IJPSR (2017), Volume 8, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 24 December, 2016; received in revised form, 10 February, 2017; accepted, 17 February, 2017; published 01 July, 2017

β-CYFLUTHRIN-INDUCED IMPAIRMENT IN NEUROTRANSMITTERS, TESTICULAR FUNCTION AND GENE EXPERISSIONS OF MALE RATS: THE PROTECTIVE ROLE OF OMEGA-3

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Keywords:

β-cyfluthrin, Omega-3, Neurotransmitters, Oxidative stress, Steroidogenesis, Genotoxicity

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ABSTRACT: The present study was designed to evaluate the protective effect of omega-3 against β-cyfluthrin (β-cyf)-induced neurotransmitters' impairment, oxidative damage, testicular toxicity and genotoxicity. Adult male Wistar rats were divided equally into four groups: Group (I): Control, Group (II): β-cyf (5 mg/kg body weight/day), Group (III): omega-3 (400 mg/kg body weight/da y) + β-cyf (5 mg/kg body weight/day), Group (IV): omega-3 (400 mg/kg body weight/day). β-cyf's treatment for 8 weeks inhibited the activities of AChE, DA, LDH, total ATPase and Na^+/K^+ATPase. Also, β-cyf resulted in an increase in oxidative stress as indicated by elevations in the levels of MDA and NO while, the levels of TAC, thiol content and TP were decreased in brain and testis. Sperm count, sperm motility and testosterone levels were reduced while, sperm abnormalities, FSH and LH levels were increased in β-cyf-treated group. Also, β-cyf administration led to a significant increase in TNFα, IL-6 and APP mRNA expressions. In addition, β-cyf caused histopathological alterations in brain and testis. The present results showed that omega-3 could improve all the measured parameters. In conclusion, omega-3 could exhibit protective effects against β-cyf induced neurological and testicular damage.

INTRODUCTION: Pyrethroids are highly active insecticides which account for 30% of insecticides used globally [1]. β-cyfluthrin (β-cyf) is a member of type II pyrethroids and is widely used in agricultural and public health and hygiene [2]. β-cyf (Cyano-(4-fluoro-3-phenoxyphenil)-methyl-3-(2,2-dichloroethenyl)-2, 2-dimethyl - cyclopropane carboxylate) is the active ingredient of insecticide formulations.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(7).2819-31

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (7).2819-31

β-cyf is well known as a hepatotoxic, neurotoxic, and teratogenic agent by causing oxidative stress. Its toxicity is exhibited by its metabolites that generate free radicals and in turn causing oxidative stress [3, 4]. These free radicals also damage the cell components including proteins, lipids and DNA [5].

Currently, the primary concerns of exposure to pyrethroids are developmental neurotoxicity [6]. β-cyf is a neurotoxic agent as it contains an alpha-cyano group which renders them more neurotoxic than their noncyano type I. It induces alterations in nerve membrane, leading to abnormal sodium and potassium ion flow [7]. Exposure to insecticide is commonly associated with restriction of spermatogenesis, destruction of seminiferous epithelium, hydrocels leading to infertility [8].

Omega-3 (Polyunsaturated fatty acids) are considered essential, because they are not synthesized by the human body that lack the natural desaturase enzymes Δ-15 and Δ-12. The traditional omega-3 supplements consist of native fish oils or fish, often cod, liver oil. Nowadays the most part of the supplements contain as much as 85% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) presented as ethyl esters (EE) or triacyglycerols. Omega-3, its members EPA and DHA, is the essential lipid class for synthesis of inflammation mediators and regulators [9, 10].

Therefore, the present study was aimed to investigate the protective role of omega-3 in amilorating the neuro- and testicular impairment induced by β-cyfluthrin in male rats.

MATERIALS AND METHODS:

Chemicals: β-cyfluthrin (Jolicoeur) in liquid form (10%) was purchased from Chema Industries - Nubaria City, Egypt. Gelatin capsules of omega-3 (1000 mg) were obtained from Arab Co. for gelatin and pharmaceutical products of Montana Pharmaceutical.

Experimental animal and treatment: Twenty eight adult male Wistar albino rats (180-200 g) were obtained from Faculty of Medicine, Alexandria University, Egypt. Animals were housed in a stainless steel wire cages placed in a well-ventilated animal house and kept on basal diet and tap water *ad libitum*. They maintained at 25±1°C with 12 hrs dark and light cycle. The local committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH).

Animals were randomly divided into four groups as follow:

Group I: Control group: rats of this group were orally received distilled water as vehicle.

Group II: β-cyfluthrin group: rats of this group were orally received β-cyfluthrin at a dose 5 mg/kg body weight/day dissolved in distilled water [7].

Group III: Omega-3 group: rats of this group were orally received omega-3 at a dose 400 mg/kg body weight/day [11].

Group IV: β-cyfluthrin and omega-3 group: rats of this group were pre-treated orally with omega-3 at a dose of 400 mg/kg body weight/day for 10 consecutive days and then in combination with β-cyfluthrin at a dose 5 mg/kg body weight/day (Omega-3 was given to rats 30 min before β-cyfluthrin). Rats were orally administered their respective doses every day for eight weeks.

Blood collection and tissue preparation: At the end of the 8th week of the experimental period, all animals of each group were anaesthetized with diethyl ether and sacrificed. Blood samples were collected from anaesthetized rats in sterile test tubes and placed immediately on ice. Serum was obtained by centrifugation of blood samples at 3000 rpm for 20 min (Hettich zentrifugen, Universal 32 R, Germany) and was stored at -80 ºC for the determination of biochemical parameters.

