Neurotoxic Effect of Lambda-Cyhalothrin, A Synthetic Pyrethroid Pesticide: Involvement of Oxidative Stress And Protective Role of Antioxidant Mixture

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Abstract: Extensive application of pesticides is usually accompanied with serious problems of pollution and health hazards. Lambda-cyhalothrin (LTC), a new generation type II synthetic pyrethroid, is widely used in agriculture, home pest control and protection of foodstuff. This study aimed to: 1. Investigate the acute toxicity (LD_{50}) and evaluate the effect of two different sublethal concentrations of LTC ($1/20^{th}$ or $1/10^{th}$ of LD_{50} values) on acetylcholinesterase (AChE), Na^+,K^+-ATPase and arginase enzyme activities, nitric oxide ($NO^•$), antioxidant status and oxidative stress biomarkers in rat brain. 2. Evaluate the possible protective role of ascorbic acid, α-tocopherol in combination with selenium (Se) at a dose of 100, 100 and 0.30 mg/kg b.w/day, p.o., respectively as antioxidant mixture (AM) during exposure to LTC. The acute toxicity test revealed that LTC has low oral LD_{50} value (84 mg/Kg) for male rats indicating its moderate cytotoxic effect. Also the results revealed that a daily exposure to LTC at a dose of 4.2 or 8.4 mg/Kg b.w, p.o. for 4 weeks was significantly inhibited the activities of AChE, Na^+,K^+-ATPase, arginase and antioxidant defense enzymes (superoxide dismutase SOD; catalase CAT; glutathione peroxidase GPx; glutathione reductase GR; gluthione-*S*-transeferase GST) and a marked decrease in reduced glutathione (GSH) and vitamins C and E a long with significantly increase the brain levels of $NO^•$, malondialdehyde (MDA) a product of lipid peroxidation (LPO) and protein carbonyl (PC), a product of protein oxidation as compared to normal control rats. Daily simultaneous administration of AM along with LTC for the same period was significantly reduced the aforementioned changes. These results provided strong evidence that oxidative stress is involved in the pathogenesis of neurotoxicity of LTC in dose dependant manner and suggested the potential synergistic and protective effects of ascorbic acid, α-tocopherol and selenium against neurotoxicity induced by LTC. However, further studies are needed in different animal models with different doses to support this novel preventive strategy to slow down the symptoms of this LTC neurotoxicity.
[Zeinab Yousef Ali. **Neurotoxic Effect of Lambda-Cyhalothrin, A Synthetic Pyrethroid Pesticide: Involvement of Oxidative Stress And Protective Role of Antioxidant Mixture.** *N Y Sci J* 2012;5(9):93-103]. (ISSN: 1554-0200). http://www.sciencepub.net/newyork. 11

Keywords: Lambda-cyhalothrin; ascorbic acid; α-tocopherol; selenium; acute toxicity; neurotoxicity; acetylcholinesterase; Na^+,K^+-ATPase; arginase; antioxidant status; oxidative stress; lipid peroxidation; protein oxidation; brain; rats

1. Introduction

Synthetic pyrethroids, a group of neurotoxic insecticides have emerged as a new class of agricultural pesticides showing high toxicity to a wide range of insects including resistance strains and low toxicity to mammoleals and birds, and rapid biodegradability (WHO, 2011). Based on the structural differences, pyrethroids are broadly classified into two types. Type I class of pyrethroids do not contain α-cyano group and type II pyrethroid with the cyano group. Type I pyrethroids generally affects the peripheral nerves and they are best suitable to small-scale personal applications. While type II affects the entire nervous system and they are preferred for large scale uses due to their higher potency (Ansari et al., 2012).

Lambda-cyhalothrin (LTC), a new generation type II synthetic pyrethroid is the most widely used pyrethroid. LTC was registered by the U.S. Environmental Protection Agency (EPA) in 1988. Figure 1 shows the structure formula of Lambda-cyhalothrin. With regard to effectiveness and toxicity, LTC appears to be the first-choice insecticide. LTC has extensive uses as an agro-pesticide in the cotton plantation, in vegetable production and to control a wide range of insect pests in a variety of crops. LTC is also used for indoor residual spraying, space spraying and treatment of mosquito nets, for the control of vectors and pests of public health importance (FAO, 1999 and WHO, 2000; 2003; 2011).

