Functional Neurotoxicity and General Toxicity of Amitraz, in Combination with Two Other Insecticides, in Subchronic Oral Administration to Rats

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Abstract: Neurotoxicity of insecticide combinations, in contrast to single substances, has not been studied sufficiently. Amitraz (**A**), a formamidine-type insecticide was, combined with the organophosphate dimethoate (**D**) and the pyrethroid cypermethrin (**C**), given to male Wistar rats 5 times a week by gavage for 12 weeks (4th to 16th weeks of life). After that, spontaneous and stimulus-evoked cortical electrical activity was recorded from the rats, which were finally dissected for organ weighing and haematological tests. In the spontaneous activity, **A** alone caused no alteration, the combinations **AD** and **ADC** shifted the activity to higher frequencies. **AD** and **ADC** also increased the latency of the somatosensory evoked potential significantly. On the visual evoked potential, all combinations, but not **A** alone, had a similar effect. In the combination groups, body weight gain was reduced. White blood cell count and the weight of several organs was reduced by A and by the combinations. A alone showed low toxicity but seemed to increase certain effects of other insecticides.

Key Words: amitraz, dimethoate, cypermethrin, combination, toxicity, neurotoxicity, rat

INTRODUCTION

Chemical plant protection has inevitably led to the presence of pesticide agents in the environment, potentially causing multiple occupational or food-borne exposure. Most of the widespread insecticide agents attack the nervous system of the target – and also non-target – species, resulting in risk of human exposure and health damage.

Amitraz (**A**) is a formamidine-type insecticide and acaricide (Tomlin, 1997). It acts in arthropods as an octopamine receptor agonist, while in the mammalian central nervous system (CNS), the primary sites of action are alpha-2 adrenoceptors and monoamine oxidase (Marrs, 2012).

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A caused depression of the CNS in humans (Atabek et al., 2002). In rats, motor hypoactivity (Florio et al., 1993) and reduced arousal (Moser, 1991) were reported. Alterations in the visual evoked potentials following a single injection of A to rats were also found (Boyes and Moser, 1987).

Organophosphates (OPs) are known to cause permanent inhibition of acetylcholinesterase (Marrs, 2012). In human OP exposure, first of all EEG abnormalities were observed (Muttray et al., 1996), even in absence of overt symptoms of poisoning. The effects of OPs on spontaneous cortical activity were reproduced in animal experiments, whereby alterations in cortical evoked potentials were also described (Papp et al., 2004). Dimethoate (D; WHO, 1989b) was chosen for the present study because it has been widely used in agriculture and hygienic insect control, and because of the previous laboratory experiences with this OP.

Pyrethroids, synthetic derivatives of pyrethrins, are broadly used as insecticides, chiefly due to their high insecticidal potency combined with low mammalian toxicity (Leahey, 1985). Their primary targets in the nervous system are Na^+ and other cation channels (Narahashi, 1996), as well as acetylcholine (Abbassy et al., 1983), GABA (Lawrence and Casida, 1983), serotonin (Oortgiesen et al., 1989) and benzodiazepine (Devaud and Murray, 1988) receptors. Cypermethrin (C; WHO, 1989a) belongs to the type II pyrethroids with predominantly central action.

The activity spectrum of individual insecticides may require the combination, or application shortly one after another, of several agents. A, for example, is often used together with C (Rodriguez-Vivas et al., 2013) but less frequently also with D. Hence, multiple occupational exposure by these insecticides, and simultaneous presence of their residues in the environment, cannot be excluded. Questions raised by expert bodies to this "cocktail" problem include possible interaction of various anticholinesterases, subgroups of elevated risk such as young children, and the implication of experimental data to human health. Among the research requirements stipulated, studies on the potential of combined actions, and assessment of the risks caused, have been mentioned (Beaumont and Buffin, 2002). In the study presented here, general toxicity and neurotoxicity of A was investigated alone, and in combination with D and/or C, in order to see if there was any – first of all positive – interaction between the agents which could result in increased toxicity and inadequacy of safety limits based on single substance effects.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats were used, obtained at the University's breeding centre, and kept under standard animal house conditions (up to 4 rats/cage; 12/12 hour light/dark cycle with light on at 6:00 a.m.; temperature 22 to 24°C) with unlimited access to food and water. The rats, 8 per group, were treated 5 days a week from their 4th to 16th week of life with the insecticide combinations shown in *Table 1*. LOEL and NOEL doses of A, D and C were determined in previous experiments on general toxicological outcomes (Institóris et al., 1999a,b). D (97% purity) was purchased from Cheminova (Lemvig, Denmark), C (97% purity) from Agrochemie (Budapest, Hungary), while A (98% purity) was kindly donated by Hockley International Ltd (Stockport, UK). A was made up as a suspension in 1% methyl cellulose mucus containing 0.1% Tween 80, and was administered in a volume of 5 ml/kg b.w., D and C were dissolved in sunflower oil and administered in 0.5 ml/kg b.w. volume. Controls were given both vehicles (sunflower oil and methyl cellulose-Tween).

