EVALUATION OF THE EFFECTS ON THE CEREBELLAR CORTEX OF CF-1 MICE EXPOSED TO A SINGLE DOSE OF CYPERMETHRIN

Germán Venegas F., Cristian Donoso, Camilo Arriaza, Omar Espinoza-Navarro, María Eugenia Castro, Cristian Torres, Felipe Lillo and Héctor Rodríguez B.

SUMMARY

Cypermethrin is a Type II pyrethroid widely used for pests control in agriculture. The principal effect of this insecticide is to alter voltage-dependent sodium channels in the nervous system, but it also affects GABA levels in the CNS of mammals. The cerebellar circuit has as its principal neurotransmitter GABA for transportation of Cl-, allowing its inhibitory and modulatory function. In this work, the effects of cypermethrin on the cerebellar cortex are studied. Thirty three adult CF-1 male mice were distributed in three groups: 1) untreated, 2) vehicle (oil) and 3) experimental (cypermethrin in oil at 1/5 DL50). The animals were euthanized at days 1, 8, 17, 25 and 34; then processed for histology (haematoxylin-eosine) and im munohistochemistry (Apaf-1 and Ki-67). The Purkinje cells and the immunoreactive Purkinje and Granular cells were quantified. The results showed a significant decrease in Purkinje cells numbers at all the times studied; also, there was an increase in the expression of Apaf-1 and Ki-67 in the Purkinje and Granular cells in acute and chronic stages. This shows that cypermethrin at 1/5 DL50 produces damage to the cerebellar cortex and induces apoptosis in the Purkinje and Granular cells.

Introduction

Cypermethrin it is a synthetic, photo stable pyrethroid with great insecticide power and a broad action spectrum, widely used for pest control in crops, with good effects on Lepidoptera, Coleoptera and Hemiptera. The inclusion of a α-cyano group classifies cypermethrin as a type II pyretroid (Soderlund *et al*., 2002). The α-cypermethrin makes up more of the 90% of the active form of the insecticide and corresponds with a rasemic mixture of the *cis* isomers (Lessenger, 1992). Cypermethrin it's considered as a class II insecticide by WHO (2010).

The pesticide has multiple ways of entrance to the body: dermal (Luty *et al*., 2000), oral (Manna *et al*., 2004), across the conjunctiva and through the respiratory tract epithelium (Martínez-Navarrete *et al*., 2008). This last one allows a high rate of absorption and is the best known (Martínez-Navarrete *et al*., 2008). Cypermethrin shows high affinity to lipids, its elimination kinetics from nervous tissue is 78% in 24h, 12% in 8 days and the remaining 10% between 18 and 24 days. The rate of excretion does not increase after repeated exposure. The half-life of *cis*-cypermethrin in fatty tissue of mice is 13 days, and the *trans* isomer has a half-life of one day (Soderlund *et al*., 2002; Wolansky and Harrill, 2008).

The principal mechanism of action is by alteration of the voltage-dependent sodium channels (VDSC) in the CNS, increasing the channel opening time, which in turn increases depolarization time (Ray and Fry, 2006). Cypermethrin also produces alterations on the gamma aminobutyric (GABA) receptor, altering the GABA dependent Cl^- channel (GDCC). GABA is the principal neurotransmitter for Cl^- transport in the CNS and the cerebellum has the highest concentration of GABA in the encephalon. Severe alterations of GABA concentration could lead to disruption of the cerebellar circuits, mainly affecting the Purkinje cells. Cypermethrin and deltamethrin produce decreases on the level of GABA in rats exposed with 1/10 of the LD_{50} (Manna *et al*., 2006). Permethrin (a Type I pyrethroid), decreases in the number of Purkinje

KEYWORDS / Apoptosis / Cell Proliferation / Cerebellar Cortex / Cerebellum / Cypermethrin / Granular Cell / Mouse / Purkinje Cells / Toxicology /

Received: 03/31/2014. Modified: 10/28/2014. Accepted: 11/03/2014.

Germán Venegas F. Veterinarian, Master in Veterinary Sciences, Doctoral Student in Veterinary Medicine, Universidad Nacional Andrés Bello (UNAB), Chile. Professor, UNAB, Chile.

Cristian Donoso. Veterinarian, Universidad de Chile (UChile). Reasearch Asistant, Universidad de Chile (UChile).

Camilo Arriaza. Veterinarian and Master of Science, Professor, UChile.