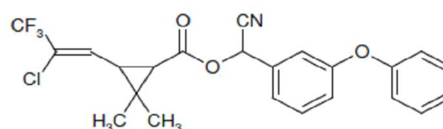

Figure 1. Structure formula of Lambda-cyhalothrin

Large-scale application of pyrethroids to crops may contribute to the presence of toxic substances in the environment. These chemical compounds can find their way into the water reservoirs, streams and rivers, thus producing an adverse impact on the aquatic biota, animals and human health. Human exposure to pesticides is reported to occur mainly occupationally during application or through pyrethroids residues such as those detected in milk and blood of dairy cows and cattle meat as well as vegetables and fruits (WHO, 2000; Turgut et al., 2011; Shen et al., 2012). Recently, pyrethroid metabolites have been detected in urine of adults, children and pregnant women (Qi et al., 2012). US Environmental Protection Agency classified LTC as a possible carcinogen (EPA, 1988). Recently, many studies have reported exposure to pesticides in humans may cause subtle to severe neuro-physiological and neurobehavioral abnormalities. Hussien, et al., (2011) showed that cypermethrin can cross the blood-brain barrier and exert its damage effect in genomic DNA. Ansari et al., (2012) reported that neurobehavioral changes may be more intense in case exposure to LTC.

LTC is rapidly metabolized in the liver via hydrolytic ester cleavage and oxidative pathways by the cytochrome P450 (CYP_{450}) enzymes yield reactive oxygen species (ROS) (Sankar et al., 2012). The over production of ROS exceeds the capacity of the cell's endogenous systems to neutralize them result in oxidative stress. The excess ROS mediate damage to proteins, lipids, mitochondria, and DNA. Cells had several biological defense mechanisms against intracellular oxidative stress. Enzymatic antioxidant defense system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione transferase (GST) and non-enzymatic antioxidants such as reduced glutathione (GSH), vitamins C and E (Evans and Halliwell, 2001; Halliwell, 2006). These mechanisms cooperate with each other to defend against reactive species that induced cellular damage.

Vitamin E (α-tocopherol), a fat soluble antioxidant is a powerful chain-breaking antioxidant and resides primarily in biologic membranes, protecting membrane phospholipids from peroxidation (Hfaiedh et al., 2012). Vitamin C (ascorbic acid), a water soluble antioxidant is the most important free radical scavenger in extra-cellular fluids, trapping radicals in the aqueous phase and protects biomembranes from peroxidative damage (Hall et al., 2010). Furthermore, vitamin C can stabilize the tocopheryl radicals that are formed during the scavenging of ROS, thereby regenerating the active form of vitamin E (Skřivan et al., 2012). However, several studies have reported that vitamin C and E levels were markedly decreased in the setting of brain injury and showing early the subsequent need for further supplementation (Hall et al., 2010, Razmkon et al., 2011, Galasko et al., 2012). Also, it was confirmed that supplementation of diets with Se was significantly increased the activity of GPx (Skřivan et al., 2012).

Therefore, the current study was aimed to investigate the acute toxicity and evaluate the effect of two different sublethal concentrations of LTC on acetylcholinesterase (AChE), Na^+,K^+-ATPase and arginase enzyme activities, antioxidant status and oxidative stress biomarkers in rat brain, as well as the possible protective role of ascorbic acid, α-tocopherol in combination with selenium at a dose of 100, 100 and 0.30 mg/kg body weight/day, respectively as antioxidant mixture (AM).

2. Material and Methods

Chemicals

Formulated lambda-cyhalothrin (LTC; 5%) was supplied from Kima Company, Egypt. Ascorbic acid, α-tocopherol and Se (sodium selenite) were obtained from Sigma Aldrich Chemicals, St. Louis, MO, USA. All other chemicals used were of analytical grade purchased from Fluka Co. (Buchs, Switzerland) or Sigma Co. (St. Louis, USA).

Animals

Sprague Dawley male adult rats (170 ± 20 g) were obtained from animal house, National Organization for Drug Control and Research, NODCAR, Giza, Egypt. The animals were kept under standard laboratory conditions of light/dark cycle (12h /12h) and temperature (25 ± 2°C). They were allowed free access to standard diet and water *ad-libitum*. All experimental protocols were approved by Biochemistry department, NODCAR, Giza, Egypt.

Acute toxicity test

Acute toxicity test of a LTC was conducted following the method of **Lorke (1983)**, and determined by the calculation of median lethal dose (LD_{50}). The doses of LTC were chosen starting from no death to 100% mortality.

Experimental design

Lambda-cyhalothrin (LTC) at two different sublethal doses equivalent to $1/20^{th}$ and $1/10^{th}$ of LD_{50} were suspended in corn oil and administered to animals by gavage in a volume of 1 ml corn oil/rat. Antioxidant mixture (AM) composite of vitamins E and C, and selenium at a dose of 100, 100 and 0.30 mg/kg body weight/day, respectively were s.c injected to animals. The antioxidant doses were selected according to the previous studies (Comin et al., 2010, Ambali et al., 2011, Skřivan et al., 2012).