Parts of the brain tissues were immediately removed and kept at −80 °C till molecular analysis. Another parts of brain and testis were immediately removed and washed using cold chilled saline solution 0.9% then, they were minced and homogenized (10%, w/v) separately, in ice-cold 50 mM potassium phosphate buffer at pH 7.4 in a Pottere Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC, and the resultant supernatants were stored at -80 ºC.

Biochemical parameters:

Brain acetylcholinesterase, dopamine and lactate dehydrogenase estimation:

Acetylcholinesterase (AChE; E.C. 3.1.1.7) [12]. Dopamine (DA) level was detected according to the manufacturer's instructions (GenWay Biotech, San Diego, USA) by using Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The activity of lactate dehydrogenase (LDH; E.C. 1.1.1.27) was assayed kinetically [13] by using commercial kits (Bio Systems S.A Costa Brava 30, Barcelona, Spain). Total ATPase activity (E.C. 3.6.1.3) was assayed using ELISA kit purchased from Immuno way. Na^+-K^+/ATPase activity (E.C. 3.6.3.9) was estimated using ELISA kit purchased from MyBioSource, USA.

Determination of Malondialdhyde, nitric oxide, total antioxidant capacity and total thiol content in brain and testis tissues: Malondialdehyde (MDA), nitric oxide (NO) and total antioxidant capacity (TAC) kits were purchased from Biodiagnostic Co. Cairo, Egypt. Total thiol content of brain and testis was also carried out [13].

Evaluation of epididymal sperm quality: Epididymal sperm analysis was carried out using a computer assisted semen analysis-Sperm Vision™ CASA System (MiniTUb, Tiefenbach, Germany) with Olympus BX 51 phase microscope (Olympus, Japan) [14].

Determination of testosterone, FSH and LH: The testosterone [15], FSH [16] and LH [17] were assayed using ELISA kits purchased from DRG International, Inc, BIOCODE–HYCEL and Elabscience Co., respectively.

Molecular analysis:

Isolation of total RNA: Total RNA was extracted from brain tissues [18] procedure using GStractTM RNA Isolation Kit II, Guanidinium Thiocyanate Method. Briefly, the tissue was homogenized in BIOZOL; total RNA Extraction Reagent (80-100 mg tissue/ml) and incubated in ice for 15 min. One hundred microliters chloroform was added to the homogenate and mixed by vigorous vortexing three times for 20 s. It was incubated in ice for 15 min then centrifuged at 12,000 rpm for 15 min at 4 ºC. The aqueous phase was transferred into new test tube and the same volume from cold isopropanol was added, mixed and incubated at -20 ºC for 25 min. The mixture was then centrifuged at 12,000 rpm for 10 min at 4 ºC and the aqueous/isopropanol solution was discarded. The pellets were washed three times with ice-cold 75% ethanol solution then were left to dry. The dried pellets were re-suspended in 50-100 µl RNAase free H_2O and stored at -80 °C.

Determination of RNA concentration and purity: RNA concentration was determined by measuring the absorbance at 260 nm (RNA solution was diluted 5/495 µl with RNAase free water). The concentration was calculated using the following equation: 1 absorbance unit at 260 nm corresponds to approximate concentration of 40 µg/ml of single-stranded RNA. Quality of RNA preparations were confirmed by calculating 260/280 ratio for detection of protein contamination and by running samples on agarose to confirm that the samples are DNA-free. Pure preparations of RNA have ratios of 1.8–2.0.

Reverse-transcriptase poly chain reaction (RT-PCR): Alteration in the steady state mRNA levels of genes relevant to neuroinflammation pathogenesis in rats is determined using reverse-transcriptase PCR analysis. Using one-step RT-PCR (RT/PCR Master Mix Gold Beads, BIORON) reaction, the cDNA was synthesized and used for amplification of the target gene(s). Briefly, total RNA (1–3 µg) and random primer (3 µM) mixture were denatured at 70 °C for 5 min and placed on ice. The incubated mixture was added to the RT/PCR Gold mix that contains all the components necessary for cDNA synthesis and amplification in one tube. The cDNA synthesis reaction was performed at 42 ºC for 60 min then 5 min at 94 °C for RTase inactivation. The primers then subjected to PCR cycles, each cycle consisting of denaturation, annealing, and extension. Annealing temperature and time was optimized for each primer/template combination. The expression of the neuroinflammatory, marker TNF-α expression was investigated by using the following primers sets. (**Table 1)**

TABLE 1: THE PRIMER SEQUENCES OF TARGET GENE IN EXPECTED PCR PRODUCTS FOR RT-PCR

Primers sequence and condition	Reference
-β-actin F: 5'-GGCATCCTGACCCTGAAGTA-3' R: 5'-GCC GAT AGT GAT GAC CTG ACC-3' 94˚C for 45s, 60˚C for 45s, 72˚C for 45s	19
-TNF-α F :5′CTCTTCTCCTTCCTGATCGTGGCA3' R:5' GAAAGCATGATCCGGGACGTGGA3' 94˚C for 30sec, 53˚C for 30sec,72˚C for 1 min Number of cycles: 35	20

Agarose Gel Electrophoresis of the amplified RT-PCR products, visualization and documentation: Products of RT-PCR were separated on agarose gel [21]. Mixture of total volume of 10 µl was prepared by mixing 7 µl of the RT-PCR product and 3 µl of the sample loading dye and electrophoresed on 1.5 % agarose gel (1.5 g/100 ml 0.5x TBE) containing 10 µg/ml ethidium bromide (EtBr) dye then visualized and documented using Chemi Doc-It®2 Imager then analyzed with Vision Works LS Acquisition and Analysis Software for determinations of relative bands intensity.

