Genotoxicity evaluation of pesticide formulations containing alachlor and atrazine in multiple mouse tissues (blood, kidney, liver, bone marrow, spleen) by comet assay

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Every year, in the European countries more than 2 million tons of pesticides are released into the environment. More than 60% of those substances appear to be herbicides. Due to extensive production and application of this chemical their putative detrimental effect on life should be known and minimized. In this study we applied the comet assay on blood and 4 mouse organs (kidney, liver, bone marrow, and spleen) to evaluate possible genome damage caused by two pesticide formulations (Bravo® and Gesaprim®) containing alachlor and atrazine as active ingredients. Five male CBA mice were assigned to each of 4 treatment groups and control group. Bravo® and Gesaprim® were injected intraperitoneally once. Two different doses of Bravo® were used: 0.031 ml/kg and 0.021 μl/kg, so that doses of alachlor mice received within the pesticide formulation given were 15 mg/kg and 0.01 mg/kg. Also Gesaprim® was given at two different doses: 1.08 ml/kg and 0.07 μl/kg so that the doses of atrazine contained within the pesticide formulation given were 540 mg/kg and $3.5x10^{-2}$ mg/kg. Mice were sacrificed 24 hours after treatment. Alkaline comet assay on the blood samples, kidney, liver, bone marrow and spleen was performed. Statistically significant ($p<0.01$) increase of tail length for all 5 tissues examined in mice treated with both Bravo® and Gesaprim® compared to the control was found. For both pesticides DNA of kidney and liver showed largest increase in migration. Also, distribution of tail length values for Bravo® and Gesaprim® for all mouse tissues examined showed a shift to the right when compared to the controls.

Key words: atrazine, alachlor, multiple mouse tissues, comet assay, genotoxicity

Pesticides of worldwide application are used in agriculture in vast amounts each year, of which herbicides are the most prominent class. Triazine herbicides constitute one of the largest groups of herbicides sold in the world. Among them, for many years atrazine has been the one most used [7]. It was developed and introduced in 1957 by J.R. Geigy S.A. in Switzerland [10] for pre-emergence and post-emergence control of annual grasses and broadleaf weeds during cultivation of maize, wheat, sorghum, sugar cane and conifers [31]. Atrazine is a member of chlorotriazines and inhibits plant photosynthesis [6]. Alachlor is a chloroacetanilide, developed and introduced in 1967 by Monsanto Company from the USA [9]. It inhibits protein synthesis in young roots and is used for pre-emergence control of a broad spectrum of grass, sedges, and broadleaf weeds in corns, soybeans, dry beans, cottons, grain sorghums, sunflowers, peanuts and other crops [9].

Due to extensive production and application of this chemical their putative detrimental effect on life should be known and minimized. Therefore, cytogenetic studies have been conducted. Some of them showed the ability of atrazine and alaclor to cause genetic damage on human and animal cells *in vitro* [4, 17, 18, 19, 20, 22, 23, 28]. Some epidemiological studies on populations occupationally exposed to pesticides showed an increased risk of cancer development among them. For the workers employed in atrazine production and application, an increased incidence of ovary tumor [2, 3] and non-Hodgkin lymphoma [11] was found. It was also shown that occupational exposure to alachlor increased the incidence of colorectal carcinoma and chronic myeloid leukemia [15].

In this study we applied the comet assay on mouse cells from blood and 4 mouse organs (kidney, liver, bone marrow, and spleen) to evaluate possible genome damage

caused by two pesticide formulations (Bravo® and Gesaprim®) containing alachlor and atrazine as active ingredients. We focused our research on possible genotoxicity of pesticide formulations and not only their active ingredients. Namely, workers handling the pesticides during their production and application are not only to exposed the active ingredients but to the complex mixture of chemicals contained in the formulation as well. Due to possible interactions between these chemicals it is important to evaluate the health risk of the entire pesticide formulation, and not only of the isolated active ingredients, which could give results that do not correspond to the actual conditions.

Material and methods

Pesticide formulation Bravo®, containing 480 mg/ml of alachlor as the active ingredient, and Gesaprim®, containing 500 mg/ml of atrazine as the active ingredient, were purchased from Herbos d.d. Sisak (Croatia).

Male CBA mice at 8 weeks of age were used. They were fed commercial pellets (Pliva d.d, Croatia) and tap water ad libidum before and throughout the experiment. The animal room was at 22–24 ˚C and 50–65% humidity with a 12-hour light-darkness cycle.