A total of 40 rats were randomly divided into following five groups each comprising of 8 rats.
Group 1: received a vehicle and served as control.
Group 2: received LTC at a dose of 1/20th of LD_{50} for 4 weeks.
Group 3: received LTC at a dose of 1/10th of LD_{50} for 4 weeks.
Group 4: received AM 30 min prior to LCT (1/20th of LD_{50}) for 4 weeks.
Group 5: received AM 30 min prior to LCT (1/10th of LD_{50}) for 4 weeks.

At the end of the experimental period, all animals were anesthetized using ether and sacrificed by cervical decapitation. Brain tissues were quickly removed, washed in cold isotonic saline and weighed. About 1 gm of brain tissue was cut for assay of vitamin C and E the remaining tissue was homogenized in cold in 50 mmole phosphate buffer (pH 7) using an electronic homogenizer to obtain 10% w/v homogenate that centrifuged at 2000×g for 15 min at 4°C. The pellets were discarded while supernatants were collected for different biochemical analysis. Protein content in brain homogenate was determined according to Lowry et al., (1951) using bovine serum albumin as a standard.

Biochemical analysis:

Determination of acetylcholine esterase (EC 3.1.1.7, AChE).

The activity of acetylcholine esterase (AChE) or acetylhydrolase was determined according to the method of Ellman et al. (1961), based on degradation of acetyl tiocholine iodide by AChE into a product which binds to 5,5-dithiobis-2-nitrobezoic acid (DTNB), forming yellow colour. One unit of AChE activity was expressed as nanomoles acetylcholine hydrolyzed/min/mg protein under experimental conditions.

Determination of Na/K activated adenosine triphosphatase (Na^+,K^+-ATPase; EC 3.6.1.37).

The reaction mixture for the Na^+, K^+-ATPase assay contained 5.0 mmole $MgCl_2$, 80.0 mmole NaCl, 20.0 mmole KCl, and 40.0 mmole Tris–HCl buffer (pH 7.4). The reaction was started by the addition of ATP (disodium salt) to a final concentration of 3.0 mmole. Controls were assayed under the same conditions with the addition of 1.0 mmole ouabain. Na^+, K^+-ATPase activity was calculated by the difference between the two assays (Chakraborty et al., 2003). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Enzyme activity was expressed as µmol Pi liberated/min/mg of protein.

Determination of arginase (EC 3.5.3.1).

Arginase activity was determined by analyzing the conversion of L-arginine to urea with the colorimetric method described by Liu et al., (2003). In brief, 10 mmole $MnCl_2$ in 50 mmole Tris–HCL (pH 7.5) was added to each sample and the arginase enzyme activated by heating at 55 °C for 10 min. The substrate, 0.5 mole L-arginine was then added and incubated at 37 °C for 1 h, after which the reaction was terminated by the addition of an acid mixture: H_2SO_4:H_3PO_4:H_2O (1:3:7). The urea formed was then assessed spectrophotometrically (Unicam-UV-Vis-Spectrometry, Japan) at 540 nm (Boyde and Rahmatullah, 1980). Arginase activity was expressed as U/ mg protein. One unit of arginase was defined as the amount of enzyme that released µmol of urea in one hour at 37°C.

Determination of nitric oxide ($NO^•$)

The brain level of the NO was estimated as nitrate/nitrite by Griess reaction after conversion of nitrate to nitrite according to the method of Montgomery and Dymock (1961). The concentration of $NO^•$ was expressed as nmole/g wet tissue.

Determination of oxidative stress markers

- ***Lipid peroxidation (LPO).*** Malondialdehyde (MDA) a marker of lipid peroxidation was assayed using thiobarbituric acid reacting substance (TBARS) (Buege and Aust, 1978). MDA level was expressed as nmole/ g wet tissue.
- ***Protein carbonyl (PC).*** As a hallmark of protein oxidation, total protein carbonyl (PC) content was determined in the brain homogenate by a spectrophotometric method described by Levine et al. (1990) and expressed as nmole/mg protein.

Determination of enzymatic antioxidants

- ***Superoxide dismutase (SOD; EC 1.15.1.1).*** SOD activity was determined by the method of Marklund and Marklund (1974). In this test, the degree of inhibition of pyrogallol autooxidation by supernatant of the tissue homogenate was measured. One SOD unit corresponds to the enzyme required to inhibit half of the oxidation of pyrogallol. SOD activity was expressed as U/mg of protein.
- ***Catalase (CAT; EC 1.11.1.6).*** CAT activity was determined according to the method of Aebi (1984). The rate of H_2O_2 decomposition was followed by monitoring absorption at 240 nm. One unit of CAT activity is defined as the amount of enzymes required to decompose1 mmoleol of hydrogen peroxide in1 min. The CAT activity was expressed as mmole H_2O_2 utilized/min/mg protein. CAT activity was expressed as U/ mg protein.
- ***Glutathione peroxidase (GPx; EC 1.11.1.9).*** GPx activity was determined as described by

Rotruck et al. (1973). This method depends on determination of the rate of glutathione oxidation by H_2O_2 as catalysed by the GPx present in the supernatant. The colour develops is read against a reagent blank at 412 nm. The activity of GPx was expressed in terms of nmole of GSH oxidized/min/mg protein. GPx activity was expressed as U/ mg protein.

