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A study on immunotoxicological effects of subacute amitraz exposure in rats

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The effects of amitraz, a formamidine pesticide, were investigated in four-week old outbred male Wistar rats on certain classic toxicological and haematological parameters as well as on specific immune functions. The animals were treated, per os by gavage for 28 days, in a five-day treatment two days break system, with 26.5, 21.1, 10.6 and 5.29 mg/kg/day amitraz. On the 29th day, organ weights of the thymus, heart, lung, spleen, liver, kidneys, adrenals, testicles and popliteal lymph node; WBC and RBC counts, Ht, MCV, haemoglobin; and cell content of the femoral bone marrow were determined. In two separate groups, the effects of amitraz on the PFC content of the spleen, and on the maximum level and time course of DTH reaction, were investigated.

Amitraz in 26.5 mg/kg dose increased relative adrenal weight, and decreased relative liver weight, MCV value, PFC content of the spleen, and the maximum level of DTH reaction. The 21.1 mg/kg dose decreased only MCV value, while 10.6 mg/kg elevated the liver-to-brain weight ratio. Based of these findings, a NOEL dose of 5.29 mg/kg was determined for amitraz in this experimental system; while the LOEL doses were 10.6 mg/kg for the general toxicological, 21.1 mg/kg for the haematological and 26.5 mg/kg for the immune function parameters. The results show that the exposure sensitivity of these immune functions to amitraz is lower than that of some other toxicological and haematological parameters. *Human & Experimental Toxicology* (*2007*) **26***, 441–445*

Key words: amitraz; immunotoxicity; rat

Introduction

The toxic effects of larger doses of pesticides on the human population are mainly well documented. There are, however, no convincing epidemiological data to prove or exclude the immunotoxic potential of repeated small doses of pesticides. Beside the number of disturbing factors (differences in lifestyle, exposure conditions, immunological history of the participants, and so on) which can affect the immunological parameters measured, there are significant differences in aim and design (duration, parameters investigated, and so on) of the epidemiological studies available.[1] As a consequence, the only way at present to obtain information about the immunotoxic or immunomodulatory potential of low dose pesticides exposures are animal studies.

The formamidine pesticide amitraz is commonly used as an animal ectoparasiticide for the control of ticks, mites and lice on cattle, dogs, goats, pigs and sheep. In the agriculture, it is applied as an insecticide against mites, pear suckers, scale insects, mealybugs, whitefly and aphids.[2] Widespread application may result in exposure of the population by lower amitraz doses for a long period of time, mainly via food. In a study with amitraz-sprayed dates the initial level of 0.34 mg/kg decreased in 21 days to 0.02 mg/kg which was still above the MRL (0.01 mg/kg).[3] In such cases, the non-observation of the restricted entry interval can result in food-borne exposure above MRL. Other commodities, including tea, were likewise of concern and were tested for amitraz residues.[4] Also food samples, taken randomly in Hungarian food markets, had occasionally pesticide residues well above the MRL, according to the reports of Hungarian authorities (www.ontsz.hu). In certain occupations – producers, pesticide workers, farm owners, animal breeders, veterinarians and so on – the risk of exposure to higher doses is also present.

Influence on the immune system has been observed in case of a number of environmental contaminants. The possible consequences include increased risk of malignant tumours, decreased defence against infectious agents, and increased occurrence of allergic and autoimmune diseases. In case of amitraz, the general toxic effects, as well as behavioural, neuro-, and

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reproductive toxicity, have already been investigated in rats and mice[4–12] but, except for potentially causing delayed contact hypersensitivity,[13] literature data on its effects on the immune system are missing. The aim of the present study was thus to investigate the effects of amitraz on certain specific immune functions (PFC assay, DTH reaction), and to compare the sensitivity of these parameters in the detection of amitraz exposure with that of certain general toxicological and haematological parameters.

Materials and methods

Amitraz of 98.2% purity was the precious donation of Hockley Int. Ltd, UK. Sheep red blood cells (SRBC) were produced by Philmaster Ltd. Budapest, Hungary; RPMI-1640 and Freund's Complete Adjuvant (FCA), by SIGMA, USA; Keyhole Limpet Haemocyanin (KLH), by Calbiochem, USA. The other materials were supplied by REANAL Factory of Laboratory Chemicals, Budapest, Hungary.

