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Evaluation of changes in monoamine levels and apoptosis induced by Cyfluthrin in rats

Dilek Guvenc[a1], Abdurrahman Aksoy[a], Ayhan Gacar[b], Enes Atmaca[a], Kursad Y. Das[a], Tolga Guvenc[b]

[a] University of Ondokuz Mayis, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Kurupelit, 55200, Atakum, Samsun, Turkey.

[b] University of Ondokuz Mayis, Faculty of Veterinary Medicine, Department of Pathology, Kurupelit, 55200, Atakum, Samsun, Turkey.

[1] **To whom corresponding should be addressed:** University of Ondokuz Mayis, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Kurupelit, 55200, Atakum, Samsun, Turkey. Tel.: +90 362 3121919 Fax: +90 362 4576922.

E-mail address: dguvenc@omu.edu.tr

Research Highlights of The Manuscript

A noticeable increase in neurodegenerative diseases was detected in recent years. Besides aging, another important risk factor for neurodegenerative diseases is long term, low level pesticide exposure. The main goal of this study was to investigate the effects of cyfluthrin on central nervous system. For this purpose we evaluated monoamine and mitochondrial cytochrome c levels and lipid peroxidation in adult male rats treated with cyfluthrin. This study also examined cyfluthrin induced-apoptosis via the signaling proteins Bcl-2, caspase-9 and caspase-3, and possible anti-apoptotic effects of Alfa-basic crystallin. These subjects have not been investigated too much by researchers.

Abstract

The aim of the study was to evaluate monoamine and mitochondrial cytochrome c levels and lipid peroxidation in adult male rats treated with cyfluthrin (14 mg/kg dose; approximately1/10 of the LD50 value) for 14 days. This study also examined cyfluthrin induced-apoptosis via the signaling proteins Bcl-2, caspase-9 and caspase-3, and possible anti-apoptotic effects of Alfa-basic crystallin (αB-c). Levels of epinephrine (E), norepinephrine (NE) and serotonin (5-hydroxytryptamine, 5-HT) in the plasma and 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were measured in the striatum to assess neurotransmitter modification. Cyfluthrin administered to the plasma significantly reduced levels of E and NE and increased 5-HT levels, with no significant increase in lipid peroxidation. In the striatum, cyfluthrin intoxication resulted in significant increase of the level of 5-HIAA but no significant increase in 5-HT. Apoptosis was detected in astrocytes without a change in the level of cytochrome c but was not detected in neurons. Immunohistochemically, caspase-9 positive and Bcl-2 negative neurons were identified. Although these neurons were also negative for both TUNEL staining and caspase-3, they were positive for αB-c. The present study may indicate that cyfluthrin toxicity appears first in neuronal supportive cells, especially astrocytes, rather than in neurons, and that in neurons, αB-c can inhibit the activation of caspase-3 and block apoptosis. In conclusion, our findings support the hypothesis that repeated exposure to cyfluthrin alters neurotransmission of E, NE and 5-HTand induces apoptosis. These data may therefore be important for the assessment of the safety of cyfluthrin.

Key words: Cyfluthrin, apoptosis, monoamine, lipid peroxidation

Introduction

Cyfluthrin is a common type II pyrethroid pesticide, frequently used in veterinary medicine, agriculture, industrial settings, public health and in some countries for the protection of stored products[1]. Cyfluthrin exposure has been reported in the humans, including the exposure of infants[2,3,4]. Voltage-sensitive sodium channels, the sites of the insecticidal action of pyrethroids, are also important target sites in mammals. Mammals, unlike insects, have multiple sodium channel isoforms that vary in their tissue distribution and their biophysical and pharmacological properties, including their differential sensitivity to pyrethroids. Pyrethroids also act on some isoforms of voltage-sensitive calcium and chloride channels, and these effects may contribute to the toxicity of some compounds[5,6].

Neurotransmitters, which are commonly found in brain and bodily fluids, are chemical messengers that transmit messages between neurons and other cells. Neurotransmitters are classified according to their chemical composition. Serotonin, epinephrine and norepinephrine are all included in the monoamine neurotransmitter group. Neurotransmitters play an important role in brain and bodily functions (e.g. behaviour patterns, sleep, waking) and deficiencies in or excess production of a neurotransmitter may cause several diseases (e.g., epilepsy, Alzheimer's disease, anxiety, depression); thus, evaluation of neurotransmitter levels or their metabolites in the plasma, urine and brain tissue is important[7]. Pesticides that have been shown to alter neurotransmission include pyrethroids, organophosphates, organochlorines and formamidines. Acute or chronic exposure to these neurotoxic insecticides could interfere with neurotransmitter systems[8].

Among the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation creates a number of secondary products; for example, malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acid peroxidation in the cells[9]. Recent experimental studies have reported that type II pyrethroids give rise to lipid peroxidation in mice plasma, and in rat liver, blood and brain tissue[10,11,12,13].

Apoptosis, or programmed cell death, is an active process that has a role in cell development, the elimination of damaged cells, and the maintenance of cell homeostasis. A subfamily of cysteine proteases known as caspases are responsible for carrying out apoptosis. Caspase activation is an important pathway initiated when

cytochrome c is released from mitochondria. Various apoptotic stimuli cause cytochrome c release, and this release then induces a series of biochemical reactions that triggers the caspase activation and results in cell death. Bcl-2, an anti-apoptotic intracellular protein, inhibits cell death by preventing cytochrome c release[14]. Although pesticide have been shown to induce apoptosis in neuronal cells[15] much less is known about the role of apoptosis in cyfluthrin and other pyrethroid neurotoxicity.