- ***Glutathione reductase (GR; EC 1.6.4.2).*** GR activity was assayed according to the modified method of Mohandas et al. (1984) by monitoring the oxidation of NADPH at 340 nm. GR activity was calculated as nmole NADPH oxidized/min/ mg protein.
- ***Glutathione S-transferase (GST; EC 2.5.1.18).*** GST activity was determined following the procedure of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The conjugation of GSH with CDNB was observed spectrophotometrically at 340 nm. The enzyme activity was expressed as nmole of CDNB formed/min/mg protein.

Determination of non-enzymatic antioxidants

- ***Reduced glutathione (GSH)***. Brain GSH content was determined by the method of Ellman (1959) modified by Jollow et al., (1974) based on the development of a yellow colour when 5,5-dithiobis-2-nitrobezoic acid (DTNB), was added to compounds containing sulfhydryl groups. The absorbance was read spectrophotometrically at 412 nm. Total GSH content was expressed as mmole GSH/g of tissue.
- ***Vitamin C (Ascorbic acid).*** Ascorbic acid concentration was assayed as described by Omaye et al. (1979). A weighted piece of brain tissue was homogenized in trichloroacetic acid and centrifuged at 2000×g for 20 min at 4^oC. DTC reagent (dinitrophenyl hydrazine, thiourea and Cu^{2+}) was added to the supernatant and mixed thoroughly. The tubes were incubated at 37°C for 3 hours and to this a solution ice cold 65% H_2SO_4 was added. The resulting colour was read at 520 nm. The results were expressed in µmole/g tissue.
- ***Vitamin E (α-Tocopherol)***. α-Tocopherol concentration was assayed as described by Backer et al. (1980). A weighted piece of brain tissue was homogenized in a mixture of petroleum ether and ethanol (2:1.6 v/v) and centrifuged at 15,680×g for 20 min at 4^oC and the supernatant was used for estimation of α-tocopherol. To one ml of supernatant, 2% 2,2-dipyridyl and 0.5% ferric chloride. The colour developed was read at 520nm.The values are expressed as nmole/g tissue.

Statistical analysis

All results are presented as mean ± S.E. The statistical comparisons were done using one way analysis of variance (ANOVA) (using SPSS 17 statistical software) and the different between means were compared by Dunnett's multiple range test (DMRT); one group as a control and compare all other groups against it. $P<0.05$ was considered significant.

3. Results and Discussions

Extensive application of pesticides is usually accompanied with serious problems of pollution and health hazards. Several studies confirmed that the central nervous system (CNS) is the principle target of synthetic pyrethroids (Agrawa and Sharma, 2010; Ansari et al., 2012). Therefore, this study aimed to investigate the acute toxicity (LD_{50}) and evaluate the repeated exposure to two sub-lethal concentrations of Lambda-cyhalothrin (LTC) on the brain enzymes, antioxidant status, nitric oxide and oxidative stress biomarkers, as well as the possible neuroprotective role of vitamins C and E in combination with selenium (100, 100 and 0.30 mg/kg body weight, for 4 weeks, respectively) as antioxidant mixture (AM).

In the present study, the acute toxicity test revealed that LTC has low oral LD_{50} value (84 mg /Kg) for male rats indicating its moderate cytotoxic effect EPA (2007). This result is in agreement with that of WHO (1990, 2003) who reported that the acute oral LD_{50} of LTC is 79 mg/kg for male rat. Consequently, LTC at two different sublethal doses 4.2 and 8.4 mg/kg body weight/day that equivalent to $1/20^{th}$ and $1/10^{th}$ of LD_{50} values, respectively were chosen in this study.

Effect on AChE, Na^+,K^+-ATPase, Arginase and Nitric Oxide

The data depicted in Table 1 revealed a significant ($P<0.001$) decrease in acetylcholinesterase (AChE), Na^+, K^+-ATPase and arginase activities along with significantly ($P<0.001$) increase in $NO^•$ in LTC- intoxicated rats compared with normal control rats. These biochemical changes were in dose dependant manner. These results are in agreement with that of Milatovic et al. (2006), Hussien, et al. (2011) and Ansari et al. (2012). Whereas co-administration of AM with LTC at two doses for 4 weeks significantly preserve these aforementioned changes to near normal levels as compared with that of LTC- intoxicated rats. The current study confirmed that the mechanism involved in brain damage by LTC appear to be linked with alteration in the activities of brain enzymes. However, co-administration of AM with LTC was able to slow the progression of this neuro-degeneration.