Omar Espinoza-Navarro. Doctor in Celullar and Genetic Biology, Universidad Autónoma of Madrid, España. Professor, Universidad de Tarapacá, Chile. Address: Department of Biology, Faculty of Science, Universidad de Tarapacá. General Velásquez Nº 1775. Arica, Chile. e-mail: oespinoz@uta.cl

María Eugenia Castro. Psychologist, Master of Science and Doctor in Psychology, Université de Paris, France. Professor, Playa Ancha University, Chile.

Cristian Torres. Veterinarian and Doctor in Veterinary Sciences, UNAB, Chile. Professor, UChile.

Felipe Lillo. Veterinarian, Master in Veterinary Sciences, Doctoral Student in Veterinary Medicine, UNAB, Chile. Professor, UNAB, Chile.

Héctor Rodríguez B. Veterinarian and Master of Science, UChile. Doctor in Biomedicine, Universidad Alcalá de Henares, Spain. Professor UChile. Address: Héctor Rodríguez. Morphology Laboratory. Faculty of Medicine, Universidad de Chile. Avenida Independencia 1027. Santiago, Chile. POBox 8389100. e-mail: hrodrigu@med.uchile.cl

EVALUACIÓN DE LOS EFECTOS DE UNA DOSIS ÚNICA DE CIPERMETRINA EN LA CORTEZA CEREBELAR DE RATONES CF-1

Germán Venegas F., Cristian Donoso, Camilo Arriaza, Omar Espinoza-Navarro, María Eugenia Castro, Cristian Torres, Felipe Lillo y Héctor Rodríguez B.

RESUMEN

La cipermetrina es un piretroide tipo II ampliamente utilizado para el control de pestes en agricultura. El principal efecto de este insecticida es alterar los canales de sodio voltaje dependientes en el sistema nervioso, pero también afecta los niveles de GABA en el SNC de mamíferos. El circuito cerebeloso tiene a GABA como su principal neurotransmisor para el transporte de Cl^-, permitiendo su función inhibitoria y moduladora. En el presente trabajo se estudian los efectos de la cipermetrina sobre la corteza cerebelosa. Treinta y tres ratones CF-1 adultos fueron distribuidos en tres grupos: 1) sin tratamiento, 2) con vehículo (aceite) y 3) experimental cipermetrina en aceite a 1/5 DL_{50}). Los animales fueron sacrificados después de 1, 8, 17, 25 y 34 días, para luego ser procesados para histología (hematoxilina-eosina)e inmunohistoquímica (Apaf-1 y Ki-67). Se cuantificaron las células de Purkinje y las células de Purkinje y Granulares inmunoreactivas. Los resultados mostraron un descenso significativo en el número de células de Purkinje en todos los tiempos del estudio, así como un aumento en la expresión de Apaf-1 y Ki-67 en las células de Purkinje y Granulares en estadio agudo y crónico. Se muestra que la cipermetrina a 1/5 D50 produce daños en la corteza cerebelosa e induce apoptosis en las células de Purkinje y Granulares.

AVALIAÇÃO DOS EFEITOS DE UMA DOSE ÚNICA DE CIPERMETRINA NO CÓRTEX CEREBELAR DE RATOS CF-1

Germán Venegas F., Cristian Donoso, Camilo Arriaza, Omar Espinoza-Navarro, María Eugenia Castro, Cristian Torres, Felipe Lillo e Héctor Rodríguez B

RESUMO

A cipermetrina é um piretróide tipo II amplamente utilizado para o controle de pestes na agricultura. O principal efeito deste inseticida é alterar os canais de sódio voltagem dependentes no sistema nervoso, mas também afeta os níveis de GABA no SNC de mamíferos. O circuito cerebeloso tem o GABA como seu principal neurotransmissor para o transporte de Cl-, permitindo sua função inibitória e moduladora. No presente trabalho se estudam os efeitos da cipermetrina sobre o córtex cerebeloso. Trinta e três ratos CF-1 adultos foram distribuídos em três grupos: 1.- sem tratamento, 2.- com veículo (óleo) e 3.- experimental cipermetrina em óleo a 1/5 DL50). Os animais foram sacrifcados depois de 1, 8, 17, 25 e 34 dias, para logo ser processados para histologia (hematoxilina-eosina)e imuno-histoquímica (Apaf-1 y Ki-67). Quantificaram-se as células de Purkinje e as células de Purkinje e Granulares imunorreativas. Os resultados mostraram um descenso significativo no número de células de Purkinje em todos os tempos do estudo, assim como um aumento na expressão de Apaf-1 e Ki-67 nas células de Purkinje e Granulares em estado agudo e crônico. Mostra-se que a cipermetrina a 1/5 D50 produz danos no córtex cerebeloso e induz apoptose nas células de Purkinje e Granulares.

cells in dermally exposed rats (Abdel-Rahman *et al*., 2001).