Alpha-basic crystallin (αB-c) belongs to a family of small heat-shock proteins (HSP) and is a major component of the eye lens. It is also found in non-lenticular tissues such as heart, skeletal muscle, skin, oesophagus, kidney, placenta, peripheral nerves and central nervous system tissues[16]. αB-c has chaperone-like properties that prevent aggregation of the damaged or misfolded proteins induced by cell stressors such as heat shock, oxidative stress, metal ions and cytokines[17]. In a normal central nervous system, a low level of αB-c is present in the glial cells, particularly in the astrocytes and oligodendrocytes, but not in the neurons[18].

This study determined the effects of cyfluthrin on plasma monoamine (E, NE, 5-HT), striatal 5-HT and its metabolite 5-HIAA levels and the importance of lipid peroxidation. We also investigated the possible mechanism of cyfluthrin-induced apoptosis, type of the apoptotic central nervous system cells and neuroprotective role of the αB-c.

Results and Discussion

Cyfluthrin and vehicle did not produce gross signs of abnormalities (i.e. abnormal posture, convulsions and tremor) during the experimental period. Compared to the corn oil control group, there were no significant changes in the weight of the bodies or brains of the rats in the experimental group at the end of the experiment period.

Plasma monoamine levels

Compared to the control group (290.09±23.50, 263.26±58.09, 51.60±10.99 µg/l plasma for E, NE, 5-HT respectively), the experimental group had a significant decrease in E (119.03±21.03 µg/l plasma, $p<0.05$), NE (96.29±29.58 µg/l plasma $p<0.01$) and significant increase in 5-HT (195.70±15.00 µg/l plasma $p<0.05$) (Fig 1A-C).

Plasma lipid peroxidation

The levels of MDA were slightly increased in the cyfluthrin-treated animals (4.08±0.45 μM) compared to the control animals (3.35±0.24 μM, $p>0.05$) (Fig 1D).

3.3. Striatal 5-HT and 5-HIAA levels

Although the levels of 5-HT were higher in the cyfluthrin group (175.5±22.57 ng/g tissue) than in the control group (138.1±8.78 ng/g tissue), this increase was not statistically significant ($p>0.05$) (Fig 2A). A significant increase of 5-HIAA levels in the striatum was observed in the cyfluthrin treated group (406.1±27.13 ng/g tissue), compared to the control group (265.8±16.16 ng/g tissue, $p<0.01$) (Fig 2B). The turnover (5-HIAA/5-HT) in the cyfluthrin treated group (2.61±0.42 ng/g tissue) was slightly higher than in the control group (1.96±0.16 ng/g tissue, $p>0.05$).

3.4. Mitochondrial cytochrome c concentration in brain tissue

The cytosolic cytochrome c concentrations, an indicator of apoptosis, were slightly higher in the cyfluthrin group (0.30±0.03 ug/mg protein) than in the control group (0.26± 0.03 ug/mg protein); the cytochrome c concentrations did not show a statistical increase ($p>0.05$) (Fig 3).

Immunohistochemical findings

The immunohistochemical staining findings for Bcl-2, caspase-9 and caspase-3, and TUNEL staining findings are summarised in Table 1 and illustrated in figure 4.

TUNEL-positive apoptotic cells were detected in the cerebellum and substantia nigra in the treated groups, but in the untreated control group the apoptotic cells were not identified with this technique (Figure 4). For the TUNEL staining, positive signals were seen in the nuclei of the glial cells. Next, to determine the presences of astrocytes, the same sections were stained for GFAP antibody using the immunoperoxidase technique and clear positive reactions were detected (Figure 5). Additionally, this combined staining technique revealed a few glial cells that were TUNEL positive, but GFAP negative; these cells were considered as oligodendrocytes.

In the cyfluthrin treated group, when the αB-c immunofluorescence staining and TUNEL technique were applied together, the neurons were αB-c positive and TUNEL negative, but some of the glial cells were αB-c negative and TUNEL positive (Figure 6).

For the immunofluorescent staining, intense positive reactions for αB-c were observed in the substantia nigra of rats from the treatment groups. In the same tissue

sections, neurons were identified by TH for dopaminergic neurons in the substantia nigra. No changes were observed in the staining intensity of the TH immunoreactivity in the control or treatment groups. The merged images demonstrated that αB-c immunopositive cells were also TH positive neurons (Figure 7).

In the present study we have shown that repeated administration of cyfluthrin affected the 5-HT and 5-HIAA concentrations in the rat striatum. The levels of 5-HT, 5-HIAA and turnover (5-HIAA/5-HT) all increased, but only the increase in the level of 5-HIAA was significant. Similar results were previously reported for 5-HT levels in rat brain exposed to single acute dose of deltamethrin[19]. The administration of cyhalothrine to rats at the dose levels of 10, 20 and 60 mg/kg caused dose-dependent increases of striatal 5-HT, but no changes were seen in the outflow of 5-HIAA[20]. In a study aimed at determining the effect of permethrin on striatum, median prefrontal cortex and hippocampal monoamine levels, a statistically insignificant increase in 5-HT levels was found in the study group treated with 34 mg/kg permethrin, whereas the 5-HIAA levels remained unchanged[21].

There are some studies showing both no change and a decrease in the 5-HT and 5-HIAA levels in the brain following pyrethroid administration. Martinez-Larranaga et al.[22] found that six days of cyfluthrin administration (14 mg/kg) decreases the 5-HT and 5-HIAA levels in the striatum, hippocampus, frontal cortex and midbrain, and increases the turnover rate of 5-HT in the midbrain. In another study, cypermethrin, which is a type II pyrethroid, was used both on adult and postnatal rats and no statistically significant increase in striatum serotonin levels was found[23]. Lazarini et al.[24] did not determine any statistically significant change in the striatum 5-HT, 5-HIAA or 5-HT levels in prenatal male and female rats that were exposed to deltamethrin (type II pyrethyroid).