Quantitative RT-PCR assay (qRT-PCR): Quantitative RT-PCR was used to measure the mRNA expression levels of IL-6 and APP genes. cDNA was synthesized by High-Capacity cDNA Reverse Transcription Kit according to the manufacture protocol. The primer for IL-6 (5'-CGAAAGTCAACTCCATCTGCC-3' and 5'-GGCAACTGGCTGGAAGTCTCT-3') [22]. The primer for APP gene (5′-TGCTGAAGATGTGGG TTCGA-3 and 5′-GACAATCACGGTTGCTAT GACAA-3′) [23]. The primers for β-actin; 5'-CCGACAGGATGCAGAAGG-3'- and 3'-GGAGTACTTGCGCTCAGGAG.5'. IL-6 and APP were normalized to β-actin, fold difference calculated by $2^{-\Delta\Delta C}$T [24].

Histological section preparation: Brain and testis specimens were obtained from rats, and immediately fixed in 10 % formalin and then treated with conventional grade of alcohol and xylol, embedded in paraffin and sectioned at 4-6 µm thickness. The sections were stained with Haematoxylin and Eosin (H&E) stain for studying the histopathological changes [25].

Statistical analysis: Data have been expressed as mean±SE Statistical analysis of data was performed using SPSS computer software package version 22 (Chicago, IL, USA). Data were analyzed by ANOVA and means were compared with post hoc multiple comparison test. A value of $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION:

Effect of β-cyfluthrin, omega-3 and their combination on AChE, DA, LDH, total ATPase and Na^+ /K^+ ATPase in brain of male rats: Pyrethroids can easily cross the blood-brain barrier and reach the central nervous system (CNS) leading to neurotoxicity [26]. Acetylcholinesterase (AChE) is a vital cholinesterase enzyme present in the neuromuscular junctions and cholinergic synapses in the CNS. AChE is considered as a key enzyme in detecting the neurotoxicity [27]. The significant ($P<0.05$) reduction in AChE activity in β-cyf - treated group **(Table 2)** indicated that β-cyf was known to block the catalytic site of AChE [28]. Moreover, cyf affects voltage sensitive sodium channels, voltage-sensitive calcium channels and alters the release of neurotransmitters [29].

In the current data, co-administration of omega-3 with β-cyf significantly ($P<0.05$) improved the inhibition in AChE activity **(Table 2)**. This result came accordance with others [30] who investigated that administration of omega-3 fatty acids alone and with lithium and aripiprazole revealed an elevation in the activity of AChE in mice brain. This may be due to the ability of omega-3 to decrease the lipid peroxidation which will manipulate a significant role in the stabilization of AChE activity in the brain. Also, it was reported that PUFAs are compounds that play an important role in cell signaling, enzymatic regulation and may also interact with the cholinergic system showing a protective effect [31].

On the basis of the present results, it might be hypothesized that the significant ($P<0.05$) lower in dopamine (DA) content **(Table 2)**, could be due to either inhibition of biosynthesis of DA, or decrease in tyrosine hydroxylase (TH) and/or decrease in aromatic L-amino-acid decarboxylase synthesis as observed after exposure to the pyrethroid deltamethrin [4, 32]. Thus, β-cyf toxicity might also be due to the release of unstable cyanohydrins. Cyanohydrins are decomposed to cyanides and aldehydes, which in turn could act as a source of free radicals [33]. This oxidative stress effect would be another explanation to the decrease of the serotonin (5-HT) and DA levels by β-cyfluthrin as observed in the present study.

Omega-3 pre- and in combination treatment with β-cyfluthrin caused a significant ($P<0.05$) increase in the activities of brain DA (**Table 2**). Among the omega-3 PUFAs, docosahexaenoic acid DHA is the most important omega-3 with physiological

significance for brain function. Omega-3 dietary deficiency affects the dopaminergic, serotoninergic and glutamatergic systems [34]. As a component of membrane phospholipids, it is documented that the percentage of omega-3 influences the physicochemical properties of the membrane and accordingly affect the function of a variety of membrane-bound proteins, including dopaminergic, GABAergic, and cholinergic receptors [35]. Dietary intake of omega-3, both absolute and relative to omega-6 status, may contribute to regulation of brain dopaminergic functioning in humans [36].

Table 2 showed a significant (*P<0.05*) decline in the brain LDH activity suggesting a decrease in the glycolytic process due to the lower metabolic rate as a result of pyrethroid exposure [37]. Pretreatment of β-cyf-intoxicated rats with omega-3 was able to significantly improve the reduction of brain LDH (*P<0.05*). This process can be restricted pharmacologically at different levels with agents that scavenge reactive oxygen metabolites, block their generation or promote endogenous antioxidant capabilities [38]. The protective effect of omega-3 against leakage of LDH may be due to the presence of docosahexaenoic acid (DHA) that protect the normal cellular architecture through its incorporation into the phospholipids of cell membranes [39].

Also, **Table 2** revealed a significant (*P<0.05*) decline in the brain total ATPase and Na^+/K^+ ATPase with β-cyf. This is may be attributed to disturbed mitochondrial energetic system due to the movement of β-cyf molecules within the mitochondria, after its access through the meninges causing a decrease in total ATPase [27]. Moreover, toxicants can alter Na^+/K^+ ATPase activity by disrupting the energy producing metabolic pathway or interacts directly with enzymes. Inactivation of Na^+/K^+ ATPase leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events like excitotoxicity [40].