The objective of this study was to evaluate the effects of a low dose (1/5 DL_{50}) of cypermethrin on the cerebellar cortex, focusing on the density of the Purkinje cell population and the expression of apoptotic (Apaf-1) and proliferation (Ki-67) biomarkers, as regulators of cellular proliferation processes, through the route of caspases and cell cycle regulatory factors, respectively.

Materials and Methods

Materials

Thirty-three mice of the CF1 strain, 2.5-3 month old, weighing 25-30g, were kept under standard feeding conditions, with a 12h light and 12h darkness cycle in the animal house of the Faculty of Medicine, Universidad de Chile. Cypermethrin (CAS Nº 52315-07-8) 92.5% p/p from Anasac was re-suspended in vegetable oil (Sunflower).

Experimental design

Animals were distributed in three groups: Group 1 (n= 3) were left untreated, Group 2 (n= 15) received 50µl vegetable oil i.p., and Group 3 (n= 15) received 50ml vegetable oil containing cypermethrin at 1/5 of the lethal i.p. dose (LD_{50}) of 485mg·kg^{-1} (IPCS, 2008). For each group, the cerebellums of three mice were dissected at 24h and 8, 17, 25, and 34 days after cypermethrin treatment, following euthanasia according to NIH protocols (Mendez, 2009). These periods coincide with the time required for metabolism and elimination of cypermethrin, and its permanence in the fatty tissue with a half-life of 9-12 and 18 days, respectively. The cerebellum was fixed for 48h in 10% buffered formalin (phosphate buffer pH 7.2) and thereafter subjected to standard histological techniques, such as paraffin embedding (56/58°C melting point). Sections of 5µm were sliced and mounted in silanised slides (Star Frost).

Purkinje cell count

After hematoxylin eosine (H-E) staining, bright field photomicrographs were taken with a 1000× objective. Cell counts were made by measuring a line in the monolayer of Purkinje cell, and an index was obtained considering number of Purkinje cells per lineal extent (µm). A total of 30 fields were read per cerebellum.

Immunohystochemistry

Apoptosis was analyzed with antibody against Apaf-1 and cell proliferation was analyzed with antibody against Ki-67 (Clon SP6. Thermo Scientific Catalog RM-9106-SO). Positive Purkinje cells were counted as the number of immune reactive cells in relation to the total of Purkinje cells. For Granular cells, positive cells were counted and tabulated as the number of immune reactive

cell per unit area (μm^2). A total of 30 fields were read per cerebellum (1000×).

Statistical analysis

Data were analyzed statistically with non-parametric ANOVA (Kruskal-Wallis test) and post test (Dunn's multiple comparison test), considering always p<0,05.

Results

The three layers of the cerebellar cortex: Molecular, Purkinje and Granular (Figure 1) were differentiated in histological sections stained with hematoxylin eosin. Morphological alterations of the tissue were not observed.

Purkinje cell count

Purkinje cells were observed with their characteristic morphology forming a monolayer in the cerebellar cortex (Figure 1). The number of Purkinje cells was significant decreased (p≤ 0.05) after the administration of cypermethrin. This was observed at all the times studied in comparison to the control group 1 (Figure 2). The decrease of the Purkinje cells was higher at days 17 and 25, which shows that the insecticide not only has an acute affect to the cerebellar tissue, but can also induce significant changes to the tissue in chronic stages (p≤ 0.05).

Apoptosis

Immunoreactive cells showed a bright brown staining in both Purkinje cells and Granular cells. There was an increase of the expression of Apaf-1 in both cell populations. In Purkinje cells there was a higher expression of Apaf-1 in days 17 and 25, but this wasn't significant (p> 0.05). Granular cells showed a significant increase of the expression of Apaf-1 at day 25 (Figure 3), which concurs with the lowest count of Purkinje cells at days 17 and 25.

