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Scientific paper

BIOLOGICAL SIGNIFICANCE OF THE OVER-EXPRESSION OF HSP70 AND ALPHA B-CRYSTALLIN IN RAT *SUBSTANTIA NIGRA* EXPOSED TO DIFFERENT DOSES OF PERMETHRIN

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The aim of this study was to investigate the possible role of the Heat Shock Protein 70 (HSP70) and Alpha B-crystallin (αBC) in the *substantia nigra* of rats exposed to permethrin at different doses on the apoptotic cell status. The orogastric gavage method was used to administer the different doses of permethrin (75 mg kg^{-1} in Group I, 150 mg kg^{-1} in group II, 300 mg kg^{-1} in group III) to the rats. Using the Western blot test, all the permethrin-treated groups showed a dose-dependent increase in the expression of HSP70 and αBC when compared to the control group. TUNEL positive apoptotic cells were not detected in the dopaminergic neurons of the substantia nigra after treatment with permethrin; however, upon immunofluorescent staining, intense positive reactions for HSP70 and αBC were observed in all of the treated groups. No immunopositive cells were detected in the tissue sections of the control group. These results suggest that the different administered doses of permethrin did not cause apoptotic cell death in the substantia nigra dopaminergic neurons; however, they did induce an increase in HSP70 and αBC expression. Thus, it appears that HSP70 and αBC could play a neuroprotective role in permethrin-induced neurotoxicity.

KEY WORDS: *Heat Shock Protein, immunohistochemistry, pyrethroid, TUNEL*

Pyrethroids are chemicals used to control indoor and agricultural pests (1). They can be divided into two classes, based on their chemical structure and biological effect after high-dose acute exposure. Type I Pyrethroids, which lack one α-cyano group, produce toxic effects, including aggressive sparring, increased sensitivity to external stimuli, whole body tremor and prostration (T syndrome). Type II pyrethroids, however, which contain the α-cyano group, produce a syndrome characterized by pawing and burrowing behaviour, salivation, and coarse tremor progressing to choreoathetosis and clonic seizures (2, 3). The toxic signs of pyrethroid insecticides set in by acting on the nervous system. Although their principal target is the voltage-gated sodium channel, recent studies propose that they target other channel and receptor systems in the neuronal tissues as well, such as calcium, chloride channels, GABA (γ-aminobutyric acid) and benzodiazepine receptors (4-6). Several studies have been conducted on the mechanisms that unveil in the neurotoxicity of pyrethroids. Type I pyrethroid permethrin was observed to cause maximum change in dopamine uptake in the striatal synaptosomes of mice (7). Kakko et al. (8) reported that synaptosomal

membrane-bound ATPases mediate the neurotoxic effects of the pyrethroids. While low-dose permethrin (3 mg kg^{-1}) significantly decreases the dopamine transporter immunoreactive protein, high-dose permethrin (200 mg kg^{-1}) significantly increases the glial fibrillary acidic protein in the striatum of C57BL/6 mice (9). The expression of tyrosine hydroxylase (TH) and the dopamine transporter protein (DAT) in the striatal dopaminergic terminals of mice did not change when subjected to long-term (3 months) and low doses (between 0.8 mg kg^{-1} and 1.5 mg kg^{-1}) of permethrin (10).

Prokaryotic and eukaryotic cells both contain Heat Shock Proteins (HSPs). A wide variety of stress factors such as heavy metals, pesticides, solvents, sodium arsenite, nitric oxide, glucose and amino acid analogues, ischemia, microbial infections and antibiotics can induce HSPs (11, 12). HSPs have a molecular-chaperone activity involving several aspects of protein synthesis including the prevention of premature protein folding, restoration of denaturing proteins, transportation and translocalization processes (13, 14). Recently, HSPs have also been found to regulate apoptosis by acting on different stages of programmed cell death machinery (15). Upregulation and overexpression of HSPs in the nervous system is associated with their neuroprotective role (16-18). The neuroprotective effects of HSPs take place by anti-apoptotic and chaperoning activities (18). They can be classified into five major categories based on their molecular weight, amino acid sequence homologies and functions. They include the HSP100 family, HSP90 family, HSP70 family, HSP60 family and the small HSP family (12).