In the current study, plasma E and NE levels were significantly reduced and plasma 5-HT levels were also significantly increased in the experimental group. Overall, studies of pyrethroid insecticides have produced inconsistent results; many studies show both an increase[25,26] and decrease[19,27] in E and NE levels.

Cyfluthrin, which is a type II pyrethroid, like the other compounds in its group, affects the sodium channels for longer periods of time than type I pyrethroids. Its effects on voltage-sensitive sodium channels alter the release of neurotransmitters; however, the effect of pyrethroids on neurotransmitter release may be dual (both stimulatory and inhibitory) or either stimulatory or inhibitory[28]. Pyrethroid effects on

isoforms of voltage-sensitive calcium channels could alter the release of neurotransmitters and hence alter pyrethroid-induced toxicity[29,30,31,31,32]. The changes in neurotransmitter levels in our study may be due to the effects of pyrethroids on ion channels, which would also change the release of neurotransmitters. Monoamine oxidase (MAO), like 5-HT, E and NE, is a mitochondria-bound isoenzyme that is responsible for the oxidative deamination of monoamine neurotransmitters. The pyrethroid pesticide deltamethrin increased the activity of MAO in male rat brains[33]. The increased 5-HIAA level in the brain does not reflect serotonin release or usage but rather MAO activity[34,35]. In addition, the intracellular metabolism of newly synthesised 5-HT could be the source of 5-HIAA[35]. In our study, significantly alterations of neurotransmitters levels suggest that the use of cyfluthrin for 14 days at the dose of 14 mg/kg may have increased the activity of MAO and catechol-O-methyltransferase (COMT, which is the enzyme responsible for the metabolisation of E and NE with MAO).

Apoptosis or programmed cell death is a physiological or pathological process that occurs through a complex signalling cascade. One of the main apoptotic pathways is related to mitochondria, and depends on the release of cytochrome c from the mitochondria into the cytosol. Cytochrome c activates the apoptotic protease activating factor-1 (Apaf-1). Subsequently, Apaf-1 and cytochrome c bind to the procaspase-9 and activate it. Next, caspase-9 activates caspase-3 as the final effector of apoptosis[36]. Here, we observed increased cytochrome c levels following cyfluthrin exposure, but the level was not statistically significant. Hossain and Richardson[37] have been also shown that deltamethrin, another type II pyrethroid, can induce apoptosis by endoplasmic reticulum (ER) stress pathway. Further studies should be done to identify the endoplasmic reticulum stress pathway as a mediator of cyfluthrin-induced apoptosis. We also identified TUNEL-positive apoptotic cells and immunohistochemically caspase-3-positive cells. The majority of these cells were also immunohistochemically GFAP positive. Therefore, it was concluded that these cells are astrocytes. Astrocytes are specialised glial cells that are ubiquitous throughout all regions of CNS. They play critical roles in normal on-going CNS functions. They are important for the regulation of blood flow, the provision of energy metabolites to neurons, and the maintenance of ion and pH homeostasis[38]. Previously, it was reported that cyfluthrin causes apoptosis in primary human astrocytes[39]. Another type II pyrethroid pesticide, cypermethrin, was also reported to

induce apoptosis in primary rat astrocytes[40]. The present study may indicate that cyfluthrin toxicity appears first in neuronal supportive cells, especially astrocytes, rather than in neurons. After a critical number of astrocytes have undergone apoptosis, neurons will have lost all of their astroglial supports. This situation could lead to slow neurodegeneration.

In the present study, immunohistochemically caspase-9 positive and Bcl-2 negative neurons were identified. Although these neurons were negative for both TUNEL staining and caspase-3, they were positive for αB-c. Recent studies confirmed that increased αB-c expression has a neuroprotective effect. This effect of αB-c occurs through several anti-apoptotic pathways. αB-c inhibits apoptosis induced by various stimuli (including DNA-damaging agents such as TNF-alpha and Fas)[41,42] and growth factor deprivation, and by disrupting the proteolytic activation of caspase-3[43,44]. Significantly, αB-c has been shown to inhibit the tumour necrosis factor that is related to apoptosis-inducing ligand (TRAIL)-induced apoptosis through the suppression of caspase-3 activation[45]. Another study showed that the inhibition of stress-induced apoptosis by αB-c could be due to suppressing the activation of caspase-3 and/or preventing the mitochondrial translocation of the pro-apoptotic Bcl-2 family members such as Bax[46]. The immunohistochemical findings of the present study may indicate that αB-c can inhibit the activation of caspase-3 and block apoptosis. This process can be considered a neuroprotective effect.

Experimental

Chemicals

The cyfluthrin, purity 97.2%, cytochrome c from equine heart, 5-HT, 5-HIAA and leupeptin were purchased from Sigma-Aldrich, St. Louis, USA. All of the other analytical grade chemicals were obtained from Merck, Darmstadt, Germany.

Animals and experimental design

Eight-week-old male Wistar rats, weighing between 250-260 g were used. The animal housings were maintained at 22±2°C, 60±5% relative humidity and a 12 h light/dark cycle. Food and water were provided *ad libitum*. The experimental protocol was approved by the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University (Hadyek-2010/60).