Four weeks old outbred male Wistar rats, obtained from the SPF breed of Charles River Ltd., Hungary, were used in the experiments. The animals were kept under conventional conditions (up to five rats per cage, 12 hour light–dark cycle, 22 ± 2°C, 70 ± 10% humidity), standard rodent food and water was available at all times. Amitraz was suspended in 1% methylcellulose containing 0.1% Tween 80 and applied per os by glass gavage in a volume of 5 mL/kg. Controls received only vehicle.

Experimental design

Acute oral LD_{50} of amitraz was determined by the Litchfield-Wilcoxon method[14] in nine-week old male Wistar rats using six doses (1000, 710, 510, 360, 260 and 190 mg/kg) and six animals per dose. After 14 days observation, the surviving animals (in the 510–190 mg/kg dose range) were dissected to determine certain general toxicological and haematological parameters (see below). In the dose-effect experiment 1/20, 1/25, 1/50 and 1/100 LD_{50} of amitraz (26.5; 21.1; 10.6; and 5.29 mg/kg, respectively) was given to 24 rats/dose in a five-day treatment two days break system for 28 days. Eight rats per dose were used for toxicological and haematological examinations, another eight per dose for PFC assay, and eight per dose for DTH reaction.

Parameters examined

Toxicological and haematological parameters The body weight of the animals was measured once a week (always on the same day). The organ weights of brain, thymus, lung, heart, liver, spleen, kidneys, adrenals, testicles and popliteal lymph nodes were determined on the 29th day. For haematological studies, blood was taken from the abdominal aorta. The absolute white blood cell (WBC) and red blood cell (RBC) count, haematocrit (Ht), mean cell volume of RBC-s (MCV), and the cell content of the femoral bone marrow were measured by a PS-5 Blood Cell Counter (Medicor, Hungary) as described.[15] The haemoglobin content was determined by a Minilab-540 photometer (Medicor, Hungary).

IgM-PFC assay The animals were immunized by 2×10^9 SRBC i.p. in 0.4 mL PBS on the 25th day of treatment. On the 29th day, the spleen was removed and the IgM-PFC number calculated for 10^6 spleen cells and for the whole spleen was determined.[15]

Delayed type hypersensitivity (DTH) The animals were immunized s.c. at the base of tail by 1 mg KLH in 0.4 mL antigen preparation (KLH was dissolved in sterile PBS and emulgated by equal volume of FCA) on the 14th day of treatment. The reaction was challenged on the 29th day by injecting 17.5 μg KLH in 50 μL PBS into the left hind footpad. Footpad thickness was measured just before, 24, and 48 hours after challenge by a Microstat micrometer (CADAR, U.K.), and specific footpad swelling (D%) was calculated as described.[15]

The distribution of data was checked for normality by the Kolmogorov–Smirnov test. Depending on the distribution, the statistical analyses were carried out by ANOVA or by Kruskal–Wallis non-parametric ANOVA setting the probability level to $P < 0.05$. In case of ANOVA, *post hoc* analysis of group differences was performed by LSD test, while group comparisons following the Kruskal–Wallis ANOVA were performed by Mann–Withney test. Statistical analysis was performed by the Statistica for Windows 4.0 software.

Results

The acute oral LD_{50} of amitraz proved to be 529 [417–670] mg/kg. Single oral doses of 190–1000 mg/kg, applied in the LD_{50} determination, resulted in excitability, aggression in response to handling, coolness to touch, hunched posture, and piloerection within 1–2 hours after administration; the symptoms subsiding with decreasing dose. Toxicological and haematological data of the surviving animals in the 190–510 mg/kg dose range are presented in Tables 1 (A, B) and 2. All four doses (510, 360, 260 and 190 mg/kg) significantly decreased the body weight gain of the animals by

the 7th day after treatment, while on the 14th day, only the body weight of the 360 and 260 mg/kg dose groups were retarded compared to controls (Table 1A). As presented in Table 1B, the higher doses elevated the relative liver, spleen, and kidney weights in a dose dependent manner; and the 510 mg/kg dose also decreased the relative weight of testicles. The three higher doses significantly diminished the cell content of the femoral bone marrow and, except the 360 mg/kg dose, the mean volume of RBCs (Table 2).

