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Effects of endogenous DHA milk and exogenous DHA milk on oxidative stress and cognition in SAMP8 mice

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ABSTRACT

In this study, Senescence Accelerated Mice (SAMP8) were supplemented with exogenous DHA milk, endogenous DHA milk, normal milk, or 0.9 % saline solution. Enzyme-linked immunosorbent assay (ELISA), gas chromatography (GC), ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI MS/MS), and Morris water maze were used to characterize the effects of diet on oxidative stress and cognition in SAMP8 mice. Supplementation endogenous DHA milk or exogenous DHA milk can enhance the antioxidant capacity of mice organs. Endogenous DHA milk increased the superoxide dismutase (SOD) activity of mice brain and serum than normal milk and 0.9 % saline solution ($P \leq 0.05$), as well as increased SOD activity of mice liver and glutathione peroxidase (GSH-Px) activity of mice brain than normal milk ($P \leq 0.05$). Exogenous DHA milk increased SOD activity of mice brain than normal milk and 0.9 % saline solution, as well as increased SOD activity of mice serum than 0.9 % saline solution ($P \leq 0.05$). Several polar lipid relative content, such as 18:0/18:2 PS, 17:0 Ceramide, and 20:4 LPC in mice brain was affected by dietary supplementation with DHA-containing milk. Lipid oxidation metabolites in mice brain were not affected by DHA-containing milk. Endogenous DHA milk increased the number of platform location crossing times of mice in the Morris water maze test, compared with Exogenous DHA milk, normal milk, and 0.9 % saline solution ($P \leq 0.05$).

1. Introduction

Oxidative damage caused by reactive oxygen species (ROS) is considered a key factor affecting the biological aging process [1]. Accumulated ROS can lead to the destruction of cellular lipids, proteins, and nucleic acids, thereby inhibiting normal cell function [2]. Oxidative stress is considered to be a significant contributor to age-related cognitive decline, as age-related memory decline is often accompanied by accumulation of oxidative damage, leading to disruption of neuronal function [3]. In response to oxidative damage, the body antioxidant system regulates the oxidative stress state in the body. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione transferase are all active components of antioxidants [4]. Superoxide radicals are dismutated by SOD to hydrogen peroxide, which is scavenged by GSH-Px in the presence of glutathione as an electron donor [2] and herewith reduce oxidative stress state in the body. Therefore, enhancing the activity of antioxidant components is an effective way to relieve the oxidative stress state of the body.

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) are characterized by the first double bond which is located on the third carbon atom from the methyl end of the fatty acid. It has been demonstrated that ω-3 PUFA are biologically functional [5], such as preventing growth retardation [6], preventing cardiovascular disease [7], reducing thrombosis [8], and have anti-inflammatory effects [6]. Docosahexaenoic acid (DHA), a ω-3 PUFA, is considered relevant not only for the growth and development of infants and young children [9], but also for maintaining cognitive performance during aging [9,10]. Docosahexaenoic acid is an important component of brain tissue, retina, and other organs [11]. Besides, it is considered important for the proper functioning of the brain, affecting neurotransmitter pathways, synaptic transmission, and signal

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transduction [12]. The current view is that the supplementation of PUFA, especially DHA, have a positive impact on cognitive ability [11,13]. Study showed that DHA supplements can alleviate age-related cognitive decline [14]. Supplementation with DHA was also able to prevent learning and memory impairment in mice [15]. However, DHA supplements appear to have no significant effect on memory performance in Alzheimer's patients [16]. The antioxidant effects of DHA and its relation to cognitive performance in the body require further investigation, particularly regarding the optimal doses, molecular structure, and the synergistic effects of other dietary components.

Based on the current literature, the effect of ω-3 PUFA on the body's oxidative stress response is still controversial. Polyunsaturated fatty acids are highly peroxidised to form 4-hydroxynonenal and isoprostanes, which are markers of lipid peroxidation and were considered potentially mutagenic and cell membrane disruptive [17,18]. However, the generally accepted view is that dietary supplementation with ω-3 PUFA could alleviate oxidative stress [19]. Cell culture experiments by Richard et al. demonstrated that ω-3 PUFA supplementation resulted in less reactive oxygen formation compared with other PUFA [20]. Additionally, studies showed that supplementation with ω-3 PUFA significantly increased total antioxidant capacity [21,22]. Specific proportions of EPA (Eicosapentaenoic acid, C20:5): DHA (1: 1 and 2: 1) in the diet reduced the production of inflammatory and oxidative stress markers in Wistar rat plasma [23], compared with EPA: DHA (1: 2).

People from all regions of the world have insufficient intake of dietary sources of ω-3 PUFA [24,25]. In most parts of the world, dairy products significantly contribute to the human diet. For infants and young children, breast milk or infant formula are the main sources of DHA. The main source of DHA in breast milk is the women's dietary intake and synthesis in vivo. Studies have shown that dietary intake of ω-3 PUFA or intake of DHA can increase DHA content in breast milk [26]. During pregnant and lactation, women with obesity require additional ω-3 PUFA supplementation to ensure adequate DHA in breast milk [27]. For adults and the elderly, dairy products based on ruminant milk contribute to their daily diet. In ruminant milk, the content of PUFA only accounts for 3–5 % of the total milk fat [28,29]. Therefore, in order to obtain dairy products containing PUFA, food additives of DHA and arachidonic acid was added to the dairy products, which is the main source of PUFA in dairy products [30]. An alternative method is to increase DHA in milk by adding microalgae to the diet of lactating cows [31].

Although dairy products containing DHA are currently available, their effects on antioxidant and cognitive effects have not yet been evaluated. The endogenous DHA milk was obtained by feeding microalgae to lactating cows. The exogenous DHA milk was obtained by adding a certain amount DHA food additive into normal milk. Due to the different ways of adding DHA to endogenous DHA milk and exogenous DHA milk, the efficacy of the two kinds of milk may be different. The aim of this research was to explore the effects of different kinds of DHA-containing milk (endogenous DHA milk and exogenous DHA milk) on alleviating peroxidation and cognitive decline caused by aging by using animal experiments. This study aims to: 1) evaluate the functionality of DHA-containing milk by detecting the antioxidant-related indicators of the mouse brain, liver, and serum, the fatty acid and phospholipid composition of the mouse brain, and Morris water maze test performance; 2) compare the efficacy of endogenous DHA milk and exogenous DHA milk.

2. Materials and methods

The study protocols were approved by the ethical committee of experimental animal care at the Institute of Food Science and Technology, Chinese Academy of Agriculture Science (Beijing, China).

