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The protective effect of eicosapentaenoic acid-enriched phospholipids from sea cucumber *Cucumaria frondosa* on oxidative stress in PC12 cells and SAMP8 mice ☆

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ABSTRACT

Alzheimer's disease (AD) is a common neurodegenerative disorders, in which oxidative stress plays an important role. The present study investigated the effect of eicosapentaenoic acid-enriched phospholipids (EPA-enriched PL) from the sea cucumber *Cucumaria frondosa* on oxidative injury in PC12 cells induced by hydrogen peroxide (H_2O_2) and tert-butylhydroperoxide (t-BHP). We also studied the effect of EPA-enriched PL on learning and memory functions in senescence-accelerated prone mouse strain 8 (SAMP8) *in vivo*. Pretreatment with EPA-enriched PL resulted in an enhancement of survival in a dose-dependent manner in H_2O_2 or t-BHP damaged PC12 cells. EPA-enriched PL pretreatment could also reduce the leakage of lactate dehydrogenase (LDH), and increase the intracellular total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) activity compared with the H_2O_2 or t-BHP group. The down-regulated Bcl-2 mRNA level and up-regulated Bax, Caspase-9, and Caspase-3 mRNA expression induced by H_2O_2 or t-BHP could be restored by EPA-enriched PL pretreatment. These results demonstrated that EPA-enriched PL exhibited its neuroprotective effects by virtue of its antioxidant activity, which might be achieved by inhibiting the mitochondria-dependent apoptotic pathway. The neuroprotective effect of EPA-enriched PL was also verified *in vivo* test: the EPA-enriched PL administration prevented the development of learning and memory impairments in SAMP8 mice. Our results indicated that EPA-enriched PL could offer an efficient and novel strategy to explore novel drugs or functional food for neuronprotection and cognitive improvement.

1. Introduction

Alzheimer's disease (AD) mainly affects elderly individuals, and is becoming an increasingly serious socioeconomic problem, the etiology of AD is thought to be complex and remains unclear (Fukasawa et al., 2012). By the year 2050, 30% of the total population will be over 65 years of age and a large number of those individuals may show signs of neurodegenerative disease. This represents a significant economic and emotional burden, not only to the health system but also to the families of those affected and the individuals themselves (Casadesus et al., 2012). Increased oxidative stress is widely accepted to be a factor in the development and progression of AD (Gotz et al., 1994). Oxidative stress occurs when the generation of free radicals of oxygen and nitrogen exceeds the endogenous antioxidant capacity of the cells, causing neuronal death and neurodegeneration in brain (Ferreiro et al., 2012). Hydrogen peroxide (H_2O_2), a natural byproduct of enzymatic oxidase action, is an endogenous source of hydroxyl free radicals that contributes to the background level of cellular oxidative stress (Halliwell, 1992). tert-Butylhydroperoxide (t-BHP), a short-chain analogue of lipid hydroperoxides, has been used as a model compound to investigate the mechanisms of cell damage initiated by oxidative stress. It can be metabolized to initiate lipid peroxidation and damage cell integrity (Lee et al., 2008; Palozza et al., 1996). Many studies have shown that these stimuli elevate oxidative stress by initiating mitochondrial dysfunction, which is

Abbreviations: AD, Alzheimer's disease; EPA-enriched PL, eicosapentaenoic acid-enriched phospholipids; H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; SAMP8, senescence-accelerated prone mouse strain 8; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; t-BHP, tert-butylhydroperoxide.

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associated with changes in the Bcl-2 family of proteins, the release of cytochrome-c, and activation of caspases (Yu et al., 2008). The rat pheochromocytomaline cell line PC12 provides a useful model system for neurological and neurochemical studies (Greene and Tischler, 1976).

The senescence-accelerated prone mouse (SAM) is an accelerated aging model that was established from the AKR/J strain. The SAM strain is a group of related inbred strains including nine strains of accelerated-senescence prone, short-lived mice (SAMP) and three strains of accelerated senescence-resistant, long-lived mice (SAMR) (Okuma and Nomura, 1998). Among SAMP mice, SAMP8 mice show age-related impairment in learning and memory that occurs as early as 2 months, which is not the case for SAMR1 mice of the same age (Takeda, 2009; Chen et al., 2004). SAMP8 mice are considered as a good model to study the fundamental mechanisms of age-related learning and memory deficits including those related to AD.

Sea cucumbers are cylinder-shaped invertebrates that live in a variety of sea floor habitats from warm tropical waters to cold deep-sea trenches. They are considered to be an important food in the Indo-Pacific region including the Philippines, Malaysia, Japan, Korea, and China. Sea cucumber contains various bioactive substances such as saponins, polysaccharides, cerebrosides, gangliosides, and phospholipids (Bordbar et al., 2011). Our previous studies have indicated that dietary saponins and cerebroside obtained from sea cucumber could improve certain metabolic parameters associated with obesity and alleviate hepatic steatosis in rats (Hu et al., 2012; Zhang et al., 2012). The sea cucumber *Cucumaria frondosa* is a widespread benthic animal inhabiting the North Atlantic and Western Arctic seas. Previous studies have reported a high eicosapentaenoic acid (EPA) content in the frigid genus *C. frondosa* (Ying et al., 2007). Xu et al., 2013 found that sea cucumber EPA phosphatidylcholine (EPA-PC) had a protective effect on renal injury in a diabetic rat model. In our previous studies, we found that sea cucumber cerebrosides have a neuroprotective effect on the oxidative damage induced by t-BHP (Wu et al., 2012). However, no study has yet been examined the neuroprotective effect of sea cucumber phospholipids.