Thirty-two rats were randomly allocated to the experimental (n=16) and control (n=16) groups. In the experiment group, the sixteen rats were administered with single daily cyfluthrin intraperitoneally (ip) at 14 mg/kg (about 1/10 of LD50 values)

for 14 consecutive days[22]. Corn oil was administered i.p. to the control group on the same schedule. All of the injections were performed in a total volume of 2 ml/kg. Twenty-four hours after the last administration, all of the animals were weighed and blood samples (3 ml) were taken from heart then euthanized by cervical dislocation and decapitation. The brains were rapidly removed and weighed. The right hemisphere of each brain was stored for 5-HT, 5-HIAA and cytochrome c analysis and the left hemisphere was used for immunohistochemical examination.

Determination of plasma monoamine levels

The E, NE and 5-HT levels were assessed with the fluorometric method described in the kit (Catecholamine, Serotonin Dual Kit, Eureka Lab Division, Ancona, Italy Analytical Sensitivities: E<8, NE<10 pg/ml and 5-HT=2 ug/l). Briefly, blood samples (3 ml) were centrifuged for 3 min, 850x*g* and the supernatants were stored at -80°C until analysis. The monoamine levels were analysed with a high performance liquid chromatography (HPLC) unit with a fluorescence detector (FLD, RF-10Axl, Schimadzu, Kyoto, Japan; excitation wavelengths were; E, NE =360, 5-HT=285 and emission wavelengths were; E, NE =490, 5-HT =344). Chromatographic separation was obtained using an Inertsil® ODS-3V, 4.6x250 mm, 5 µm (GL Sciences Inc., Tokyo, Japan) analytical column. The flow rate was 1.2 ml/min. The monoamine concentrations were expressed as µg/l plasma.

Lipid peroxidation measurements in plasma

The plasma MDA levels were determined with the thiobarbituric acid method[47,48]. All standards were prepared in the range of 0.125-5 µM using 1-1-3-3-tetraethoxypropane (TEP) in ethanol. The linear regression was R^2=0.999. The limits of detection (LOD) and limits of quantification (LOQ) of MDA were 0.25 and 0.78 nM, respectively. The standards and samples of plasma were treated with 20% trichloroacetic acid and 0.67% thiobarbituric acid, then derivatised at 95°C for 30 min. The plasma samples were rapidly cooled and extracted with 4 ml of n-buthanol. Twenty microliter supernatants were injected onto the HPLC-FLD system (excitation wavelength=515, emission wavelength=553) and separated on an Inertsil® ODS-3V, 4.6x250 mm, 5 µm (GL Sciences Inc., Tokyo, Japan) analytical column. The MDA concentrations were expressed as µM in plasma.

5-HT and 5-HIAA levels in striatum

The 5-HT and 5-HIAA concentrations were determined in the striatum by the method described in Li et al.[49]. The striatum in the right hemisphere of 16 rats (8

control and 8 experimental group) were dissected on an ice-cold plate. The tissues were weighed and homogenised in a solution of 0.1 M $HClO_4$ by MagNA Lyser (Roche Applied Science, Penzberg, Germany). The homogenates were centrifuged at 16,000x*g* for 15 min at 4°C. The resulting supernatant was decanted and filtered through a PVDF filter with a pore size of 0.22 µm, and stored at -80°C until assay. The samples were analysed for 5-HT and 5-HIAA using reverse-phase HPLC (LC-20A Prominence; Schimadzu, Kyoto, Japan) with an electrochemical detector (ECD, Coulochem III, 5020 Guard Cell and 5010A Analytical Cell, ESA, Inc., Chelmsford, MA, USA). Separation was obtained using an Inertsil® C4, 4.6x150 mm, 5 µm (GL Sciences Inc., Tokyo, Japan) analytical column. The mobile phase consisted of 50 mM sodium acetate and 10% methanol; the final pH was adjusted to 5.4. The flow rate was 0.8 ml/min. The injection volume was 20 µl. The 5-HT and 5-HIAA concentrations were expressed as ng/g wet weight of striatum. A standard calibration curve was set between 12.5 ng/ml and 40 ng/ml for both 5-HT and 5-HIAA. The linear regression was R^2=0.99. The LOD for 5-HT and 5-HIAA were 0.27 ng/ml and 0.22 ng/ml, respectively. The LOQ of 5-HT and 5-HIAA were 0.82 ng/ml and 0.69 ng/ml, respectively.

Mitochondrial cytochrome c measurement in brain tissue

Right hemispheres of the other 16 (8 control and 8 experimental group) rats brains (0.5 g) were homogenised using a homogenisation buffer (0.32 M sucrose, 0.3 mM phenylmethylsulfonylfluoride, 10 µg/l leupeptin) in a Dounce homogeniser at 4°C. The brain homogenates were centrifuged (Nüve NF 800R) at 1,000x*g* for 20 min and then the supernatants were centrifuged at 12,000x*g* for 25 min at 4°C. The resulting supernatants were centrifuged (Optima™ L-100 XP, Beckman Coulter, Inc.) again at 100,000x*g* for 30 min. Ten microliter cytosolic fractions were injected onto the HPLC system with a Diode Array Detector (DAD, Schimadzu SPD-M 20A, Kyoto, Japan). The cytochrome c was detected at 393 nm. Chromatographic separation was obtained using an Inertsil® C4, 4.6x150 mm, 5 µm (GL Sciences Inc., Tokyo, Japan) analytical column. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid (pH 4.5, solvent A) and acetonitrile (solvent B). The program used was as a follows. First, there was a linear gradient step from 20% to 60% (solvent B) in 8 min. The column was then washed with 60% acetonitrile for 5 min followed by re-equilibration for 5 min in the 20% acetonitrile buffer. The flow rate was 1 ml/min[50,51]. The protein content was determined by Bradford's method[52], with bovine serum albumin as a protein

standard (Sigma-Aldrich, St. Louis, MO). The results were expressed as µg/mg protein. The standards were prepared within a range of 0.5-50 µg/ml. The linear regression was R^2=0.999. The LOD and LOQ of cytochrome c were 14.9 ng/ml and 45.2 ng/ml, respectively.