During the cage-side observations in the subacute studies, no abnormalities (such as those seen in the LD_{50} determination) were observed. Neither of the doses applied affected the body weight gain or the absolute brain weight. The weight of adrenals related to 100 g body weight increased only at the highest (26.5 mg/kg) dose ($P < 0.05$). The liver to brain weight ratio was elevated at the 10.6 mg/kg ($P < 0.05$), and diminished at the 26.5 mg/kg dose ($P < 0.001$; Figure 1). Among the haematological parameters measured, only MCV decreased at the two higher doses ($P < 0.05$ in both cases; Table 3). The 26.5 mg/kg dose significantly reduced the spleen cell number of the animals immunised with SRBC ($P < 0.005$) but had no effect on the number of plaques formed from 10^6 spleen cells. As a consequence, the PFC content of the spleen also decreased ($P < 0.002$; Figure 2). The two higher doses decreased the maximum and shortened the decay of DTH reaction but the difference was statistically significant only at the highest dose ($P < 0.05$; Figure 3).

Discussion

The results of the present study are comparable with several findings reported in the literature. The acute oral LD_{50} of amitraz was 529 mg/kg in this study. In the literature, data between 523 and 800 mg/kg are

Table 1 Effect of a single amitraz dose on body weight gain (A), and on relative organ weights (B) related to 100 g body weight or to brain in the surviving animals (X ± SE)

	Body weight (g) ± SE		
Dose (mg/kg)	*Day 0*	*Day 7*	*Day 14*
510 (3)	176 ± 0.88	160 ± 3.33*	280 ± 5.77
360 (5)	162 ± 0.86	160 ± 3.74*	240 ± 8.37*
260 (6)	180 ± 0.76	188 ± 4.47*	245 ± 4.01*
190 (6)	173 ± 0.76	202 ± 3.33*	292 ± 3.07
Control (6)	170 ± 0.65	230 ± 4.08	282 ± 7.07

		Organ weight to brain (g/g or mg/g) ± SE				*Organ weight to 100 g body weight (g/100 g)*			
Dose (mg/kg)	*Abs. brain weight*	*Liver*	*Spleen*	*Kidneys*	*Testicles*	*Liver*	*Spleen*	*Kidneys*	*Testicles*
510 (3)	1.75 ±0.04	7.46 ±0.28*	0.597 ±0.08*	1.34 ±0.06	1.45 ±0.09	4.66 ±0.07*	0.372 ±0.04	0.836 ±0.02	0.911 ±0.08*
360 (5)	1.77 ±0.04	6.35 ±0.27	0.543 ±0.03	1.21 ±0.03	1.64 ±0.03	4.69 ±0.10*	0.403 ±0.03*	0.894 ±0.03*	1.22 ±0.06
260 (6)	1.91 ±0.04	5.83 ±0.32	0.408 ±0.02	1.25 ±0.06	1.71 ±0.07	4.55 ±0.17*	0.319 ±0.01	0.976 ±0.02*	1.34 ±0.07
190 (6)	1.85 ±0.05	6.15 ±0.15	0.478 ±0.01	1.19 ±0.01	1.72 ±0.06	3.89 ±0.11	0.303 ±0.01	0.752 ±0.01	1.09 ±0.04
Control (6)	1.86 ±0.01	5.50 ±0.35	0.467 ±0.02	1.14 ±0.03	1.74 ±0.02	3.71 ±0.24	0.316 ±0.01	0.770 ±0.01	1.18 ±0.02

n = Number of survivors; *$P < 0.05$ related to control; the day 0 values involve only the starting body weight of the surviving animals.