Table 1. LD_{50} data and actual doses of the insecticides (top), and treatment groups with their corresponding identification codes (bottom).

Insecticide agent	LD_{50} (mg/kg b.w.)	Dose applied (mg/kg b.w.)
Amitraz (A)	529	LOEL dose, 26.5
Dimethoate (D)	460	NOEL dose, 7.04
Cypermethrin (C)	554	NOEL dose, 11.1

Treatment	Group code
control	CON
amitraz	A
amitraz + cypermethrin	AC
amitraz + dimethoate	AD
amitraz + dimethoate + cypermethrin	ADC

Administration was done by gavage. In the combination groups, A was given first, and the other substances, 30 min later. During the treatment period, the animals were observed daily for signs of toxicity, and their body weight was measured each Monday. During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed.

Electrophysiological recording

On the day following the last insecticide administration, the rats were prepared for electrophysiological recording. In urethane anaesthesia (1000 mg/kg ip.; Koblin, 2002) the left hemisphere was exposed by a sagittal cut in the head skin, blunt removal of connective tissues, and drilling around the left parietal bone. Following recovery (at least 30 min), recording electrodes were placed on the primary somatosensory (SS), visual (VIS) and auditory (AUD) areas, and electrocorticogram (ECoG) was recorded for 6 min. Then, sensory stimuli (SS: electric shocks [3-4 V, 0.05 ms] to the contralateral whisker pad, VIS: flashes, AUD: clicks through the hollow ear bar of the stereotaxic frame) were applied, and cortical evoked potentials (EPs) were recorded from the same sites. One train of 50 stimuli was given with 1 Hz frequency, but SS stimulation was repeated with 2 and 10 Hz to see any frequency effect.

Analysis of the ECoG records provided the power spectrum by bands (traditional human EEG bands as defined in Kandel and Schwartz (1985). On the cortical evoked responses, latency and duration of the main waves was measured manually after averaging. All stimulations, recording and analyses were done using the NEUROSYS 1.11 software (Experimetria, Budapest, Hungary). For further details of electrophysiological recording and analysis, see Papp et al. (2004).

General toxicological investigation

Immediately after the recordings, the rats were sacrificed with an overdose of urethane, and dissected. The organ weight of brain, thymus, lung, heart, liver, spleen, kidneys, adrenals, testicles, and popliteal lymph node was determined, and relative organ weights were calculated

as [organ weight / brain weight]. Due to the effect of the treatments on body weight, brain-based relative organ weights were supposed to be more reliable (Schärer, 1977). White blood cell (WBC) count, red blood cell (RBC) count, haematocrit (Ht) and other haematological indices were measured by a PS-5 Blood Cell Counter (Medicor, Budapest, Hungary) as described in Institóris et al. (1999a,b).

Data evaluation

From the primary data, group averages were obtained and compared by one-way ANOVA after the Kolmogorov-Smirnov normality test. For post hoc analysis, LSD (least squared differences) test was used with $p<0.05$ as criterion of significance.

RESULTS

General toxicity

No overt signs of toxicity (such as rough fur, hunched back or sudden aggressive behaviour) were observed in the treated rats. The animals' body weight gain (between the starting week and the 12th week) was affected in the combination groups only. In AC and AD, body weight gain was reduced vs. control and A, and in the ADC group, only vs. control (Table 2). Among the organs weighed, only the brain-relative weight of the lungs, kidneys, adrenals and the popliteal lymph node were significantly altered in the treated rats. Except for the popliteal lymph node in group A, all organ weights were decreased vs. control and/or vs. A *(Table 2)*.