Immunohistochemical studies

Left hemispheres of the brain (16 control and 16 experimental group) were fixed with 10% neutral-buffered formalin solution, and embedded in paraffin according to standard histological techniques. The specimens were sectioned (5 µm) and placed on 3-aminopropyltriethoxysilane (Sigma, St.Louis, MT, USA) coated slides, and were then stained using the streptavidin-biotin-peroxidase complex (SBPC) technique (Invitrogen, Histostain Plus Kit, USA). Endogenous peroxidase was blocked with 3% H2O2 for 5 minutes. The irradiation of the sections was done at 600W in 0.1 M citrate buffer of pH 6.0 for 20 min, in a microwave oven. Next, the tissues were incubated with protein blocking solution for 10 minutes at room temperature. Immediately afterward, the tissues were incubated with the primary antibody for 60 minutes at room temperature. Anti-Bcl-2 antibody (1/100; ab7973, Abcam, USA), active caspase 3 (1/100; NB100-56113, Novus Biological, USA) and active caspase 9 (1/10; PA1-26435, Thermo scientific, USA) were used as the primary antibodies**.** Two negative controls were applied, first by omitting the primary antibody, and then replacing with PBS. All slides were incubated with the secondary antibody, and the horseradish peroxidase–streptavidin solution, respectively, for 30 minutes at room temperature. Amino ethyl carbazole (AEC) was used as the chromogen in H_2O_2 for 10 min. The sections were counterstained with Mayer's haematoxylin for 1 min and rinsed with tap water. Subsequently, the sections were mounted with an aqueous mounting medium. After each step was completed, the slides were washed twice with phosphate-buffered saline solution for 5 minutes, except the protein-blocking step.

The number of immunopositive cells was assessed using a microscopy image analysis system (Bs200Pro Image analysis system, BAB software, Ankara, Turkey). The number of immunohistochemically positive cells was scored by counting 1000 cells in randomly selected ×10 high-power magnification fields per brain sample. Every stained cell was considered positive regardless of the intensity of the staining. The number of immunopositive cells was recorded as follows: negative (-), weak, less than 5% (−/+); mild, 6% to 25% (+).

Detecting apoptosis

To identify the DNA fragmentation, the brain sections were stained with the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) method (*in situ* cell death detection kit, Roche Diagnostics, GmbH, Germany). This was performed according to the manufacturer's directions. The paraffin-embedded sections were dewaxed and rehydrated. Next, irradiation of the sections was done at 350 W in 0.1 µM citrate buffer, pH 6.0 for 5 min, in a microwave oven. After washing twice in PBS, the sections were covered with 50 µL of the TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP (2´-deoxyuridine 5´-triphosphate). The slides were incubated with anti-fluorescein-horseradish peroxidase conjugated antibody for 40 min at room temperature. After washing three times for 15 min each in PBS, the sections were stained through incubation with the chromogenic substrate AEC at room temperature and counterstained with Mayer's haematoxylin and mounted with an aqueous mounting medium. The number of TUNEL positive cells was evaluated in the same manner with immunohistochemical studies as previously described.

Detection of apoptosis in GFAP-immunoreactive astrocytes

Apoptosis was detected firstly by TUNEL assay using the in situ cell death detection kit previously described. To investigate whether the apoptotic cells were astrocytes, the sections were incubated with mouse GFAP antibody (1/100; ab7779, Abcam, USA) at 4°C overnight. After rinsing in 0.1 M PBS, the sections were stained using the SBPC technique as previously described (Invitrogen, Histostain Plus Kit, USA). AEC and or 3-3' Diaminobenzidine (DAB) were used as chromogenic substrates for the GFAP and TUNEL staining, respectively.

Alpha B-crystallin immunoreactive cells and apoptosis

The tissue sections were incubated with the anti-αB-c antibody (1/200, ab13497, Abcam, USA) followed by the TRITC-linked anti-rabbit antibody (1/400, T6778, Sigma, USA). Next, the sections were stained with the TUNEL technique except for incubated with anti-fluorescein-horseradish peroxidase conjugated antibody., and the sections were visualised under a fluorescence microscope (Nikon, E-600).

Detection of alpha basic crystallin in tyrosine hydroxylase-immunoreactive dopaminergic neurons

For double immunofluorescence staining, the tissue sections were incubated with the anti-αB-c antibody (1/200, ab13497, Abcam, USA) followed by the FITC-labelled anti-rabbit antibody (1/160, F7512, Sigma, USA). Next, the sections were incubated with the anti-tyrosine hydroxylase antibody (1/200, MAB318, Millipore, USA), followed by the rhodamine-linked anti-mouse antibody (1/100, AP124R, Millipore, USA). Subsequently, the sections were mounted with an aqueous mounting medium. The

Statistical analysis

All of the statistical analyses were performed with SPSS version 21 (IBM Corp., Armonk, NY, USA). The striatal 5-HT, 5-HIAA and brain mitochondrial cytochrome c levels were evaluated with a Student T-test. The plasma E, NE, 5-HT and MDA levels were analysed with a nonparametric analysis of Mann-Whitney-U. The results are expressed as mean±SE. Values with $p<0.05$ were considered statistically significant.