2.1. Animals, experimental design, and treatments

Male Senescence Accelerated Mouse-Prone 8 (SAMP8) mice (3–5-month-old at the start of the experiment, from Beijing HFK Bioscience CO., LTD., Beijing, China) were housed under a 12 h/12 h light/dark cycle at 22 ± 1 °C with 60 % ± 5 % humidity. All mice were fed with a AIN93 standard diet (Mediscience Ltd, Jiangsu, China) ad libitum. The mice were raised in single cages (290×178×160 mm). Mice were given free access to water and on the standard diet for one week before the experiment, in order to adapt to the environment.

The mice (N = 39) were randomly divided into 4 groups, including 3 treatment groups (1) Endogenous DHA milk powder group (n = 10); (2) Exogenous DHA milk powder group (n = 10); (3) Normal milk powder group (n = 10); and (4) Control group fed with 0.9 % saline solution (n = 9).

2.2. Milk treatments

Endogenous DHA milk powder, exogenous DHA milk powder, and normal milk powder were provided by Heilongjiang Feihe Dairy Co., Ltd. (Heilongjiang, China). Endogenous DHA milk was obtained by feeding microalgae (*Schizochytrium sp.*) supplement (Xiamen Huison Biotech Co., Ltd) to lactating Holstein cows. Exogenous DHA milk was obtained by adding DHA additive (CABIO Biotech (Wuhan) Co, Ltd) to normal milk. Milk powder was made by spray drying cow milk. The DHA content in endogenous DHA milk powder and exogenous DHA milk powder accounted for about 0.33 % of the total fatty acid (Table S1).

Milk powders were dissolved at a concentration of 1 g/mL with deionized water before gavage. The gavage dose of milk solution was 0.1042 g dry milk/(10 g.BW) (body weight) according to the BW of mice. The gavage dose of control group was 0.3 mL/(10 g.BW) saline solution. The time of gavage was 10 a.m. every day for 6 weeks. The weekly milk gavage volume of the mice was adjusted according to the weekly body weight.

2.3. Morris water maze test

After 42 days of feeding treatments, mice were subjected to the Morris water maze test to measure cognitive ability. The Morris water maze (Zhenghua Bio Instrument Ltd., Anhui, China) was made up of a trajectory recording device and a circular pool with a platform. The pool (1.5 m in diameter and 0.5 m height) was divided into four quadrants which were named quadrant I, II, III, and IV. With the water 0.3 m depth and temperature at 23 ± 1°C, the platform (9 cm in diameter) was submerged 1.5 cm under the water level in the centre of quadrant III. Black ink was added to the water to contrast the mice against the background. A camera connected to a computer was located 2 m above the centre of the pool, and the movement of the mouse trajectory was automatically recorded and analysed by Animal Behavioural Video Analysis System Ver 20.0919 (Zhenghua Bio Instrument Ltd., Anhui, China)

Morris water maze test included a 4-day training session trail and a 1-day test session. During each training session (day 1–4): each mouse was trained for 4 times per day and entered the water from the fixed position of the fourth, third, second and first quadrants facing the pool wall. The escape latency time defined as time from entering the water to finding the platform was recorded. After the mice climbed onto the platform, they remained on the platform for 15 s. If the mouse did not find the platform within 60 s, the experimenter would pull it to the platform and allowed it to stay on the platform for 20 s. The escape latency time was then recorded as 60 s, and the interval between two training sessions was 30 s. The average of the 4 training latencies was recorded as the escape latency of the day. On the test session (day 5): a spatial probe test. For this session, the platform was removed, and each mouse was placed in the water on quadrant I. The over-head cameras were used to directly monitor the mouse in the pool and record the images for subsequent

analysis. From the recordings, the number of times of crossing the original platform position (in quadrant III) within 60 s was collected. The escape latency time and platform crossing times of mice were used to detect the learning and memory ability of the mice.

2.4. Measurements, sampling, and analysis

2.4.1. Weight data and sample collected

The weight of the mice was measured on days 0, 7, 14, 21, 28, 35, and 42. After the Morris water maze test, mice were anesthetized using pentobarbitals and orbital blood was collected 0.5–1 mL, and the serum was extracted by centrifugation (3000 rpm, 20 min). Brain and liver were prepared as a 10 % (*w/v*) tissue homogenate in 0.9 % saline solution after washing with PBS (pH 8.3). All samples were stored at −80 °C until analysis.

2.4.2. Detection of antioxidant indicators

The activity of SOD and GSH-Px in brain homogenate, liver homogenate, and serum were measured by using assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) respectively. All these measurements were performed according to the operating instructions.

2.4.3. Detection of fatty acids

To determine fatty acid composition, total lipids were extracted by the Bligh and Dyer method [32]. Analytical reagents methanol, sulfuric acid, and chloroform used for lipid extraction were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Endogenous DHA milk powder and exogenous DHA milk powder dissolved before lipid extraction. To be more detailed, 0.5 g powder was mixed with 5 mL 65 °C of steamed water. From each mouse brain homogenate sample, 250 μL of brain homogenate was taken for lipid extraction. The extracted lipid was methylated by adding 2.5 M sulfuric acid methanol solution at 70 °C for 30 min. After the reaction, 0.5 mL of n-hexane (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) was added to extract fatty acid methyl esters, and after centrifugation (3750 rpm/min, 5 min), the supernatant was taken for fatty acid detection by gas chromatography. Each sample was analysed by GC with a hydrogen flame ionization detector (Agilent 8890 B) and a capillary column (DB-23 60 m × 0.25 mm × 0.25 μm; Sigma-Aldrich). Both the injector and detector temperatures were 250 °C. The fatty acid methyl ester (FAME) was identified by comparison of the retention times of the sample peaks with those of a mixture of FAME standards (Supelco 37-component FAME mix, Sigma-Aldrich Co. LLC Burlington, MA, United States).