TABLE 2. General toxicological parameters: body weight gain, relative organ weights, and white blood cell counts.

	Groups				
Parameters	**Control**	**A**	**AC**	**AD**	**ADC**
Body weight gain (g)	336.2 ± 23.2	316.2 ± 41.4	292.5 ± 20.6*°	287.5 ± 26.0*°	303.7 ± 28.3*
Relative organ weights					
Lungs	0.918 ± 0.234	0.773 ± 0.089*	0.726 ± 0.133*	0.660 ± 0.038*	0.791 ± 0.043
Kidneys	1.478 ± 0.338	1.248 ± 0.085*	1.174 ± 0.106*	1.059 ± 0.078*°	1.212 ± 0.096*
Adrenals	33.793 ± 11.033	28.191 ± 3.741	24.827 ± 2.793*	24.112 ± 3.662*	29.301 ± 4.733
Popliteal lymph node	8.147 ± 1.336	9.955 ± 2.713*	6.953 ± 1.229°	7.532 ± 1.360°	7.598 ± 1.195°
WBC count ($10^3/\mu L$)	9.175 ± 2.209	6.413 ± 2.388*	8.175 ± 2.622	5.675 ± 2.117*	11.938 ± 2.018*°

The table shows only those parameters where significant alterations were observed in any of the treated groups.
**: $p<0.05$ vs. control; ° $p<0.05$ vs. A*

Neurotoxicity

The changes of ECoG in the three areas were not fully in parallel. In the SS area, **A** alone had no noteworthy effect on the spectrum. In the **AC** group, alpha activity increased significantly vs. control. In the **AD** and **ADC** groups, delta activity decreased, and beta1 and beta2 activity increased *(Fig. 1A),* the latter two changes being significant. In the VIS and AUD area, there was significant increase of delta activity in the **AC** treated group *(Fig. 1B,C)*. The effect of **AD** was similar in the SS area, albeit insignificant. In the **ADC** group, beta1 and beta2 band increased significantly vs. **A** group in the VIS area, and alpha and beta1 band vs. control in the AUD area.