In the present study, we first evaluated the protective effect of eicosapentaenoic acid-enriched phospholipids (EPA-enriched PL) obtained from the sea cucumber *C. frondosa* against H_2O_2 or t-BHP-induced cytotoxicity in PC12 cells *in vitro*, and the possible mechanisms thereof. Predicated on the *in vitro* results, we further verified the effect of the EPA-enriched PL on SAMP8 mice *in vivo*. Our results contributed to the development of marine organisms as functional foods for neuroprotection and preventing the cognitive decline associated with aging.

2. Materials and methods

2.1. The preparation and fatty acid compositions analysis of EPA-enriched PL

The fresh sea cucumber, *C. frondosa*, were collected on the Nanshan aquatic market of Qingdao, and EPA-enriched PL was extracted from the body wall of *C. frondosa*, which was carried out in Food Science and Human Health Laboratory of Ocean University of China (Qingdao, China). The total lipids were extracted from the samples according to the modified method of Folch et al. (1957) and then mixed with one-fifth volume of 0.15 M NaCl solution. The mixture was placed into a separatory funnel and kept for 24 h and then the chloroform solution was evaporated to dryness under vacuum. Then phospholipids were separated from other lipids by a silica-gel column chromatography using chloroform, acetone and methanol, and the fatty acid compositions of EPA-enriched PL were determined by the method of Lou et al. (2012). The main fatty acid composition was 20:5, 18:0, and 20:1, the contents were 57.86%, 9.59%, and 8.65%, respectively, and other compositions were in a minor content.

For *in vitro* experiments, the EPA-enriched PL was prepared in liposome referring to the method of Hossain et al. (2006), and diluted to the appropriate concentration by medium for following experiments.

2.2. Cell culture and treatment

Rat pheochromocytoma cells (PC12) purchased from Shanghai institute of biochemistry and cell biology (Shanghai, China) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The medium was changed every other day. To study the effects of EPA-enriched PL on PC12 cells, cells were preincubated with EPA-enriched PL at the indicated concentrations for 24 h, and 500 μmol/L of H_2O_2 or 300 μmol/L of t-BHP was added to the medium for 4 h.

2.3. Determination of cell viability

For cell viability determination, the MTT assay was conducted after exposure to H_2O_2 or t-BHP. At the end of incubation time, the supernatant was discarded and the cells were rinsed with phosphate-buffered saline (PBS). Then MTT (0.5 g/L), dissolved in DMEM, was added to each well. After additional 4 h incubation, the medium was removed, and 200 μL acidated dimethylcarbinol was added to each well. The absorbance at 570 nm of solubilized MTT formazan products was measured using microplate reader (Model 680, Bio-Rad, Tokyo, Japan). Cell viability was expressed as a percentage of the value in the control.

2.4. Lactate dehydrogenase (LDH) release assay

PC12 cells were seeded in a 96-well culture plate at 1.0×10^5 cells/mL and LDH leakage in the conditioned medium, an indicator of cellular injury, was detected with a colorimetric assay that measured the conversion of lactic acid to pyruvic acid by LDH, the protocol was supplied by manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). The absorbance of the samples was read at 440 nm. The data was expressed as percentage of the fluorescence values in the normal group.

2.5. Measurement of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) activity

PC12 cells were seeded in a 6-well plate at a density of 5.0×10^5 cells/mL. At the end of the sample treatment, the cells were washed with PBS, scraped from the plate into ice-cold PBS and homogenized. T-AOC was determined with a commercial T-AOC assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). This method was based on the oxidation of intracellular antioxidants with Fe^{3+} in acidic medium. The liberated Fe^{2+} reacts with 1, 10-phenanthroline to form a colored complex, which was measured at 520 nm. One unit of T-AOC was defined as the capability of increasing 0.01 optical density values per mg protein per min at 37 °C.

SOD activity was measured using SOD activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China) according to the manufacture's protocol. This method was based on the production of superoxide radicals during the conversion of xanthine to nitrite by xanthine oxidase. The calculated SOD activity

was expressed as U/mg protein in the cells. The protein content was measured using the Bradford method (Bradford, 1976).

2.6. RNA extraction and quantitative RT-PCR

Total cellular RNA was isolated from PC12 cells using TRIzol reagent (Invitrogen, USA). The concentration of total RNA was assessed by Nanodrop2000 (Thermo Scientific, USA). 1 μg of total RNA from each sample were reverse-transcribed to cDNA using random primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Madison, WI). After cDNA synthesis, quantitative RT-PCR was performed in the Bio-Rad iCycler iQ5 system as described previously (Du et al., 2012). The gene expression was determined by relative quantification using the standard curve method. Target gene mRNA concentration was normalized to the mRNA concentration of the housekeeping gene β-actin. The prime sequences for analyzed genes are shown in Table 1.

2.7. Animals and diets

One-month-old male SAMP8 mice and SAMR1 mice were purchased from Nanjing qingzilan Co. Ltd. (Nanjing, China). All animals were used according to the guidelines of ethical committee of experimental animal care at Ocean University of China. Mice were housed in an air-conditioned room with a 12-h light/dark cycle, a constant temperature of 23 ± 2 °C, and relative humidity of 65 ± 15%. At 4 months old, the SAMP8 mice were randomly assigned to two experimental dietary groups: one control group and one EPA-enriched PL group, each group contained 10 animals. Male SAMR1 mice (n = 10), which showed normal characteristics, were used as the external control. All animals were allowed free access to drinking water. SAMP8 control group and SAMR1 group mice were supplemented with AIN-93G diet and EPA-enriched PL group mice were supplemented with AIN-93G diet plus 0.5% EPA-enriched PL. The composition of the diets and EPA-enriched PL concentration are shown in Table 2. After 12 weeks of treatment, the mice were test in water maze or open-field.