Table 1. Effect of different treatments on AChE and Na^+, K^+-ATPase, arginase and nitric oxide and percentage change from the normal control

Groups	AChE	Na^+, K^+-ATPase	Arginase	$NO^{\bullet}$
Control	955.7 ± 7.42	1.31 ± 0.047	11.2 ± 0.47	79.5 ± 1.99
LTC (4.2 mg/kg/day)	699.7 ± 10.9 *** -29.9 %	1.07 ± 0.023 *** -18.8 %	9.14 ± 0.24 *** 15.5 %	109.1 ± 2.12 *** 37.1 %
LTC (8.4 mg/kg/day)	545.8 ± 16.3 *** -42.9 %	1.01 ± 0.052 *** -22.7 %	8.56 ± 0.19 *** 23.3 %	125.9 ± 2.25 *** 58.3 %
AM + LTC (4.2 mg/kg/day)	879.8 ± 11.8 * -7.94 %	1.24 ± 0.026 ^{ns} -5.54 %	10.8 ± 0.34 ^{ns} 7.74 %	87.6 ± 2.94 ^{ns} 10.2 %
AM + LTC (8.4 mg/kg/day)	843.1 ± 20.0 ** -11.8 %	1.18 ± 0.030 * -10.2 %	10.4 ± 0.23 ^{ns} 10.6 %	92.6 ± 2.14 * 16.4 %

Each value represents the mean of 8 rats ± S.E. ns : Non-significant; * : Significant at $p < 0.05$; **: Highly significant at $p<0.01$; ***: Very highly significant at $p<0.001$. Acetylcholine esterase (AChE, U/mg protein); Na^+, K^+-ATPase (µmol Pi liberated/min/mg protein); arginase (U/mg protein); Nitric oxide ($NO^{\bullet}$, nmole/g tissue)

Acetylcholinesterase (AChE) is an important cholinesterase enzyme present in the neuromuscular junctions and cholinergic synapses in the CNS. AChE terminates the signal transmission by hydrolyzing acetylcholine (ACh), a neurotransmitter that conducts nerve impulses across neuromuscular junctions in the nervous system of vertebrates as well as insects (Periasamy et al., 2009). ACh is also implicated in brain plasticity and disease (Ljubisavljevic et al., 2012). Therefore, AChE is considered as a key enzyme in detecting the neurotoxicity. Numerous studies have suggested that LTC exert its neurotoxicity toxicity primarily by virtue of AChE inhibition (Singh et al., 2012).

The obtained results in Table 1 showed that administration of LTC at two doses for 4 weeks was significantly ($p<0.001$) inhibited the enzymatic activity of AChE in dose dependant manner suggested that the impairment of AChE activity could be one of the LTC-neurotoxicity mechanism. However, co-administration of AM with LTC was able to preserve the AChE activity near healthy state. The inhibition of AChE enzyme was attributed to the occupation of its active sites by pollutants that could lead to decrease the cellular metabolism, disturb metabolic and nervous activity and lead to ionic refluxes and differential membrane permeability in addition to increase in lipid peroxidation (El-Demerdash, 2011). In this context, Podolska and Napierska (2006) observed that the inhibition of AChE enzyme causes accumulation of ACh resulting in rapid, uncontrolled twitching of voluntary muscles which eventually leads to cholinergic hyperactivity, convulsion, status epilepticus, paralysis and finally respiratory failure and death. Furthermore, Ansari et al. (2012) reported that neurobehavioral changes may be more intense in exposure to pyrethroids and associated with environmentally induced Parkinson's disease.

