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Protective role of Krill oil against estrogen deficiency induced neurodegeneration in ovariectomized rats

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Marine oils are rich in long-chain polyunsaturated omega-3 fatty acids and known to be associated with health promoting effects, particularly on learning memory and prevention of neurodegenerative diseases by decelerating cognitive decline. Krill oil (KO) is novel marine oil rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the antioxidant astaxanthin (ASTA) which play central role in oxidative stress in neuronal metabolism. In the present study, the possible protective role of KO against learning and memory impairment as well as brain oxidative damage induced by ovariectomized (OVX) rats either alone or combined with γ-radiation was investigated. Our data revealed that OVX rats, alone or with γ-radiation, induced a significant decrease in the levels of estrogen (E), serotonin (SER), dopamine (DA), insulin growth factor-1 (IGF-1) and the gene expression of brain-derived neurotrophic factor (BDNF) mRNA, Selective AD Indicator-1 (SELADIN-1) mRNA associated with a significant elevation in malondialdehyde (MDA), amyloid precursor protein (APP) mRNA and glycogen synthase kinase-3beta (GSK-3β) mRNA, acetylcholinesterase (AChE) and norepinephrine (NE). Treatment with KO to OVX rats, alone or with γ-radiation, resulted in significant amelioration of all investigated parameters. This study has confirmed the protective effect of Krill oil against memory impairment and thereby preventing the development of Alzheimer disease.

Keywords: Acetylcholinesterase, Alzheimer disease, Amyloid precursor protein, Cognitive decline, Dopamine, Estrogen, γ-radiation, Insulin growth factor-1, Krill oil, Learning memory, Oxidative damage, Malondialdehyde, Neuroprotection, Norepinephrine, Serotonin

Menopause is a period marked by numerous significant changes resulting from the cessation of ovarian hormone secretion that has been linked to increased risks of many diseases[1]. Oxidative stress is a disparity between the rate of free radical production and the rate of elimination, occurring when antioxidant mechanisms are repressed. Oxidative stress increases in women after menopause[2]. There is growing evidence that the oxidative stress and estrogen deprivation after menopause or ovariectomy are two main risk factors which are closely related to the pathological development of Alzheimer disease (AD)[1].

Further, women at the stage of menopause are often exposed to ionizing radiation during X-ray breast examination (mammogram), undergoing routine diagnostic or therapeutic radiation treatment procedures and occupationally (for e.g. research laboratories with radiological facilities). Exposure to ionizing radiation, particularly in higher doses (above some cGy), may result in increased rates of genetic mutations and cell death[3]. Radio-protectors are important in protecting cells from deleterious radiation-induced side effects[4].

The neurological benefits associated with seafood consumption can be attributed to the adequate amounts of polyunsaturated fatty acids (PUFAs) and antioxidants present therein. Krill oil (KO) is extracted from Antarctic krill, *Euphausia superba*, a zooplankton crustacean. At least three KO components could be responsible either individually or in combination for its potential health benefits. They are: (a) high amounts of the powerful antioxidant carotenoid ASTA, (b) high amounts of n-3 PUFAs (mostly EPA and DHA[5] and (c) phosphatidylserine (PS). PS is a member of natural phospholipids and an essential cell membrane building-block. It has been known that PS is one of the important brain phospholipids and plays a key role in maintaining the structure and function of normal neurons. PS and other essential fatty acids especially DHA, may also play an important role in many functions of neuron membranes, such as release of neurotransmitters, signal transduction, cell's

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communication, cell growth and apoptosis[6]. Krill oil exerted antioxidant, anti-inflammatory effect at a level greater than that of fish oil because of the presence of ASTA[7]. ASTA can directly cross the blood-brain barrier to reach different mammalian brain regions. Krill oil consumption has been increasingly suggested to be a potentially healthy nutrition strategy, especially with regard to immune response enhancement, lowering the risk of cardiovascular diseases, and neuroprotection against progressive cognitive loss[5].

Recently, Simonyan and Chavushyan[8] who investigated the effects of hydroponic *Teucrium polium* L., popularly known as felty germander, on hippocampal neuronal activity and morpho-histochemistry of bilateral ovariectomized rats, have revealed that it ameliorates hippocampal cells spike activity and morphological impairments induced by the estrogen deficiency. Essentially, the ethanolic extract of hydroponic *T. polium* reduces the OVX-induce neurodegenerative alterations in entorhinal cortex-hippocamp circuitry and facilitates the neuronal survival by modulating the activity of neurotransmitters and network plasticity.