Ki-67

Both cell populations showed immunoreactive cells to the antibody. There was an increased of the expression of Ki-67 in the Purkinje cells at days 17 and 25, but this was not significant (p> 0.05). Granular cells showed a significant increase in the expression of Ki-67 at day 25 (Figure 4).

Discussion

The effect of cypermethrin on the CNS is principally through an increase of the opening time of the voltage-dependent sodium channels, but also affects the GABA receptors and GABA encephalic

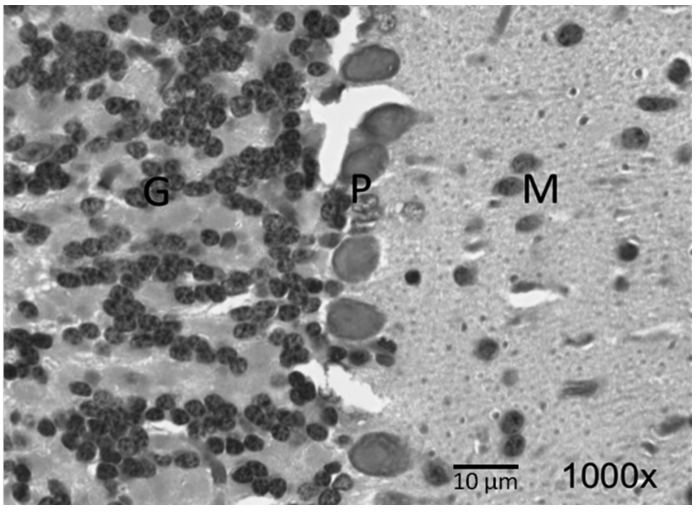

Figure 1. Cerebellar cortex, H-E staining showing the three layers of the cortex, Granular (G), Purkinge (P) and Molecular (M).

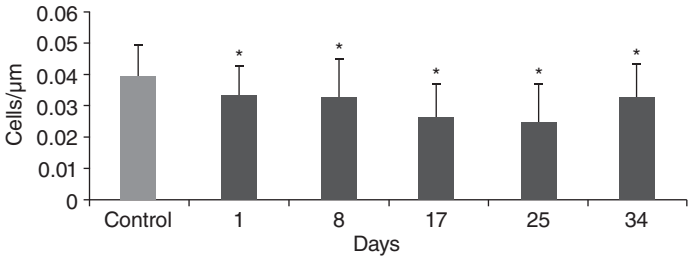

Figure 2. Effect of cypermethrin on the Purkinje cells in the cerebellum at different times. The bars show the means and standard deviation. (*) p≤ 0.05.

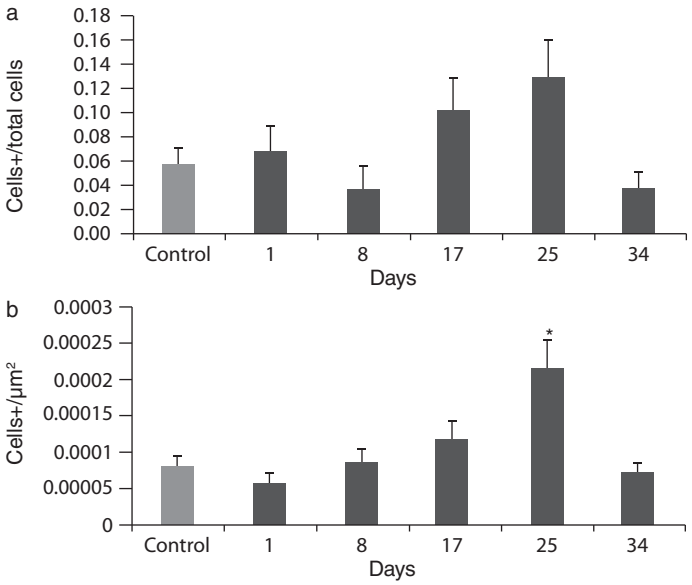

Figure 3. Expression of Apaf-1 in Purkinje (a) and Granular (b) cells in mice cerebellums after a single dose (1/5 DL_{50}) of cypermethrin. The bars show the means and standard error. (*) p≤0.05.