2.8. Behavioral test

2.8.1. Morris water maze test

The procedures were similar to that described by Morris, (1981) with minor modifications. Briefly a circular stainless steel pool (130 cm in diameter and 50 cm in height) was divided into four quadrants which were marked with a triangle, square, diamond and circle, respectively. Ink staining water was filled into the pool and the temperature was set at 22 ± 1 °C. A circular black escape platform (9 cm in diameter and 29 cm in high) located 1 cm beneath the surface of the water in the middle of one of the quadrants. SAMR1 and SAMP8 mice were trained to find the platform with three trails on the first day, and then tested to find the hidden platform for 6 consecutive days. Each mouse was released and faced the wall of the maze. If the animal found the platform within 60 s, it was allowed to remain there for 10 s, if the mouse failed to locate the platform within 60 s, it was gently guided to the platform and allowed to stay there for 10 s and its escape latency was recorded as 60 s. The swim paths, distances and latencies taken to swim to the platform were monitored with a video camera linked to computer system. Probe tests were performed on the 7th day to evaluate spatial memory retention. The platform was removed from the pool and the mice were then placed in a position opposite the location of platform position and allowed to swim for 60 s. The number of crossings over the previous position of the platform and the time spent in the target quadrant in which the platform was hidden during the acquisition trails were recorded as measures for spatial memory.

Table 1
Sequences of the primers used in the quantitative RT-PCR.

Gene	Forward primers	Reverse primers
Bax	TCATCCAGGATCGAGCAGA	AAAGTAGAAGAGGGCAACCAC
Bcl-2	TGGGATACTGGAGATGAAGACT	CCACCGAACTCAAAGAAGG
Caspase-9	GCCTCATCATCAACAACGTG	CCTGGTATGGGACAGCATCT
Caspase-3	GACGACAGGGTGCTACGAT	ACAGACCAGTGCTCACAAGG
β-Actin	GCAGATGTGGATCAGCAAGC	GTCAAAGAAAGGGTGTAAAACG

Table 2
Composition of experimental diets (g/kg diet).

Ingredients	Control	EPA-enriched PL
Casein	200	200
Starch	499.5	499.5
Sucrose	100	100
Soy oil	50	48.3
Lard	50	48.3
Mineral mix[a]	35	35
Vitamin mix[b]	10	10
Cellulose	50	50
L-Cystine	3	3
Choline bitartrate	2.5	2.5
EPA-enriched PL	–	5

[a] AIN-93G mineral mix.
[b] AIN-93G vitamin mix.

2.8.2. Open-field test

The open-field test fear response to novel stimuli was used to assess locomotion, exploratory behavior, and anxiety. Open-field test protocols were modified from that of Lukacs et al. (1995). The apparatus consisted of a wooden box (diameter 50 cm, height 30 cm) with a video camera fixed at the top. After a pre-adaption period of 2 min, the animals were placed in the centre of the apparatus. The mice were video recorded and analyzed to calculate the total distance and average speed of each mouse over 5 min.

2.9. Statistical analysis

The data were expressed as mean ± standard error mean (SEM) and analyzed using the SPSS 10.0 software package. Differences between the groups were evaluated using one-way analysis of variance (ANOVA), followed by a Student's test. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effect of EPA-enriched PL on the cell viability in PC12 cells

As Fig. 1A shows, increasing doses of EPA-enriched PL not only had no toxic or inhibitory effect on the growth of PC12 cells, rather EPA-enriched PL promoted the growth rate of the cells at the test concentrations. The results demonstrated that EPA-enriched PL was not harm to PC12 cells, and had a positive effect on PC12 cells at the tested concentration range.

The MTT assay showed that, H_2O_2 and t-BHP induced cytotoxicity in the PC12 cells ($P < 0.05$, Fig. 1B). However, when the cells were treated with the EPA-enriched PL concentrations of 10 and 40 μg/mL for 24 h, and then incubated with H_2O_2 or t-BHP for another 4 h, the cell viability values reversed significantly and in a dose-dependent manner compared with the model group.

