Genotoxicity of mixture of imidacloprid, imazalil and tebuconazole

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A B S T R A C T

Genotoxicity of the mixture of generic pesticides imidacloprid + imazalil + tebuconazole in a ratio of 14.0/1.7/1.0 by weight was assessed using Ames test (*Salmonella typhimurium*) and micronucleus test *in vivo* on mammalian bone marrow erythrocytes (CD-1 mice) supporting the data creation for the Real Life Risk Simulation (RLRS) approach. This pesticides' combination is used in the commercial formulation for seed treatment in advance of or immediately before sowing. Tested pesticides' technical grade active ingredients (TGAIs) showed no evidence of genotoxicity upon separate treatments. In combination, the three pesticides demonstrated negative results in the Ames test but induced a statistically significant, dose-depended increase in MN-PCEs in mice bone marrow at doses lower than those used separately. The observed effect may be mediated by the synergistic action of the tested TGAIs, their metabolites or impurities.

1. Introduction

Modern agriculture almost all over the world cannot manage without the integrated use of fertilizers and pesticides. In recent years, more and more pesticide formulations containing several active ingredients have been entering the market of plant protection products. This is due to the need to overcome resistance to certain pesticides and increase their effectiveness. Furthermore, pesticides are often used as tank mixtures, and several treatments of the same crop are carried out during the season using pesticides of different chemical classes. Therefore, food products may contain residual amounts of several pesticides simultaneously [1,2]. Such pesticide mixtures can cause numerous toxic effects in the organisms, including genetic damage. For assessing such effects under the global risk assessment process, the Real-Life Risk Simulation (RLRS) approach was developed. RLRS approach refers to the necessity of assessing health risk under real-life conditions such as long-term exposure to combinations of chemical and non-chemical stimuli in low doses [3,4]. Under the frame of the RLRS approach a number of studies, testing approaches, and methodologies are already published [5–14]

The presence of more than one pesticide active ingredient in mixtures

can lead to additive, synergistic or antagonistic genotoxic effects. For example, the synergism of endosulfan and chlorpyrifos was shown in cultured human peripheral blood lymphocytes by chromosomal aberration test and comet assay [15]. Combinations of monocrotophos + carbofuran, endosulfan + chlorpyrifos, monocrotophos + carbofuran [16], parathion methyl + carbofuran + alpha-hexachlorocyclohexane [17] and a mixture of triclosan + carbendazim [18] also acted synergistically. The mixture of carbaryl and metaldehyde increased the frequency of chromosomal aberrations in *Allium cepa* cells [19]. Combinations of commercial formulations of paraquat + linuron [20,21], captan + carbendazim [22], atrazine + pendimethalin, and thiram + thiophanate methyl [23] were studied for genotoxicity using the micronucleus and alkaline elution tests, indicating interactions in the cases of mixtures of paraquat + linuron and captan + carbendazim. Seven benzimidazole pesticides combined at sub-threshold levels induce micronuclei *in vitro*. The effects of the mixtures were explained by the concentration addition model [24]. Acetamiprid and propineb separately were genotoxic in mice bone marrow *in vivo*, but in combination these pesticides caused the antagonistic effect, decreasing the incidence of micronucleated PCE in comparison to separate treatments [25]. It is clear that mixtures of pesticides can induce genotoxic effects even

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if each of them separately does not manifest such activity in testing. Therefore, it is necessary to evaluate the genotoxicity of pesticide combinations to ensure the safety of pesticides for public health.

The aim of this study was to assess the genotoxicity of the combination of three active ingredients: insecticide imidacloprid and fungicides imazalil and tebuconazole. Formulation based on this combination is used in agriculture for seed treatment in advance of or immediately before sowing.

2. Materials and methods

2.1. Materials

TGAIs of generic imidacloprid (97,0%), imazalil (97,8%), tebuconazole (98,4%) were used in the study. The studied combination of TGAIs contained imidacloprid, imazalil and tebuconazole in a ratio of 14.0/1.7/1.0 by weight, which corresponded to the ratio most commonly used in commercial formulations.

Salmonella typhimurium strains TA97, TA98, TA1535, TA100 and TA102 purchased from the Russian National Collection of Industrial Microorganisms were used in Ames test.

CD-1 mice were purchased from the Federal Government Budgetary Establishment of Science "Scientific Center of Biomedical Technologies" of the Federal Bio-Medical Agency of the Russian Federation.

2.2. Methods

2.2.1. Ames test

TGAIs imidacloprid, imazalil and tebuconazole, separately and in combination, were studied using Ames test (*Salmonella typhimurium)* according to OECD Guideline № 471 [26]. The plate incorporation method both in the absence and in the presence of an exogenous metabolic activation system (S9) was used as described in one of our previous publications [27].