Table 2 Changes in the haematological parameters following single large doses of amitraz (X ± SE)

Dose (mg/kg)	*WBC ($\times 10^6$/mL)*	*RBC ($\times 10^9$/mL)*	*MCV (fl)*	*Ht (%)*	*Cell/femur ($\times 10^8$)*	*Hb (g/L)*
510 (3)	4.40 ± 0.88	6.09 ± 0.87	63.3 ± 0.63*	38.5 ± 3.77	1.68 ± 0.06*	14.1 ± 2.60
360 (5)	8.52 ± 0.86	6.21 ± 0.44	66.0 ± 0.71	40.9 ± 2.98	1.40 ± 0.18*	12.5 ± 0.39
260 (6)	7.52 ± 0.76	6.51 ± 0.61	63.4 ± 1.25*	46.5 ± 2.54	1.61 ± 0.17*	13.3 ± 0.40
190 (6)	7.52 ± 0.99	6.90 ± 0.24	65.2 ± 0.50	45.0 ± 1.35	1.89 ± 0.15	12.7 ± 0.31
Control (6)	6.10 ± 0.89	6.87 ± 0.14	67.2 ± 0.82	46.2 ± 1.07	2.17 ± 0.07	12.5 ± 0.35

( ) Number of survivors; *$P < 0.05$ related to control.

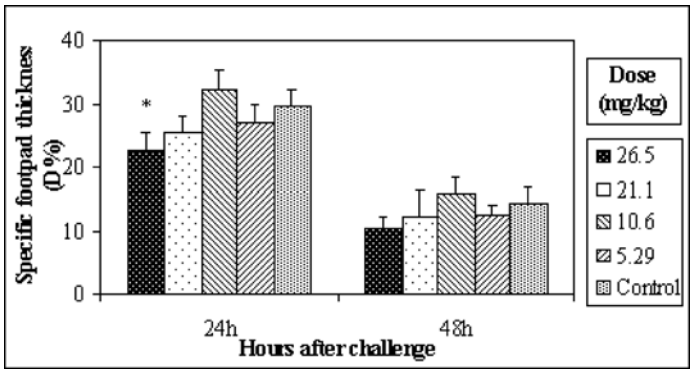

Figure 1 Effect of four-week amitraz treatment on the relative liver and adrenal weights (X ± SE, $n = 8$,* $P < 0.05$).

Table 3 Effect of four-week amitraz treatment on certain haematological parameters (X ± SE)

Dose (mg/kg)	*WBC (±106/mL)*	*RBC (±109/mL)*	*MCV (fl)*	*Ht (%)*	*Cell/femur (×108)*	*Hb (g/L)*
26.5	8.63 ± 1.86	7.57 ± 0.38	65.8 ± 0.97*	43.8 ± 1.65	2.25 ± 0.29	13.0 ± 0.25
21.1	11.1 ± 2.01	6.31 ± 0.52	64.3 ± 1.35*	42.7 ± 2.79	1.71 ± 0.28	13.4 ± 0.93
10.6	8.65 ± 1.57	6.57 ± 0.08	68.9 ± 0.47	45.3 ± 0.75	1.99 ± 0.13	13.8 ± 0.90
5.29	7.33 ± 0.94	6.53 ± 0.15	69.1 ± 0.83	44.8 ± 1.21	2.17 ± 0.08	12.7 ± 0.19
Control	7.31 ± 0.51	6.80 ± 0.09	68.2 ± 0.56	46.3 ± 0.72	2.19 ± 0.11	12.8 ± 0.28

*$P < 0.05$.

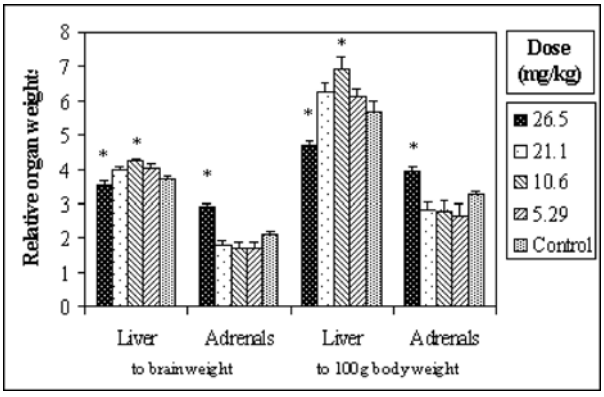

Figure 2 Changes of the PFC content of the spleen following a four-week oral treatment with amitraz (X ± SE, $n = 8$, *$P < 0.05$).