2.4.4. Detection of polar lipids and lipid metabolites

To determine polar lipids and lipid metabolites composition, brain homogenates of 3 or 4 mice in each group were pooled in equal proportions and then subjected to total lipid extraction. Analytical grade sulfuric acid and chloroform used for lipid extraction were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Three mixed samples were tested in each group. Total lipids of the brain were extracted by the Bligh and Dyer method [32]. The lipid in the brain tissue of SAMP8 mice was detected by LC-MS according to Li [33]. Internal l-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (12:0 LPC) (Avanti Polar Lipids, Alabaster, AL, U.S.A.) were used quality control for the detection of Lysophosphatidylcholine (LPC), Phosphatidylcholine (PC), Sphingomyelin (SM), Lysophosphatidylethanolamine (LPE), Phosphatidyl-ethanolamine (PE), Lysophosphatidylinositol (LPI), Phosphatidylinositol (PI), Cerebrosides (Cb), Ceramide, 1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), 1-Palmitoyl-2-(9-oxononanoic acid)-sn-glycero-3-phosphocholine (PONPC), and 1-(Palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine (KOdiAPC). Internal 1-myristoyl-2-hydroxy-sn-glycero-3-phosphate (14:0 LPA) was used for quality control for the detection of Lysoglycerophosphatidic acid (LPA), Glycerophosphatidic acid (PA), Sphingosine-1-phosphate (S1P), Lysophosphatidylglycerol (LPG), Phosphatidylglycerol (PG), Lysophosphatidylserine (LPS), Phosphatidylserine (PS), DHA, AA, Prostaglandin (PG)A2, PGJ2, PGD2, PGE2, PGF2α, 11,12- Epoxyeicosatrienoic acid (EET), and 14,15-EET. For the quantitative analyses, we referred to the previous study which the peak area ratio (internal lipid standard) and molar ratio (external lipid standard) were plotted and fitted as linear regressions [33]. Brain lipid samples were loaded through a LC system (I-class Acquity UPLC, Waters Corporation, Milford, United States) with an autosampler. The mobile phase A was isopropanol/acetonitrile (9:1, *v/v*) with 10 mM ammonium formate and 0.1 % formic acid and the mobile phase B was acetonitrile/H_2O (7:3, *v/v*) with 10 mM ammonium formate and 0.1 % formic acid and mobile phase C was methanol/H_2O (9:1, *v/v*) with 0.1 % ammonia. Chromatographic-grade acetonitrile, methanol (MeOH), isopropanol (IPA), chloroform ($CHCl_3$), formic acid, ammonium formate, and ammonia were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Sample were injected for analysis by MS (API 4500 Q-Trap, AB SCIEX, Framingham, MA, United States) equipped with an ESI source. In positive ion mode, the mobile phase A and B, and a BEH C18 column (1.7 μm, 2.1 mm i.d. × 100 mm, Waters) was used for detection of lipids. The column was maintained at 55°C. The UHPLC separations were 2 min/-sample using the following scheme: (1) 0–2 min, 80 % A: 20 % B. All the changes are linear, and the flow rate was set at 400 μL/min. The pressure was around 7200–9800 psi in this process. In negative ion mode, the mobile phases A and C, and a short C18 HPLC column (5 μm, 2.1 mm i.d. × 20 mm, TR-0121-C185, Higgins Analytical, Southborough, MA) were used for separation of lipids. The separations were 12 min/sample using the following scheme: (1) 0–3 min, 100 % C, constant flow rate: 100 μL/min; (2) 3–4 min, 100 % C decreased to 50 % C, flow rate: increased from 100 to 300 μL/min; (3) 4–10 min, 50 % C, constant flow rate: 300 μL/min; (4) 10–11 min, 50 % C increased to 100 %, flow rate: decreased from 300 to 100 μL/min; (5) 12–12 min, 100 % C, constant flow rate: 100 μL/min. The pressure was around 450–760 psi in this process.

MS analysis was performed using the API 4500 QTRAP mass spectrometer (Applied Biosystems/MDS SCIEX) with the Analyst data acquisition system. Both the nebulizer and desolation gases were nitrogen. The curtain gas, ion source gas 1, and ion source gas 2 were set as 25, 45, and 20, respectively. Moreover, the positive ion multiple reaction monitoring (MRM, Q1 = Q3) and negative ion MRM mode were set singly at electrospray voltage 5500 and −4500 under the temperature 500°C or 250°C. When MRM-information correlation acquisition (IDA)-enhanced production (EPI) was performed, the IDA threshold was set at 200 counts per second, the collision energy in EPI mode was set at 35, the collision energy spread was set at 15, and the dynamic fill time function was used to against the space charges effect.

2.5. Statistical analysis

Before statistical analysis, all data were tested for normal distribution and homogeneity of variances. Data distribution was assessed using the Shapiro-Wilk test with the UNIVARIATE procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC), with $P > 0.05$ indicating normality. A UNIVARIATE procedure of SAS (SAS Institute Inc., Cary, NC) was used to check the group data homogeneity of the variances. For normally distributed data with homogenous variance, the ANOVA procedure was used for testing, otherwise, the Kruskal-Wallis procedure was used for testing.

The PROC MIXED procedure of SAS (SAS version 9.4, SAS Institute Inc., Cary, NC) and Kruskal-Wallis Test (PROC NPAR1WAY, SAS version 9.4, SAS Institute Inc., Cary, NC) for repeated measures analysis was used to analyse the effects of treatment on SOD and GSH-Px activity in brain, liver, and serum (n = 39 in total), as well as on the fatty acid (n = 39 in total) and phospholipids (n = 12 in total) relative content in brain, including body weight as a covariate. Preliminary result showed that bodyweight did not have a significant impact on the above experimental

indicators ($P > 0.05$) (Table S2) and was therefore excluded from the model. Mouse was considered as the repeated subject. Tukey-Kramer post-hoc analyses (ANOVA) and Dwass, Steel, Critchlow-Fligner (DSCF) post-hoc analyses (Kruskal-Wallis) evaluated between group differences after adjusting for multiple comparisons. The data presented in the tables are expressed as least-square means (LSM) with their maximal standard error of the mean (SEM). Differences are regarded as significant if $P \leq 0.05$, and trends are discussed if $0.05 < P \leq 0.10$.

Furthermore, R studio (R version 4.0.5, R Core Team) including the package of ggplot2 was used for visualizing the plots escape latency time and crossing times. R studio including the package of ggplot2 [34] and corrplot [35] was used for visualizing the correlation of SOD and GSH-Px activity in brain, liver, and serum and crossing times. The Spearman correlation coefficients have been used to evaluate correlation between indicators. R studio including the package of ggplot2 [34] was used for visualizing the relative content of brain polar lipids and principal component analysis (PCA) of lipid oxidation metabolites.

3. Results

3.1. Effects of DHA supplementation on oxidative stress variables in brain, liver, and serum

Oxidative stress-related metabolites in the brain tissue, liver tissue and serum of the mice in the dietary DHA supplemented group were significantly different from those without DHA supplemented group (Table 1). The brain SOD activity of exogenous DHA and endogenous DHA groups were higher than that of normal milk and control group ($P \leq 0.05$). The brain GSH-Px activity of the endogenous DHA group was higher than that of the Normal milk group ($P \leq 0.05$). The SOD activity in liver tissue of mice supplemented with endogenous DHA milk was higher than that of control mice ($P \leq 0.05$). The activity of liver GSH-Px tended to be higher in mice fed diets supplemented with endogenous DHA milk compared with mice in the control group ($P = 0.06$). For serum, supplementation of both exogenous DHA milk and endogenous DHA milk increased SOD activity compared with control group ($P \leq 0.05$).