Bacteria were exposed to individual pesticides in concentrations up to 5.0 (imidacloprid), 1.25 (imazalil), 2.5 (tebuconazole) mg/plate and to their mixture in concentrations of 0.05, 0.16, 0.5, 1.6 and 2.5 mg/plate. Maximum concentrations were chosen based on the results of preliminary experiments for the cytotoxicity assessment on TA100 strain. 2-aminoantracene (Sigma-Aldrich), sodium azide (Sigma-Aldrich), 2-nitrofluorene (Sigma-Aldrich), 9-aminoacridine (Fluka), methyl methanesulfonate (Sigma-Aldrich) were taken as a positive control. The incubation of bacteria was conducted at $37 \pm 2 \,°C$ for 48−72 h. For revertant counting SCAN® 500 Interscience was used.

2.2.2. Micronucleus assay

TGAIs imidacloprid, imazalil and tebuconazole and their combination were tested using the micronucleus test *in vivo* on mammalian bone marrow erythrocytes according to OECD Guideline № 474 [28].

The experiments on CD-1 mice were performed in accordance with the ethical guidelines of Directive 2010/63/EU and OECD TG 474. Mice were housed in standard cages with access to drinking water and feed *ad libitum*. Temperature (22−23 °C), humidity (36–40 %) and photoperiod (12 h light/12 -h dark) were maintained in an animal room.

For the assessment of genotoxic activity of pesticide mixture 5 groups of females and 5 groups of male mice (5 animals per group) were used. As a positive control cyclophosphamide (Sigma) at dose 40 mg/kg b.w. was chosen. Vehicle (sunflower oil) served as a negative control. The pesticides were administered orally by intragastric gavage twice at intervals of 24 h at three dose levels. Maximum doses of TGAIs upon separate treatments were 120 (imidacloprid), 300 (imazalil), and 1000 (tebuconazole) mg/kg b.w./d. The mixture of TGAIs was administered at doses 15/1.82/1.07 (Low dose), 30/3.64/2.14 (Middle dose) and 60/7.29/4.29 mg/kg b.w./d (High dose) of imidacloprid/imazalil/tebuconazole, respectively. The doses of TGAIs and their mixture were selected based on the results of preliminary experiments for finding maximum tolerated doses.

Bone marrow samples were harvested from mice sacrificed by cervical dislocation at 22 h after the second administration. Suspension of femoral bone marrow cells in fetal bovine serum was dropped on microscope slides (2 slides per animal), dried, fixed and stained using "Leucodif 200" kit (Erba Lachema s.r.o., CZ). All slides were coded and examined microscopically (Nikon Eclipse Ci-L, Japan). The proportion of PCEs among total (PCEs + NCEs) erythrocytes was determined by counting at least 500 total erythrocytes. 4000 PCEs were counted per animal for the assessment of micronucleated PCE incidence.

2.2.3. Statistical analysis

Statistical analysis was conducted in SPSS Statistics v.22.0 (IBM Corporation, New York, USA). Analysis of variance with post-hoc comparisons (Dunnett t-test) and rank correlation (Spearman) were used for the Ames method data. Generalized Poisson log-linear regression model with an animal as the unit of analysis [29] and the Mantel-Haenszel method were used for the micronucleus test data [30].

3. Results

3.1. Ames test

All separately tested TGAIs were negative in all *Salmonella* strains (data not shown). The results of mutagenicity assessment for the combination of imidacloprid, imazalil and tebuconazole TGAIs are given in Table 1.

The combination of imidacloprid, imazalil and tebuconazole showed no evidence of mutagenicity in the Ames test either in the presence or in the absence of S9.

3.2. Micronucleus assay

TGAIs of imidacloprid, imazalil and tebuconazole individually at doses up to 120, 300 and 1000 mg/kg b.w./d, respectively, did not induce micronucleus formation in PCE of mice bone marrow (data not shown). The combination of imidacloprid, imazalil and tebuconazole caused a statistically significant increase in MN-PCE incidence at the middle and high doses in comparison to the negative control. No suppression of erythropoiesis in the bone marrow of mice was observed (Table 2, Fig. 1). In addition, a significant linear dose-effect dependence was found (p = 0.000).