Five male CBA mice were assigned to each of the 4 treatment groups and the control group. Bravo® and Gesaprim® were injected intraperitoneally once. Bravo® was given at the dose of 0.031 ml/kg so that the dose of alachlor contained within the pesticide formulation given was 15 mg/kg and 0.021 μl of Bravo®/kg so that the dose of alachlor contained within the pesticide formulation given was 0.01 mg/kg. Gesaprim® was given at the dose of 1.08 ml/kg so that the dose of atrazine contained within the pesticide formulation given was 540 mg/kg and 0.07 μl of Gesaprim®/kg so that the dose of atrazine contained within the pesticide formulation given was $3.5x10^{-2}$ mg/kg. For both pesticides used lower concentration of active ingredient represents oral chronic Referent Dose (RfD) of alachlor/atrazine [12]. The higher dose of Bravo® corresponds to the concentration of pesticide used by RIBAS et al [23] which gave positive results in chromosomal aberration, sister chromatid exchange and micronucleus test on human lymphocytes *in vitro*. Higher dose of Gesaprim® used was the dose compatible with survival and almost the same as used by TENNANT et al [30] in mice *in vivo*. Mice treated with methyl methanesulfonate (MMS) (Sigma) at the concentration of 70 mg/kg were used as positive controls. The control group was treated intraperitonelly with a 0.8% NaCl solution (Kemika, Croatia). The mice were sacrificed 24 hours after treatment, and blood and 4 organs (kidney, liver, bone marrow and spleen) were taken.

After weighing, the organs were minced and suspended at the concentration of 1 g/ml in a chilled homogenization buffer (pH 7.5) containing 0.0075 M NaCl and 0.0024 M Na_2EDTA (Sigma, Germany). Organs were homogenized using a Potter-type homogenizator, in ice [25]. Blood was taken into heparinized vacutainers (Becton-Dickinson, UK).

The alkaline comet assay on the blood samples was performed according to the standard protocol [27]. For kidney, liver, bone marrow and spleen, the alkaline comet assay protocol was modified according to SASAKI et al [25, 26]. All chemicals were obtained by Sigma. 4 μl of whole blood or 6 μl of organ homogenate were suspended in 0.5% low melting agarose and sandwiched between a layer of 0.6% normal melting agarose and a top layer of 0.5% low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each gel-layer. After the solidification of the 0.6% agarose layer the slides were immersed in a lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%) at 4 ˚C. After one hour, the slides were placed into an electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA, pH 13) at 0 ˚C to allow for the DNA to unwind (blood samples for 20 minutes; kidney, liver, bone marrow and spleen samples for 10 minutes). The electrophoresis was performed at 300 mA and 25 V in a horizontal electrophoresis platform, 20 minutes for the blood samples and 15 minutes for the kidney, liver, bone marrow and spleen samples. The slides were neutralized with a Tris-HCl buffer (pH 7.5) and stained with 10% ethidium-bromide for 10 minutes. Each slide was analyzed by using the Leitz Orthoplan epifluorescence microscope. For each organ and pesticide concentration, 100 cells were analyzed by the Comet assay II automatic digital analysis system (Perceptive Instruments Ltd., Suffolk, Halstead, UK), determining tail length and tail moment.

Possible differences of comet assay endpoints between the control blood and organs and the blood and organs of treated mice were evaluated using the Mann-Whitney U-test.

Results

No death, morbidity, or distinctive clinical signs were observed after any treatment. Necropsy of the animals revealed no effect of treatment on any organs examined. Mean values of the comet assay endpoints for blood, kidney, liver, bone marrow, and spleen, for the control group of mice and the groups treated with different concentrations of Bravo® and Gesaprim® are shown in the Table 1.

Bravo® (alachlor). A statistically significant ($p<0.01$) increase of tail length for all 5 tissues examined in mice treated with 0.031 ml/kg and 0.021 μl/kg compared to the control group was found. For both concentrations given, the DNA

Table 1. Mean values of comet assay endpoints for lymphocytes and four organs of control, mice treated with pesticides and positive control (MMS)