Na^+, K^+-ATPase, a membrane bound lipid dependent enzyme catalyzes the active transport of Na^+ and K^+ in CNS that necessary to maintain cellular homeostasis and the ionic gradient for neuronal excitability. Also, Na^+, K^+-ATPase is a key enzyme implicated in neural excitability, metabolic energy production and uptake and release of catecholamines and serotonin (Hussien, et al., 2011). Results in Table 1 showed that LTC intoxication significantly ($P<0.001$) inhibited the enzymatic activity of Na^+, K^+-ATPase, suggested that impairment of Na^+, K^+-ATPase activity could be one of the underlying biochemical mechanism that leading to CNS dysfunctions. However, co-administration of AM with LTC was able to prevent the inhibition of this enzyme. In this context, Singh et al., (2012) suggested that the enhanced susceptibility of brain membranes to LPO can lead to loss of membrane bound ATPases activity that modulates the cell functions. Moreover, it was well established that the primary effect of pyrethroids occurs on the voltage sensitive sodium channels; disrupting the functioning of the CNS both in insects and mammalians (Ansari et al., 2012). Inactivation of Na^+, K^+-ATPase leads to partial membrane depolarization allowing excessive Ca^{2+} entry inside neurons with resultant toxic events like excitotoxicity (Swamy et al., 2005). This membrane bound enzyme requires phospholipid for its activity and is highly vulnerable to oxidative insult. The mechanism of inactivation under such conditions also involves disruption of phospholipid microenvironment of the enzyme or direct damage to enzyme protein by ROS or LPO products that associated alteration of fluidity or other membrane properties (Billimoria et al., 2006; Hossain and Richardson, 2011). Therefore, LTC

decline of rat brain Na^+, K^+-ATPase activity is presumably the consequence of an enhanced oxidative damage in brain. Consequently, the present study demonstrated that LTC exerts its neurotoxic effect through voltage dependent sodium channel and integral protein ATPase in the neuronal membrane (Hussien, et al., 2011; Ljubisavljevic et al., 2012) while, co-administration of AM with LTC- was markedly restores the activities of membrane bound ATPases, which denotes that AM conserves the integrity of cell membrane, strength the antioxidant capacity and normal physiological functions of brain.

Arginase, the final enzyme in the urea cycle is responsible for the hydrolysis of L-arginine to urea and ornithine (Ahn et al., 2012). Arginase shares the substrate, L-arginine, with nitric oxide synthase (NOS) that catalyze the production of nitric oxide ($NO^•$) from L-arginine. The mutual regulation of arginase and NOS in L-arginine-metabolizing pathways has recently been demonstrated (Yanik et al., 2004). $NO^•$ is a gaseous highly reactive free radical that can diffuse freely across biological membranes (North and Scott, 2011) and acts as neurotransmitter, implicated in brain plasticity (Choi et al., 2012). It plays an important role in neuronal tissue damage, mitochondrial dysfunction, lipid peroxidation (LPO), protein nitration, ion channel disability and electrolyte imbalance (Ljubisavljevic et al., 2012). The obtained results in Table 1, demonstrated that LTC intoxicated rats exhibited a significant increase in NO content with concomitant decrease in the arginase activity that indicating the imbalance between arginase and NOS as a result of LTC neurotoxicity. However simultaneous administration of AM with LTC for 4 weeks was significantly maintained the balance between arginase and NOS in brain. The present results are in agreement with previous studies, Lim et al. (2007) showed that arginase and NOS compete for L-arginine, thus arginase modulates NOS activity by regulating intracellular L-arginine bioavailability.

Effect on Oxidative Stress Markers

The brain tissue is highly susceptible to LPO because of its high rate of oxygen utilization, an abundant supply of polyunsaturated fatty acids, a deficient antioxidant defense and a high content of transition metals like copper and iron (Singh et al., 2012). Table 2 illustrated that the brain markers of LPO and protein oxidation (MDA and PC) were significantly increased ($p<0.001$) in LTC-intoxicated rats in dose dependant manner as compared with control rats, confirmed the susceptibility of brain tissues to oxidative stress. However, co-administration of LTC for 4 weeks with AM was significantly reinforcing the resistance of the brain towards LTC toxicity by direct scavenging ROS.

Table 2. Effect of different treatments on the oxidative stress markers and antioxidant enzyme activities and percentage change from the normal control

Groups	Oxidative Stress Markers		Antioxidant Enzyme Activities				
	MDA	PC	SOD	CAT	GPx	GR	GST
Control	17.6 ± 0.41	1.45 ± 0.065	4.07 ± 0.118	85.3 ± 1.96	88.9 ± 2.60	8.24 ± 0.25	16.9 ± 0.25
LTC (4.2 mg/kg/day)	32.2 ± 1.63*** (82.5%)	2.31 ± 0.085*** (58.7%)	2.87 ± 0.120*** (-29.4%)	68.5 ± 2.15*** (-19.7%)	75.0 ± 1.50*** (-15.6%)	6.88 ± 0.17*** (-16.5%)	11.9 ± 0.39*** (-29.6%)
LTC (8.4 mg/kg/day)	34.6 ± 1.59*** (96.5%)	2.75 ± 0.064*** (89.3%)	2.47 ± 0.076*** (-39.4%)	61.9 ± 2.11*** (-27.4%)	72.2 ± 2.79*** (-18.7%)	6.29 ± 0.11*** (-23.6%)	11.2 ± 0.32*** (-33.6%)
AM + LTC (4.2 mg/kg/day)	18.9 ± 1.82ns (7.24%)	1.60 ± 0.085 ns (9.97%)	4.68 ± 0.119* (15.0%)	90.3 ± 2.50ns (5.83%)	99.8 ± 2.95* (12.3%)	8.41 ± 0.16ns (2.12%)	17.0 ± 0.29ns (0.59%)
AM + LTC (8.4 mg/kg/day)	20.6 ± 0.99* (17.0%)	1.65 ± 0.076* (13.8%)	4.20 ± 0.114ns (3.16%)	87.1 ± 3.15ns (2.11%)	89.0 ± 2.64ns (0.17%)	8.03 ± 0.23ns (-2.58%)	16.0 ± 0.46ns (-5.25%)