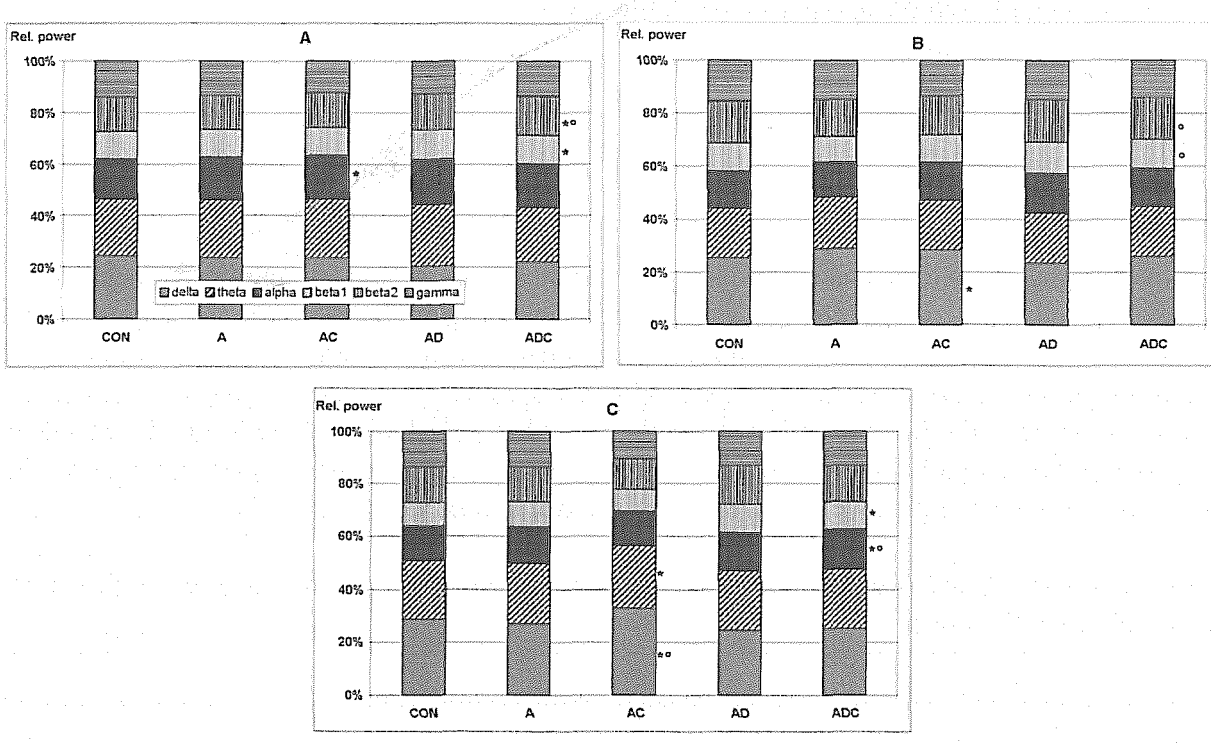

*Fig. 1. Power spectrum of the electrocorticogram from the somatosensory (A), visual (B) and auditory (C) area. Abscissa, groups (**CON**, control; **A**, amitraz; **AC**, amitraz + cypermethrin; **AD**, amitraz + dimethoate; **ADC**, triple combination). Ordinate, relative power of the ECoG bands (see insert in A). Mean+SD, n=8.*
** $p<0.05$ vs. control, ° $p < 0.05$ vs. amitraz-only; always comparing the same bands.*

On the measured parameters of the cortical EPs, the effects were partly dissimilar to the alterations of the ECoG. When given alone, **A** had no significant effect on the latency of any of the EPs recorded. The duration of the SS EP, at 2 and 10 Hz stimulation (Fig. 2) was significantly increased vs. control. In the **AC** combination group, latency of the SS EP vs. group **A** was shortened at 2 and 10 Hz. The latency of the VIS EP (Fig. 3) was increased vs. control, and its duration, vs. **A**. In the **AD** and **ADC** combination groups, latency of the SS EP was significantly increased at 1, 2 and 10 Hz stimulation frequency, both vs. control and vs. **A**. The same held true for the VIS EP, the duration of which was decreased (significantly in **AD** vs. **A**). There were no significant alterations in the latency and duration of the AUD EPs.

As seen in *Fig. 2,* the latency of the SS EP was lengthened with increasing stimulation frequency. This frequency dependence itself was, however, not significantly altered by the insecticides.

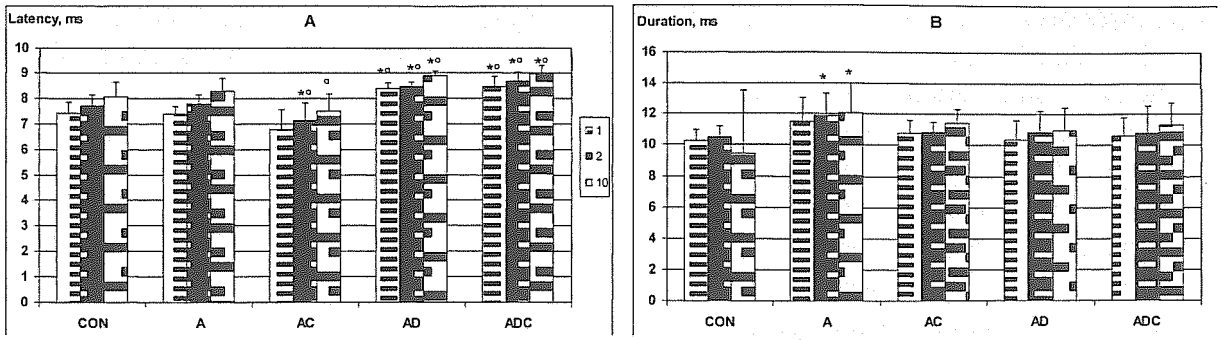

Fig. 2. Latency (A) and duration (B) of the somatosensory cortical evoked potential in the control and treated groups (abscissa, as in Fig. 1) at different stimulation frequencies (see insert in A). Ordinate, latency and duration, ms. Mean+SD, n=8.
** p<0.05 vs. control, ° p < 0.05 vs. amitraz-only; always comparing values at the same stimulation frequency.*