In this context, the present data indicated that omega-3 fatty acids in combination with β-cyf significantly (*P<0.05*) increased total ATPase and Na^+/K^+ ATPase in brain tissue (**Table 2**) similar to previous results [41]. The protective effect of omega-3 may be attributed to a direct protection of the enzyme molecule by modification of the lipid microenvironment surrounding the Na^+/K^+ ATPase molecule [42].

TABLE 2: EFFECT OF β-CYFLUTHRIN, OMEGA-3 AND THEIR COMBINATION ON AChE, LDH, DA, TOTAL ATPase AND Na^+/K^+ ATPase OF MALE RATS

Parameters	Experimental groups			
	Control	β-cyfluthrin	Omega-3	β-cyfluthrin+omega-3
AChE (U/mg protein)	171.00±1.345^b	61.85±0.857^d	178.43±2.202^a	111.86±1.121^c
DA (ng/g tissue)	189.14±1.335^b	91.28±1.847^d	201.71±1.643^a	159.14±1.183^c
LDH (U/mg protein)	210.57±1.192^b	89.14±0.986^d	218.00±0.899^a	140.29±0.969^c
Total ATPase (U/mg protein)	57.68±0.378^a	28.12±0.270^c	58.53±0.384^a	45.55±0.392^b
Na^+/K^+ ATPase (pg/mg protein)	32.15±0.340^a	12.44±0.281^c	32.71±0.498^a	24.22±0.214^b

Values are expressed as means±S.E, n=7 for each treatment group.
Mean values within a row not sharing a common superscript letter (a, b, c and d) were significantly different *P*<0.05.

Effect of β-cyfluthrin, omega-3 and their combination on the oxidative stress markers: MDA, NO, TAC, total thiol content and total protein (TP) levels in brain and testis of male rats: β-cyf administration exihibeted significant (*P<0.05*) increase in MDA and NO levels in brain and testis tissues (**Table 3**). Similar results were obtained by [43, 44] who reported elevation in the levels of MDA and NO after exposure to mixture of type II pyrethroid. The increase in MDA induced by the pyrethroid could be as a result of the generation of ROS that attacked the unsaturated lipids, thereby causing the generation of lipid peroxides leading to alteration of membrane permeability and cell function. Pyrethroids indirectly generate various ROS such as superoxide radical and hydroxyl radical, and reactive nitrogen species (RNS) like peroxynitrite and nitric oxide [40]. Moreover, **Table 3** manifested significant (*P<0.05*) reduction in the total antioxidant capacity (TAC), total thiol and total protein (TP) content in brain and testis of β-cyf- treated group. The decline in the total thiol content may be due to excessive free radical generation, which might attack the thiol

group of cysteine residues and polyunsaturated fatty acids of biological membranes [45]. Additionally, the accumulation of H_2O_2 in response to lipid peroxidation leads to a decreased in intracellular GSH and antioxidant enzyme activities [46]. Also, the reduction in total protein contents may be attributed to the inhibition of protein synthesis and low cell survival due to defective antioxidant enzyme [47].

Omega-3 treatment in combination with β-cyf significantly (*P<0.05*) improved the oxidant and antioxidant markers in the brain and testis tissues **(Table 3)**. Similarly, it was reported that omega-3 fatty acids improved the oxidative status of tissues which was confirmed by reduction in the MDA, NO level and elevation in GSH levels. In addition, EPA as a member of omega-3 essential fatty acid may cause stabilization of membrane structure, decrease ROS generation and hence lipid peroxidation [48].

Furthermore, omega-3 is a source of naturally available antioxidant, might make the neural tissues less susceptible to lipid peroxidation leading to its beneficial effects [49]. Likewise, it was deduced that omega-3 fatty acids may be useful in the prevention and treatment of methotrexate-induced testicular damage that may be due to its antioxidant properties [50].

TABLE 3: EFFECT OF β-CYFLUTHRIN, OMEGA-3 AND THEIR COMBINATION ON MDA, NO, TAC AND TP LEVELS IN BRAIN AND TESTIS TISSUES OF MALE RATS

Parameters	Experimental groups			
	Control	β-cyfluthrin	Omega-3	β-cyfluthrin+omega-3
Brain MDA (nmol/g tissue)	13.12±0.165^{c}	72.14±0.675^{a}	12.13±0.165^{c}	31.53±0.208^{b}
NO (nmol/g tissue)	8.31±0.104^{c}	25.53±0.240^{a}	8.42±0.153^{c}	13.12±0.207^{b}
TAC (mM/g tissue)	65.42±0.350^{b}	16.91±0.236^{d}	75.74±0.385^{a}	48.90±0.441^{c}
Thiol content (mM/g tissue)	51.25±0.356^{a}	17.50±0.317^{c}	51.32±0.260^{a}	32.41±0.404^{b}
TP (mg/g tissue)	117.00±0.816^{b}	92.50±0.645^{d}	127.57±0.649^{a}	109.00±0.816^{c}
Testis MDA (nmol/g tissue)	12.12±0.132^{c}	60.42±0.545^{a}	11.31±0.194^{c}	23.01±0.277^{b}
NO (nmol/g tissue)	7.31±0.081^{c}	28.41±0.363^{a}	7.28±0.085^{c}	14.14±0.210^{b}
TAC (mM/g tissue)	88.34±0.313^{a}	20.62±0.215^{c}	87.58±0.625^{a}	57.21±0.532^{b}
Total thiol (mM/g tissue)	46.75±0.322^{a}	19.94±0.254^{c}	46.68±0.291^{a}	32.72±0.305^{b}
TP (mg/g tissue)	138.57±0.649^{b}	115.71±0.680^{d}	142.00±0.816^{a}	131.57±0.649^{c}