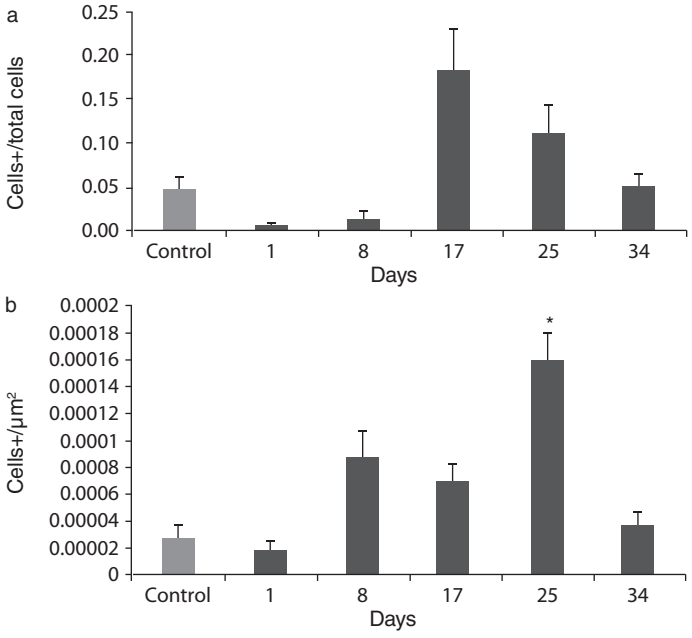

Figure 4. Expression of Ki-67 in Purkinje (a) and Granular (b) cells in the cerebellum after a single dose (1/5 DL_{50}) of cypermethrin. The bars show the means and standard error. (*) p≤0.05.

levels. In the normal cerebellar circuits the Purkinje cells acts as their axis (Sarna and Hawkes, 2003), exerting an inhibitory function through GABA dependent Cl^- channels. This implies that an alteration on the GABA receptors can affect the cerebellar circuits and the cerebellar cell population, which can induce a decrease in the Purkinje cell population, as observed in the present study. A cypermethrin effect on GABA levels was showed in rats exposed to the insecticide to a single dose of 145mg·kg^{-1}, producing a significant decrease in the concentrations of the neurotransmitter (Manna *et al.*, 2006b).

The main afferent pathway of the normal cerebellar circuit is through the mossy and the climbing fibers. The mossy fibers forms glutamatergic synapses on the Granular cells, and these cells in turn project their axons to the molecular layer, to synapse on the dendritic spines of Purkinje cells. This means that damage to Purkinje cells, as observed in this study, could alter the population of Granular cells. The climbing fibers come from the olivar complex and synapse directly on the Purkinje cells. Finally, the Purkinje cell axons synapse on neurons in deep cerebellar and vestibular nuclei that are constantly modulated by inhibition from the Purkinje cells, which are exclusively GABAergic (Sarna and Hawkes, 2003). This circuit can be altered by the presence of pyrethroids that reduce GABA levels (Manna *et al.*, 2006b) and also reduce the number of Purkinje cells (Abdel-Rahman *et al.*, 2001). In the present study a significant decrease in the number of Purkinje cells was observed after a single dose of cypermethrin.

The euthanasia of the animals was carried out at days 1, 8, 17, 25 and 34, which correspond with the biotransformation and excretion of cypermethrin. The *trans* isomer is almost completely eliminated in the first 24h, while the *cis* isomer is eliminated by day 8 from liver and kidney, and in days 12, 17, 24 and 28 from fatty tissue (Crawford *et al.*, 1981; Rhodes *et al.*, 1984; Soderlund *et al.*, 2002).

There was a larger decrease in the Purkinje cell number at days 17 and 25; this concurs with previous results in cerebral cortex, where a significant decrease in the neuronal density was observed at day 25 in mice exposed to a 1/5 DL_{50} of cypermethrin (Jiménez *et al.*, 2008). This allows to determinate that cypermethrin has a significant effect in acute and chronic stages after exposure.

Other authors also described the chronic effect of cypermethrin in the CNS; this was shown in rats exposed to 1/10 and 1/20 of the LD_{50}, where the animals had a significant decrease of the GFAP protein present in astrocytes at days 2 and 21 after exposure, the reduction being more pronounced in day 21 (Malkiewicz *et al.*, 2006). This shows that cypermethrin not only leads to damage of neurons, but also affects the glial cells in the CNS.