Treatment	Organ	Tail length/μm	Tail moment
Control	Lymphocytes	14.6 ± 0.70	10.6 ± 0.77
	Kidney	14.5 ± 1.14	10.5 ± 1.16
	Liver	14.9 ± 1.14	10.6 ± 1.22
	Bone marrow	14.7 ± 0.92	10.4 ± 0.98
	Spleen	14.7 ± 0.80	10.5 ± 0.89
Bravo® 0.021 μl/kg 0.01 mg/kg b.w. of alachlor[b]	Lymphocytes	17.4 ± 2.58[a]	14.4 ± 2.57[a]
	Kidney	18.8 ± 4.67[a]	16.0 ± 4.34[a]
	Liver	18.5 ± 3.69[a]	15.7 ± 3.57[a]
	Bone marrow	17.2 ± 2.40[a]	14.6 ± 2.34[a]
	Spleen	16.7 ± 2.93[a]	14.2 ± 2.83[a]
Bravo® 0.031 ml/kg 15 mg/kg b.w. of alachlor[b]	Lymphocytes	18.2 ± 3.72[a]	15.5 ± 3.54[a]
	Kidney	20.5 ± 5.51[a]	17.5 ± 5.29[a]
	Liver	19.4 ± 4.51[a]	16.6 ± 4.33[a]
	Bone marrow	18.4 ± 3.46[a]	15.5 ± 3.28[a]
	Spleen	18.2 ± 3.65[a]	15.4 ± 3.32[a]
Gesaprim® 0.07 μl/kg 540 mg/kg b.w. of atrazine[b]	Lymphocytes	17.4 ± 3.02[a]	14.2 ± 2.88[a]
	Kidney	19.2 ± 7.70[a]	16.3 ± 7.08[a]
	Liver	19.5 ± 7.26[a]	16.7 ± 6.53[a]
	Bone marrow	16.9 ± 2.76[a]	14.6 ± 2.52[a]
	Spleen	17.3 ± 3.74[a]	14.3 ± 3.52[a]
Gesaprim® 1.08 ml/kg $3.5x10^{-2}$ mg/kg b.w. of atrazine[b]	Lymphocytes	19.1 ± 4.64[a]	15.5 ± 4.18[a]
	Kidney	22.0 ± 10.07[a]	19.4 ± 8.53[a]
	Liver	22.4 ± 9.74[a]	19.2 ± 8.68[a]
	Bone marrow	18.6 ± 4.80[a]	15.7 ± 4.40[a]
	Spleen	18.8 ± 4.99[a]	15.5 ± 4.46[a]
MMS 70 mg/kg	Lymphocytes	43.3 ± 2.71[a]	19.2 ± 1.58[a]
	Kidney	50.9 ± 3.77[a]	23.7 ± 2.64[a]
	Liver	51.0 ± 3.18[a]	24.9 ± 3.02[a]
	Bone marrow	41.4 ± 2.53[a]	18.7 ± 2.28[a]
	Spleen	40.1 ± 2.30[a]	20.1 ± 2.15[a]

[a]Statistically significant compared to the negative control ($p<0.01$), [b]concentration of active ingredient contained within the pesticide formulation given.

of kidney and liver showed the largest increase in migration. Also, tail length for all 5 tissues of mice treated with Bravo® at 0.031 ml/kg of alachlor/kg was significantly ($p<0.01$) higher compared to the mice treated with 0.021 μl/kg. For the mice given 0.021 μl/kg, the tail length values of kidney and liver were significantly higher than those of blood, bone marrow and spleen from the same group, but there were no significant differences between the tail length values of kidney and liver. The tail length values of kidney and liver

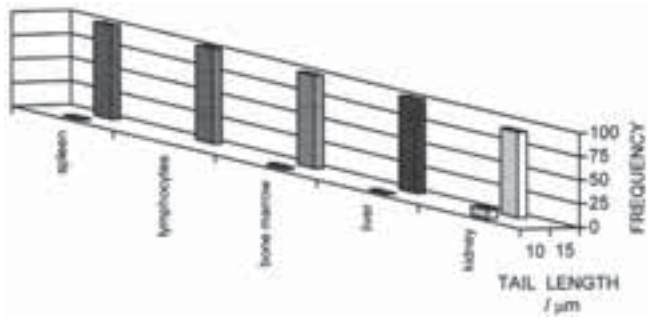

Figure 1. Tail length distribution for different organs of the control mice.

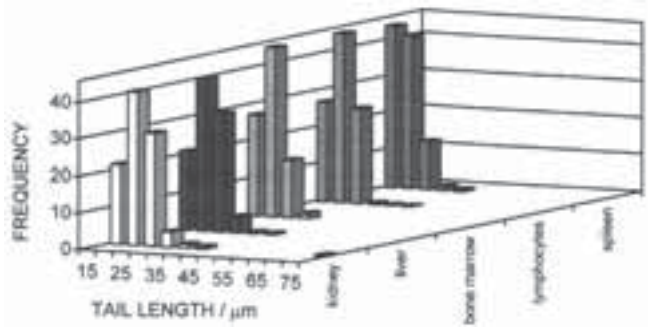

Figure 2. Tail length distribution for different organs of mice treated with Bravo® 0.021 μl/kg b.w. for 24 hours.