Values are expressed as means ±S.E, n=7 for each treatment group.
Mean values within a row not sharing a common super script letter (a, b, c, d) were significantly different *P*<0.05

Effect of β-cyfluthrin, omega-3 and their combination on sperm count, sperm motility, sperm abnormalities, testosterone, FSH and LH of male rats: β-cyf exhibited significant (*P<0.05*) decline in sperm count, sperm motility and significant elevation in sperm abnormalities along with a significant (*P<0.05*) decrease in testosterone level (**Table 4)**. This decline was attributed to either direct effect of toxicant on androgen biosynthesis pathway in testis or its effect on hypothalamus pituitary gland which might have indirectly affected the testis and sexual function [47]. It was also suggested that pyrethroid insecticides may cause mitochondrial membrane impairment in Leydig cells and disrupt testosterone biosynthesis by diminishing the delivery of cholesterol into the mitochondria and decreasing the conversion of cholesterol to pregnenolone in the cells resulting in reducing subsequent testosterone production [51]. Hence, the reduced testosterone might be responsible for the decreased sperm counts and motility and also morphological abnormality of testis. It is hypothesized that the decrease in serum testosterone concentrations suppressed spermatogenesis [52]. Besides, β-cyf-treated group showed a significant (*P<0.05)* increase in FSH and LH **(Table 4)**. The increase in FSH secretion may be principally due to a feedback signal from the damaged seminiferous tubules. Under such situation, the Sertoli cells are found to produce less inhibin B, and then FSH released from the pituitary is increased significantly due to a negative feedback action [53].

Fortunately, omega-3 administration alone or in combination with β-cyf resulted in a significant (*P<0.05*) amelioration of the disturbances in the sex hormone levels as well as the sperm quality **(Table 4)**. As concerned that feeding fish oil improved all semen characteristics including sperm motility, progressive motility and concentration. This is mainly attributed to the high concentration

of PUFAs in fish oil that can be incorporated in the sperm lipids, and cause alterations in the fluidity and flexibility of the sperm membrane [54]. There is an evidence that feeding PUFAs can also affect the biosynthetic pathways involved in steroidogenesis, which have multiple roles in the regulation of reproductive function. Moreover, the presence of oleic acid, monounsaturated fatty acids (MUFAs) also lowers the susceptibility of the test is to lipid peroxidation [55]. Another reason may be acceptable to give a reliable explanation that omega-3 have a powerful initiation effect on acetylcholine which is a neurotransmitter regulating sexual desire, so stimulation of this neurotransmitter has a positive effect on cognitive functioning, especially memory and attention, and also increase semen volume [56].

TABLE 4: EFFECT OF β-CYFLUTHRIN, OMEGA-3 AND THEIR COMBINATION ON SPERM COUNT, SPERM MOTILITY, SPERM ABNORMALITIES, TESTOSTERONE, FSH AND LH OF MALE RATS

Parameters	Experimental groups			
	Control	β-cyfluthrin	Omega-3	β-cyfluthrin+omega-3
Sperm count (million/ml)	98.42±0.649^{b}	17.42±0.751^{d}	104.00±0.816^{a}	62.00±0.534^{c}
Sperm motility (%)	87.71±0.680^{b}	19.00±0.534^{d}	92.71±0.680^{a}	76.42±0.480^{c}
Sperm abnormalities (%)	10.28±0.420^{c}	69.14±0.911^{a}	8.28±0.420^{d}	26.28±0.680^{b}
Testosterone (ng/ml)	7.03±0.056^{b}	1.09±0.069^{d}	8.01±0.044^{a}	4.91±0.050^{c}
FSH (ng/ml)	2.05±0.095^{c}	6.34±0.057^{a}	1.34±0.064^{d}	3.30±0.081^{b}
LH (mlU/ml)	1.05±0.040^{c}	3.70±0.058^{a}	0.52±0.021^{d}	1.61±0.066^{b}

Values are expressed as means ±S.E, n=7 for each treatment group.
Mean values within a row not sharing a common superscript letter (a, b, c and d) were significantly different $P<0.05$.

Effects of β-cyfluthrin, omega-3 and their combination on the brain TNF-α, IL-6 and APP mRNA expressions of male rats: The current results revealed significant elevations (P<0.05) in TNF-α, IL-6 and APP gene expressions in β-cyf group compared to the control group (**Fig. 1** and **Table 5)**. Similarly, it was reported that β-cyf caused the up-regulation of interleukin-6 receptor (IL-6R) and tumor necrosis factor receptor super family (TNFRSF10A) genes accompanied with brain inflammation [57].

The NO pathway is involved in the regulation of IL-6 expression in human. At its early stage, it may accelerate the process by stimulating the synthesis of pro-inflammatory cytokines [58]. The modulation of cytokine production by monocytes and macrophages depends on INF-γ, which stimulates TNFα synthesis. Furthermore, oxidative stress as free radicals generated by pyrethroids is a paramount player in the induction of pro-inflammatory cytokines that stimulate IL-12, INF-γ and TNFα release [59]. ROS are major activators of the NF-κB transcription factor involved in innate immune or inflammation responses. Activation of NF-κB upregulates the expression of many inflammation-related genes, including; TNF-α, IL-6, IL-8 and vascular endothelial growth factor [60]. Furthermore, it was reported that cypermethrin stimulated a typical pro amyloidogenic processing of APP through sequential activation of β-secretase (BACE) and PS, elevating both isoforms of Aβ [61]. The amyloid hypothesis postulates a proteolytic cleavage of APP by BACE to release C-terminal fragment (CTF-β) that is then cleaved by gamma (γ)-secretase to generate $Aβ_{1-42}$ and less amyloidogenic, Aβ1-40 [62]. Otherwise, administration of omega-3 alone showed insignificant (*P>0.05*) increase in TNF-α (**Fig. 1**), while, IL-6 and APP gene expressions were insignificantly (*P>0.05*) decreased compared to the control group.