3.2. Fatty acid composition in brain

The relative content of SFA in the brain tissue of mice supplemented with exogenous DHA milk, endogenous DHA milk, and normal milk were significantly higher ($P \leq 0.05$; Table 2) than that of control group mice. The relative contents of SFA, MUFA, and PUFA in mouse brain tissue were not different ($P > 0.05$) between exogenous DHA milk, endogenous DHA milk, and normal milk groups. Relative content of monounsaturated and PUFA in the brain tissue from three milk-feeding treatments were lower ($P \leq 0.05$) than control group. The increase of relative content of C16:0 and C18:0 in mice brain tissue is the main reason for the increase of total SFA content. The content of fatty acid C16:0 in exogenous DHA milk, endogenous DHA milk, and normal milk treatment was 42.5 %, 45.4 %, and 45.8 %, respectively, while it only accounted for 35.6 % of the total fatty acid in the control group. The unsaturated fatty acids, including monounsaturated-rated fatty acids (MUFA) and PUFA, in the brain tissue of mice from exogenous DHA milk, endogenous DHA milk, and normal milk groups were lower than unsaturated fatty acid content in the control group ($P \leq 0.05$), mainly shown in the relative contents of fatty acid C18:1 ω-9 cis, C20:4 ω-6, and C22:6 decreased. There was no difference in the ratio of 22:6–20:4 among the four groups ($P > 0.05$).

In addition, the relative content of ω-3 PUFA and ω-6 PUFA in the brain tissue of mice fed exogenous DHA milk, endogenous DHA milk, or normal milk groups were lower than that of mice in control group ($P \leq 0.05$). The ratio of ω-3PUFA to ω-6 PUFA did not differ among the three treatment groups and the control group ($P > 0.05$).

Table 1
Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activity in 10 % brain homogenate, 10 % liver homogenate, and serum of SAMP8 mice supplemented with exogenous DHA milk, endogenous DHA milk, normal milk, or 0.90 % saline solution (control group). Values represent LSMEANS with SD and maximal SE.

Metabolites	Group				SE	P-value
	Exo (n = 10)	End (n = 10)	Nor (n = 10)	Con (n = 9)		
Brain SOD (U/mL)	95.9 ± 3.52^{a}	89.7 ± 6.11^{b}	81.3 ± 1.99^{c}	77.8 ± 1.93^{d}	1.76	< 0.01
Brain GSH-Px (U/mL)	172 ± 42.4^{ab}	234 ± 87.5^{a}	144 ± 38.8^{b}	163 ± 52.5^{ab}	27.0	0.03
Liver SOD (U/mL)	113 ± 3.77^{ab}	115 ± 2.55^{a}	107 ± 11.9^{b}	104 ± 14.3^{ab}	4.31	< 0.01
Liver GSH-Px (U/mL)	76.5 ± 6.97^{a}	90.3 ± 18.2^{a†}	79.6 ± 9.26^{a}	76.4 ± 7.21^{a†}	5.27	0.34
Serum SOD (U/mL)	101 ± 6.95^{ac}	105 ± 4.49^{a}	93.8 ± 7.86^{bc}	91.7 ± 4.41^{b}	2.83	< 0.01
Serum GSH-Px (U/mL)	88.1 ± 13.6^{a}	94.6 ± 19.8^{a}	94.0 ± 7.18^{a}	98.2 ± 11.9^{a}	6.40	0.47

Exo, Exogenous DHA milk group; End, Endogenous DHA milk group; Nor, Normal milk group; Con, Control group. a, b, c different letters in the same row with different subscripts differ ($P \leq 0.05$). † values in the same row with this symbol tend to differ ($0.05 < P \leq 0.1$).

3.3. Polar lipids composition in brain

A total of 103 polar lipids were determined in brain (Table S3), including LPI, PI, LPE, PE, Cb, Cer, LPC, PC, SM, LPA, PA, LPG, PG, LPS, PS, S1P, AA, and DHA (Table S4). Furthermore, the correlation coefficient (R^2) of the standard curve of 12:0 LPC and 14:0 LPA was 0.9532 and 0.9627, respectively. And the recovery rate of sample mixed with the standard was between 82.68 % and 101.80 %.

The content of PC in the brain tissue of SAMP8 mice was the highest. Among them, the relative content of PC in the exogenous DHA group tended to be different ($P = 0.07$), compared with the control group. Among all Lys phospholipids, the content of LPC is higher than others, accounting for over 1 % of the total phospholipids.

After statistical analysis, the proportion of some polar lipids with intergroup differences ($P \leq 0.05$) or tendency differences ($0.05 < P \leq 0.10$) in the total polar lipids is shown in Fig. 1. The relative content of 17:0 Ceramide ($P = 0.04$) in the exogenous DHA group was higher than that in the control group, while the relative content of 20:4 LPC ($P = 0.04$) was lower than that of the control group. In the exogenous DHA group, 18:0/22: 6 PG ($P = 0.07$) and 18:2 LPA ($P = 0.09$) tended to be higher than in the control group, but d18:1/24:0 SM ($P = 0.06$) tended to be lower than that of the control group. Endogenous DHA milk also had effects on the composition of polar lipids in mice brain. Compared with normal milk, the relative content of 18:0/18:2 PS ($P = 0.04$) in endogenous DHA group was increased, and the relative content of 18:0/20:4 PS ($P = 0.08$) showed an increasing trend. In endogenous DHA group, the 20:4 LPC ($P = 0.06$) tended to be lower than the control group. The relative content of 18:0/18:0 PE ($P = 0.08$) in endogenous group tended to be higher than that of exogenous DHA group. In addition, the relative content of 20:4 LPC ($P = 0.01$) in the normal milk group was lower than that in the control group. The relative content of 22:0 Ceramide ($P = 0.08$) and 18:0/18:0 PC ($P = 0.06$) in the normal milk group tended to be lower and higher than those in the control group, respectively.

3.4. Lipid oxidation metabolite composition in brain

A total of 10 lipid oxidation metabolites were detected (Table S5), including POVPC, PONPC, KOdiAPC, prostaglandin, and EET. Principal-component analysis was conducted on lipid oxidation metabolites from 4 groups (Fig. 2). The results showed that the top three principal

Table 2
Relative quality (×100 %) of fatty acids in brain of SAMP8 mice supplemented with exogenous DHA milk, endogenous DHA milk, normal milk, or 0.90 % saline solution (control group). Values represent LSMEANs with SD and maximal SE.