Each value represents the mean of 8 rats ± S.E. ns: Non-significant; *: Significant at $P<0.05$; ***: Very highly significant at $P<0.001$. Malondialdehyde (MDA, nmole/g tissue), Protein carbonyl (PC, nmole/mg protein); Superoxide dismutase (SOD, U/mg protein); Catalase (CAT, U/mg protein); Glutathione peroxidase (GPx, U/mg protein); Glutathione reductase (GR, nmole NADPH oxidized/min/mg protein); Glutathione transferase (GST, nmole of CDNB formed/min/mg protein)

According to WHO (2000), pyrethroids indirectly generates various ROS such as superoxide radical and hydroxyl radical, and reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide. Also, Piner and Ünerb (2012) reported that LTC metabolism generates reactive oxygen species (ROS) which in turn lead to enhanced LPO and oxidative stress. Therefore the present study suggested that oxidative damage induced by pyrethroid is attributed to their lipophilicity nature, whereby they could penetrate the cell membrane easily and accumulate in biological membranes leading to increased production of ROS and oxidative damage to its lipids and oxidative modification of proteins as confirmed from significant increased in the brain MDA and PC levels. In this aspect, Piner and Ünerb (2012) showed that these radicals can destroy proteins; lipids and DNA by oxidation and attack the cell membrane lead to destabilization and disintegration of cell membrane and decrease its fluidity as a result of lipid peroxidation and protein oxidation.

Effect on Enzymatic Antioxidant

The detoxification of ROS in brain involves the co-operative action of all intracellular antioxidant enzymes. Superoxide dismutase (SOD) is the first line of antioxidant enzymatic defense catalyzes the conversion of superoxide radicals to less toxic H_2O_2. Then catalase (CAT) metabolizes H_2O_2 to water. When this mechanism is saturated, the second line of antioxidant enzymatic defense mainly GPx that regulated by selenium availability is activated (Duntas, 2012). GPx are a family of selenium-containing enzymes that responsible for detoxification of H_2O_2 and lipid peroxides at the membrane level into less reactive species using cellular GSH as substrate thus preventing the progressive formation of free radicals and provide the cell important protection aginst oxidative stress and LPO (Sankar and Telang, 2012). Many cells contain both CAT and GPx, while the brain GPx seems to be the major importance (Sun et al., 2012).

The data in Table 2 illustrated that a daily administration of LTC at two doses for 4 weeks were significantly (P<0.001) decreased the activity of the antioxidant defense enzymes (CAT, SOD, GPx, GR and GST) in a dose dependant as compared with that of the normal rats. While simultaneous administrated of AM (vitamins E and C + Se) with LTC for the same period significantly enhanced the activity of these antioxidant enzymes in brain tissue as compared to the control group. This depression of antioxidant enzyme activities reflects failure of the antioxidant defense mechanisms to overcome the influx of ROS induced by LTC exposure that leads to the accumulation of free radicals and facilitate the enhancement of LPO, which in turn increases the oxidative damage to the brain tissue (Hfaiedh et al., 2012). The current data are in accordance with previous results which reported a decreased activity of antioxidant enzymes in pyrethroids exposed rats (Hussien, et al., 2011; Brzóska et al., 2012). The present results provided strong evidence that oxidative stress is involved in the pathogenesis of neurotoxicity of LTC. Therefore, supplementation with anti-oxidants vitamins E and C and selenium was able to support the activities of antioxidant enzymes during exposure to different doses of LTC for 4 weeks leading to delay the development of oxidative disease. Furthermore, the enhanced activity of GR is responsible for maintenance the stable ratio of GSH/GSSG by catalyzing the recycling of GSH from the toxic oxidized form (GSSG) to the reduced form (GSH) (Halliwell, 2006; Piner and Ünerb, 2012).