In this study, we examined the relationship between the developments of Alzheimer's risk factors at the stage of menopause under the condition of radiation exposure and explored the potential benefit of krill oil in checking the disease progress. In order to accomplish this goal, we measured the levels of estrogen (E), malondialdehyde (MDA), insulin growth factor-1 (IGF-1), and neurotransmitters [norepinephrine (NE), serotonin (SER) and dopamine (DA)], acetylcholinesterase (AChE) activity, and gene expression of, amyloid precursor protein (APP), glycogen synthase kinase-3beta (GSK-3β), brain-derived neurotrophic factor (BDNF) and Selective AD Indicator-1 (Seladin-1) mRNA in OVX and OVX -irradiated rats.

Materials and Methods

Materials

Krill oil was obtained from (Superba™ Krill oil, Aker BioMarine ASA, Oslo, Norway).

Experimental animals

Virgin Sprague-Dawley female rats aged 12 month weighing (230-250 g), obtained from the Egyptian Holding Company for Biological Products and Vaccines (Cairo, Egypt). Animal experimentation were consistent with the guidelines of Ethics by the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) in accordance with the recommendations for proper care and use of laboratory animals approved by the animal care committee of the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Surgeries

The rats were ovariectomized under ketamine (150 mg/kg, i.p.) anesthesia[9]. After confirmation of anesthesia, ventral incision was made and ovaries and ovarian fats were removed. Ovaries were isolated by ligation of the most proximal portion of the oviduct before removal. These procedures were performed on the sham operated animals except, laparotomy was done without removing the ovaries. The animals were reversed to their cages to recover from the anesthesia[10]. After the surgery, the rats lived in animal house for one month before conducting the experiment for diminution of the endogenous sex hormones.

Radiation facility

Whole body γ-irradiation of rats was performed using a Canadian gamma cell-40, (^{137}Cs) at the NCRRT, Cairo, Egypt. The dose rate was 0.45 Gy/min.

Animal groups

Rats were divided into five equal groups (10 rats/group): *Sham group:* The surgical procedure for the sham-operated rats was the same except that the ovaries were not removed; OVX group: ovariectomized rats; *OVX +R group*: OVX rats exposed to 2Gy; *OVX+KO group*: OVX rats which received krill oil (KO) (200 mg/kg) [11] for two months; and *OVX+KO+R group*: OVX rats that received KO and also exposed to R. At the end of treatment, the animals were sacrificed; serum and brain tissues were collected. Brains were removed, rapidly frozen on dry ice, and stored at −80°C for biochemical examinations.

Biochemical assay

Measurement of E, IGF, AChE, MDA and neurotransmitters

Serum E was detected by BioVendor research and diagnostic USA ELISA kit and brain IGF-1 were detected by quantikine R and D system ELISA kit according to the manufacturer's instruction using ELISA microplate reader (DV 990 BV 4/6; Gio.De Vita & Co., Rome, Italy). Brain AChE was determined calorimetrically according to the method of Ellman[12] and the extent of lipid peroxidation (LPO) was assayed by the measurement of brain MDA according to the procedure described by Yoshioka[13] using spectrophotometer (Thermo Electron UV-Visible spectrophotometers USA) at 405 nm. Brain NE, SER and DA content were determined according to the method of Ciarolone[14] by fluorometric method (Biotech, USA).

Detection of APP, GSK-3β, BDNF and SELADINE-1

APP, GSK-3β, BDNF and Seladine-1 were detected by gene expression using real time PCR (RT–PCR) according to method stated by Livak and Schmittgen[15].

RNA extraction

Total RNA was isolated from brain tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to the manufacturer's instruction. The purity (A260/A280 ratio) and the concentration of RNA were obtained using spectrophotometer (GeneQuant 1300, Uppsala, Sweden). RNA quality was confirmed by gel electrophoresis.

cDNA synthesis

First-strand cDNA was synthesized from 4 µg of total RNA using an Oligo (dT)12-18 primer and Superscript™ II RNase Reverse Transcriptase. This mixture was incubated at 42°C for 1 h [the kit was supplied by SuperScript Choice System (Life Technologies, Breda, Netherlands)].