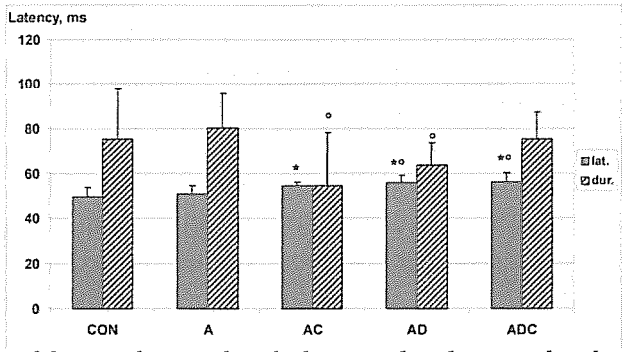

Fig. 3. Latency and duration of the visual cortical evoked potential in the control and treated groups (abscissa, as in Fig. 1). Ordinate, latency and duration, ms. Mean+SD, n=8. Insert, bar pattern for latency (lat.) and duration (dur.).
** p<0.05 vs. control, ° p < 0.05 vs. amitraz-only.*

DISCUSSION

Amitraz is generally mentioned in the toxicological literature as causing CNS depression. In human poisoning cases (affecting mostly children: Atabek et al., 2002), the calculated single dose was ca. 90 to 160 mg/kg (Yilmaz and Yildizdas, 2003). A comparable acute dose in rats caused, among others, decreased motility (Moser and McPhail, 1989; Florio et al., 1989) and delayed onset of the visual evoked potentials (Boyes and Moser, 1987). The principal way of CNS action of A seems to be inhibition of synaptic noradrenalin release by a partial agonist action on the presynaptic alpha-2 adrenoceptors (Altobelli et al., 2001). This clearly could lead to reduced release of noradrenaline, and so, to the observed hypomotility (Moser and MacPhail, 1989) and other signs of CNS depression. This central depression was, however, short-lived (Yilmaz and Yildizdas, 2003), and in the animal experiments no cumulative effect of A on behavioural (Moser, 1991) or neurochemical (Costa et al., 1989) parameters was observed, at least not in the dose range corresponding to that applied by us. The lack of significant changes in the spontaneous and evoked cortical activity in the A-treated group vs. control in our study was in line with that. This also meant that the dose, which was chosen as LOEL based on its significant effect on organ weights in this study (see Table 2) and on immunotoxicity parameters in an earlier one, was in terms of neurotoxicity NOEL; indicating that neurotoxicity was probably not a leading feature in the sub-chronic (in contrast to acute) mammalian toxicity of A.

The alterations seen in the AD combination groups were similar to those seen in D-treated rats in previous studies. The decrease of delta and increase of beta2 activity in the ECoG, obtained with 7.04 mg/kg D and 26.5 mg/kg A, showed the same trend (although non-significantly)

which was seen in another experiment with 18.0 mg/kg (but not with 4.5 mg/kg) **D**, given also for 12 weeks (Lengyel et al., 2006). In the latency of the SS and VIS EP, the increase caused by the **AD** combination was significant and was comparable to that obtained by Lengyel et al. with a ca. 2.5 times higher dose of **D**. The positive interaction suggested by these results may be explained by the modulatory systems influenced by **A** and **D**, respectively. Reduced release of noradrenalin, an effect of **A**, may diminish the responsiveness of cortical neurons to the glutamatergic thalamocortical input (Mouradian et al., 1991). Cortical neurons also have a cholinergic modulation where higher than normal ACh levels, due to the AChE inhibition by **D**, can act to depress cortical responses to, e.g, somatosensory (Donoghue and Caroll, 1987) or visual (DeBruyn et al., 1991) inputs.

In spite of the alleged non-toxicity of pyrethroids in warm-blooded animals (Bradberry et al., 2005), there are data in the literature about the convulsive effect of pyrethroids in humans and animals (Condés-Lara et al., 1999). In our work, done with a substantially lower dose (11 mg/kg b.w. in contrast to 300 mg/kg b.w. in Condés-Lara et al.) of **C**, a somewhat depressed ECoG with increased delta activity was seen, more likely related to the elevated AChE activity observed in brain samples of **C**-treated rats by Rao and Rao (1995). This would raise a possibility of interaction between **C** and **A**, at least at the level of final outcome, but this cannot be verified on the basis of our results. The changes in the EP parameters in the **AC** group had no clear trend.