The HSP70 family comprises two major members, viz., HSP70, an inducible form, and Hsc70, the heat shock cognate protein, a constitutively expressed form. HSP70 is never expressed in the brain under non-stressed conditions (19). Therefore, due to the fact that inducible HSP70 cannot be detected under normal conditions, it serves as a useful sensitive marker for neuronal injury (20). Several studies have indicated the expression of HSP70 to be a response to various neurotoxic stimuli, including hyperthermia, cerebral and focal ischemia, seizures, excitotoxicity, subarachnoid haemorrhage and spinal cord injury (20, 21).

Primarily defined as a major component of the eye lens, αBC belongs to the family of small HSPs. It is also found in non-lenticular tissues such as the heart, skeletal muscle, skin, oesophagus, kidney, placenta, peripheral nerves and nervous system (22). Interestingly, αBC expression has also been detected in heat shock, anticancer drugs, radiation and oxidative stress (23). In a normal central nervous system, αBC is present in glial cells, particularly in astrocytes and oligodendrocytes, although not in the neurons (24). The neuronal expression of αBC has been investigated in Alexander's disease, Alzheimer's disease, Pick's disease, Creutzfeldt-Jakob disease, multiple sclerosis (25), astrocytoma, glioblastoma multiforme and oligodendroglioma (26). Thus, αBC is considered to be a good molecular marker for neurodegenerative disorders and brain tumours (25, 27).

Relatively little information is available on the possible dose-related toxicity of permethrin, despite its wide application in practice. Also, not much is known on permethrin-induced apoptotic cell death in the *substantia nigra*. Therefore, the aim of this study is to investigate the apoptosis in the substantia nigra of rats exposed to different doses of permethrin, as well as the interaction of permethrin with HSP70 and αBC.

MATERIAL AND METHODS

Animals and Treatments

Approval for the experimental protocol involved in this study was granted by the Experimental Animal Studies Ethics Committee of the Ondokuz Mayis University (HADYEK-2008/51). Thirty-two adult male Spraque-Dawley rats, about 8 weeks old, and approximately 270 g in weight, (supplied by Kobay Inc., Ankara, Turkey) were used. The animal room was maintained at 22 °C ± 2 °C, 60 % ± 5 % relative humidity, and a 12 h/12 h light/dark cycle. Food and water were given *ad libitum*. Thirty-two rats were randomized into three experimental groups and one control group (n=8 for each group). Permethrin was given orally, three times in the experimental groups, on days 1, 7 and 14, respectively; group I rats (n=8), 75 mg kg^{-1} permethrin (1/20 of the LD_{50} value); group II rats (n=8) 150 mg kg^{-1} permethrin (1/10 of the LD_{50} value, group II) and group III rats (n=8) 300 mg kg^{-1} (1/5 of the LD_{50} value, group III) (28-30). Corn oil (vehicle/one millilitre per animal) was orally administered to the control group (n=8) on the same days.