Fatty acid (wt%)	Group				SE	P-value
	Exo (n = 10)	End (n = 10)	Nor (n = 10)	Con (n = 9)		
C8:0	1.92 ± 0.403^{a}	1.58 ± 0.567^{a}	1.7 ± 0.287^{a}	1.03 ± 0.675^{a}	0.230	0.05
C11:0	0.533 ± 0.14^{a}	0.48 ± 0.15^{a}	0.495 ± 0.112^{a}	0.273 ± 0.159^{b}	0.0647	< 0.01
C14:0	0.825 ± 0. 235^{a}	0.981 ± 0.155^{a}	0.833 ± 0.138^{a}	0.453 ± 0.154^{b}	0.0815	< 0.01
C15:0	0.0721 ± 0.0553^{a}	ND^{b}	ND^{b}	0.0873 ± 0.0358^{a}	0.0151	< 0.01
C16:0	42.5 ± 2.26^{b}	45.4 ± 2.31^{a}	45.8 ± 1.53^{a}	35.6 ± 6.27^{c}	1.59	< 0.01
C16:1	0.245 ± 0.0979	0.203 ± 0.0718	0.175 ± 0.0493	0.521 ± 0.315^{a}	0.0762	< 0.01
C17:0	0.0602 ± 0.0455^{bc}	0.109 ± 0.0612^{ab}	0.0409 ± 0.0224^{c}	0.140 ± 0.0581^{a}	0.0225	< 0.01
C18:0	34.0 ± 3.17^{a}	33.3 ± 2.68^{ab}	35.2 ± 1.34^{a}	27.4 ± 5.97^{b}	1.62	< 0.01
C18:1 ω-9 cis	6.41 ± 0.597^{b}	6.27 ± 1.07^{b}	5.58 ± 0.876^{b}	9.85 ± 2.81^{a}	0.715	< 0.01
C18:2 ω-6 trans	0.153 ± 0.406^{a}	ND^{a}	ND^{a}	0.00992 ± 0.0169^{a}	0.0947	0.07
C18:2 ω-6 cis	1.23 ± 1.74^{ab}	1.09 ± 1.44^{ab}	0.678 ± 0.591^{b}	3.77 ± 5.21^{a}	1.28	0.03
C18:3 ω-6	ND^{b}	ND^{b}	ND^{b}	0.043 ± 0.541^{a}	0.0119	< 0.01
C18:3 ω-3	ND^{a}	ND^{a}	ND^{a}	0.0802 ± 0.142^{b}	0.0313	< 0.01
C20:0	0.164 ± 0.096^{a}	ND^{b}	ND^{b}	0.193 ± 0.051^{a}	0.0249	< 0.01
C20:1 ω-9	0.933 ± 1.47^{a}	0.46 ± 0.142^{a}	0.363 ± 0.0828^{a}	0.717 ± 0.445^{a}	0.348	0.06
C20:2	0.0518 ± 0.135^{a}	ND^{b}	ND^{b}	0.179 ± 0.181^{a}	0.0412	< 0.01
C21:0	0.205 ± 0.109^{a}	ND^{b}	ND^{b}	0.439 ± 0.292^{a}	0.0713	< 0.01
C20:4 ω-6	4.89 ± 2.23^{b}	3.84 ± 1.09^{b}	3.48 ± 0.52^{b}	7.93 ± 2.44^{a}	0.764	< 0.01
C22:0	ND^{a}	ND^{a}	ND^{a}	0.0851 ± 0.167^{b}	0.0367	< 0.01
C20:5 ω-3	0.124 ± 2.13^{a}	0.419 ± 0.133^{a}	0.45 ± 0.0937^{a}	0.112 ± 0.0783^{a}	0.0846	0.59
C22:1 ω-9	0.089 ± 0.112^{ab}	0.265 ± 0121^{a}	0.154 ± 0.0628^{b}	0.135 ± 0.0805^{b}	0.0457	< 0.01
C24:0	0.118 ± 0.0891^{ab}	0.24 ± 0.111^{a}	0.116 ± 0.0599^{b}	0.245 ± 0.134^{a}	0.0462	< 0.01
C22:6 ω-3	6.11 ± 1.18^{b}	5.43 ± 1.57^{b}	5.03 ± 0.678^{b}	10.5 ± 3.56^{a}	0.921	< 0.01
C24:1 ω-9	0.119 ± 0.0929^{ab}	0.185 ± 0.0811^{a}	0.0939 ± 0.0584^{b}	0.226 ± 0.207^{ab}	0.0554	0.04
22:6/20:4	1.21 ± 0.271^{a}	1.41 ± 0.128^{a}	1.45 ± 0.0931^{a}	1.33 ± 0.217^{a}	0.122	0.42
SFA[1]	80.4 ± 4.24^{b}	82.1 ± 4.68^{b}	84.2 ± 2.32^{b}	65.9 ± 12.5^{a}	3.16	< 0.01
MUFA[2]	7.79 ± 1.45^{b}	7.38 ± 1.24^{b}	6.36 ± 1^{b}	11.5 ± 3.51^{a}	0.920	< 0.01
PUFA[3]	12.5 ± 4.01^{b}	10.6 ± 3.7^{b}	9.46 ± 1.57^{b}	22.6 ± 9.51^{a}	2.47	< 0.01
ω-3 PUFA	6.23 ± 1.24^{b}	5.64 ± 1.61^{b}	5.30 ± 0.64^{b}	10.7 ± 3.63^{a}	0.939	< 0.01
ω-6 PUFA	6.27 ± 3^{ab}	4.93 ± 2.35^{b}	4.16 ± 1.05^{b}	11.8 ± 6.93^{a}	1.78	< 0.01
ω-6 PUFA/ω-3 PUFA	0.980 ± 0.305^{a}	0.869 ± 0.229^{a}	0.78 ± 0.133^{a}	1.08 ± 0.474^{a}	0.140	0.09

Exo, Exogenous DHA milk group; End, Endogenous DHA milk group; Nor, Normal milk group; Con, Control group. wt%: weight percentage ND: Not detected.
[1]SFA Saturated fatty acid.
[2]MUFA monounsaturated fatty acid.
[3]PUFA polyunsaturated fatty acid.
a, b, c letters in the same row with different superscripts differ (P ≤ 0.05).

components (PC1: 35.9 %, PC2: 18.3 %, and PC3: 12.1 %) could distinguish endogenous DHA milk group from exogenous DHA milk group, normal milk group, and control group (Fig. 2a). The main contributing metabolites that distinguish the endogenous milk group from the other three groups are PGE2, PGF2α, 14, 15-EET, and PONPC (Fig. 2b). However, according to statistical analysis, the contents of these 10 lipid oxidation metabolites did not differ among groups.

3.5. Morris water maze test

During the four-day training session, the escape latency time of mice of endogenous DHA milk group, exogenous DHA milk group, and control group tended to decrease (Fig. 3a), but there is no difference among 4 groups (P > 0.05).

The crossing times of platform location at the test day of mice in the endogenous DHA milk group was more than that of mice in the other three groups (P ≤ 0.05, Fig. 3b). However, the crossing times of platform location at the test day of the exogenous DHA milk group was less than normal milk group and control group. Among them, although the content of DHA in endogenous DHA milk and exogenous DHA milk was similar, the effects of the two treatments on the cognitive ability of mice were different (P ≤ 0.05). The Fig. 4 presents the Spearman correlations of metabolites in brain tissue, serum, and liver tissue with the mice crossing times of 4 groups. According to Fig. 4, the crossing times of platform location of mice were related with liver GSH-Px activities (P ≤ 0.05) but not related to SOD and GSH-Px activities in brain (P > 0.05).