Body weight gain, and some of the relative organ weights, indicated a positive interaction between **A** and the other two insecticides studied. In case of **AD**, this was supported by the electrophysiological findings and could, at least in part, be explained from the known effects of the two insecticides, while the case of **AC** was less clear.

The combination of LOEL and NOEL doses for 12 weeks seems to be a correct model of low-level, long-term human exposure applied successfully in several previous studies (Institóris et al., 1999a,b, 2004). Such experiments are capable of detecting both synergistic and antagonistic (protective) interactions of xenobiotics, and may contribute to the development of more adequate limits, ensuring a higher level of safety. The results of the present study, indicating synergism between insecticide agents potentially exposing humans simultaneously from various sources, emphasize the need of revised limit values, taking interactions into account.

REFERENCES

ABBASSY, M.A., ELDEFRAWI, M.E. and ELDEFRAWI, A.T. (1983). "Pyrethroid action on the nicotinic acetylcholine receptor/channel." Pestic. Biochem. Physiol. 19:299-308.

ALTOBELLI, D., MARTIRE, M., MAURIZI, S. And PREZIOSI, P. (2001). "Interaction of formamidine pesticides with the presynaptic alpha2-adrenoceptor regulating [^{3}H] noradrenaline release from rat hypothalamic synaptosomes." Toxicol. Appl. Pharmacol. 172:179-185.

ATABEK, M.E., AYDIN. K. and ERKUL, I. (2002). "Different clinical features of amitraz poisoning in children." Hum. Exp. Toxicol. 21:13-16.

BEAUMONT, P. and BUFFIN, D. (2002). "A cocktail of problems." Pesticide News 55:10-11.

BOYES, W.K. and MOSER, V.C. (1987). "Investigations of amitraz neurotoxicity in rats, II Effects on visual evoked potentials." Fundam. Appl. Toxicol. 9:140-153.

BRADBERRY, S.M., CAGE, S.A., PROUDFOOT, A.T. and VALE, J.A. (2005). "Poisoning due to pyrethroids." Toxicol. Rev. 24:93-106.

CONDÉS-LARA, M., GRAFF-GUERRERO, A. and VEGA-RIVEROLL, L. (1999). "Effect of cypermethrin on the electroencephalographic activity of the rat: A model of chemically induced seizures." Neurotoxicol. Teratol. 21:293-298.

COSTA, L.G., OLIBET, G., WU, D.S. and MURPHY, S.D. (1989). "Acute and chronic effects of the pesticide amitraz on alpha 2-adrenoceptors in mouse brain." Toxicol. Lett. 47:135-143.

DEBRUYN, E.J., CORBETT, G.K. and BONDS, A.B. (1991). "Pretreatment with physostigmine, mecamylamine and atropine reduces the impact of soman on the cortical visual evoked potential of the cat." Life Sci. 48:1277-1282.

DEVAUD, L.L. and MURRAY, T.F. (1988). "Involvement of peripheral-type benzodiazepine receptors in the proconvulsant actions of pyrethroid insecticides." J. Pharmacol. Exp. Ther. 247:14-22.

DONOGHUE, J.P. and CAROLL, K.L. (1987). "Cholinergic modulation of sensory responses in rat primary somatic sensory cortex." Brain Res. 408:367-371.

FLORIO, J.C., SAKATE, M. and PALERMO-NETO, J. (1993). "Effects of amitraz on motor function." Pharmacol. Toxicol. 73:109-114.

INSTITÓRIS, L., PAPP, A., SIROKI, O. and BANERJEE, B.D. (2004). "Comparative investigation of behavioral, neurotoxicological, and immunotoxicological indices in detection of subacute combined exposure with methyl parathion and propoxur in rats." Ecotox. Environ. Saf. 57:270-277.

INSTITÓRIS, L., SIROKI, O. and DÉSI, I. (1999a). "Immunotoxicity study of repeated small doses of dimethoate and methylparathion administered to rats over three generations." Hum. Exp. Toxicol. 14:879-883.