Immunofluorescence microscopy

Five animals from each experimental group were anesthetized with pentobarbital (100 mg kg^{-1}, *ip*) and perfused through the heart with phosphate-buffered saline followed by 2 % paraformaldehyde and 1.5 % glutaraldehyde in phosphate-buffered saline. The brains were removed, postfixed, and embedded in paraffin according to standard histological techniques. Next, the specimens were sectioned (5 μm) and placed on 3-aminopropyltriethoxysilane (Sigma, St. Louis, MT, USA) coated slides. The sections were stained using the immunofluorescence technique. For double immunostaining, tissue sections were incubated with anti-alpha B-crystallin antibody diluted 1:200 (ab13497, Abcam, USA) or anti-HSP70 antibody diluted 1:100 (SPA-810, Stressgen, USA), followed by FITC-labelled anti-rabbit antibody (1:160, F7512, Sigma, USA) or FITC-labelled anti-mouse antibody (1:50, AP300F, Chemicon, USA). Tissue sections were incubated with anti-tyrosine hydroxylase antibody (1:200, AB152, Millipore, USA), followed by rhodamine-linked anti-mouse antibody (1:100, AP124R, Chemicon, USA). Two negative controls were prepared, first by omitting the primary antibody, and then by replacing them with PBS. The sections were then mounted with an aqueous mounting medium. Slides were evaluated with a fluorescence microscope (Nikon, E-600) equipped with appropriate filter systems (Nikon, B-2A for FITC and G-2A for rhodamine). A total of 10 high-power fields were randomly chosen and analysed at high magnification (200x) by two independent pathologists (TG and YBK). Image analysis and merged images were carried out with the Bs200P Image Analysis System software (BAB software, Ankara, Turkey).

TUNEL Staining

To identify DNA fragmentation, brain sections were stained using the terminal deoxynucleotidyl transferase-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 instructions. The paraffin-embedded sections were dewaxed and rehydrated. Next, the irradiation of the sections was done at 350 W in 0.1 μmol L^{-1} citrate buffer, pH 6.0 for 5 min, in a microwave oven. After washing in PBS twice, the sections were covered with 50 μL of the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-dUTP (2′-deoxyuridine 5′-triphosphate). Tissue sections were incubated with anti-tyrosine hydroxylase antibody (1:200, AB152, Millipore, USA), followed by rhodamine-linked anti-mouse antibody (1:100, AP124R, Chemicon, USA). Tissue sections were mounted with an aqueous mounting medium, and evaluated with a fluorescence microscope as described previously.

Analysis of stress protein expression on Western blot

Non-fixed brain tissues of three animals were homogenized for 2 min by using a tissue homogenizator (20.000 rpm, SilentCrusher M, Heidolph Instruments, Germany) in a lysis buffer (50 mmol L^{-1} Tris, pH 7.4, containing 0.15 mol L^{-1} NaCl, 10 % glycerol, 1 % NP-40, protease inhibitor cocktail tablets, Roche Diagnostics, GmbH, Germany) in a tissue: buffer ratio of 1:5. After homogenization, the samples were centrifuged at 10,000 *g* for 15 min, and the supernatants were collected and stored at −70 °C. The protein concentration was determined by the method used by Lowry et al. (31), and equal quantities of protein were loaded per lane and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (4 % stacking gel and 12 % separating gel) as described by Laemmli (32). Electrophoresis was performed at 75 V and the proteins resolved were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics, GmbH, Germany) in a transfer buffer (0.2 mol L^{-1} glycine, 25 mmol L^{-1} Tris and 20 % methanol). Successful transfer was confirmed by Ponceau S staining of the blots. The membranes were incubated in a blocking buffer (phosphate-buffered saline containing 0.1 % Tween 20 and 5 % non-fat dry milk powder) for 5 h, at room temperature, followed by incubation in the respective primary antibodies (1:200 anti-alpha B-crystallin and 1:100 anti-HSP70 antibody). Incubation with the primary antibodies was done overnight at 4 °C. The next day, the blots were washed in phosphate-buffered saline and incubated for 1 h at room temperature, with either horseradish peroxidase-conjugated anti-mouse IgG (1:8000, A9044, Sigma, USA) or anti-rabbit IgG (1:12000, A9169, Sigma, USA). Immunodetection of proteins was done using the 3-amino-9-ethylcarbazole (AEC) Staining Kit (Sigma) as the substrate. Quantification of band intensity of the blots from four independent experiments was performed on scanned Western blot images with an image analysis system (Bs200P Image Analysis System, BAB software, Ankara, Turkey).

