

## 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 ( $\alpha$ BC) 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<sup>-1</sup> in Group I, 150 mg kg<sup>-1</sup> in group II, 300 mg kg<sup>-1</sup> 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  $\alpha$ BC 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  $\alpha$ BC 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  $\alpha$ BC expression. Thus, it appears that HSP70 and  $\alpha$ BC 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  $\alpha$ -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  $\alpha$ -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 ( $\gamma$ -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 ( $3 \text{ mg kg}^{-1}$ ) significantly decreases the dopamine transporter immunoreactive protein, high-dose permethrin ( $200 \text{ mg kg}^{-1}$ ) 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  $0.8 \text{ mg kg}^{-1}$  and  $1.5 \text{ mg kg}^{-1}$ ) 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 translocation 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,  $\alpha$ BC 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,  $\alpha$ BC expression has also been detected in heat shock, anticancer drugs, radiation and oxidative stress (23). In a normal central nervous system,  $\alpha$ BC is present in glial cells, particularly in astrocytes and oligodendrocytes, although not in the neurons (24). The neuronal expression of  $\alpha$ BC 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,  $\alpha$ BC 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  $\alpha$ BC.

## 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 Mayıs University (HADYEK-2008/51). Thirty-two adult male Sprague-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  $22 \text{ }^{\circ}\text{C} \pm 2 \text{ }^{\circ}\text{C}$ ,  $60 \% \pm 5 \%$  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 ( $n=8$ ),  $75 \text{ mg kg}^{-1}$  permethrin (1/20 of the  $\text{LD}_{50}$  value); group II rats ( $n=8$ )  $150 \text{ mg kg}^{-1}$  permethrin (1/10 of the  $\text{LD}_{50}$  value, group II) and group III rats ( $n=8$ )  $300 \text{ mg kg}^{-1}$  (1/5 of the  $\text{LD}_{50}$  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<sup>-1</sup>, *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<sup>-1</sup> 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 homogenizer (20,000 rpm, SilentCrusher M, Heidolph Instruments, Germany) in a lysis buffer (50 mmol L<sup>-1</sup> Tris, pH 7.4, containing 0.15 mol L<sup>-1</sup> 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<sup>-1</sup> glycine, 25 mmol L<sup>-1</sup> 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 (Figure 1A) and  $\alpha$ BC (Figure 1C) 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  $\alpha$ BC 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  $\alpha$ BC 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  $\alpha$ BC expression was observed on a dose-dependent manner ( $P < 0.05$ ) (Figures 4A and 4B).

