promising resources for the treatment of neurological diseases, Inter. J. Mol. Sci. 19 (2018) 1970. http://doi.org/10.3390/ijms19071970.

[31] N. Lian, S. Zhang, J. Huang, et al., Resveratrol attenuates intermittent hypoxia-induced lung injury by activating the Nrf2/ARE pathway, Lung 198 (2020) 323-331. http://doi.org/10.1007/s00408-020-00321-w.

[32] J. Lenart, E. Bratek, J.W. Lazarewicz, et al., Changes in the expression of SNAP-25 protein in the brain of juvenile rats in two models of Autism, J. Mol. Neurosci. 70 (2020) 1313-1320. http://doi.org/10.1007/s12031-020-01543-6.

[33] Q. Wang, Y. Wang, W. Ji, et al., SNAP25 is associated with schizophrenia and major depressive disorder in the Han Chinese population, J. Clin. Psychiatry 76 (2015) E76-E82. http://doi.org/10.4088/JCP.13m08962.

[34] W.J. Lin, C. Jiang, M. Sadahiro, et al., VGF and its C-terminal peptide TLQP-62 regulate memory formation in hippocampus *via* a BDNF-TrκB-dependent mechanism, J. Neurosci. 35 (2015) 10343-10356. http://doi.org/10.1523/jneurosci.0584-15.2015.

[35] S.Y. Lee, J.H. George, D.A. Nagel, et al., Optogenetic control of iPS cel-derived neurons in 2D and 3D culture systems using channelrhodopsin-2 expression driven by the synapsin-1 and calcium-calmodulin kinase II promoters, J. Tissue Eng. Regen. Med. 13 (2019) 369-384. http://doi.org/10.1002/term.2786.

[36] Y.H. Meng, Y. Zhang, V. Tregoubov, et al., Regulation of spine morphology and synaptic function by LIMK and the actin cytoskeleton, Rev. Neurosci. 14 (2003) 233-240.

[37] C.C. Wang, Y. Guo, M.M. Zhou, et al., Comparative studies of DHA-enriched phosphatidylcholine and recombination of DHA-ethyl ester with egg phosphatidylcholine on ameliorating memory and cognitive deficiency in SAMP8 mice, Food Funct. 10 (2019) 938-950. http://doi.org/10.1039/c8fo01822g.