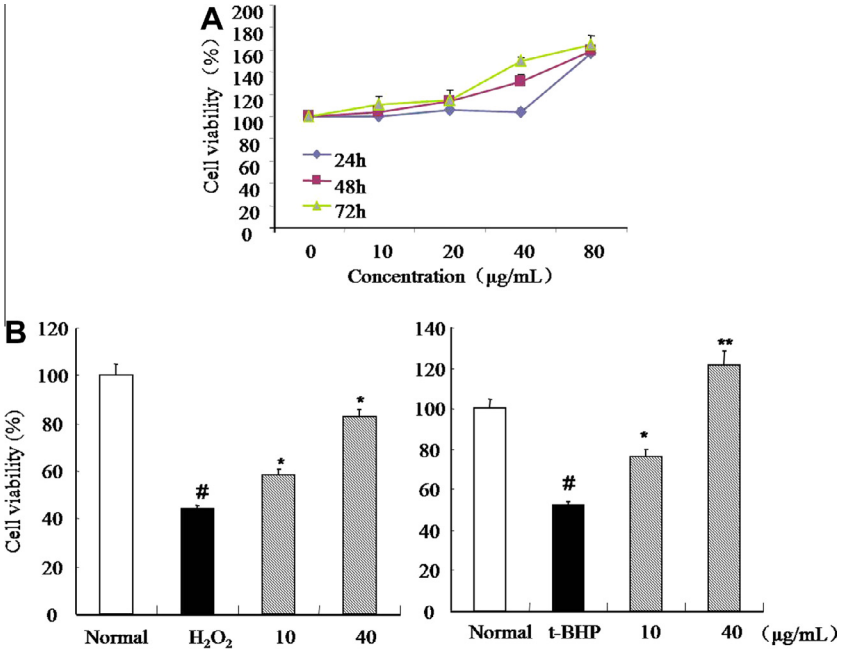

Fig. 1. Effect of EPA-enriched PL on the cell viability in PC12 cells. (A) PC12 cells were treated with various concentrations of EPA-enriched PL liposome for 24, 48 and 72 h, then MTT assay was used to determine the cell viability. There is no toxiferous effect of EPA-enriched PL on PC12 cells at the tested concentration range. (B) PC12 cells were pretreated with EPA-enriched PL (10 and 40 μg/mL) for 24 h, then the cells were exposed to 500 μM H_2O_2 or 300 μM t-BHP for 4 h, then MTT assay was used to measure the cell viability. Results showed that 10 or 40 μg/mL EPA-enriched PL exhibited the neuroprotective effects against both H_2O_2 and t-BHP-induced toxicity. Results were obtained from three independent experiments and were expressed as mean ± SEM. $^{\#}P < 0.05$, versus normal group, $^{*}P < 0.05$, $^{**}P < 0.01$, versus H_2O_2 or t-BHP group.

3.2. Effect of EPA-enriched PL on H_2O_2 or t-BHP-induced cytotoxicity

To further investigate the protective effects of EPA-enriched PL, an LDH assay was performed. As shown in Fig. 2, when PC12 cells were incubated with H_2O_2 or t-BHP for 4 h, the percentage of LDH leakage increased compared to the normal group ($P < 0.01$). In contrast, when the cells were pretreated with different concentrations of EPA-enriched PL, the LDH leakage dropped significantly, in a dose-dependent manner.

3.3. Effect of EPA-enriched PL on H_2O_2 or t-BHP-induced oxidative stress

To examine whether the effect of EPA-enriched PL on H_2O_2 or t-BHP-induced cytotoxicity was mediated through antioxidant actions, intracellular T-AOC and SOD activities were measured. As shown in Fig. 3, compared to the normal group, the H_2O_2 or t-BHP treatment groups showed largely reduced T-AOC and SOD activities, suggests that the cytotoxic effect of H_2O_2 and t-BHP in PC12 cells may be mediated by oxidative stress. Interestingly, EPA-enriched PL (10 and 40 μg/mL) strongly prevented the H_2O_2 or t-BHP-induced decrease in T-AOC and SOD activities, in a dose-dependent manner.

3.4. Effect of EPA-enriched PL on the levels of Bcl-2, Bax, Caspase-3 and Caspase-9

To investigate the protective mechanisms of EPA-enriched PL, the expression levels of Bcl-2, Bax, Caspase-3 and Caspase-9 were determined. Consistent with previous reports, the present study showed that H_2O_2 and t-BHP significantly decreased the Bcl-2 mRNA levels but upregulated the mRNA levels of Bax (1.4 and

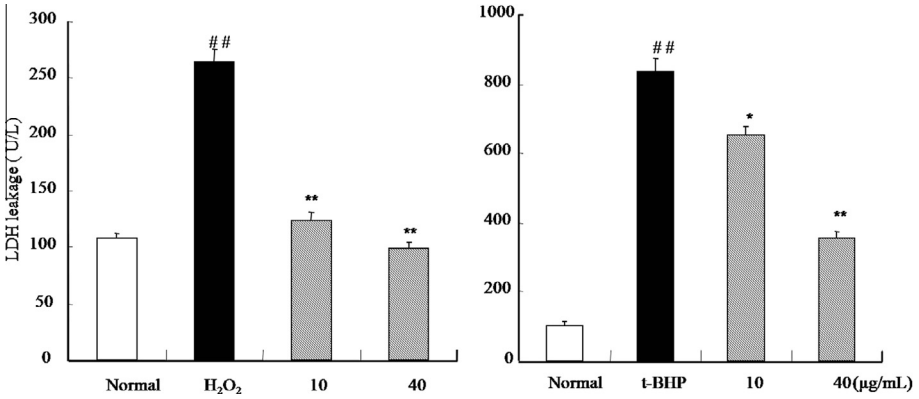

Fig. 2. Effects of EPA-enriched PL on the extracellular LDH levels in PC12 cells. PC12 cells were added 10 or 40 μg/mL EPA-enriched PL for 24 h, then exposed to 500 μM H_2O_2 or 300 μM t-BHP for 4 h. The medium was collected and determined using LDH assay kit according to the manufacturer's protocol. Treatment with EPA-enriched PL could reduce the leakage of LDH induced by H_2O_2 or t-BHP. Results were obtained from three independent experiments and were expressed as mean ± SEM. $^{\#\#}P < 0.01$, versus normal group, $^{*}P < 0.05$, $^{**}P < 0.01$, versus H_2O_2 or t-BHP group.