Co-addition of omega-3 with β-cyfluthrin significantly (*P<0.05*) reduced TNF-α, IL-6 and APP gene expressions compared to β-cyf group (**Fig. 1** and **Table 5).** Omega-3 fatty acid supplements can affect cytokine synthesis and can modulate inflammation-induced transcription of TNF-α by inhibition of NF-kB [63]. This could be due to omega-3 derived lipid mediators, the resolvins and protectins, which have been shown to be potent anti-inflammatory mediators [64]. EPA exerts anti-inflammatory effects through a group of pro-resolving mediators, E-series resolvins, which are derived from EPA by cycloxygenase (COX1) and 5-lipoxygenase (5-LOX) [65].

Previous studies have established that APP processing depends on the local membrane environment, whereas can be altered by

manipulating the membrane lipid composition. Since fatty acids modulate membrane organization and functions, they may affect APP processing. In addition, DHA has been shown to increase membrane fluidity and PUFAs play a central role in the normal development and functioning of brain [66]. DHA decreased the amount of vascular Aβ deposition and reduced Aβ burden in aged Alzheimer mouse model. In Alzheimer disease mouse model, DHA modulated APP processing by decreasing both α- and β-APP C terminal fragment products and full-length APP [67].

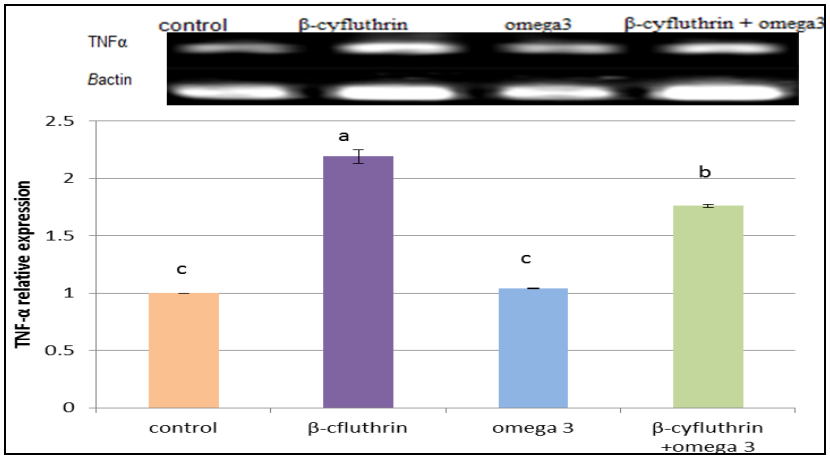

FIG. 1: BRAIN TNFα RELATIVE EXPRESSION IN DIFFERENT EXPERIMENTAL GROUPS (CHANGE CALCULATED IN REFERENCE TO CONTROL

TABLE 5: EFFECT OF β-CYFLUTHRIN, OMEGA-3 AND THEIR COMBINATION ON THE BRAIN IL-6 AND APP mRNA EXPRESSION OF MALE RATS (FOLD CHANGE CALCULATED IN REFERENCE TO CONTROL

Parameters	Experimental groups			
	Control	β-cyfluthrin	Omega-3	β-cyfluthrin +omega-3
IL-6	1.00±0.00^{c}	4.20±0.56^{a}	0.85±0.07^{c}	1.95±0.07^{b}
APP	1.00±0.00^{c}	3.09±0.15^{a}	0.98±0.01^{c}	2.1 ±0.26^{b}

Values are expressed as means ± SD, n=7 for each treatment group.
Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different $P<0.05$.

Histopathological analysis: Histopathological examination of brain sections through cerebral cortex of control and omega-3 groups (**Figs. 2 A & C**) showed normal histoarchitecture of cerebral cortex tissues; normal pyramidal and granular cells. However, sections in cerebral cortex of β-cyf-treated group exhibited loss of normal structure, neuronal degeneration, encephalomelacia with plaque, presence of cytoplasmic vacuolization, congested blood vessels with edema and neurofibrillary tangles (**Figs. 2 B1 & B2**) compared to those of control rats.

Cypermethrin induced histopathological alterations; perinuclear cytoplasmic vacuoles in neurons and mild degenerative changes of nerve fibers with congestion of blood vessels [68]. This may be due to the intense oxidative stress motivated by cypermethrin which can lead to lipid peroxidation and neurotoxic effect accompanied with inhibition of AChE activity and impairment of neural conductivity in the central and peripheral nervous system. Further, the histopathological alterations were markedly reduced in omega-3 plus β-cyf-treated group manifested more or less normal neurons with few pyknotic nuclei and residual fine vacuolations compared to those of β-cyf-treated group (**Fig. 2 D**). These observations were coincidence with previous results which indicated that omega-3 supplementation exerts a neuroprotective effect against hyperoxic brain injury in the developing brain [69].