Real-time quantitative polymerase chain reaction (*PCR*)

Real-time PCR (RT-PCR) amplification was carried out using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers, the sequences of PCR primer pairs used for each generate shown in Table 1. Reactions were carried out in the ABI 7500 quantitative PCR (qPCR) (Applied Biosystems) or Eppendorf Realplex instrument. A first cycle of 10 min at 95°C was followed by 40 cycles of 15 s at 94°C, 20 s at 55°C, and 20 s at 68°C. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA was normalized to GAPDH genes. The threshold C_t value is the cycle number selected from the logarithmic phase of the PCR curve in which an increase in fluorescence can be detected above background. The ΔC_t is determined by subtracting the C_t of GAPDH from the C_t of the target (ΔC_t = C_t -target - C_t- GAPDH) Livak and Schmittgen[15].

Statistical analysis

The SPSS/PC computer program was used for the statistical analysis of the results. Data were analyzed using one-way analysis of variance (ANOVA). Values were expressed as mean values ± SE. Differences were considered significant at P <0.05.

Results

Effects of estrogen deficiency on neurotransmitters, MDA, IGF-1 and AChE

After ovariectomy, serum E levels declined significantly (P <0.05) below the baseline compared to the control rats (Fig. 1A). Brain neurotransmitters (DA and SER) (Fig. 2 C & D) and IGF-1 (Fig. 1B)

Table 1—Primer sequences used for RT-PCR

Primer	Sequence
APP	Forward: 5'-GGA TGC GGA GTT CGG ACA TG-3'
	Reverse: 5'-GTT CTG CAT CTG CTC AAA G-3'
GSK-3β	Forward: 5'-CTACCTTGCGGCTCACTTTC-3'
	Reverse: 5'-ATCCAAGTGCGAAACCAAAC-3'
BDNF	Forward primer 5'-ACC CTG AGT TCC ACCAGG TG-3'
	Reverse primer 5'-TGG GCG CAG CCT TCA T-3'
Seladin-1	Forward primer: 5'-ATCGCAGCTTTGTGCGATG-3'
	Reverse primer: 5'-CACCAGGAAACCCAGCGT-3'
GAPDH	Forward: 5'-CTCCCATTCTTCCACCTTTG-3'
	Reverse: 5'-CTTGCTCTCAGTATCCTTGC-3'

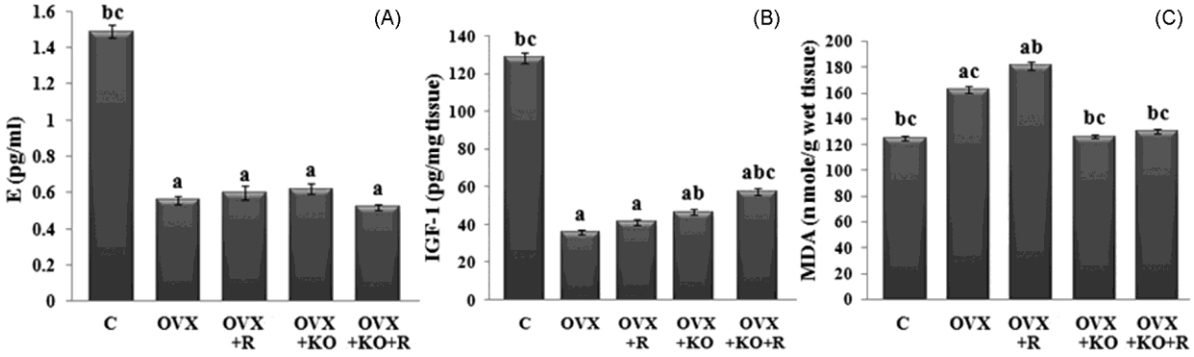

Fig. 1—Effect of KO on (A) Estrogen (E) (pg/mL); (B) insulin growth factor-1 (IGF-1) (pg/mg tissue); and (C) malondialdehyde (MDA) (n mole/g wet tissue) levels in brain tissue. [Each value represents the mean ± SE (n=6). a significantly different from control at P <0.05. b: significantly different from OVX at P <0.05. c: significantly different from OVX+R at P <0.05]