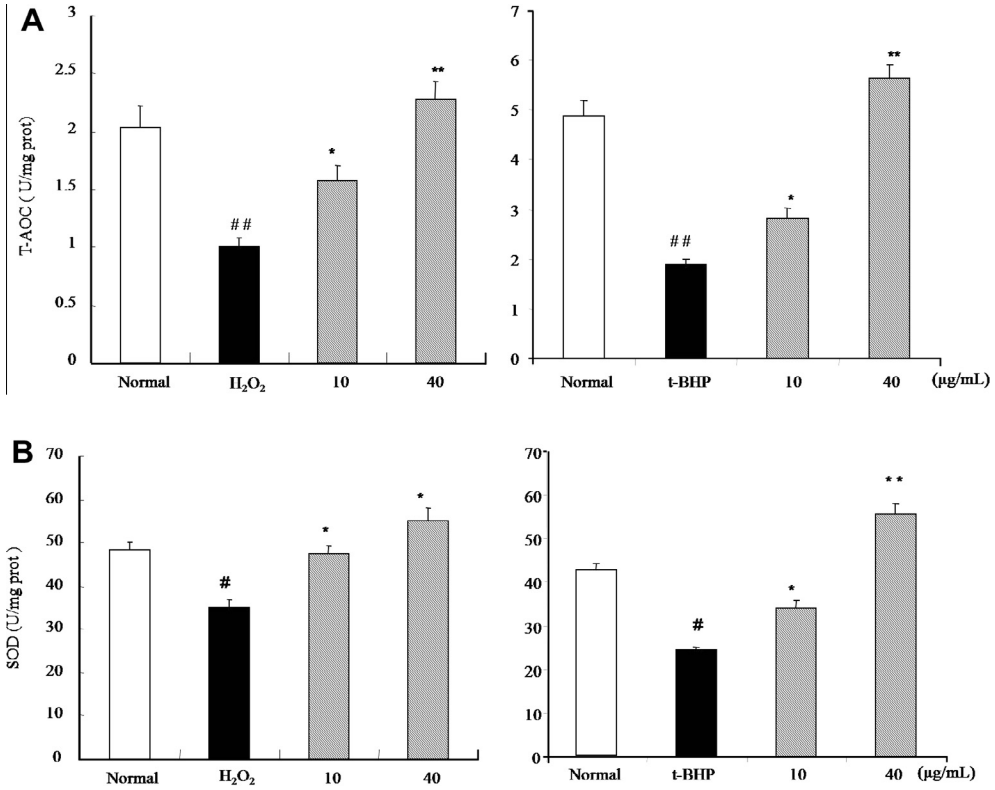

Fig. 3. Effects of EPA-enriched PL on T-AOC and SOD activities in PC12 cells. PC12 cells were pretreated with EPA-enriched PL (10 and 40 μg/mL) for 24 h, then the cells were exposed to 500 μM H_2O_2 or 300 μM t-BHP for 4 h, then the cells were collected and homogenized. Intracellular T-AOC and SOD activities were measured using T-AOC and SOD activity assay kit according to the manufacturer's protocol. (A) Effects of EPA-enriched PL on T-AOC activity in PC12 cells. (B) Effects of EPA-enriched PL on SOD activity in PC12 cells. Note that 10 and 40 μg/mL EPA-enriched PL strongly prevented the H_2O_2 or t-BHP-induced decrease in T-AOC and SOD activities in a dose-dependent manner. Data represent the mean ± SEM. of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group, $^{*}P < 0.05$, $^{**}P < 0.01$, versus H_2O_2 or t-BHP group.

4.0-folds), Caspase-3 (1.93 and 1.57-folds) and Caspase-9 (3.77 and 3.76-folds), respectively (Fig. 4). EPA-enriched PL at the concentrations of 10 and 40 μg/mL markedly inhibited the H_2O_2 or t-BHP-induced increase in Bax, Caspase-3 and Caspase-9 expression levels and, decrease in Bcl-2 mRNA expression in comparison to the model group and showed a dose-dependent manner.

3.5. Effect of EPA-enriched PL on learning and memory in SAMP8 mice

In order to assess the effects of EPA-enriched PL on learning and memory, we used an *in vivo* model of aging, SAMP8, and a control counterpart strain, SAMR1. The body weight of all the animals before the behavioral test did not differ between the SAMR1, SAMP8, and EPA-enriched PL groups (31.1 ± 1.8 g, 30.3 ± 1.5 g, and 30.2 ± 1.7 g, respectively). We determined the time required to find the platform (escape latency) as a function of days of training in the Morris water maze, and observed that the escape latency of SAMP8 mice was significantly longer than that of SAMR1 animals (Fig. 5A). However, SAMP8 animals treated with EPA-enriched PL showed improved performance, as indicated by a marked reduction of their escape latency compared to that of untreated SAMP8 mice.

A probe trail was conducted to assess spatial memory in all mice. As shown in Fig. 5B, there was a significant overall group difference in the number of platform crossings and time spent in the target quadrant, both of which were significantly lower for SAMP8 mice compared to SAMR1 mice. However, SAMP8 mice treated with EPA-enriched PL showed markedly increased number of platform crossings, and lengthened time spent in the target quadrant, compared to untreated SAMP8 animals.

3.6. Effect of EPA-enriched PL on the locomotor activity of SAMP8 mice

The water-maze results showed that the cognitive function of SAMP8 mice was worse than that of SAMR1 mice and, EPA-enriched PL could improve their cognitive function. However, EPA-enriched PL might affect behavior and how animals respond to different stimuli, generally. Therefore, we performed an open field test to examine the animals' locomotor activity. We found no significant difference in the total distance travelled among the three groups (Fig. 6A), suggesting that EPA-enriched PL treatment did not affect the locomotor performance of SAMP8 mice. We also recorded the ratio of the distance travelled in the central area to that in the total area in the open-field, which is an indirect measure of exploratory behavior and anxiety. This ration was higher for SAMP8 mice treated with EPA-enriched PL than for the untreated SAMP8 mice, which had a lower ratio than SAMR1 mice (Fig. 6B), demonstrating that EPA-enriched PL promoted exploratory behavior and diminished anxiety in SAMP8 mice.