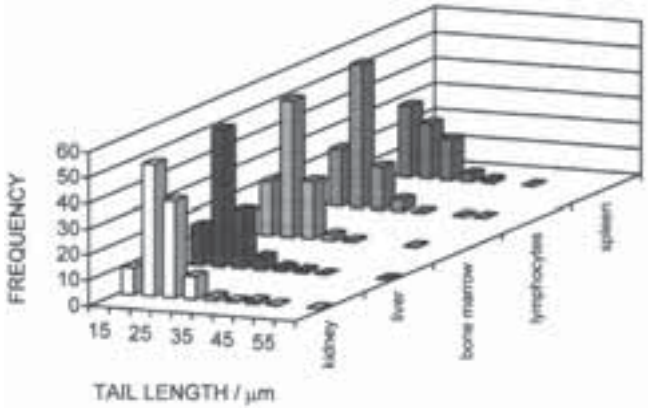

Figure 3. Tail length distribution for different organs of mice treated with Bravo® 0.031 ml/kg b.w. for 24 hours.

for mice given 0.031 ml/kg were significantly higher than those of blood, bone marrow and spleen from the same group. Within the same group, the tail length values of kidney were significantly higher than those of liver ($p<0.01$).

Distribution of tail length values for Bravo® for all mouse tissues examined showed a shift to the right when compared to the controls, regardless of the dose. The shifts were bigger for kidney and liver samples than for the lymphocytes, bone marrow and spleen. For Bravo®, in both kidney and liver at the concentration of 0.031 ml/kg of alachlor/kg, comets with tail length up to 57 μm and for the

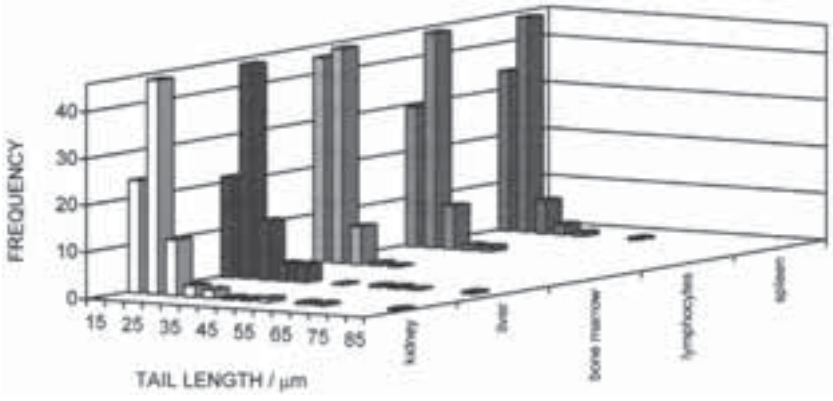

Figure 4. Tail length distribution for different organs of mice treated with Gesaprim® 0.07 μl/kg b.w. for 24 hours.

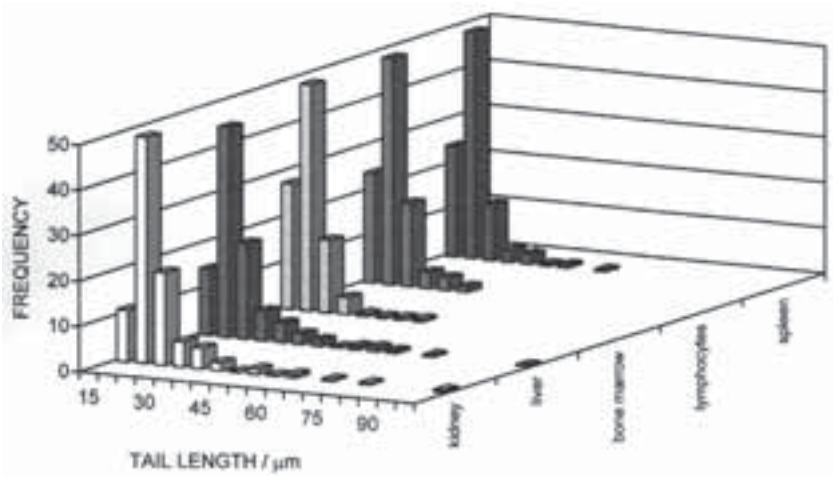

Figure 5. Tail length distribution for different organs of mice treated with Gesaprim® 1.08 ml/kg b.w. for 24 hours.