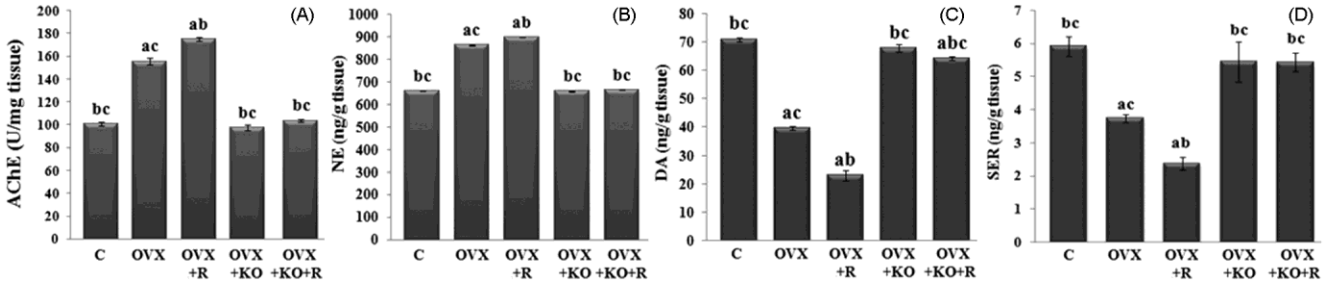

Fig. 2—Effect of krill oil (KO) on (A) acetylcholinesterase (AChE) activity (U/mg tissue); (B) norepinephrine (NE); (C) dopamine (DA); and (D) serotonin (SER) (ng/g tissue) levels in brain tissue. [Each value represents the mean ± SE (n=6). a significantly different from control at P <0.05. b: significantly different from OVX at P <0.05. c: significantly different from OVX+R at P <0.05]

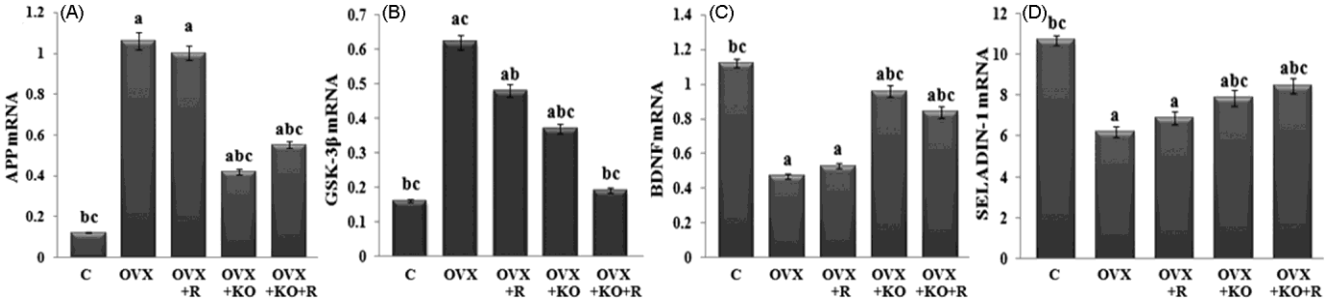

Fig. 3—Effect of krill oil (KO) on gene expression of (A) amyloid precursor protein (APP) mRNA; (B) glycogen synthase kinase-3beta (GSK-3β) mRNA; (C) brain-derived neurotrophic factor (BDNF) mRNA; and (D) Selective AD Indicator-1 (SELADIN-1) mRNA in brain tissue. [Each value represents the mean ± SE (n=6). a significantly different from control at P <0.05. b: significantly different from OVX at P <0.05. c: significantly different from OVX+R at P <0.05]

significantly (P <0.05) decrease in OVX rats compared to the control rats. Whereas, significant (P <0.05) increase in brain AChE activity (Fig. 2A), NE level (Fig. 2B) and MDA (Fig. 1C) content observed in OVX rats compared to the control rats. In OVX rats, exposed to γ-radiation showed significant decrease in serum E levels (Fig. 1A) and IGF (Fig. 1B) compared to the control group but observed a non-significant effect compared with those OVX rats. Whereas, revealed a significant (P *<0.05*) decrease in DA (Fig. 2C) and SER levels (Fig. 2D) and significant increase in brain AChE activity (Fig. 2A), NE (Fig. 2B) and MDA content (Fig. 1C) as compared to the control and OVX rats. Administration of krill to OVX rats (alone or with γ-radiation) led to significant (P <0.05) elevation in IGF-1 (Fig. 1B) as well as DA (Fig. 2C) and SER levels (Fig. 2D) as compared with those OVX rats. Also, treatment of OVX rats with KO revealed significant decrease in brain MDA content (Fig. 1C), AChE activity and NE in comparison with those OVX rats (Fig. 2 A & B).