4. Discussion

A variety of phospholipids can exert beneficial actions against dementia including that associated with Alzheimer's disease and age-related cognitive decline. Recent studies have reported that bovine brain cortex-derived phosphatidylserine (BC-PS) improved cognitive deficits in patients with senile dementia (Delwaide et al., 1986), soybean lecithin transphosphatidylated phosphatidylserine ameliorated amnesia in mice (Furnshiro et al., 1997), and phosphatidylcholine increased brain acetylcholine concentrations

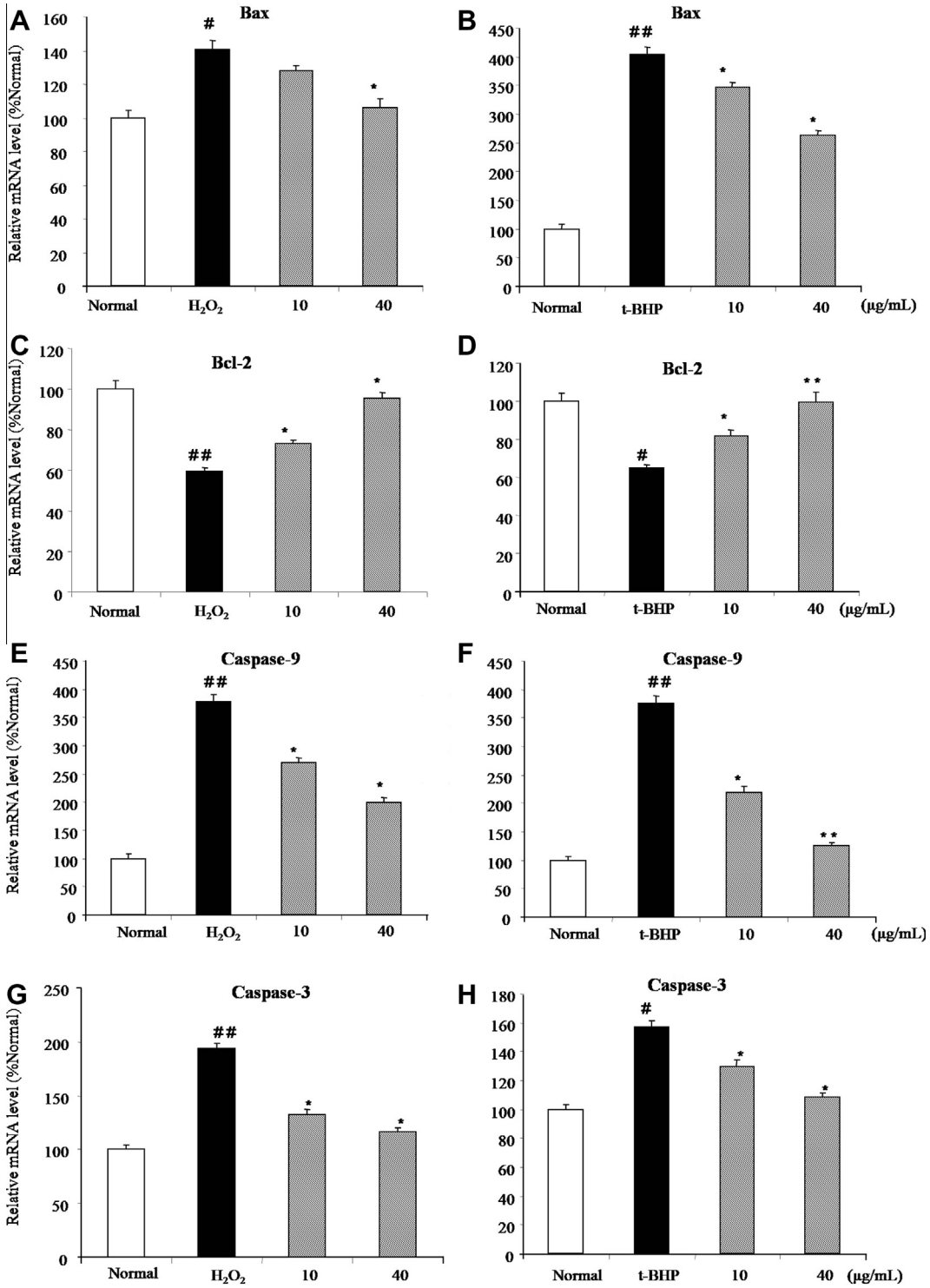

Fig. 4. Effect of EPA-enriched PL on the levels of Bcl-2, Bax, Caspase-3 and Caspase-9. PC12 cells were pretreated with EPA-enriched PL (10 and 40 μg/mL) for 24 h, and then exposed to 500 μM H_2O_2 or 300 μM t-BHP for another 4 h. The mRNA expression of Bax (A and B), Bcl-2 (C and D), Caspase-9 (E and F) and Caspase-3 (G and H) were measured by quantitative RT-PCR, which were normalized with that of β-actin. Pretreatment with EPA-enriched PL could reverse the decrease of the Bcl-2 gene level and increase of the Bax, Caspase-3 and Caspase-9 gene expression induced by H_2O_2 or t-BHP. Results were obtained from three independent experiments and were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group, $^{*}P < 0.05$, $^{**}P < 0.01$, versus H_2O_2 or t-BHP group.

and improved memory in mice with dementia (Chung et al., 1995). Recently, *t* very-long-chain omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are increasingly becoming nutritional exposures of interest in observational research on chronic disease risk. Zhao et al., (2012) reported that fish oil, a rich source of EPA and DHA, could improve the learning performance of diabetic rats. In additions, Antarctic krill oil, a phospholipid, containing a relatively high amount of EPA and DHA, improved spatial cognition of adult rats (Gamoh et al., 2011). Moreover, it has recently been reported that DHA connecting phospholipids augment learning and memory performance in rats (Hiratsuka et al., 2009). However, the effect of EPA-enriched PL on oxidative stress and cognitive disorders is far from completely understood.