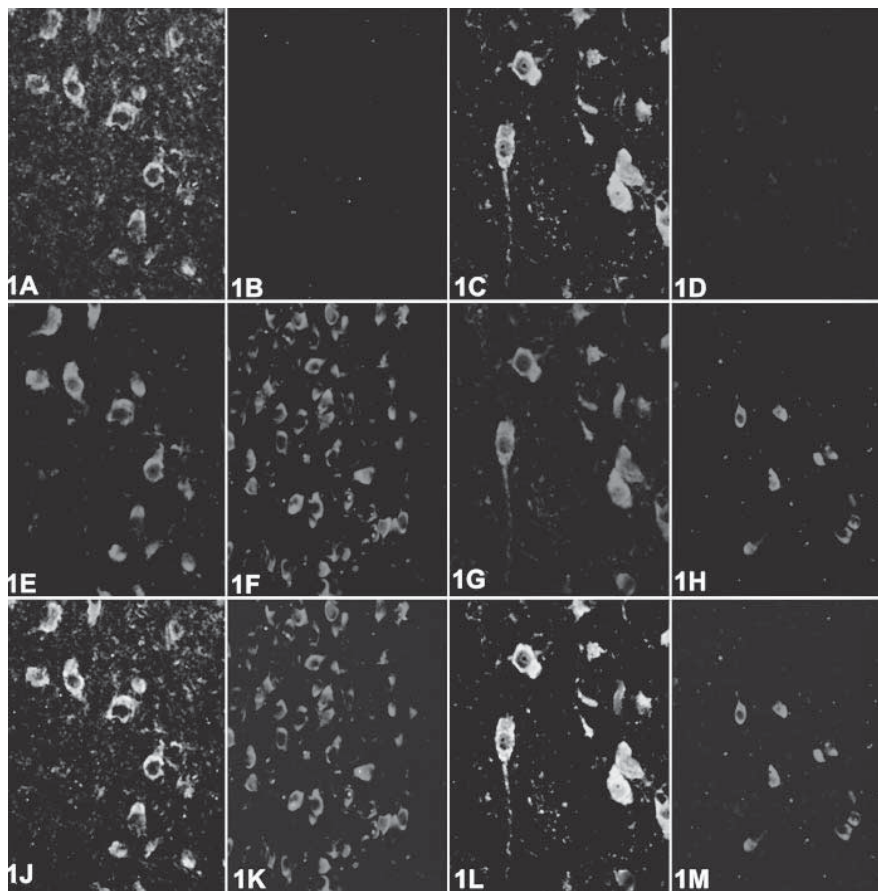
## DISCUSSION

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

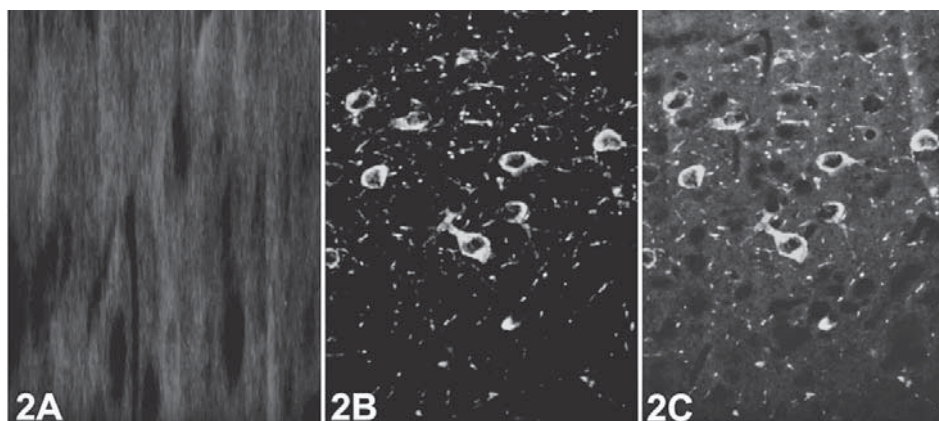
(Apaf-1). Subsequently, Apaf-1 and cytochrome-c bind to procaspase-9 and activate it. Afterwards, caspase-9 activates the effector caspase, caspase-3. Thus, the mitochondria-dependent apoptotic pathway is initiated (33, 34). Earlier studies have reported that a single dermal application of permethrin 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  $\mu$ mol L<sup>-1</sup>) 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  $\mu$ L, equivalent to 1100 mg kg<sup>-1</sup> 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<sup>-1</sup>, 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  $\alpha$ BC expression possesses a neuroprotective effect. This effect of  $\alpha$ BC occurs through several anti-apoptotic pathways.  $\alpha$ BC 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



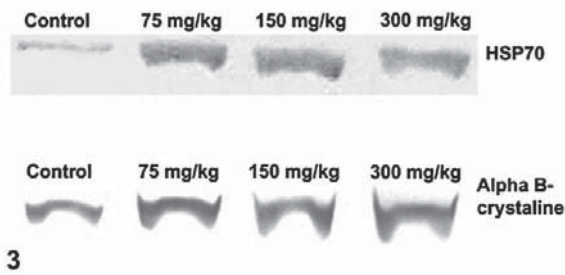
**Figure 1** Brain sections were double immunofluorescence staining with HSP70,  $\alpha$ BC and TH in permethrin treatment (group III, 300 mg kg<sup>-1</sup>) and control group. [1A] HSP70 (green) in group III (300 mg kg<sup>-1</sup>); [1B] HSP70 in control group, no Immunofluorescence staining; [1C]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