Statistical Analysis

Statistical differences in the Western blot bands at certain experiment times were determined by a One-Way Analysis of Variance (ANOVA) followed by a Tukey's post-hoc Test. Differences were considered significant only when the *P*-values were less than 0.05.

RESULTS

Immunofluorescent staining demonstrated intense positive reactions for HSP70 (Figure1A) and αBC (Figure1C) in the *substantia nigra* of rats from the treatment groups; however, no immunopositive cells were detected in the control tissue sections (Figures 1B and 1D). 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 TH immunoreactivity in the control or treatment groups (Figures 1E to 1H). The merged images strongly suggested that HSP70 and αBC immunopositive cells were also TH positive neurons (Figures 1J to 1M). As expected, TUNEL staining was negative in the nuclei of control substantia nigra cells. The same sections were also stained for TH antibody using the immunofluorescent technique to determine the dopaminergic neurons in the substantia nigra, and clear positive reactions were detected. A similar finding was observed in the case of the treated groups (Figures 2A to 2C).

We also explored the possible induction of HSP70 and αBC after treatment of permethrin at the protein level determined by the Western blotting technique. Anti-alpha B crystallin antibody and HSP 70 antibody were detected as major bands of 21 kD and 70 kD, respectively (Figure 3). A significant increase in the HSP70 and αBC expression was observed on a dose-dependent manner ($P<0.05$) (Figures 4A and 4B).

DISCUSSION

Apoptosis develops through a complex signalling cascade which can occur under pathological or specific physiological conditions. One of the main apoptotic pathways (the so-called endogenous-dependent programmed cell death) is related to the mitochondrial release of cytochrome-c into the cytosol. Cytochrome-c activates the apoptotic protease-activating factor 1 (Apaf-1). Subsequently, Apaf-1 and cytochrome-c bind to procaspase-9 and activate it. Afterwards, caspase-9 activates the effector caspase, caspase-3. Thus, the mitochondria-dependent apoptotic pathway is initiated (33, 34). Earlier studies have reported that a single dermal application of permethrin did not significantly increase the release of cytochrome c. However, a combined single dermal application of DEET (*N,N*-Diethyl-*m*-toluamide) and permethrin significantly increased the release of brain mitochondrial cytochrome c, which has been correlated with inducing apoptosis (35, 36). Elwan et al. (37) showed that a lower concentration of permethrin (5 μmol L^{-1}) can induce increased DNA fragmentation in the cultured neuroblastoma cells. These data, therefore, indicate that apoptosis can be induced by permethrin. Other reports have shown that the topical application of permethrin (25 μL, equivalent to 1100 mg kg^{-1} bw) in mice significantly increased apoptosis in the CD4(-) 8(-) and CD4(-)8(+) thymocytes (38). Contrary to these reports, in the present study, TUNEL-TH co-labelling of cells was not detected in the nigral dopaminergic neurons in spite of the rats being treated with higher doses of permethrin (300 mg kg^{-1}, orally). This finding suggests that the loss of nigral dopaminergic neurons might not be caused by apoptosis due to permethrin treatment.

The major stress-inducible protein HSP70 is produced by any kind of stressful stimulus, such as hyperthermia or cerebral ischemia (21, 39). It has been recognized as a molecular chaperone, whose main functions include folding up proteins and protecting the tertiary protein structure (20, 21, 39). Furthermore, the cytoprotective effects of HSP70 are not reduced to these functions; they also block the apoptotic mechanism at various points of cell death. HSP70 has been reported to be capable of inhibiting caspase activation by interfering with Apaf-1, and preventing the participation of procaspase-9 apoptosome. Nevertheless, the role of HSP70 against apoptosis is not limited to caspases. HSP70 can also prevent apoptosis in a caspase-independent but mitochondrial-dependent manner by direct interaction with the AIF (Apoptosis inducing factor) (40).