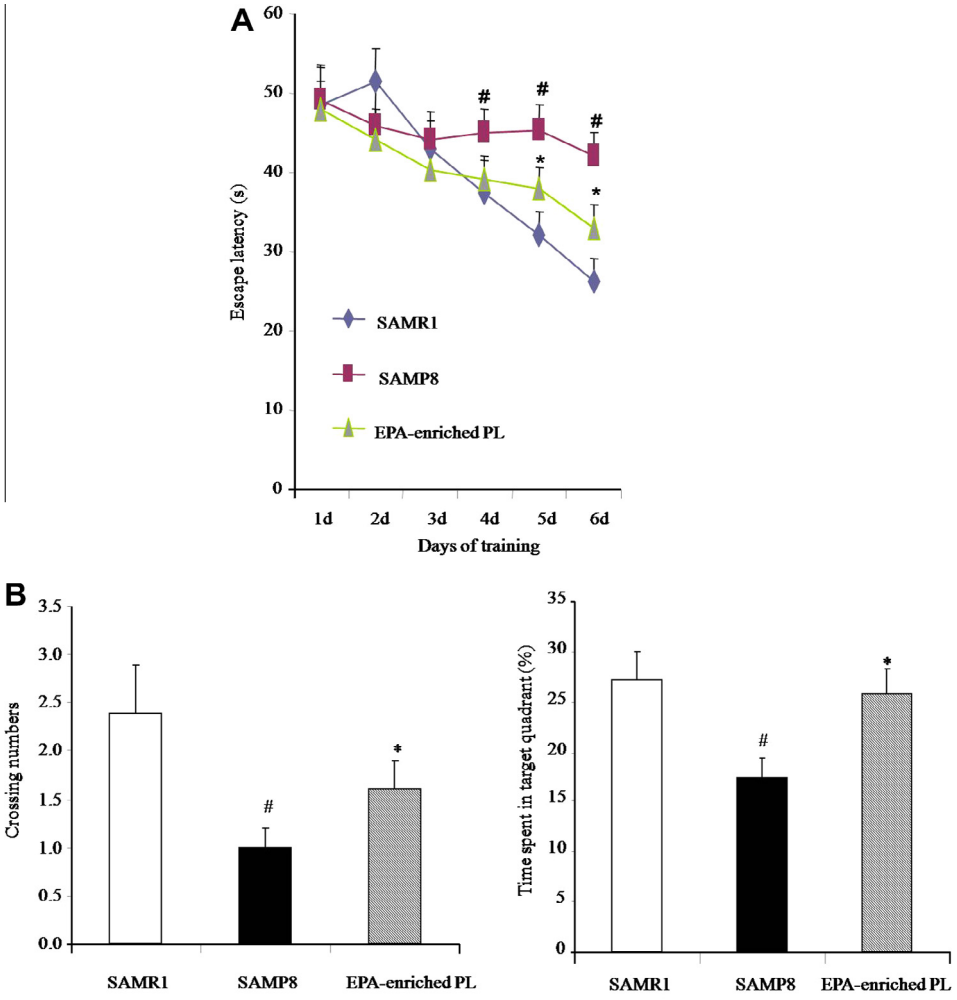

Fig. 5. Effects of EPA-enriched PL on the spatial learning and memory in SAMP8 mice in water maze test. At 12 weeks after the operation, all the mice were first subjected to the maze test. The results were expressed as the mean ± SEM of 10 animals in each group. (A) Effect of EPA-enriched PL on the escape latency in SAMP8 mice. SAMP8 mice treated with EPA-enriched PL improved the performance by a significant reduction of the escape latency in comparison to that of untreated SAMP8 mice, which was longer than that of SAMR1 mice. (B) Effect of EPA-enriched PL on the number of crossing of the platform and the time spent in the target quadrant (%) in SAMP8 mice in probe trail. The number of crossing platform and the time spent in the target quadrant in SAMP8 mice were significantly lowered than that of SAMR1 mice. However, EPA-enriched PL reversed the changes in SAMP8 mice significantly. $^{\#}P < 0.05$, versus SAMR1 group, $^{*}P < 0.05$, versus SAMP8 group.