INSTITÓRIS, L., ŰNDEGER, Ű., SIROKI, O., NEHÉZ, M. and DÉSI, I. (1999b). "Comparison of detection sensitivity of immuno- and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats." Toxicology 137:47-55.

KANDEL, E.R. and SCHWARTZ, J.H. (1985). Principles of Neural Science. Elsevier, New York, pp. 643-644.

KOBLIN, D.D. (2002). "Urethane: help or hindrance?" Anesth. Analg. 94:241-242.

LAWRENCE, L.J. and CASIDA, J.E. (1983). "Stereospecific action of pyrethroid insecticides on the gamma-aminobutyric acid receptor-ionophore complex." Science 221:1399-1401.

LEAHEY, J.P. (1985). The Pyrethroid Insecticides. Taylor and Francis, London

LENGYEL, Zs., LUKÁCS, A., SZABÓ, A. and NAGYMAJTÉNYI, L. (2006). "General toxicity and neurotoxicity of lead and mercury, in combination with dimethoate, in rats after subchronic oral exposure." Trace Elem. Electrol. 23:242-246.

MARRS, T. C. (2012). „Toxicology of insecticides to mammals Pest. Manag. Sci . 68:1332–1336.

MOSER, V.C. (1991). "Investigations of amitraz neurotoxicity in rats IV Assessment of toxicity syndrome using a functional observational battery." Fundam. Appl. Toxicol. 17:7-16.

MOSER, V.C. and MACPHAIL, R.C. (1989). "Investigations of amitraz neurotoxicity in rats III Effects on motor activity and inhibition of monoamine oxidase." Fundam. Appl. Toxicol. 12:12-22.

MOURADIAN, R.D., SESSLER, F.M. and WATERHOUSE, B.D. (1991). "Noradrenergic potentiation of excitatory transmitter action in cerebrocortical slices, evidence for mediation by an alpha I receptor-linked second messenger pathway." Brain Res. 546:83-95.

MUTTRAY, A., PADBERG, F., JUNG, D., ROHLFING, H.R., SCHULZ, M. and KONIETZKO, J. (1996). "Acute changes in human EEG after exposure to low doses of oxydemeton methyl." Centr. Eur. J. Occup. Environ. Med. 2:367-378.

NARAHASHI, T. (1996). "Neuronal ion channels as the target sites of insecticides." Pharmacol. Toxicol. 78:1-14.

OORTGIESEN, M., VAN KLEEF, R.G.D.M. and VIJVERBERG, H.P.M. (1989). "Effect of pyrethroids on neurotransmitter-operated ion channels in cultured mouse neuroblastoma cells." Pestic. Biochem. Physiol. 34:164-173.

PAPP, A., PECZE, L., and VEZÉR, T. (2004). "Comparison of the effect of subacute organophosphate exposure on the cortical and peripheral evoked activity in rats." Pestic. Biochem. Physiol. 79:94-100.

RAO, G. V. and RAO, K. S. (1995). "Modulation in acetylcholinesterase of rat brain by pyrethroids in vivo and an in vitro kinetic study." J. Neurochem 65:2259-2266.

RODRIGUEZ-VIVAS, R.I., LI, A. Y., OJEDA-CHI, M. M., TRINIDAD-MARTINEZ, I., ROSADO-AGUILAR, J. A., MILLER, R.J. and PÉREZ DE LEÓN, A. A. (2013). „In vitro and in vivo evaluation of cypermethrin, amitraz, andpiperonyl butoxide mixtures for the control of resistant *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) in the Mexican tropics. Vet. Parasitol. 197:288–296.

SCHÄRER, K. (1977). "The effect of chronic underfeeding on organ weights of rats." Toxicology 7:45-56.

TOMLIN, C.D.S, (Ed.) (1997). Amitraz. In: Pesticide Manual, 11th ed. British Crop Protection Council, Alton, U.K., pp. 38-40.

WHO (1989a). Cypermethrin. Environmental Health Criteria 82. World Health Organization, Geneva.

WHO (1989b). Dimethoate. Environmental Health Criteria 90. World Health Organization, Geneva.

YILMAZ, H. L. and YILDIZDAS, D. R. (2003). "Amitraz poisoning, an emerging problem, epidemiology, clinical features, management, and preventive strategies." Arch. Dis. Child. 88:130-134.