Recent studies confirmed that the increase in αBC expression possesses a neuroprotective effect. This effect of αBC occurs through several anti-apoptotic pathways. αBC inhibits apoptosis induced by various stimuli, including DNA-damaging agents such as TNF-alpha and Fas (41, 42), as well as growth factor deprivation by disrupting the proteolytic activation of

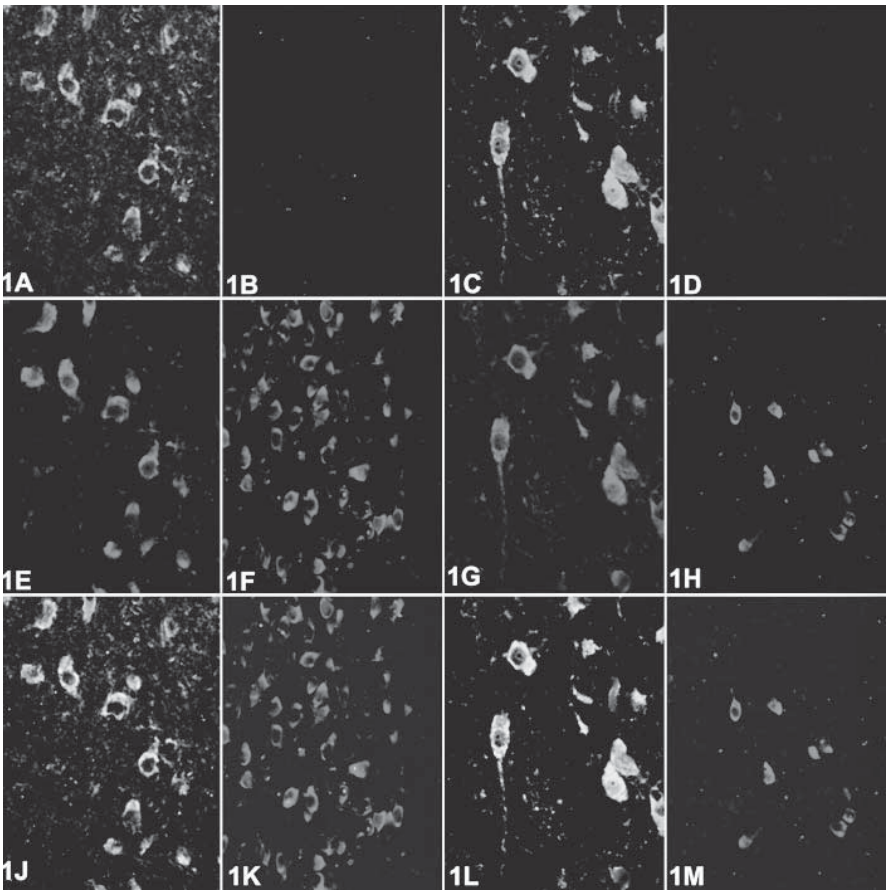

Figure 1 *Brain sections were double immunofluorecence staining with HSP70, αBC and TH in permethrin treatment (group III, 300 mg kg^{-1}) and control group. [1A] HSP70 (green) in group III (300 mg kg^{-1}); [1B] HSP70 in control group, no Immunofluorescence staining; [1C] αBC (green) in group III (300 mg kg^{-1}); [1D] αBC in control group, no Immunofluorescence staining; [1E-H] Immunofluorescence staining of TH (red) in permethrin treatment group III (300 mg kg^{-1}) [1E,G] and control group [1F,H]; [1J-M] Merged images shows TH-positive neurons co-express HSP70 and αBC in treatment group [1J,L] and negative results in control group (1K,M). Magnification 200x.*