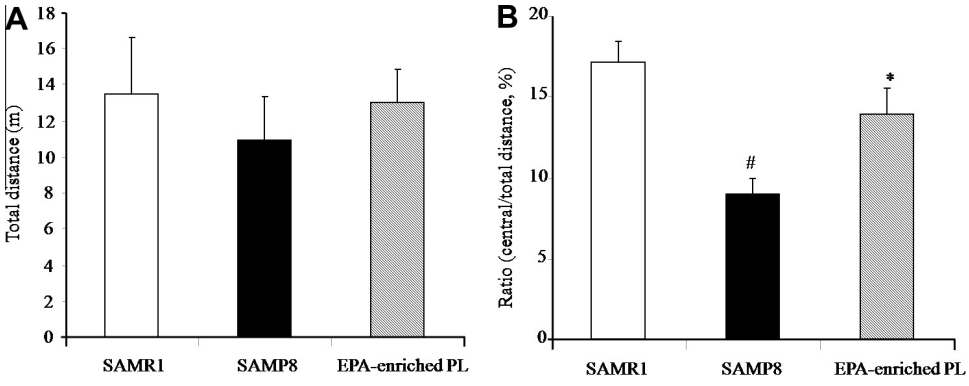

Fig. 6. Effect of EPA-enriched PL on locomotor activity of SAMP8 mice in open-field test. After the water maze test, we continued to carry out the open-field test for all the mice. (A) Total distance and (B) the ratio of central/total distance. There was no significant difference on the total distance among the three groups, while, EPA-enriched PL increased the ratio of the distance travelled in the central area to that in the total area compared with the SAMP8 mice. Value is a mean ± SEM of 10 animals in each group. $^{\#}P < 0.05$, versus SAMR1 group, $^{*}P < 0.05$, versus SAMP8 group.

Oxidative stress has been implicated as a major cause of cellular injuries in neurodegenerative disorders, such as AD (Beal, 2002; Du et al., 2003). Both H_2O_2 and t-BHP are considered as agents causing major injury to neurons. In the present study, pretreatment with EPA-enriched PL could significantly prevent the negative effects of these toxins in PC12 cells, showing the neuroprotection on

PC12 cell injury. T-AOC is an indicator of total intracellular antioxidant status, and SOD is an important constituent in the oxidation system, which can catalyze disproportionation reactions and clear free radicals. T-AOC and SOD activities reflect cell damage indirectly, and can be used to determine the degree of cell damage (Gilgun-Sherki et al., 2002; Mugge et al., 1991). EPA-enriched PL significantly increased the T-AOC and SOD activities in H_2O_2 and t-BHP-treated cells, suggesting that the protective effect of EPA-enriched PL might be partly associated with its antioxidative function.

Given the neuroprotective effects of EPA-enriched PL on PC12 cells, we further investigated the effect of EPA-enriched PL in SAMP8 mice *in vivo*. SAM has been established as a murine model for accelerated aging, and SAM shows various age-associated disorders as observed in humans (Hosoi et al., 2005). SAMP8 mice exhibit age-related learning and memory deficits, as well as amyloid-like deposits in the brain (Del Valle et al., 2010). Spatial learning and memory, impairment in SAMP8 mice can be observed in the Morris' water maze task, where the mouse was trained to escape from the water by locating a hidden platform (Miyamoto, 1997). SAMP8 mice also show age-related emotional disorders characterized by reduced anxiety-like behavior (Miyamoto et al., 1992). In the present study, SAMP8 mice exhibited severely impaired learning and memory performances in the water-maze test. These findings agree with previous studies on the same strain of mice (Miyamoto, 1997). Moreover, SAMP8 mice showed weaker exploratory behavior and higher anxiety than SAMR1 mice. Treatment with EPA-enriched PL improved the learning and memory deficits, and prevented the cognitive decline and anxiety behaviors in SAMP8 mice, which might depend on the neuroprotective effects of EPA-enriched PL.

To further investigate the mechanisms underlying the protective actions of EPA-enriched PL both *in vitro* and *in vivo*, we focused on the mitochondria-dependent apoptotic pathway. Mitochondria are central to the oxidative stress regulation in many physiological and pathological conditions. The mitochondria-dependent apoptotic pathway is known to be involved in H_2O_2 and t-BHP induced cytotoxicity in PC12 cells, the Bcl-2 family (a group of apoptosis regulatory genes) plays a pivotal role in this pathway (Saito et al., 2007). As a mitochondrial membrane-associated protein, Bcl-2 exerts its anti-apoptotic effect by inhibiting Bax expression, cytochrome c release from mitochondria, and the subsequent activation of Caspase-3 and Caspase-9 (Yu et al., 2008). In our study, H_2O_2 and t-BHP significantly decreased Bcl-2 mRNA level and markedly increased the levels of Bax, Caspase-3 and Caspase-9, leading to mitochondrial dysfunction. Treatment with EPA-enriched PL, however, significantly and dose-dependently inhibited the H_2O_2 or t-BHP mediated mitochondrial dysfunction. These data indicated that EPA-enriched PL exerts its neuroprotective property via inhibition of the mitochondria-dependent apoptotic pathway.

5. Conclusions

In conclusion, we first demonstrated that EPA-enriched PL from the sea cucumber *C. frondosa* had a neuroprotective effect on oxidative stress in an *in vitro* experiment and ameliorated the learning and memory deficits in an *in vivo* model. The neuroprotective activity of EPA-enriched PL might be mediated, in part, via inhibition of the mitochondria-dependent apoptotic pathway. Future studies are necessary to thoroughly understand the mechanisms involved in the effects of EPA-enriched PL in PC12 cells and SAMP8 mice. Our results may provide a new insight to explore novel drugs or functional foods for protecting against neuronal damage and treating cognitive disorders.

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F.J.W. performed all the cell and animal experiments and wrote the manuscript. Y.X. and X.F.L. were in charge of the sample preparation and discussing the results. X.H.X., J.F.W. and Y.M.W. all participated in the study design and statistical analysis. L.D. and K.T. conceived the study and helped draft the manuscript. All authors have read and approved this manuscript.

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