The cellular mechanisms implied the neuroprotective effect of DHA have been identified as free radical scavenger via regulation of gene expression involved in diverse pathways such as cell signaling, division, growth and apoptosis. DHA is the precursor of neuroprotectin D1, which is a lipid mediator demonstrated to protect neurons and retinal cells from oxidative stress-induced apoptosis [70].

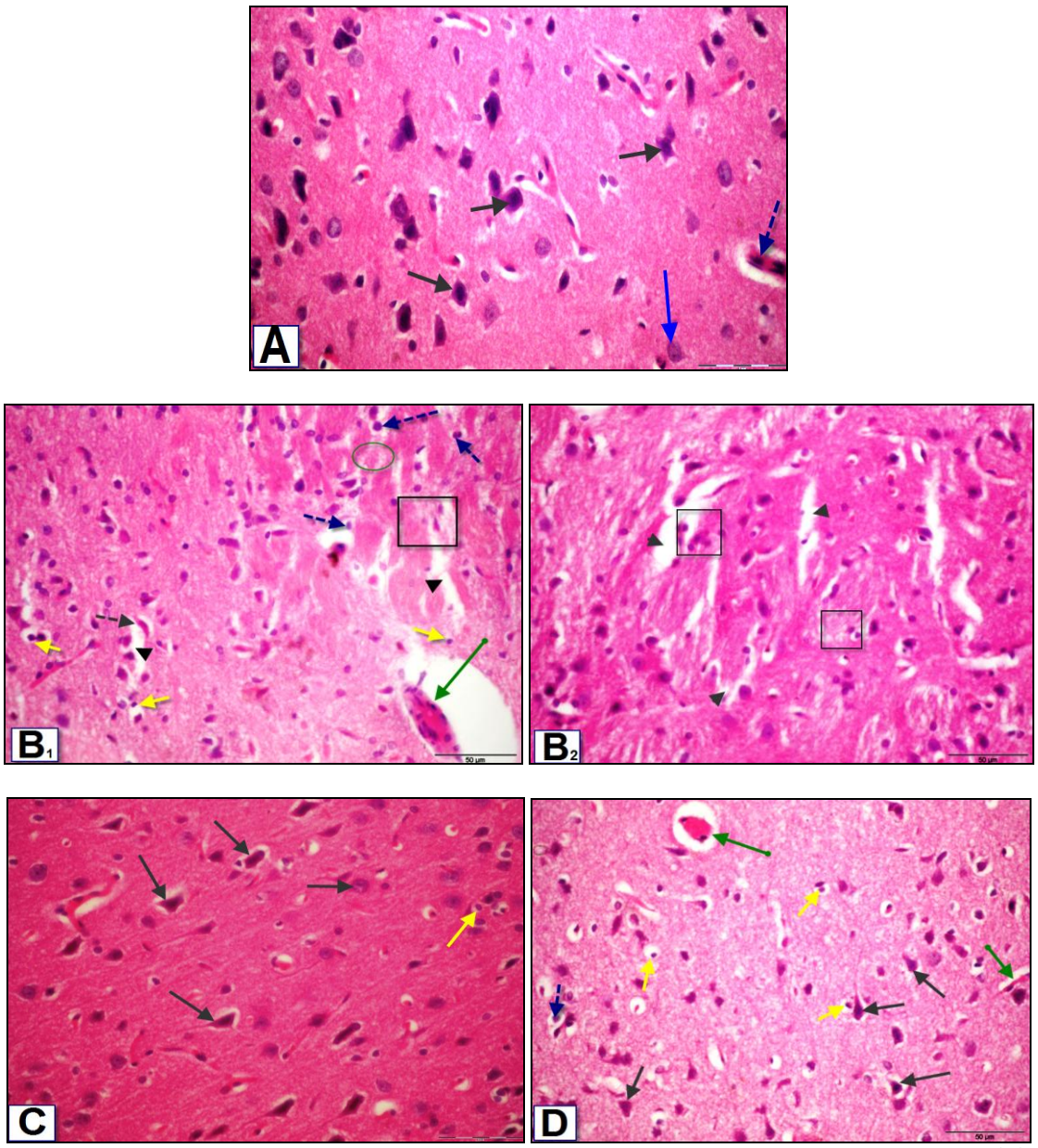

FIG. 2: LIGHT MICROGRAPH OF THE BRAIN SECTIONS IN MALE RAT CEREBRAL CORTEX: (A) control rat, normal histo-architecture; normal pyramidal cells (black arrow), normal granular cells(blue arrow) & normal blood vessel(blue dotted arrow). (B_1 & B2): Sections of β-cyf –treated rats, showing pericellular edema (black head arrow), dilatation of blood capillary with congestion (green arrow), neurofibrillary tangle(black dotted arrow), pyknotic nuclei (blue dotted arrow), encephalomalacia with plaque formation (green circle), neuronal degeneration&cytoplasmic vaculation (black square) and more glial cells (yellow arrow). (C): Section of the cerebral cortex of rat treated with omega 3-treated group showing, normal histo-architecture with normal histology of the pyramidal cells & granular cells (black arrow). (D): Section of the cerebral cortex of rat treated with omega + 3β-cyf –treated rats, showing slightly restores of the pyramidal cells (black arrow) to near normal structure with residual fine vacuolations (green arrow) and glail cells (yellow arrow)" H&E, X 400".