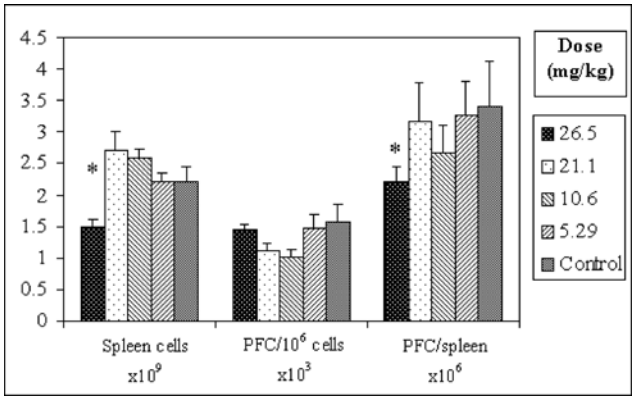

Figure 3 Effect of four-week amitraz treatment on the DTH reaction measured by footpad swelling assay (X ± SE, $n = 8$).

found, depending on the rat strain used and the purity of amitraz.[5,6,13]

A 90 days treatment of male Ash-Wistar rats with 12 mg/kg amitraz resulted in 8% retardation of body weight gain and in reduction of relative liver weight by 6%.[13] Ueng *et al.*[16] found that 25 and 50 mg/kg amitraz, administered on three consecutive days i.p., elevated the relative liver weight of female Wistar rats by 12 and 18%, respectively. The higher dose also decreased the body weigh by 14%, and increased the relative lung (30%), kidney (13%) and adrenal (26%) weight. In the present study, 10.6 mg/kg amitraz elevated the relative liver weight by 22% while 26.5 mg/kg resulted in a 20% reduction. The latter dose also increased the relative adrenal weight by 37%. According to these results, the direction of change of the relative liver weight seems to be dose-dependent.

The haematological effects were investigated following a three-week inhalation (six hours a day) exposure to 0.01, 0.1 and 1.0 mg/L amitraz concentrations.[13] Beside the characteristic toxic signs and body weight loss at the highest concentration, reduced packed cell volume, haemoglobin, and red cell

number was observed. In our system, which represents a lower exposure level, neither toxic signs nor body weight loss were observed, and only the mean volume of the red blood cells (MCV) decreased at the two higher doses. The reduction of haematocrit was less pronounced and not statistically significant.

The effect of amitraz on the humoral immune response was detected by PFC assay. The reduced PFC content of the spleen was the consequence of the decreased cell count of the spleen in the animals immunised with SRBC, as the number of plaques formed from 10^6 spleen cells did not change. This finding can be explained by cell loss or cell damage in the spleen rather than by a direct effect on the specific immune response. The maximum and time course of DTH reaction, which was applied for characterisation the effect of amitraz on one of the main routes of cellular immune response, also decreased at the two higher doses but it was statistically significant only at the 26.5 mg/kg dose.

The NOEL dose (the dose not affecting any parameter investigated) proved to be 5.29 mg/kg in the present system. In different experimental systems in rats, NOAEL (non observed adverse effect level) values of oral amitraz exposure varied between 1.3 and 12 mg/kg/day, depending on the aim of studies and duration of treatment.[13] In our experiments, 10.6 mg/kg amitraz elevated the relative liver weight versus control but this effect was not dose-dependent. In addition, the relative liver weight in this group was not significantly different from that in the lower (2.29 mg/kg) and the higher (21.1 mg/kg) dose groups. The 21.1 mg/kg dose altered only the MCV value but had no effect on the other parameters investigated. The 26.5 mg/kg dose decreased relative liver weight, MCV value, the cellularity of the spleen of the animals immunized by SRBC, and the maximum of DTH reaction; and increased the relative adrenal weight. So, in respect of the effect on the specific immune functions, 26.5 mg/kg can be regarded as LOEL in the present experimental system. The exposure sensitivity of the immune function assays in detection of effects after four weeks oral amitraz treatment proved to be lower than that of certain toxicological and haematological parameters investigated.

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