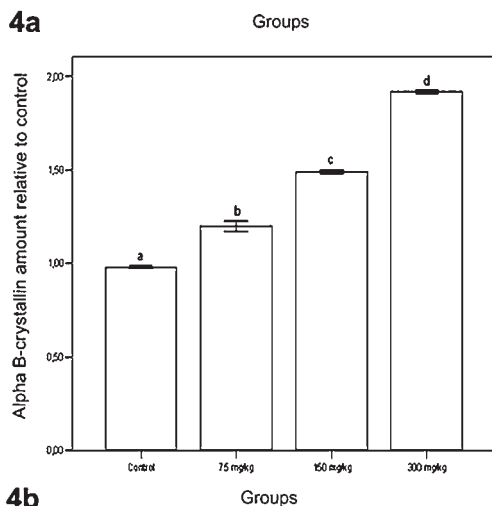
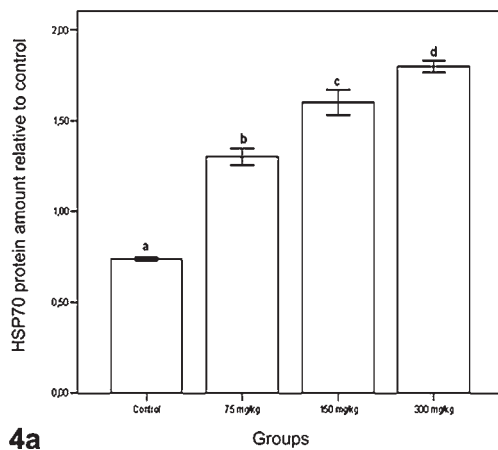
**Figure 2** TUNEL and immunofluorescence staining with TH antibody in permethrin treatment (group III, 300 mg kg<sup>-1</sup>). [2A] Negative staining for TUNEL methods; [2B] Immunofluorescence staining of TH (red); [2C] Merged images 2A and 2B. Magnification 200x.

caspase-3 (43, 44). Moreover, it has been shown that  $\alpha$ BC 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  $\alpha$ BC 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  $\alpha$ BC. Equal amounts of protein (20  $\mu$ g) were resolved by SDS-PAGE on 12 % polyacrylamide gels. Brain tissue from permethrin treatment groups shows increased protein levels of HSP70 and  $\alpha$ BC relative to control.



**Figure 4** Protein levels of HSP70 (4a) and  $\alpha$ BC (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  $\alpha$ BC could be neuroprotective by blocking apoptosis. Additionally,

Kirbach and Golenhofen (47) reported a significant increase in  $\alpha$ BC (HspB5) in cultured rat hippocampal neurons after heat shock. This finding indicates that  $\alpha$ BC might protect neurons from heat stress. We showed that HSP70 and  $\alpha$ BC-TH co-labelled cells were seen in the substantia nigra of the permethrin treated rats. Also, a significant increase in both HSP70 and  $\alpha$ BC 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  $\alpha$ BC 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  $\alpha$ BC. The increase in HSP70 and  $\alpha$ BC 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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#### REFERENCES

1. Kaneko H. Pyrethroid chemistry and metabolism. In: Kreiger R, editor. Handbook of pesticide toxicology. San Diego: Academic Press; 2010. p. 1635-63.
2. Aldridge WN. An assessment of the toxicological properties of pyrethroids and their neurotoxicity. Crit Rev Toxicol 1990;21:89-104.
3. Verschoyle RD, Aldridge WN. Structure-activity relationships of some pyrethroids in rats. Arch Toxicol 1980;45:325-9.
4. Ray DE. Pyrethroid insecticides: mechanisms of toxicity, systemic poisoning syndromes, paraesthesia, and therapy. In: Kreiger R, editor. Handbook of pesticide toxicology. San Diego: Academic Press; 2001. p. 1289-303.
5. Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. Mechanisms of

- pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology* 2003;171:3-59.
6. Soderlund DM. Toxicology and mode of action of pyrethroid insecticides. In: Kreiger R, editor. *Handbook of pesticide toxicology*. San Diego: Academic Press; 2010. p. 1665-86.
  7. Karen DJ, Li W, Harp PR, Gillette JS, Bloomquist JR. Striatal dopaminergic pathways as a target for the insecticides permethrin and chlorpyrifos. *Neurotoxicology* 2001;22:811-7.
  8. Kakko I, Toimela T, Tähti H. The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin. *Chemosphere* 2003;51:475-80.
  9. Pittman JT, Dodd CA, Klein BG. Immunohistochemical changes in the mouse striatum induced by the pyrethroid insecticide permethrin. *Int J Toxicol* 2003;22:359-70.
  10. Kou J, Bloomquist JR. Neurotoxicity in murine striatal dopaminergic pathways following long-term application of low doses of permethrin and MPTP. *Toxicol Lett* 2007;171:154-61.
  11. Kiang GJ, Tsokos GC. Heat Shock Protein 70 kDa: Molecular Biology, Biochemistry, and Physiology. *Pharmacol Ther* 1998;80:183-201.
  12. Gupta SC, Sharma A, Mishra M, Mishra RK, Chowdhuri DK. Heat shock proteins in toxicology: How close and how far? *Life Sci* 2010;86:377-84.
  13. Samali A, Orrenius S. Heat shock proteins: regulators of stress response and apoptosis. *Cell Stress Chaperones* 1998;3:228-36.
  14. Goldbaum O, Landsberg RC. Stress proteins in oligodendrocytes: differential effects of heat shock and oxidative stress. *J Neurochem* 2001;78:1233-42.
  15. Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem Biophys Res Commun* 2001;286:433-42.
  16. Landsberg CR, Goldbaum O. Stress proteins in neural cells: functional roles in health and disease. *Cell Mol Life Sci* 2003;60:337-49.
  17. Brown IR. Heat shock proteins and protection of the nervous system. *Ann N Y Acad Sci* 2007;1113:147-58.
  18. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Hyperthermia* 2005;21:379-92.
  19. Suzuki T, Usuda N, Murata S, Nakazawa A, Ohtsuka K, Takagi H. Presence of molecular chaperones, heat shock cognate (Hsc) 70 and heat shock proteins (Hsp) 40, in the postsynaptic structures of rat brain. *Brain Res* 1999;816:99-110.
  20. Yenari MA, Giffard RG, Sapolsky RM, Steinberg GK. The neuroprotective potential of heat shock protein 70 (HSP70). *Mol Med Today* 1999;5:525-31.
  21. Rajdev S, Sharp FR. Stress proteins as molecular markers of neurotoxicity. *Toxicol Pathol* 2000;28:105-12.
  22. Iwaki T, Kume-Iwaki A, Goldman JE. Cellular distribution of  $\alpha$ B-crystallin in non-lenticular tissues. *J Histochem Cytochem* 1990;38:31-9.
  23. Parcellier A, Schmitt E, Brunet M, Hammann A, Solary E, Garrido C. Small heat shock proteins HSP27 and  $\alpha$ B-crystallin: cytoprotective and oncogenic functions. *Antioxid Redox Signal* 2005;7:404-12.
  24. Iwaki T, Wisniewski T, Iwaki A, Corbin E, Tomokane N, Tateishi J, Goldman JE. Accumulation of  $\alpha$ B-crystallin in central nervous system glia and neurons in pathologic condition. *Am J Pathol* 1992;140:345-56.
  25. van Rijk AF, Bloemendal H. Alpha-B-crystallin in neuropathology. *Ophthalmologica* 2000;214:7-12.
  26. Aoyama A, Steiger RH, Fröhli E, Schäfer R, von Deimling A, Wiestler OD, Klemenz R. Expression of alpha B-crystallin in human brain tumors. *Int J Cancer* 1993;55:760-4.
  27. Head MW, Goldman JE. Small heat shock proteins, the cytoskeleton, and inclusion body formation. *Neuropathol Appl Neurobiol* 2000;26:304-12.
  28. Bloomquist JR, Barlow RL, Gillette JS, Li W, Kirby ML. Selective effects of insecticides on nigrostriatal dopaminergic nerve pathways. *Neurotoxicology* 2002;23:537-44.
  29. Cantalamessa F. Acute toxicity of two pyrethroids, permethrin, and cypermethrin in neonatal and adult rats. *Arch Toxicol* 1993;67:510-3.
  30. Kirby ML, Castagnoli K, Bloomquist JR. *In Vivo* effects of deltamethrin on dopamine neurochemistry and the role of augmented neurotransmitter release. *Pestic Biochem Physiol* 1999;65:160-8.
  31. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
  32. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
  33. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007;35:495-516.
  34. Vermeulen K, Van Bockstaele DR, Berneman ZN. Apoptosis: mechanisms and relevance in cancer. *Ann Hematol* 2005;84:627-39.
  35. Abu-Qare AW, Abou-Donia MB. Combined exposure to DEET (N,N-diethyl-m-toluamide) and permethrin-induced release of rat brain mitochondrial cytochrome c. *J Toxicol Environ Health A* 2001;63:243-52.
  36. Martinou JC, Desagher S, Antonsson B. Cytochrome c release from mitochondria: all or nothing. *Nat Cell Biol* 2000;2:E41-3.
  37. Elwan MA, Richardson JR, Guillot TS, Caudle WM, Miller GW. Pyrethroid pesticide-induced alterations in dopamine transporter function. *Toxicol Appl Pharmacol* 2006;211:188-97.
  38. Prater MR, Gogal RM Jr, Blaylock BL, Longstreth J, Holladay SD. Single-dose topical exposure to the pyrethroid insecticide, permethrin in C57BL/6N mice: effects on thymus and spleen. *Food Chem Toxicol* 2002;40:1863-73.
  39. Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG. Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. *Ann N Y Acad Sci* 2005;1053:74-83.
  40. Ravagnan L, Gurbuxani S, Susin SA, Maise C, Daugas E, Zamzami N, Mak T, Jäättelä M, Penninger JM, Garrido C, Kroemer G. Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat Cell Biol* 2001;3:839-43.
  41. Mehlen P, Kretz-Remy C, Préville X, Arrigo AP. Human hsp27, *Drosophila* hsp27 and human alphaB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNF $\alpha$ -induced cell death. *EMBO J* 1996;15:2695-706.