Effect on Non-Enzymatic Antioxidant

There is a co-operative action between enzymatic and non-enzymatic antioxidants have been shown to scavenge free radicals and ROS. Non enzymatic antioxidants include vitamins A, C and E, GSH and trace elements like zinc and selenium (Halliwell, 2006). The results depicted in Table (3) revealed that administration of LTC at both doses were significantly decreased GSH, ascorbic acid and vitamin E levels as compared with normal control. Co-administration of AM with either two doses of LTC recovered these antioxidants close to normal. These results indicated that repeated administration of LTC result in inadequate detoxification mechanism to prevent the overproduction of reactive radicals that induce oxidative damage in brain tissue, and the ability of AM to restore the original redox state and attenuate LTC-induced oxidative damage. These results are in agreement with that of the previous studies of Agrawa and Sharma (2010), Hussien et al. (2011) and Skřivan et al. (2012).

Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. Brain glutathione and other thiol containing proteins play a crucial role in cellular defense against toxicity of pesticides (Halliwell, 2006). GSH acts as a direct free radical scavenger as well as co-substrate for GPx and GST to react with the highly reactive free radicals and organic peroxides. Recent studies have shown that LTC treatment depletes GSH levels that enhanced LPO as one of the mechanisms of LTC toxicity (Ansari et al., 2012). In the current study, the significant (P<0.001) decrease of GSH content in LTC-treated group could be a result of decreased synthesis or increased utilization, leading to increased susceptibility of the brain tissue to free radical

damage (Singh et al., 2012). The direct scavenging property of the AM was able to neutralize the overproduction of reactive radicals that induced by LTC. Thus the present results demonstrated that the depletion of intracellular GSH by pyrethroid insecticides resulted from excess generation of ROS.

Table 3. Effect of different treatments on the non- enzymatic antioxidants and percentage change from the normal control

Groups	GSH	Ascorbic Acid	α-tocopherol
Control	1.33 ± 0.022	862.3 ± 11.6	32.9 ± 1.57
LTC (4.2 mg/kg/day)	0.99 ± 0.046*** (-25.4%)	682.7 ± 16.2*** (-20.8%)	25.3 ± 1.39** (-23.2%)
LTC (8.4 mg/kg/day)	0.82 ± 0.027*** (-38.4%)	627.3 ± 14.3*** (-27.3%)	22.1 ± 0.92*** (-32.8%)
AM + LTC (4.2 mg/kg/day)	1.40 ± 0.041ns (5.57%)	907.8 ± 12.4 ns (5.28%)	35.4 ± 1.24 ns (7.60%)
AM + LTC (8.4 mg/kg/day)	137 ± 0.036 ns (3.62%)	881.7 ± 13.8 ns (2.25%)	34.0 ± 1.09 ns (3.35%)

Each value represents the mean of 8 rats ± S.E. ns : Non-significant;
** : Highly significant at $P < 0.01$; *** : Very highly significant at $P < 0.001$
Reduced glutathione (GSH, nmole of CDNB formed/min/mg protein); Ascorbic acid (μmole/g tissue); α-tocopherol (nmole/g tissue)

In accordance with the present study, it was shown that vitamins C and E levels are decreased in the setting of brain injury and the subsequent needed for further supplementation (Hall et al., 2010; Hfaiedh et al., 2012). α-Tocopherol (vitamin E) is the most important lipophilic antioxidant and is residing mainly in the cell membranes, and thus helping to maintain membrane stability (Sun et al., 2012). It is a powerful chain-breaking antioxidant, inhibits ROS-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids and from oxidative damage of cellular proteins (Shrivastava, 2012). While, ascorbic acid (vitamin C) the most abundant hydrophilic antioxidant in the body acts primarily in cellular fluid of particular in combating free radical and protecting biomembranes from peroxidative damage induced by pollution (Hassan and Jassim, 2010; Skřivan et al., 2012). Furthermore vitamin C helps vitamin E to return to its active in the cell membrane. Because of this regeneration process, the combination of vitamins C and E provides better antioxidant protection than vitamin C or vitamin E alone. Indeed, combination of vitamins C and E was shown to have protective effects in many diseases associated with enhanced oxidative stress (Hfaiedh et al., 2012). In addition, Selenium is involved in cellular antioxidant defense via the activity of Se-dependent glutathione peroxidase (GPx). There is considerable evidence in the literature that Se and vitamin E may act synergistically (Skřivan et al., 2012). Consequently, the present study proved a synergistic neuroprotective effect of α-tocopherol, ascorbic acid, and selenium against neurotoxicity induced by LTC.

In conclusion, this study supported the involvement of oxidative stress in neurotoxicity of LTC in dose dependant manner and the potential synergistic and protective effects of ascorbic acid, α-tocopherol and selenium against neurotoxicity induced by LTC. However, further studies are needed to clarify this hypothesis and, if confirmed in the human, might support a novel preventive strategy to slow down the symptoms of this LTC neurotoxicity.

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7/25/2012