Conclusion

Our results show that repeated exposure to cyfluthrin changed the plasma concentrations of E and NE, but did not affect lipid peroxidation. The brain 5-HT levels were unchanged; however, 5-HIAA levels were increased in the experimental group suggesting an increase in MAO and COMT activity. This argument should be confirmed in future studies. Additionally, apoptosis was detected in astrocytes without any change in the levels of cytochrome c. Although apoptosis was clearly observed in this study, further investigation is necessary to determine the mechanisms of ER-mediated apoptosis.

Declaration of interest

The authors declare that there are no conflicts of interest.

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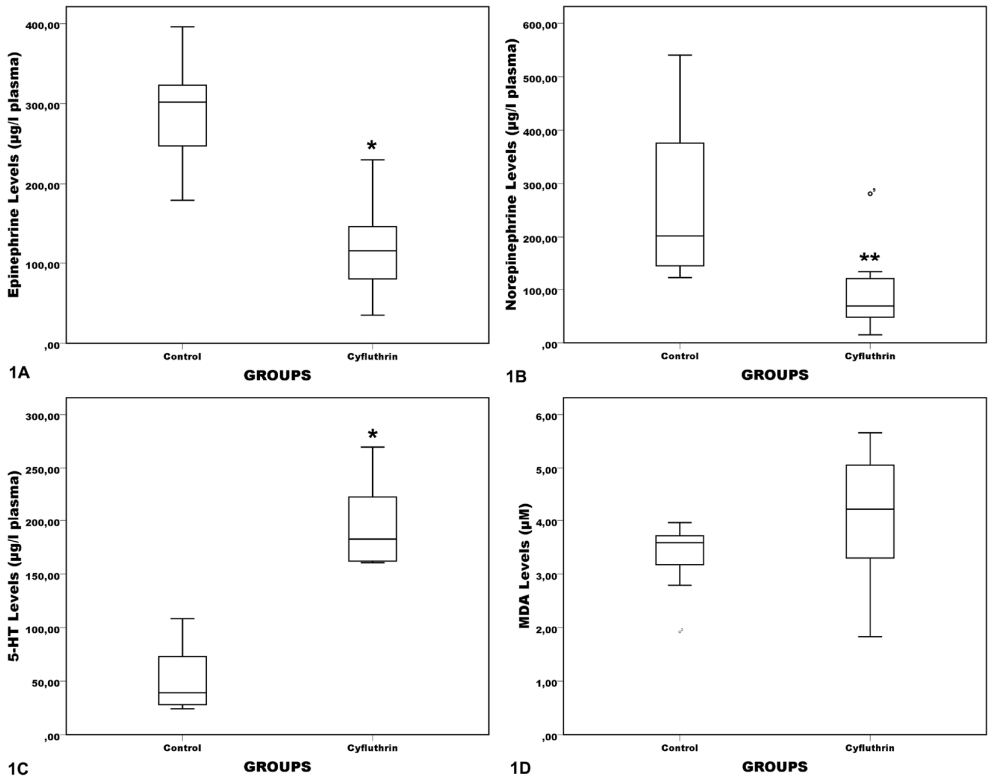
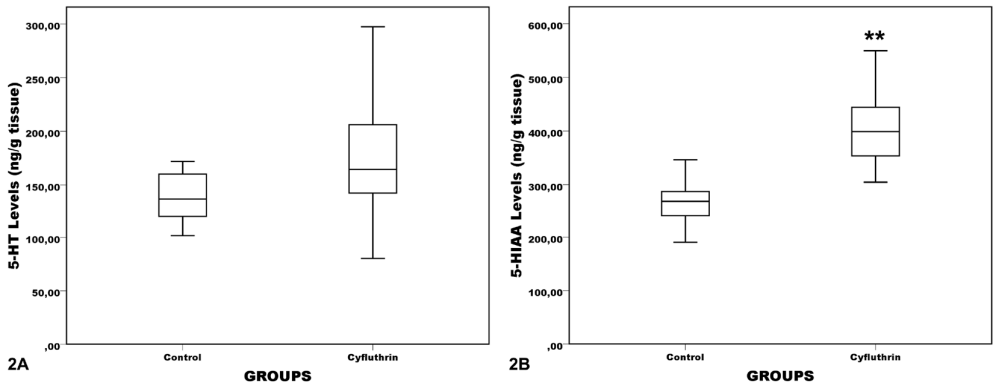


Figure 1. Effect of cyfluthrin on plasma monoamine and MDA levels in control and cyfluthrin-treated rats. High performance liquid chromatography (HPLC) analyses of (1A) epinephrine (E), (1B) norepinephrine (NE), (1C) Serotonin (5-HT), (1D) malondialdehyde (MDA) were performed in the plasma of control and treated rats. The data are expressed as µg/l and µM for monoamine and MDA, respectively. * $p<0.05$, ** $p<0.01$



Figuere 2. Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels measured in brain control and cyfluthrin treated rats. The data are expressed as ng/g of tissue. * $p<0.05$, ** $p<0.01$

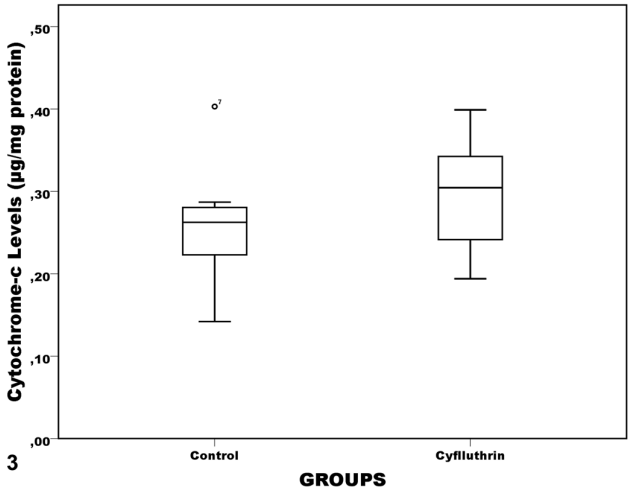


Figure 3. Mitochondrial cytochrome c levels measured in brain control and cyfluthrin treared rats. The data are expressed as µg/mg protein.