Effects of estrogen deficiency on the genes expression of APP, GSK-3β, BDNF and SELADIN-1

After ovariectomy, the expression levels of APP mRNA (Fig. 3A) and GSK-3β mRNA (Fig. 3B) in brain were significantly (P <0.05) higher compared to those of control rats. The expression of the BDNF mRNA (Fig. 3C) and SELADIN-1 mRNA (Fig. 3 D) in brain tended to decrease significantly (P <0.05) post ovariectomy compared to that of control rats. In OVX rats, exposed to γ-radiation a significant (P <0.05) increase in expression of APP mRNA and GSK-3β mRNA (Fig. 3 A & B) compared to that of control rats but slightly reduction occurred in expression of GSK-3β mRNA compared to OVX rats. In addition, expression of the BDNF mRNA and SELADIN-1 mRNA decrease significantly (P <0.05) compared to control rats but non-significant change was observed in the BDNF mRNA (Fig. 3C) and SELADIN-1 mRNA (Fig. 3 D) compared to those OVX rats. However, the expression of APP mRNA and GSK-3β mRNA (Fig. 3 A & B) in brain significantly (P <0.05) decreased in the OVX rats (alone or with γ-radiation) administrated with krill compared with those OVX rats, whereas showed a significant increase in the level of brain BDNF mRNA and SELADIN-1 mRNA (Fig. 3 C & D).

Discussion

There is growing evidence that estrogen deprivation and oxidative stress after menopause or

ovariectomy represent two main causes closely related to the development of neurodegeneration (AD)[1]. One of the hallmarks of AD is deposition of amyloid β-peptide (Aβ) which is derived from the two-step enzymatic processing of APP[16]. In the present study, the data obtain revealed APP mRNA over expressed in the brain of ovariectomized rats. Estrogen deficiency is known to induce amyloid deposition of Aβ, production of neurofibrillary tangles and spatial memory deficit in ovariectomized rats[17]. Memory deficits caused by Aβ, are due to intracellular oxidative stress and neuronal apoptosis promoted by Aβ formation and subsequent aggregation and deposition[18]. The over expression of GSK-3β mRNA, decrease of IGF-1 concentration and the increase in oxidative stress observed by high MDA concentration could be interpreted in the view that the accumulation of Aβ exacerbates the problem. Aβ disrupts insulin signalling by competing with insulin, or reducing the affinity of insulin binding to its own receptor[19]. On a cellular basis, inhibition of insulin/IGF signalling contributes to neurodegeneration followed by increasing the expression of APP, generation of ROS and reactive nitrogen species (RNS) that damage proteins, RNA, DNA and lipids and elevate of the activity of GSK-3β that aberrantly phosphorylate tau[19]. Pathological hyperphosphorylation of tau is associated with several neurodegenerative diseases[20]. Our result demonstrates that seladin-1 mRNA was decrease significantly in OVX rats as compared to control. Seladin-1 (a new neuroprotective gene was identified and found to be down regulated in AD vulnerable brain regions) was originally found to confer resistance against Aβ and oxidative stress-induced apoptosis and to effectively inhibit the activation of caspase-3, a key mediator of the apoptotic process[21]. The down regulation of seladin-1 gene could be attributed to the interplay between seladin-1, E and IGF-1. Estrogen stimulates the expression of the seladin-1 gene via functionally active half-palindromic estrogen responsive elements present in its promoter region. In addition, E increases the release of IGF1. IGF1, on turn, induced seladin-1 expression by a direct consequence of IGF1/ IGF1 receptors (IGF1R) binding or is mediated via an interaction between IGF1R and estrogen receptor[21]. The seladin-1 deficiency could reduce the levels of cholesterol that were associated with increased cleavage of APP by β-secretase and high levels of Aβ. Conversely, seladin-1 over expression increased cholesterol levels and reduced APP processing in neuroblastoma cells[21].

Neurotrophins are molecules that promote the development, health and survival of neurons. These signalling molecules exert considerable control over the switch between life and death pathways in cells. Among the neurotrophins, BDNF has emerged as a major regulator of synaptic plasticity, neuronal survival and differentiation, and also as a key molecular target for drug development in neurological disorders[22]. The deprivation of E after ovariectomy might responsible for observed decrease expression of BDNF mRNA. E could regulate the expression of BDNF via the E response element on the BDNF gene[1,23]. The down regulation of BDNF signalling may be an early and possibly primary event in AD (*i.e.*, mild cognitive impairment) [24].