Light microscopic evaluation of testicular tissue from the control and omega-3 groups showed the normal histology of the testis with complete stages of spermatogenesis and high concentration of sperms in the lumen of the seminiferous tubules (**Fig. 3 A & C**). While, the testis of the β-cyf group depicted degeneration and atrophy of seminiferous tubules, incomplete spermatogenic cycles, a few numbers of sperms in the lumen and Leydig cells atrophy (**Fig. 3 B**). These changes may be in the same line with the others who stated that deltamethrin might induce lipid peroxidation and decline in testosterone hormone, since testosterone is required for the attachment of different generations of germ cells in seminiferous tubules.

Therefore, minimal level of testosterone might have led to detachment of germ cells from seminiferous epithelium leading to germ cell apoptosis and reproductive toxicity [51]. Treatment with omega-3 plus β-cyf revealed partial improvement of testis architecture, degenerative changes in the germinal cells and sperm numbers (**Fig. 3 D**). Pretreatment of omega-3 significantly alleviated the abnormalities caused by doxorubicin and largely counteracted the unfavorable effects and preserved the integrity of spermatogenic structures [71].

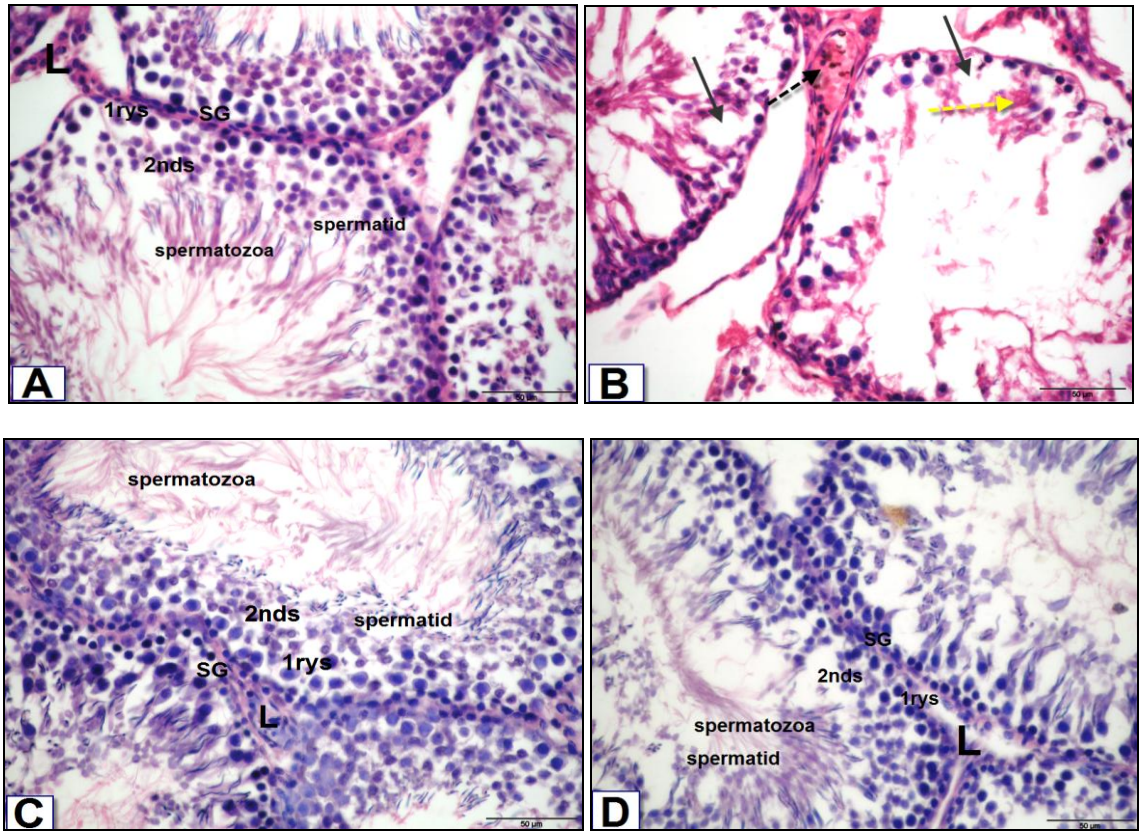

FIG. 3: LIGHT MICROGRAPH OF THE TESTIS SECTIONS IN MALE RATS: (A) control group, the normal histo - architecture of seminiferous tubules and Leydig cell (L), the different stages of spermatogenesis: spermatogonia (SG), primary spermatocyte (1ry S), secondary spermatocyte (2nd S), spermatids and lumen filled with spermatozoa. (B): β-Cyf –treated group, showing seminiferous tubules lost its shape and appeared with irregular outline with pyknotic nuclei, vacuolations (black arrow) and hemorrhage (yellow arrow). Interstitial tissues showed hemorrhage and enlargement (black dotted arrow). (C) omega3-treated group, showing the normal structure of seminiferous tubules and Leydig cells (L) with regular spermatogenic cycle. Lumen was full of spermatozoa. (D) Omega 3+ β-Cyf –treated group showing slightly improved architecture of semniferous tubules. More or less normal distribution of the spermatogenic cells. "H&E, X 400".

CONCLUSION: In conclusion, omega-3 could exhibit protective effects against β-cyf-induced neurological and testicular damage.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

ACKNOWLEDGEMENTS: The authors are thankful to the histopathological lab at the High Institute of Public Health, Alexandria University, Egypt. Also, grateful to molecular lab at Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt.

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How to cite this article:
Abdou HM, Mohamed NA, Awad D and El-Qazaz I: β-cyfluthrin-induced impairment in neurotransmitters, testicular function and gene experissions of male rats: the protective role of omega-3. Int J Pharm Sci Res 2017; 8(7): 2819-31. doi: 10.13040/IJPSR.0975-8232.8(7).2819-31.

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