4. Discussion

Imidacloprid, imazalil and tebuconazole separately did not induce reverse gene mutations in *Salmonella typhimurium* strains and MN formation in mouse bone marrow erythrocytes. Their mixture in the ratio of 14.0/1.7/1.0 by weight was also negative in the Ames test. However, a statistically significant genotoxic effect of the mixture was found in the MN *in vivo* experiment. The tested combination caused a statistically significant increase in the incidence of MN-PCE in mice bone marrow at doses lower than the doses of these TGAIs used separately: 4, 80 and 466 times lower (imidacloprid, imazalil and tebuconazole, respectively) than the maximum doses of these pesticides upon separate treatments.

It should be noted that the upper control limit of the MN-PCE incidence distribution even at the high dose reached only 0.34 %, which is slightly higher the value for historical negative control in the laboratory (0.20 %). However, given the statistically significant difference to the concurrent negative control and the linear dose-response relationship with some results outside the distribution of the historical negative control data (Poisson-based 95 % control limits), it may be concluded that the combination of the three active ingredients showed weak genotoxic activity under the experimental conditions. The effect may be mediated by synergism of TGAIs, since it was detected at lower dose levels than used upon separate treatments.

Table 1

Summary data of mutation testing of the combination of imidacloprid, imazalil and tebuconazole using *Salmonella* strains.

Concentration, mg/plate	Strains									
	TA97		TA98		TA100		TA102		TA1535	
	mean ± SD		mean ± SD		mean ± SD		mean ± SD		mean ± SD	
	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
DMSO	126 ± 7	109 ± 10	30 ± 6	28 ± 6	140 ± 16	133 ± 8	174 ± 11	157 ± 13	29 ± 4	27 ± 7
0.05	114 ± 8	102 ± 14	34 ± 4	28 ± 4	141 ± 10	161 ± 5	172 ± 24	191 ± 12	28 ± 1	36 ± 8
0.16	133 ± 5	103 ± 13	36 ± 3	31 ± 5	161 ± 15	144 ± 10	162 ± 15	173 ± 6	28 ± 6	31 ± 5
0.5	116 ± 10	88 ± 5	35 ± 5	28 ± 5	146 ± 10	139 ± 17	167 ± 14	155 ± 8	32 ± 6	28 ± 3
1.6	113 ± 5	75 ± 11	33 ± 3	27 ± 7	155 ± 12	135 ± 8	171 ± 11	154 ± 17	25 ± 5	19 ± 2
2.5	114 ± 12	92 ± 9	27 ± 2	14 ± 4	160 ± 17	143 ± 13	141 ± 14	130 ± 8	25 ± 6	2 ± 2
Positive control	501 ± 33	1111 ± 47	266 ± 32	1417 ± 58	1046 ± 68	1009 ± 19	556 ± 47	837 ± 30	144 ± 24	1558 ± 54

Positive controls: 2AA – 50 µg/plate, 2NF - 10 µg/plate, 9AA- 30 µg/plate, MMC – 5 µg/plate, NaN3-10 µg/plate.

Table 2

Results of the MN assay for both male and female animals (N = 10).

Treatment group	MN-PCE incidence			Cytotoxicity%
	Poisson-based 95 % control limits	Mean MN-PCE incidence and 95 % Poisson CI		2PCE/(PCE + NCE) ± SD
		Wald, %	Profile likelihood*, arbitrary units	
Concurrent negative control	0.00−0.17	$_{0.06}$ 0.08 $_{0.12}$	$_{0.616}$ 1.000 $_{1.326}$	0.58 ± 0.09
Low dose	0.01−0.24	$_{0.10}$ 0.13 $_{0.17}$	$_{1.003}$ 1.545 $_{2.415}$	0.54 ± 0.07
Middle dose	0.04−0.30	$_{0.13}$ 0.17 $_{0.21}$	$_{1.349}$ 2.030 $_{3.116}$	0.57 ± 0.05
High dose	0.06−0.34	$_{0.16}$ 0.20 $_{0.25}$	$_{1.632}$ 2.424 $_{3.684}$	0.57 ± 0,06
Positive control	0.91−1.63	$_{1.16}$ 1.27 $_{1.39}$	$_{11.011}$ 15.394 $_{22.318}$	0.58 ± 0.04
Historical negative control	0.00−0.20	$_{0.08}$ 0.10 $_{0.11}$	–	–

* Relative to the mean MN-PCE incidence in concurrent negative control.

Although it is known that some pesticides and their mixtures, induce not only genotoxic but also cytotoxic effects in various tissues [31], the combination of imidacloprid, imazalil and tebuconazole did not cause a toxic effect in bone marrow.