4. Discussion

In the current study, supplementation of DHA milk, whether it is exogenous or endogenous DHA, increased the SOD activity in the brain of SAMP8 mice (Table 1). The activity of brain GSH-Px was increased in the endogenous DHA group. Supplementing DHA can alleviate peroxidation, not because DHA have antioxidant functions directly. Instead, it acts by activated PPAR expression. In vivo, DHA synthesized primarily in the liver, but also in the brain, testicles, and kidneys. The synthesis is limited by δ-5 desaturase and δ-6 desaturase and substrate [36]. During peroxidation status, the δ-5 desaturase and δ-6 desaturase enzymes required for the synthesis of polyunsaturated fatty acids in the liver are downregulated, and attack by ROS causes free radical reactions in ω-3 PUFAs such as DHA. This results in a decrease in the abundance of ω-3 PUFA in the body [19]. Low levels of ω-3 PUFA were associated with downregulation of PPAR-α and upregulation of SREBP-1c and NF-κB [37]. Dietary supplementation of DHA upregulates the activities of PPARα and Nrf2, accompanied by downregulating the activities of NF-kB and SREBP-1c, thus exerting antioxidant effects [38]. Activation of PPAR expression increases SOD levels and maintain the overall homeostasis of the glutathione system [39–41]. In previous study, DHA-supplemented diet increased SOD2 activity in the rat brain [18]. In addition, DHA could up-regulate γ-glutamyl-cysteinyl ligase, glutathione reductase, and glutathione S-transferase in human fibroblast cells [42]. As the precursor of DHA in vivo synthesis, EPA has the effect of restoring liver SOD activity in rats with non-alcoholic fatty liver disease [43]. In clinical trials, supplementation with ω-3 fatty acids increased GSH-Px levels in serum [22].

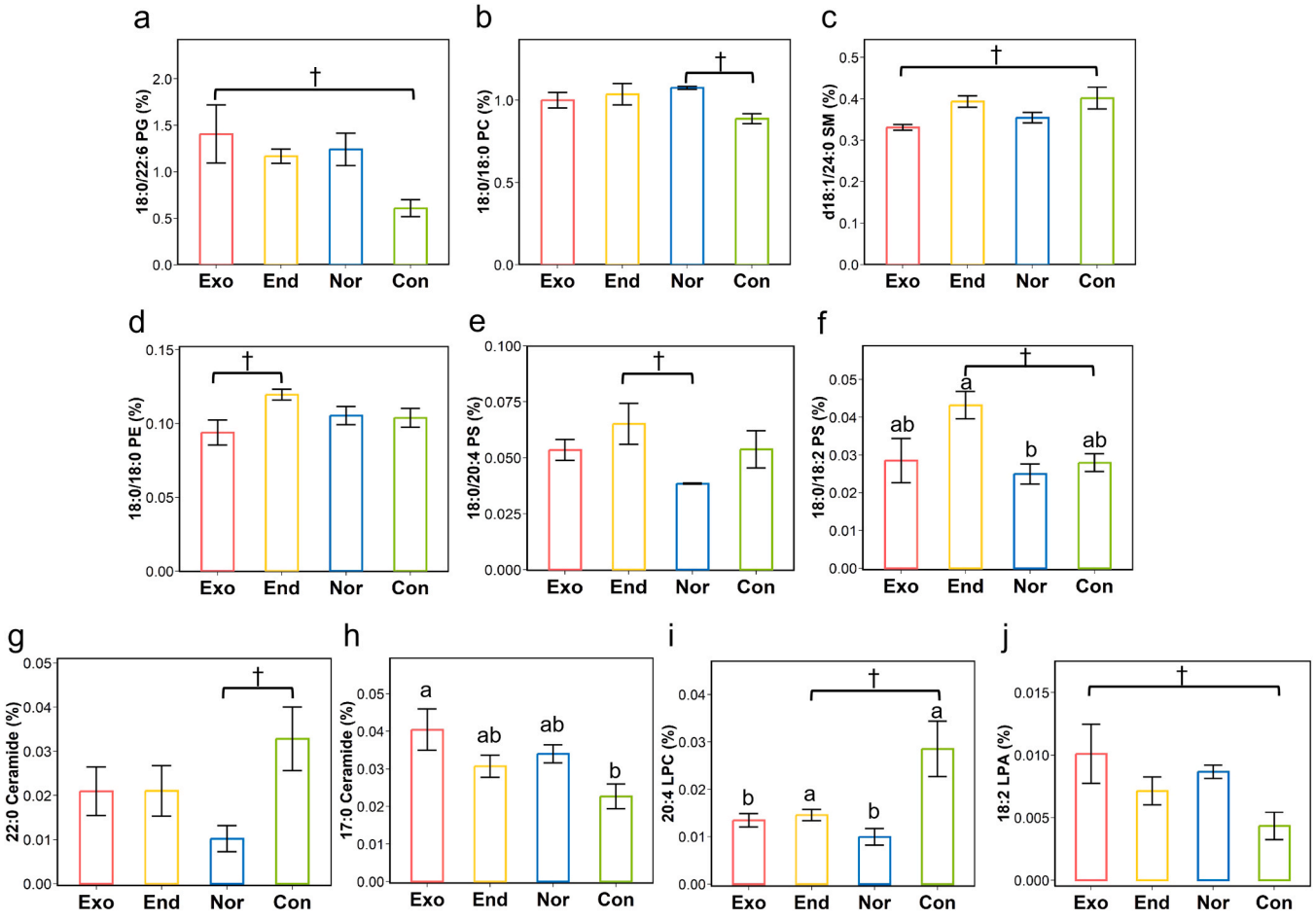

Fig. 1. Composition details for each phospholipid in brain of mice supplemented with exogenous DHA milk, endogenous DHA milk, normal milk, 0.9 % saline solution (control group). Exo, Exogenous DHA milk group; End, Endogenous DHA milk group; Nor, Normal milk group; Con, Control group; PG, Phosphatidylglycerol; PC, Phosphatidylcholine; SM, Sphingomyelin; PE, Phosphatidyl-ethanolamine; PS, Phosphatidylserine; LPC, Lysophosphatidylcholine; LPA, Lysoglycerophosphatidic acid. Bars with different letter differ ($P \leq 0.05$). Bars with † means trend of difference ($0.05 < P \leq 0.1$).