42. Mehlen P, Schulze-Osthoff K, Arrigo AP. Small stress proteins as novel regulators of apoptosis. Heat shock protein 27 blocks Fas/APO-1- and staurosporine-induced cell death. *J Biol Chem* 1996;271:16510-4.
43. Kamradt MC, Chen F, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. *J Biol Chem* 2001;276:16059-63.
44. Kamradt MC, Chen F, Sam S, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation. *J Biol Chem* 2002;277:38731-6.
45. Kamradt MC, Lu M, Werner ME, Kwan T, Chen F, Strohecker A, Oshita S, Wilkinson JC, Yu C, Oliver PG, Duckett CS, Buchsbaum DJ, LoBuglio AF, Jordan VC, Cryns VL. The small heat shock protein alpha B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. *J Biol Chem* 2005;280:11059-66.
46. Mao YW, Liu JP, Xiang H, Li DW. Human alphaA- and alphaB-crystallins bind to Bax and Bcl-X(S) to sequester their translocation during staurosporine-induced apoptosis. *Cell Death Differ* 2004;11:512-26.
47. Kirbach BB, Golenhofen N. Differential expression and induction of small heat shock proteins in rat brain and cultured hippocampal neurons. *J Neurosci Res* 2011;89:162-75.



### **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 ( $\alpha$ BC) 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 ( $75 \text{ mg kg}^{-1}$  u skupini I,  $150 \text{ mg kg}^{-1}$  u skupini II,  $300 \text{ mg kg}^{-1}$  u skupini III). Nakon provođenja analize *Western blot* sve skupine kojima je dan permetrin pokazale su, ovisno o dozi, povećanje ekspresije HSP70 i  $\alpha$ BC 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  $\alpha$ BC 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  $\alpha$ BC. Stoga bi HSP70 i  $\alpha$ BC mogli imati pozitivan neuroprotektivni učinak pri neurotoksičnosti izazvanoj permetrinom.

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

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