An increase in the expression of Apaf-1 in the Purkinje and Granular cells was observed at all the times studied, with a significant increase in day 25, which agrees with the lowest numbers in the Purkinje cell population. This shows that the decrease in the cell number could be due top the apoptotic cell death induced by cypermethrin. Apoptosis induced by pyrethroids in Purkinje cells was also shown in rats exposed for 30 days to permethrin at daily doses of 0.14mg·kg^{-1} (Abdel-Rahman *et al.*, 2004). Also, cypermethrin-induced apoptosis was observed in telencephalon of tadpoles of *Physalaemus biligonigerus*, 96h after exposure to 193µg·l^{-1} of the insecticide (Izaguirre *et al.*, 2000). The increase in the expression of Apaf-1 in Granular cells can be a direct effect of cypermethrin, or due to disruption of the cerebellar circuit after the death of the Purkinje cells, in turn inducing the death of Granular cells.

There was an increase in the expression of Ki-67 in the Purkinje cells at days 17, 25 and 34. This was not statistical significant in comparison with the control groups, althoug it was significant at day 25 in comparison with days 1 and 8. Granular cells also showed a significant increase in the expression of Ki-67 in day 25. This change can be a response to the decreased cellular population resulting from the expose to cypermethrin, causing activation of the cell cycle leading to an increase in the expression of Ki-67. However, the cells do not complete the cycle; it has been shown that cells can return to a quiescent state even after having synthesized DNA (Scholzen and Gerdes, 2000). Cells can undergo cellular arrest and apoptosis after entering the cell cycle if they do not successfully past the checkpoints of the cycle (Liu and Greene, 2001). Neurons are highly differentiated cells, and they do not have the adequate cellular machinery to successfully complete a cell cycle, so that after they start it they can end in cellular arrest and apoptosis.

Cypermethrin has a significant effect on the cerebellar cortex, altering both Purkinje and Granular cells. This is established by the reduction in density of Purkinje cell population and the increases in the expression of Apaf-1 and Ki-67 in both Purkinje and Granular cells. Ki-67 is a unique peptide that appears in the G1 and G2 phases of the cell cycle, and has a short half life. Meanwhile, Apaf-1 brings together , through the caspase pathway, the intrinsic and extrinsic apoptosis pathways.

Cypermethrin produces acute and chronic effects in the cerebellar cortex. The insecticide can remain in fatty tissue even 28 days after the exposure (Rhodes *et al.*, 1984) and can cause a chronic effect to the cerebellar neurons. Such chronic effect of cypermethrin can be produced by two processes: 1) as mentioned, the insecticide can remain in fatty tissue after 28 days of exposure, and 2) because of the damage to cerebellar circuits the intact neurons activate the cellular machinery trying to compensate this lost circuits, ending in cellular arrest (Liu and Greene, 2001; Migliorini *et al.*, 2002). It is difficult to determine which one of the proposed processes has more importance in the damage produced by cypermethrin. Also, this damage has been observed in other tissues under the similar experimental conditions (Jiménez *et al.*, 2008; Rodriguez *et al.*, 2009).

Conclusion

In adult mice, cypermethrin at a 1/5 LD_{50} produces acute and chronic effects on the cerebellar cortex, causing a decrease in the number of Purkinje cells. It also increases the expression of apoptotic biomarkers such as Apaf-1 in Purkinje and Granular cells, and increases the expression of Ki-67 in these cells. Cypermethrin can cause subclinic damage to the cerebellar cortex due to direct damage and induction of apoptosis.

ACKNOWLEDGMENTS

This work was supported by the Major Research Project UTA Nº 4712-13 from the Universidad de Tarapacá, Chile and Fondecyt Nº 1101046.

REFERENCES

Abdel-Rahman A, Dechkovskaia AM, Goldstein LB, Bullman SH, Khan W, El-Masry EM, Abou-Donia MB (2004) Neurological deficits induced by malathion, DEET, and permethrin, alone or in combination in adult rats. *J. Toxicol. Environ. Health A 67*: 331-356.

Abdel-Rahman A, Shetty AK, Abou-Donia MB (2001) Subchronic dermal application of N,N-diethyl m-toluamide (DEET) and permethrin to adult rats, alone or in combination, causes diffuse neuronal cell death and cytoskeletal abnormalities in the cerebral cortex and the hippocampus, and Purkinje neuron loss in the cerebellum. *Exp. Neurol. 172*: 153-171.

Crawford MJ, Croucher A, Hutson DH (1981) Metabolism of

cis- and trans-cypermethrin in rats. Balance and tissue retention study. *J. Agric. Food Chem. 29*: 130-135.