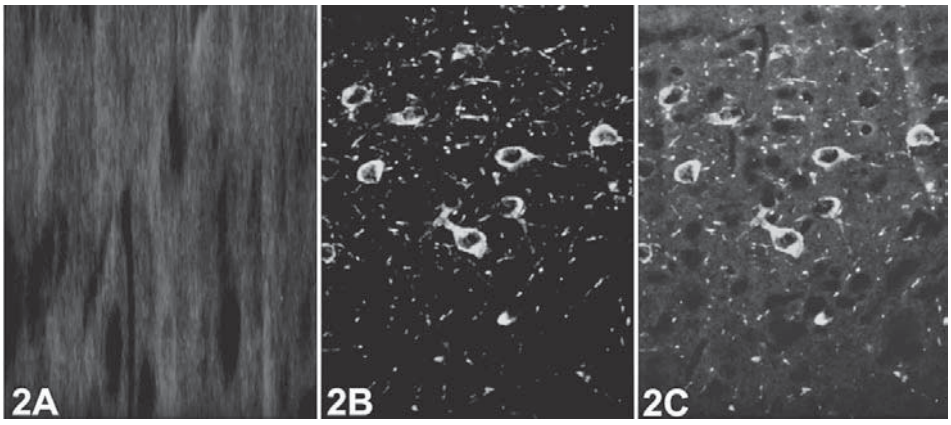

Figure 2 *TUNEL and immunofluorecence staining with TH antibody in permethrin treatment (group III, 300 mg kg^{-1}). [2A] Negative staining for TUNEL methods; [2B] Immunofluorescence staining of TH (red); [3C] Merged images 2A and 2B. Magnification 200x.*

caspase-3 (43, 44). Moreover, it has been shown that αBC inhibits TRAIL-induced (tumour necrosis factor involved in apoptosis-inducing ligand) apoptosis through the suppression of caspase-3 activation (45). Another group showed that the inhibition of stress-induced apoptosis by αBC could suppress the activation of caspase-3 and/or prevent the mitochondrial translocation of the proapoptotic Bcl-2 family

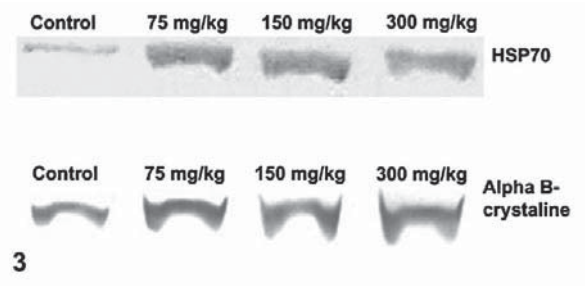

Figure 3 *Western blot analysis of the effects of permethrin on the expression of HSP70 and αBC. Equal amounts of protein (20 μg) were resolved by SDS-PAGE on 12 % polyacrylamide gels. Brain tissue from permethrin treatment groups shows increased protein levels of HSP70 and αBC relative to control.*