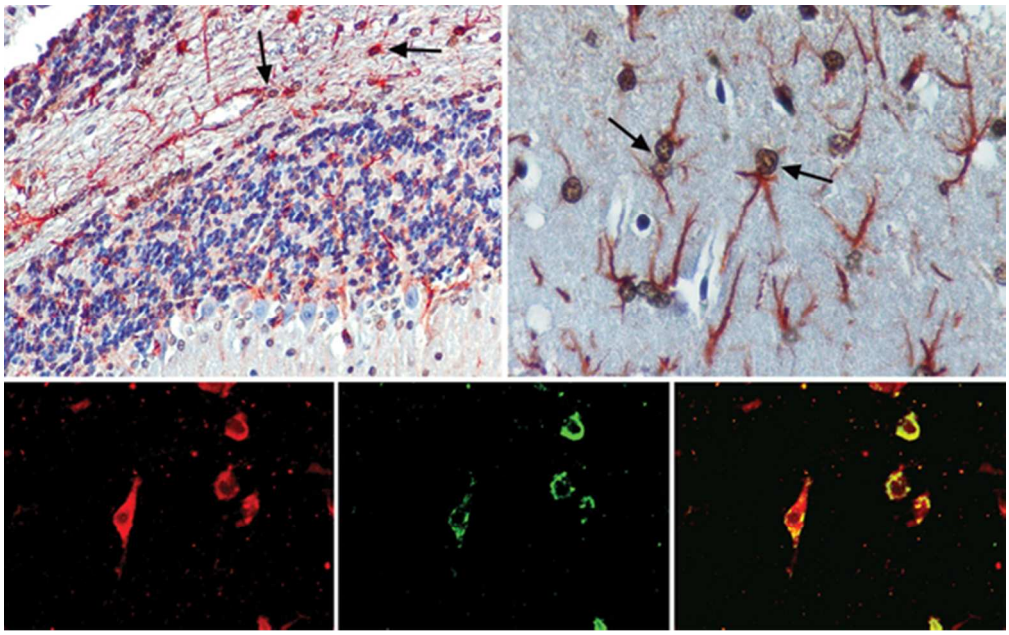
Figure Legends:

Figure 4. Bcl-2, Caspase-9, Caspase-3 immunostaining and TUNEL staining of cerebellum and substantia nigra. Caspase-3 immunostaining and TUNEL staining was clearly visible in Cyfluthrin treatment gruop but both caspase-3 immunoreactivity and TUNEL staining was not observed in control group. Arrows show positive cells.

Figure 5. Immunohistochemical labelling of GFAP and TUNEL staining. AEC (red) and DAB (brown) was used as chromogen for GFAP and TUNEL staining, respectively. Arrows indicate positive cells.

Figure 6. TUNEL and immunofluorecence staining with αB-c antibody in cyfluthrin treatment group. 6A. Immunofluorescence staining of αB-c (red); 6B. Positive nuclear staining for TUNEL methods (green); 6C. Merged images 6A and 6B, in the merged image there was not overlapping with TUNEL and αB-c immunofluorecence staining.

Figure 7. Brain sections were double immunofluorecence staining with αBC and TH in cyfluthrin treatment group. The neurons of the substantia nigra were identified as dopaminergic by rhodamine labeling of tyrosine hydroxylase (7A) and fluorescein labeling of αB-c (7B). Merged image shows TH-positive neurons co-express αB-c (7C).



Graphical Abstract
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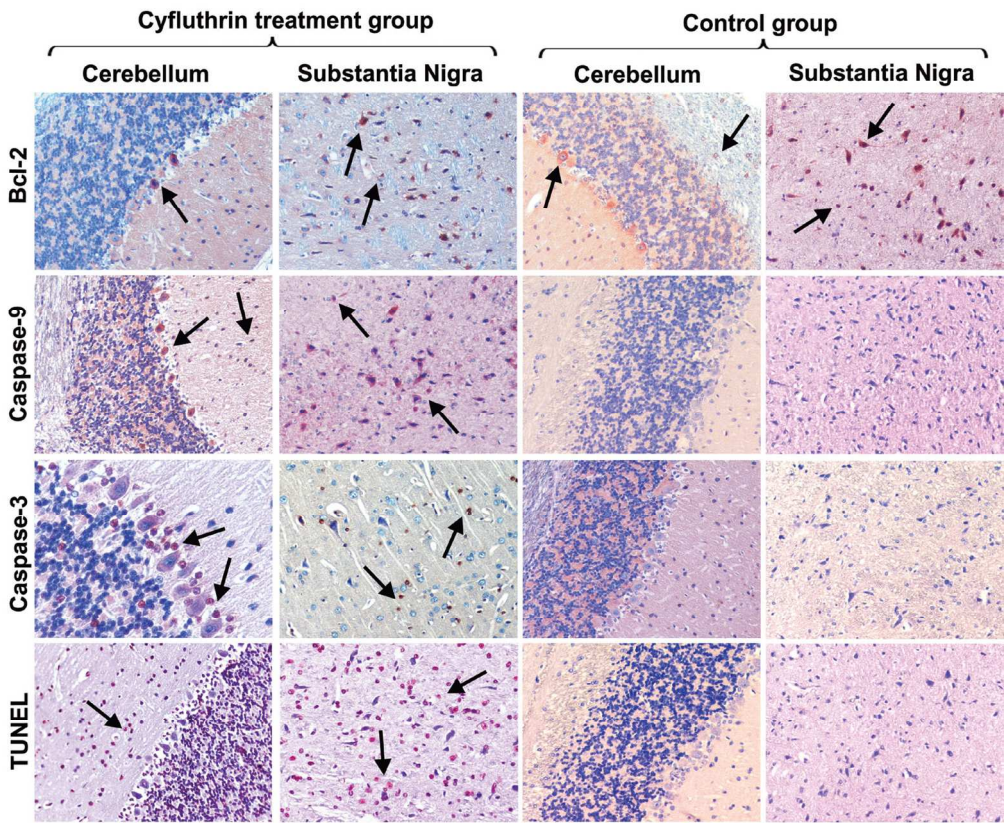