IPCS (2008) *Data Sheet on Pesticide Nº 58. Cypermethrin*. International Programme on Chemical Safety. World Health Organization. Geneva, Switzerland.

Izaguirre MF, Lajmanovich RC, Peltzer PM, Soler AP, Casco VH (2000) Cypermethrin-induced apoptosis in the telencephalon of *Physalaemus biligonigerus* tadpoles (Anura: Leptodactylidae). *Bull. Environ. Contam. Toxicol. 65*: 501-507.

Jiménez L, Quilodrán J, Miranda JP, Rodríguez H (2008) Efecto de dosis única intraperitoneal de cipermetrina en la corteza cerebral somatosensorial de ratones CF-1. *Int. J. Morphol. 26*: 19-26.

Lessenger JE (1992) Five office workers inadvertently exposed to cypermethrin. *J. Toxicol. Environ. Health A 35*: 261-267.

Liu DX, Greene LA (2001) Neuronal apoptosis at the G1/S cell cycle checkpoint. *Cell Tiss. Res. 305*: 217-228.

Luty S, Latuszynska J, Obuchowska-Przebirowska D, Tokarska M, Haratym-Maj A (2000) Subacute toxicity of orally applied alpha-cypermethrin in Swiss mice. *Ann. Agric. Environ. Med. 7*: 33-41.

Malkiewicz K, Koteras M, Folkesson R, Brzezinski J, Winblad B, Szutowski M, Benedikz E (2006) Cypermethrin alters Glial Fibrillary Acidic Protein levels in the rat brain. *Environ. Toxicol. Pharmacol. 21*: 51-55.

Manna S, Bhattacharyya D, Mandal TK, Das S (2004) Repeated dose toxicity of alfa-cypermethrin in rats. *J. Vet. Sci. 5*: 241-245.

Manna S, Bhattacharyya D, Mandal T, Dey S (2006a) Neuropharmacological effects of deltamethrin in rats. *J. Vet. Sci. 7*: 133-136.

Manna S, Bhattacharyya D, Mandal TK, Das S (2006b) Sub-chronic toxicity study of alpha-cypermethrin in rats. *Iran. J. Pharmacol. Therap. 5*: 163-166.

Martínez-Navarrete J, Loria-Castellanos J, Nava-Ocampo AA (2008) Accidental poisoning with"Chinese chalk". *Acta Biomed. Ateneo Parm. 79*: 36.

Méndez G (2009) Aspectos Bioéticos de la Experimentación Animal. Principales pautas de refinamiento en experimentación animal. Comité Asesor Comisión Nacional de Investigación Científica y Tecnológica. Chile. pp 61-112.

Migliorini D, Lazzerini Denchi E, Danovi D, Jochemsen A, Capillo M, Gobbi A, Helin K, Pelicci PG. Marine JC (2002) Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol. Cell. Biol. 22*: 5527-5538.

Ray DE, Fry JR (2006) A reassessment of the neurotoxicity of pyrethroid insecticides. *Pharmacol. Therap. 111*: 174-193.

Rhodes C, Jones BK, Croucher A, Hutson DH, Logan CJ, Hopkins R, Hall BE, Vickers JA (1984) The bioaccumulation and biotransformation of cis, trans-cypermethrin in the rat. *Pest. Sci. 1*: 471-480.

Rodriguez H, Tamayo C, Inostroza J, Soto C, Bustos-Obregon E, Paniagua R (2009) Cypermethrin effects on the adult mice seminal glands. *Ecotoxicol. Environ. Saf. 72*: 658-662.

Sarna JR, Hawkes R (2003) Patterned Purkinje cell death in the cerebellum. *Progr. Neurobiol. 70*: 473-507.

Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. *J. Cell. Physiol. 182*: 311-322.

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML (2002) Mechanisms of pyrethroid neurotoxicity: implications for cumulative risk assessment. *Toxicology 171*: 3-59.

WHO IPoCS (2010) *The WHO Recommended Classification of Pesticides by Hazard and Guidelines to Classification*. 2009 ed. World Health Organization. Geneva, Switzerland. 81 pp.

Wolansky M, Harrill J (2008) Neurobehavioral toxicology of pyrethroid insecticides in adult animals: a critical review. *Neurotoxicol. Teratol. 30*(2): 55-78.