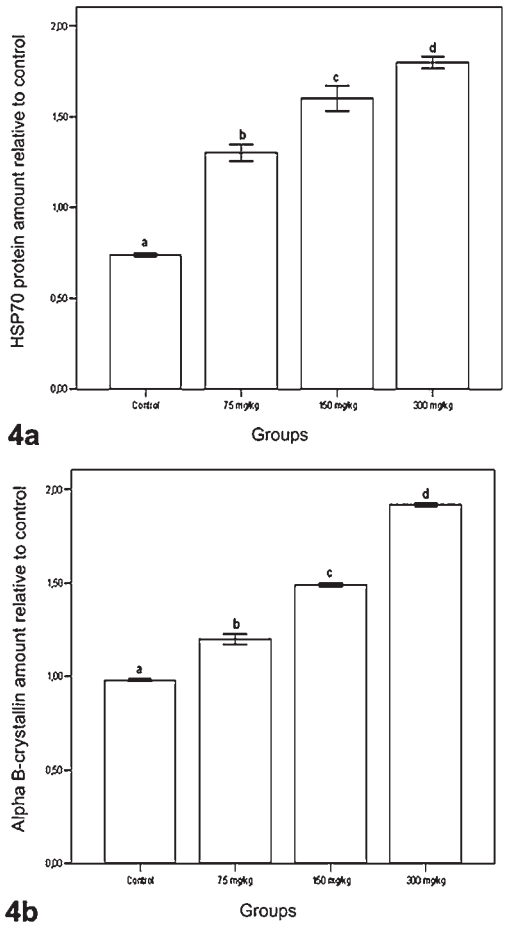

Figure 4 *Protein levels of HSP70 (4a) and αBC (4b) in the brain of permethrin-treated rats (n=3). Each data point represents the average values from four independent experiments. Superscripts (a, b, c, d) are significantly different (P < 0.05).*

members such as Bax (46). All of these data strongly suggest that the overexpression of αBC could be neuroprotective by blocking apoptosis. Additionally, Kirbach and Golenhofen (47) reported a significant increase in αBC (HspB5) in cultured rat hippocampal neurons after heat shock. This finding indicates that αBC might protect neurons from heat stress. We showed that HSP70 and αBC-TH co-labelled cells were seen in the substantia nigra of the permethrin treated rats. Also, a significant increase in both HSP70 and αBC expressions were observed in a dose-dependent manner. No TUNEL positive signals were detected in the substantia nigra dopaminergic neurons of the control and treated groups. Taken together, these findings suggest that an over-expression of both HSP70 and αBC will inhibit pro-apoptotic events, exerting a neuroprotective effect.

It has previously been reported that TH and DAT protein expression in mice striatal dopaminergic terminals did not change when they were subjected to long-term and low doses of permethrin (10). Similarly, no changes in the staining intensity of TH immunoreactivity were observed in our study, either.

In conclusion, our findings demonstrated that relatively high doses of permethrin did not cause apoptotic cell death in the substantia nigra dopaminergic neurons. Nevertheless, the present study also observed an over-expression of HSP70 and αBC. The increase in HSP70 and αBC expression suggests that these stress proteins may have played a role as anti-apoptotic factors in our experimental model of permethrin-induced neurotoxicity.

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Sažetak

BIOLOŠKA VAŽNOST PRETJERANE EKSPRESIJE HSP70 I ALFA-B KRISTALINA U SUPSTANCIJI NIGRI ŠTAKORA IZLOŽENIH RAZLIČITIM DOZAMA PERMETRINA

Svrha ove studije bila je istražiti moguću ulogu proteina toplinskog stresa 70 (HSP70) i alfa-B kristalina (αBC) u supstanciji nigri (lat. *substantia nigra*) štakora izloženih različitim dozama permetrina na apoptotske stanice. Metoda orogastričnog hranjenja upotrijebljena je kako bi se štakorima dale različite doze permetrina (75 mg kg^{-1} u skupini I, 150 mg kg^{-1} u skupini II, 300 mg kg^{-1} u skupini III). Nakon provođenja analize *Western blot* sve skupine kojima je dan permetrin pokazale su, ovisno o dozi, povećanje ekspresije HSP70 i αBC u usporedbi s kontrolnom skupinom. Apoptotske stanice pozitivne na TUNEL-test nisu otkrivene u dopaminergičkim neuronima supstancije nigre nakon tretmana permetrinom. Međutim nakon imunofluorescentnog bojenja za HSP70 i αBC primijećene su snažne pozitivne reakcije u svim tretiranim skupinama. U tkivu kontrolne skupine nije bilo imunopozitivnih stanica. Naši rezultati upućuju na to da različite doze permetrina nisu uzrokovale apoptozu dopaminergičkih neurona supstancije nigre, ali su izazvale povećanje ekspresije HSP70 i αBC. Stoga bi HSP70 i αBC mogli imati pozitivan neuroprotektivni učinak pri neurotoksičnosti izazvanoj permetrinom.

KLJUČNE RIJEČI: *protein toplinskog stresa, imunohistokemija, piretroid, TUNEL-test*

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