Figure-4. Bcl-2, Caspase-9, Caspase-3 immunostaining and TUNEL staining of cerebellum and substantia nigra. Caspase-3 immunostaining and TUNEL staining was clearly visible in Cyfluthrin treatment gruop but both caspase-3 immunoreactivity and TUNEL staining was not observed in control group. Arrows show positive cells.
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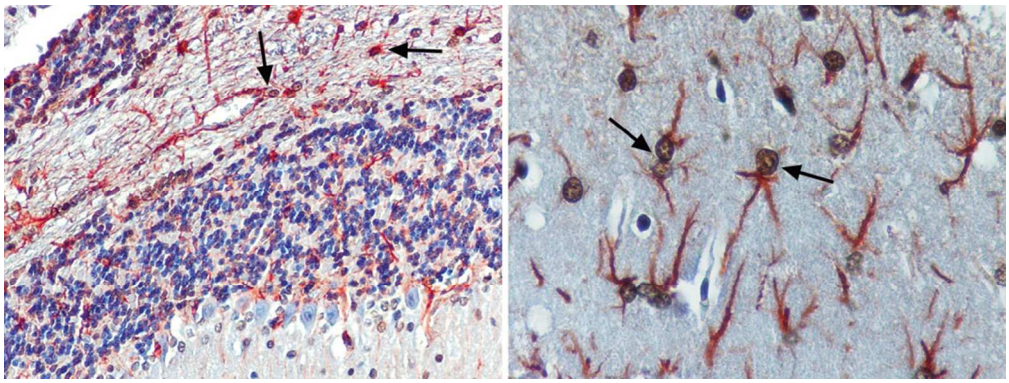

Figure-5. Immunohistochemical labelling of GFAP and TUNEL staining. AEC (red) and DAB (brown) was used as chromogen for GFAP and TUNEL staining, respectively. Arrows indicate positive cells.
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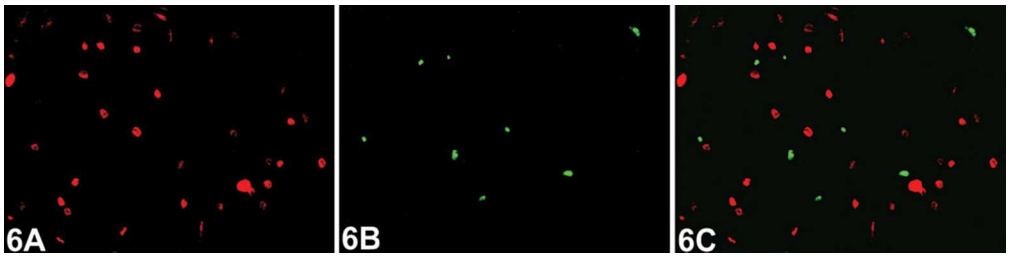

Figure-6. TUNEL and immunofluorecence staining with αB-c antibody in cyfluthrin treatment group. 6A. Immunofluorescence staining of αB-c (red); 6B. Positive nuclear staining for TUNEL methods (green); 6C. Merged images 6A and 6B, in the merged image there was not overlapping with TUNEL and αB-c immunofluorecence staining.
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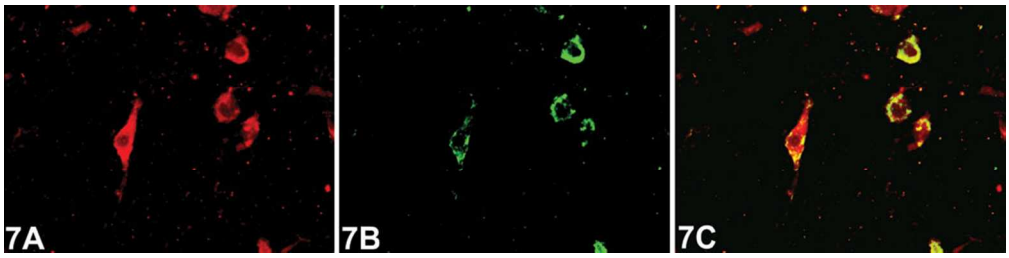

Figure-7. Brain sections were double immunofluorecence staining with αBC and TH in cyfluthrin treatment group. The neurons of the substantia nigra were identified as dopaminergic by rhodamine labeling of tyrosine hydroxylase (7A) and fluorescein labeling of αB-c (7B). Merged image shows TH-positive neurons co-express αB-c (7C).
90x22mm (300 x 300 DPI)