Published data on genotoxicity of imidacloprid, imazalil and tebuconazole are contradictory. According to the EFSA no evidence for genotoxicity of tebuconazole could be observed in an adequate test battery [32]. However, a number of studies have reported the genotoxic effects of tebuconazole and its commercial formulations. For example, tebuconazole was genotoxic at 3 and 6 mg/l for two species of green algae [33]. It also caused cyto- and genotoxic effects to B. laevis at environmentally relevant levels. [34]. Statistically significant increase

Fig. 1. Effect of pesticide mixture (imidacloprid + imazalil + tebuconazole) on the incidence of MN-PCE in mice bone marrow. Bars – 95 % Wald CI. (2-column-fitting image).

in the chromosomal aberration frequency after tebuconazole exposure was found in bovine peripheral lymphocytes *in vitro*. No statistically significant increase was demonstrated in the induced MN after the exposure to the fungicide formulation [35]. According to FAO, imidacloprid was negative in most tests. Positive results were obtained only with sister chromatid exchange analysis [36]. There are some in vitro studies of the genotoxic activity of imidacloprid in human cells: HepG2-cells, peripheral lymphocytes and SHSY-5Y cells, using the comet assay or the micronucleus test. Genotoxic effects of imidacloprid at µM-concentrations were shown by the comet assay or the micronucleus test [37–42]. Imidacloprid caused hepatotoxicity, oxidative renal and DNA damage in rats [43,44] and induced iNOS, 8−OHdG and TNF-α activation in different tissues of common carp [45].

The cytogenetic results showed that imazalil caused dose-dependent increases in the frequency of the structural chromosomal aberrations and the incidence of MN in comparison to negative controls in zebrafish [46]. Assessment of imazalil on cultured human lymphocytes with chromosomal aberration analysis and MN test as cytogenetic endpoints also demonstrated genotoxicity [47].

In regards to combinations, our findings are in agreement with some of the published data. Genotoxic effects of the neonicotinoid insecticide imidacloprid in combination with other pesticides were described in the literature. For example, the synergistic effect of imidacloprid and the organophosphorus insecticide methamidophos caused an increase in genetic damage to non-target organisms: bacteria *S. typhimurium* and *Wistar albino* rats [48]. The *in vivo* micronucleus assay showed a statistically significant effect induced in rat bone-marrow PCE by each of the pesticides: imidacloprid and metalaxyl at doses of 300 mg/kg b.w., while genotoxicity was detected at a lower dose of their combination (100 + 100) mg/kg b.w. [49]. On the other hand, it was also shown that imidacloprid and sulfentrazone separately interact with the DNA in HepG2 cells and cause irreversible damage. However, it was shown their antagonistic effect in combination mixture with less pronounced DNA
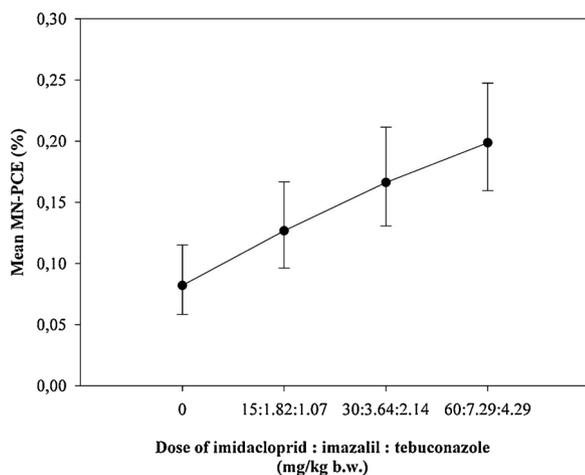
damage [50]. The mixture of imazalil, cypermethrin and carbendazim caused DNA damage in hepatocytes evaluated by comet assay due to synergistic effects; in this case, carbendazim potentiated the effects of imazalil and cypermethrin [51].

The revealed weak genotoxic effect of imidacloprid, imazalil and tebuconazole in combination may be due to the mutual influence of the active substances or their metabolites. In addition, impurities that are present in generic technical products in low concentrations can also contribute to the genotoxicity of the mixture. Additional studies are required to clarify the mechanism(s) of the observed synergistic effect. Our data confirm the need for testing pesticide mixtures in relation to genotoxicity for their safe use.

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CRediT authorship contribution statement

Nataliya A. Ilyushina: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Olga V. Egorova:** Validation, Investigation, Writing - original draft, Writing - review & editing. **Gleb V. Masaltsev:** Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization. **Nataliya S. Averianova:** Investigation. **Yulia A. Revazova:** Writing - review & editing. **Valerii N. Rakitskii:** Resources, Supervision. **Marina Goumenou:** Writing - review & editing. **Alexander Vardavas:** Writing - review & editing. **Polychronis Stivaktakis:** Writing - review & editing. **Aristidis Tsatsakis:** Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest

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