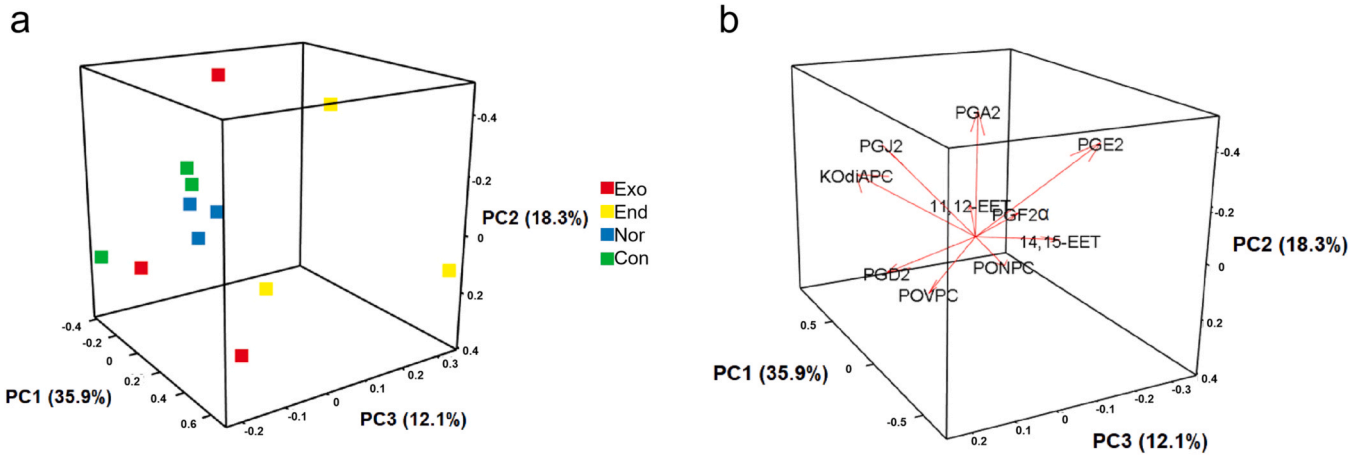

Fig. 2. PCA score plot (a) and PCA loading plot (b) of Lipid metabolites in brain of mice supplemented with exogenous DHA milk, endogenous DHA milk, normal milk, 0.9 % saline solution (control group). Exo, Exogenous DHA milk group; End, Endogenous DHA milk group; Nor, Normal milk group; Con, Control group; EET, Epoxye-icosatrienoic acid; PG, Prostaglandin; POVPC, 1-Palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine; PONPC, 1-Palmitoyl-2-(9-oxononanoic acid)-sn-glycero-3-phosphocholine; KOdiAPC, 1-(Palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine.

By comparing the activity values of GSH-Px and SOD in brain tissue, liver tissue and serum, it can be seen that endogenous DHA milk performed better than exogenous DHA milk in enhancing the antioxidant status of mouse organs. This may be due to the higher bioavailability of DHA in endogenous DHA milk than exogenous DHA milk. Our previous study showed that milk obtained by feeding goats with *Schizochytrium sp.* had a greater content of DHA at the sn-2 position of triglycerides [44]. DHA located at the sn-2 position in triglycerides may have higher biological activity [45].

Although exogenous DHA milk and endogenous DHA milk contained

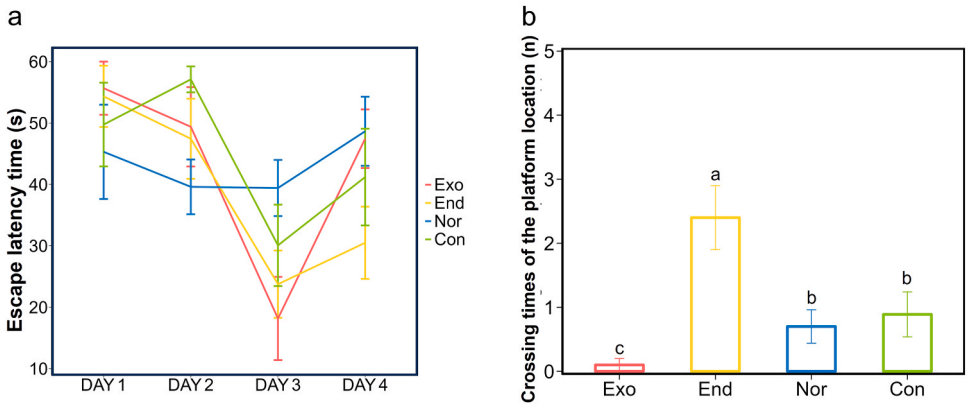

Fig. 3. Escape latency time during the training days (a) and crossing times of the platform location during the test day (b) in the Morris water maze test of mice supplemented with exogenous DHA milk, endogenous DHA milk, normal milk or 0.9 % saline solution (control group). Exo, Exogenous DHA milk group; End, Endogenous DHA milk group; Nor, Normal milk group; Con, Control group. Values represent means ± SEM. Bars with different letters differ ($P \leq 0.05$).

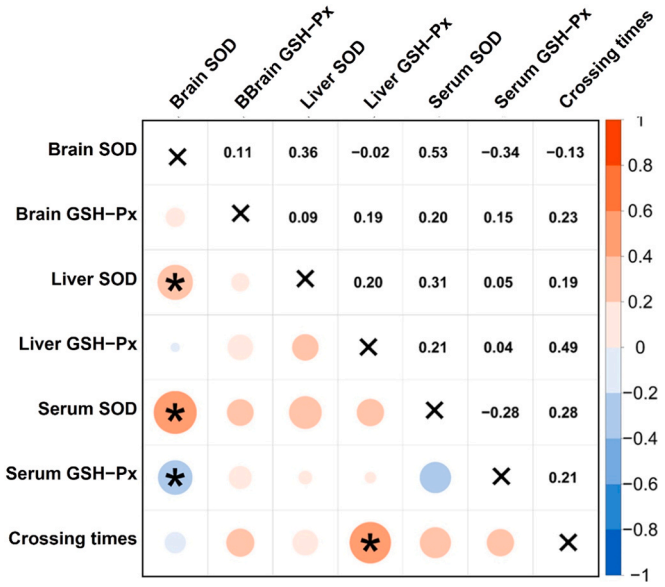

Fig. 4. Spearman correlation of SOD, GSH-Px from brain, liver, and serum and crossing times in the Morris water maze test; Red dot means positive correlation; Blue dot means negative correlation; Values represent R-value, with * indicating $P \leq 0.05$.

a similar dose of DHA, this content had limited effect on the composition of total fatty acids in milk. The dominant fatty acids in milk are still SFA. Compared with the control group, the relative contents of C16:0, C18:0, and C14:0 in the exogenous DHA group, the endogenous DHA group, and the normal milk group were increased, indicating that dietary supplementation of milk affected the brain tissue of the mice, as these three fatty acids are the main fatty acids in milk fat. In addition, the relative contents of 20:4 and 22:6 in brain tissue of mice in the exogenous DHA group, endogenous DHA group, and normal milk group were lower than those in control group. This is reasonably presumed to be influenced by the increase in C16:0 and C18:0 content. After milk supplementation, the fatty acid composition of the mice brain was more affected by the high content of SFA in milk than by the low content of DHA. According to another study, dietary DHA supplementation could significantly increase the levels of DHA in the plasma and liver [46]. This may be because of oral supplementation in that study was algae oil, in which DHA is the dominant fatty acid component. As shown in Table 2, the ratio of 22:6–20:4 content in brain tissue was not different among the four groups ($P > 0.05$), which also indicated that at this dose dietary supplementation with milk would not alter the balance of the ratio of these two fatty acids in brain tissue. Also, it is noteworthy that while C18:1 ω-9 cis is the major fatty acid in milk fat (about 20 %) [28], the relative content of C18:1 ω-9 cis in the exogenous DHA group, endogenous DHA group, and normal milk group was lower than the control group. This may also be influenced by SFA in milk, which is higher than C18:1 ω-9 cis in milk.