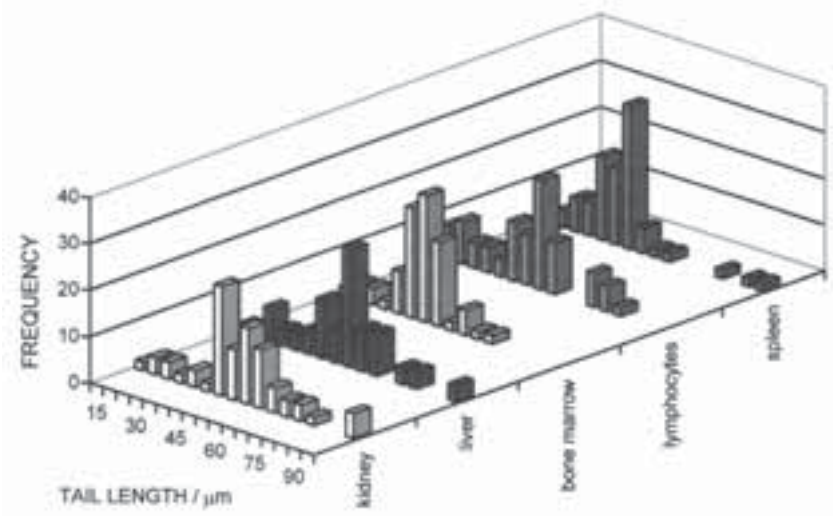

Figure 6. Tail length distribution for different organs of mice treated with MMS 70 mg/kg b.w. for 24 hours (positive control).

0.021 μl/kg up to 38 μm were observed (data not shown).

Gesaprim® (atrazine). A statistically significant ($p<0.01$) increase of tail length for all 5 tissues examined in mice treated with 1.08 ml/kg and 0.07 μl/kg compared to the control group was found. For both concentrations given, the DNA of kidney and liver showed the largest increase in migration. Also, tail length for all 5 tissues of mice treated with Gesaprim® at 1.08 ml/kg (540 mg/kg b.w. of atrazine contained within the pesticide formulation given) was significantly ($p<0.01$) higher compared to the mice treated with 0.07 l/kg ($3.5x10^{-2}$ mg/kg of atrazine contained within the pesticide formulation given). For the mice given 0.07 μl of Gesaprim®/kg, the tail length values of kidney and liver were significantly higher compared to those of blood, bone marrow and spleen from the same group, but there was no significant difference between tail length values for kidney and liver. The tail length values of kidney and liver for mice given 1.08 ml of Gesaprim®/kg were significantly higher than those of blood, bone marrow and spleen of the same group, but there was no statistically significant difference between them.

Tail lenghts up to 99 μm were observed in the kidney DNA from mice treated with Gesaprim® at the concentration of 1.08 ml/kg, and up to 84 μm for the concentration of 0.07 μl/kg (data not shown). For the liver, at the dose of 1.08 ml/kg the maximum tail length value was almost the same as for the kidney (97 μm), whereas at the concentration of 0.07 μl/kg it was much lower compared to the kidney (39 μm).

Discussion

In this paper we tested two different pesticide formulations: Bravo®, containing alachlor as the active ingredient, and Gesaprim® containing atrazine as the active ingredient. We focused our study on pesticide formulations instead on purified active ingredients because workers producing and applying pesticides are exposed to such formulations and not only to active

ingredients. In occupational exposure to pesticides, all chemicals contained in the formulation, not only active ingredients, affect human health, so all of them should be considered in cytogenetical research simulating the exposure [32, 33].

Although neither pharmacotoxic signs nor any gross changes were found at necropsy, there is a possibility that due to the disruption of cell membranes by the homogenization the comet assay results were affected by some secondary cellular effects. To minimize the effect of the homogenization on the DNA migration, the non-enzymatic technique proposed by SASAKI et al [25] was used.