Furthermore, the deprivation of estrogen (E) which resulted in Aβ deposition is closely associated with the dysfunction of central cholinergic neuronal circuits in the basal forebrain**.** The dysfunction of central cholinergic neuronal systems, including decreased brain acetylcholine levels and an upregulation of AChE activity may be related to the degrees of Aβ deposition[18]. In addition, AChE activity is involved in mediating the processing and deposition of Aβ by formation of stable AChE-Aβ complexes as showed in the brain of AD patients[25]. In addition, the obtained results pointed to disturbance in NE, SER and DA neurotransmission system. This disturbance might be due to continuous infusion of Aβ as result of estrogen deprivation[18].

The exposure of OVX rats to gamma radiation (2 Gy) augment the effect of estrogen deprivation on MDA, AChE and NE while DA, SER and the expression of GSK3β mRNA undergoes significant decrease compared to OVX rats. This could be attributed to the increase of oxidative stress resulted after radiation exposure. Where, the abundance presence of PUFA in the myelin components of axons of the millions of neurons in the brain provide a ready source of target for the gamma radiation bombarding the brain to release free radicals that will cause lipid peroxidation of these membranes[26] resulting from the interaction of •OH, a bi-product of water radiolysis, with PUFA which is substantiated by the significant increase of MDA[27,28]. The gamma-irradiation caused nuclear degeneration, formation of enucleated cells and intercellular spaces in the amygdala and cortex; neuronal loss and neuronal dispersion in rat brain tissue[29] and down regulate the gene expression and enzyme activity[30]. Previous study showed that

γ-irradiation of erythroleukemic K562 cells caused an increase in AChE activity accompanied by cell differentiation and cessation of cell proliferation[31]. However, the inhibition of GSK3β expression after exposure to 2 Gy gamma radiations could be due to DNA damage[27]. This damage might be induces phosphorylation of GSK3β through NF-kappa B and Akt pathway[32]. Furthermore, alterations of brain neurotransmitters in irradiated rats may be attributed to the increased rate of formation of O_2^- and H_2O_2 and may be responsible for exerting neurodegenerative diseases and Lewy bodies'aggregations[27,33]. The KO administration to OVX or OVX irradiated rats significantly recovers the damage result from estrogen deprivation by significant amelioration in APP mRNA. The DHA significantly decreases the levels of Aβ peptides[34]. Consumption of n-3 PUFA might prevent the accumulation of Aβ via action mechanisms including the following: positive regulation of neurotrophic factors, inhibition of the inflammatory cascade, reduction in oxidative damage (especially through a reduction in oxidized protein accumulation, lipid peroxidation, and ROS levels), and effects on cellular membrane properties and cellular signalling pathways[5]. In addition, the results showed significant improvement in IGF-1, GSK-3β mRNA, BDNF mRNA, seladin-1 mRNA, neurotransmitters and reduction in MDA and AChE after KO administration. This could be attributed to the upregulation of IGF-1 expression after krill-PS[6]. However, the inactivation of GSK-3β might results from upregulation of nuclear factor (erythroid-derived 2)-like 2 regulated phase II enzymes through activation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway by ASTA[35]. In addition, ASTA serves as a safeguard against oxidative damage by various mechanisms, like quenching of singlet oxygen; scavenging of radicals to prevent chain reactions; preservation of membrane structure by inhibiting lipid peroxidation; enhancement of immune system function and regulation of gene expression[36]. Furthermore, n-3 PUFAs can induce serotonergic neurotransmission and neurotrophin expression such as increase BDNF mRNA level via increased transcription or an effect on RNA turnover[37]. Moreover, with regard to neurodegenerative diseases, DHA and EPA are the strongest inhibitors involved in AChE inhibition[38].

Conclusion

With the results of this study, it can be concluded that Krill oil (KO) protects against neurodegenerative due to synergistic action of its various components such as ASTA, n-3 PUFAs and PS. It is also suggested that Krill oil could serve as a novel approach to alleviate neurological injury (development of Alzheimer risk factors) and thus become a potentially healthy nutrition strategy for postmenopausal women.

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