Supplementing DHA containing milk had a positive impact on alleviate oxidative status in the brain of SAMP8 mice, as indicated by increased SOD in brain. This did not increase the percentage of DHA in total fat content of brain tissue. Based on this result, it was derived that the relative content of DHA is not related to the degree of oxidative stress in the brain.

The overall composition of polar lipid in SAMP8 mice was not affected by dietary supplementation of DHA milk powder, and the content of some polar lipid molecules increased or decreased. This is very different from the changes in mouse brain fatty acid composition. Maintaining the stability of polar lipid composition in brain tissue is critical to brain health. Brain polar lipid are composed mostly of cholesterol, glycerophospholipids (PC, PE, and PI), and sphingolipids (SM, Cb, sulfatides, and gangliosides) [47]. In this study, the effect of exogenous DHA milk on polar lipid in mice brain tissue was greater than that of endogenous DHA milk. However, these changes did not have a significant effect on the total content of polar lipid. In terms of polar lipid species, the content of PC tended to be affected by dietary supplementation with DHA-containing milk (Table S4). Particularly, the composition of 20:4 LPC in mice brain was decrease ($P \leq 0.05$) due to the influence of exogenous DHA milk or tend to be decrease ($0.05 < P \leq 0.1$) due to the influence of endogenous DHA milk than control group (Fig. 1). LPC induces NO overproduction, which may increase oxidative stress in endothelial cells [48].

Lipid metabolism is closely related to the degree of oxidative stress [49]. Lipid peroxidation is one of the manifestations of oxidative stress in the body. In this study, the contents of POVPC, PONPC, KOdiAPC, prostaglandin, and EET were not affected by dietary supplementation with both endogenous DHA milk and exogenous DHA milk.

It is worth noting that although exogenous DHA milk and endogenous DHA milk contained the same dose of DHA in this study, they had similar effects on oxidative stress-related metabolites and different effects on cognitive performance in SAMP8 mice. This may be related to the difference in bioavailability due to the DHA in the two kinds of milk. The absorption and utilization of different forms of DHA are different. It is now known that DHA produced in microalgae is esterified on triglyceride [50], so the DHA in exogenous DHA milk is DHA-TG (DHA esterified on triglyceride). The DHA in endogenous DHA milk may be DHA-TG and DHA-PL (DHA esterified on phospholipids). DHA-PL could be cross the blood-brain barrier for brain uptake due to Mfsd2a transport

[51]. Antonio Gázque et al. [52] reported that there was no difference between 2.5 % DHA-TG (DHA esterified on triglycerides) group (n = 10), 2.5 % DHA-PL group (n = 9) and 9 % DHA-PL group (n = 9) in the aggregation of DHA in rat fetal brains. However, it also report that DHA-TG supplementation has no impact on cognition in the elderly with Alzheimer's disease [53]. Therefore, to clearly evaluate the nutritional properties of endogenous DHA milk and exogenous DHA milk, lipid composition analysis will be an important breakthrough point.

The cognitive performance is not only determined by the level of oxidative stress in the brain, but also related to mouse brain phospholipids and lipid metabolite composition. Accumulation of damage caused by oxidative stress, such as oxidized proteins, glycation products, and lipid peroxidation, can lead to neuronal degeneration, which can affect cognitive performance [54]. Peroxidation and oxidative stress of brain tissue lipids are thought to cause cognitive and behavioural impairments [55]. Compared with endogenous DHA milk, exogenous DHA milk influenced the polar lipid composition and lipid metabolites of mice brain, which may be one of the reasons for the significant differences in the results of cognitive and behavioural tests between the two groups. Furthermore, this study focused on the nutritional functionality of foods containing functional ingredients, rather than high-purity and high-dose functional ingredients, which may also be the reason of the poor performance of SAMP8 mice in the exogenous DHA milk group.

5. Conclusions

Supplementation of milk containing 0.33 % DHA of the total fatty acid alleviated the level of oxidative stress in brain and serum of SAMP8 mice as indicated by increased SOD activity. Compared with normal milk, endogenous DHA milk, but not exogenous milk, increased the activity of liver SOD. Exogenous DHA milk and endogenous DHA milk supplementation did not increase the relative contents of DHA and ω-3 PUFA in the brain tissue of mice, nor did they change ω-3 ratio of PUFA to ω-6 PUFA. A total of 113 polar lipid and lipid metabolites were detected in mice brain tissue. Several polar lipid relative content, such as 18:0/18:2 PS, 17:0 Ceramide, and 20:4 LPC in mice brain were affected by dietary supplementation with DHA containing milk. The contents of lipid oxidation metabolites POVPC, PONPC, KOdiAPC, prostaglandin, and EET were not affected by dietary supplementation with exogenous DHA milk and endogenous DHA milk. Endogenous DHA milk increased cognitive ability of mice as indicated by a greater number of crossing times of the platform location of mice in the water maze test. In the current study, endogenous DHA milk had better effects on oxidative stress and cognition than exogenous DHA milk. Future studies should focus on the composition and molecular structure identification of lipids in endogenous and exogenous DHA milk to increase the functional properties of milk enriched with DHA.

CRediT authorship contribution statement

Bas Kemp: Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis. **Shilong Jiang:** Project administration. **Xiaoyang Pang:** Project administration, Conceptualization. **Shuwen Zhang:** Project administration, Conceptualization. **Ariette van Knegsel:** Writing – review & editing, Supervision, Methodology, Formal analysis, Data curation. **Akke Kok:** Supervision, Software, Methodology, Formal analysis. **Yumeng Zhang:** Writing – review & editing, Writing – original draft, Data curation. **Baorong Chen:** Writing – review & editing, Validation. **Huiquan Zhu:** Writing – review & editing, Writing – original draft, Validation. **Jiaping Lv:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Xiaodan Wang:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Jing Lu:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declared that they have no conflicts of interest to this work.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116467.

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