As Table 1 shows, both Bravo® and Gesaprim® induced comet assay detectable DNA damage in all tissues examined, regardless of the dose. Nevertheless, genome damage for both pesticides was found to be higher for the higher dose given. The most affected organs were kidney and liver, expressing DNA migration significantly higher than in lymphocytes, bone marrow and spleen. Distribution of tail length values for both Bravo® and Gesaprim® for all mouse tissues examined showed a shift to the right when compared to the control group, regardless of the dose (data not shown). The shifts were bigger for kidney and liver samples compared to the lymphocytes, bone marrow and spleen, suggesting that these two organs could be the primary targets for alachlor and atrazine genotoxicity. Thus, the *in vivo* genotoxicity of these pesticide formulations was detected in spite of the difference in targeted organs.

There are studies of other authors aiming to show possible genotoxic effects of alachlor *in vivo* and *in vitro*. Some of them gave positive results. RIBAS et al [23], MEISNER et al [18], DUNKELBERG et al [4], and GREGORIAN et al [8] suggest that alachlor might have genotoxic effects on human lymphocytes *in vitro*. BONFANI et al [1] reported that alachlor was able to induce DNA strand breakage in rat hepatocytes, and LIN et al [16] determined its clastogenic potency on CHO cells. Still, some other authors reported negative results on alachlor genotoxicity *in vitro* [5, 21].

Studies of alachlor genotoxicity *in vivo* have also been contradictory. GEORGIAN et al [8] reported positive results on rat bone marrow, whereas results of GEBEL et al [6] on mouse bone marrow micronucleus test, and TANINGHER et al [29] on DNA strand breakage test in rats and mice gave no evidence for alachlor *in vivo* genotoxicity.

In our study, Bravo®, containing alachlor, also showed genotoxic potential in bone marrow cells, which could be in agreement with results of the epidemiological study showing that occupational exposure to alachlor increased the incidence of colorectal carcinoma and chronic myeloid leukemia [15].

Genotoxic studies of atrazine *in vivo* and *in vitro* also gave contradictory results. RIBAS et al [23] found atrazine to cause alkaline labile sites on human lymphocytes using the comet assay. LIOI et al [17] also found it to be genotoxic on human lymphocytes using chromosomal aberration and sister chromatid exchange assay and MEISNER et al [19] using chromosome breakage assay. On the contrary, in some other studies on human lymphocytes, atrazine did not appear to be genotoxic [4, 13, 24].

Using alkaline elution assay on rat liver, kidney and stomach, PINO et al [20] reported atrazine to be able of causing DNA damage *in vivo* which is in agreement with results of our study. Similar results were obtained by GEBEL et al [6] using mouse bone marrow assay. Using comet assay, TENNANT et al [30] showed that atrazine in concentrations of 250 and 500 mg/kg significantly increased DNA migration in leukocytes of mice treated *in vivo*. The concentration used (500 mg/kg) was similar to the one used in this paper (540 mg of atrazine/kg b.w. within the dose of 1.08 ml of Gesaprim®/kg b.w.). Nevertheless, the same concentrations of atrazine in *in vivo* mouse bone marrow micronucleus assay did not increase the micronucleus frequency [14]. Also, ROLOFF et al [24] were not able to report an increase in chromosomal aberrations in mouse bone marrow cells but they were able to find it in spleen cells of treated animals.

In the epidemiological studies atrazine was found to be able of increasing the incidence of ovary tumor [2, 3] and non-Hodgkin lymphoma [11] in workers employed in its production and application. These findings could be partially in agreement with results presented in this paper showing increased DNA migrations in bone marrow nuclei of mice treated with Gesaprim®, regardless of the dose.

The finding that for both pesticides tested, their genotoxic activity slightly increased at the higher doses compared to the RfD, could be due to removal of highly damaged cells by apoptosis pathway which was spotted while analyzing the comet slides (data not shown). Nevertheless, there were high interindividual differences in the number of apoptotic cells for the same organ among mice treated. So the other apoptosis detection techniques should be used before presenting those results.

One of the reasons for contradictions in atrazine and alachlor genotoxicity results might be the usage of active ingredients produced by different manufacturers. Some of them might produce lower quality alachlor or atrazine containing higher levels and numbers of impurities that could affect the results [9]. The results of all those studies would be much different and more similar to the field exposure conditions if pesticide formulations were used instead of active ingredient, as it was the case in the presented paper.

In conclusion, by using the alkaline comet assay on different tissue samples, genotoxic effects of pesticide *in vivo* can be detected and more knowledge on the specificity of its mutagenic action for different organs could be gained. Also, used on different organs in *in vivo* genotoxicity studies, the comet assay could provide a good assessment of potential pesticide carcinogenicity.

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