***In vivo* Genotoxic Evaluation of Biological and Organic Pesticides and Fertilizers**

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ABSTRACT

The substitution or reduction of the use of chemical pesticides and fertilizers, by means of the introduction of biological and organic agents, is a mandatory need for environmental safety. In spite of the supposed low toxicity of these biological and organic products, an evaluation system to assess its possible damage to man and environment must be used. A well-structured battery of assays must include the genotoxic evaluation. We developed the genotoxic assessment through a battery of *in vivo* assays in rodents. These studies include Mammalian Erythrocyte Micronucleus Test, and Mammalian Bone Marrow Chromosomal Aberration Test. In this work, we used these two assays to perform the genotoxic evaluation of four pesticides and three fertilizers: NeoNim 60, OleoNim 80, Bactivec, Griselesf, Gluticid, Indolacetic acid, Jasmonic acid. No chromosome aberrations induction and increase of micronucleus formation were detected in six of the products. In the case of Gluticid, was found to be a causative agent of genotoxic and citotoxic effects in somatic cells of female mice at the tested dose. It could be concluded that the two assays included in our battery test has proved to be useful to obtain the genotoxic profile of tested products, allowing this results a more safety utilization of the products.

Key words: Pesticides, fertilizer, genotoxic evaluation, micronucleus, chromosome aberration

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INTRODUCTION

The substitution or reduction of the use of chemical pesticides and fertilizers, by means of the introduction of biological and organic agents, is a mandatory need for environmental safety. In spite of the supposed low toxicity of these biological and organic products, an evaluation system to assess its possible damage to man and environment must be used.

A well-structured battery of assays must include the genotoxic evaluation. In the Center of Experimental Toxicology of CENPALAB, Cuba, we have developed genotoxicity evaluation through a battery of *in vivo* assays in rodents.

The Mammalian Erythrocyte Micronucleus Test (MN test)^{1,2} and the Mammalian Bone Marrow Chromosomal Aberration Test (CA test)³ are *in vivo* assays often included in almost all the genotoxic assessment batteries. These are short-term assays widely used for the detection of induced cellular damage to chromosomes or mitotic apparatus⁴. This cellular damage is clearly

associated to cancer origin¹, giving an adequate estimation of human risks⁵.

Our center has a history of genotoxic evaluation of more than 10 years, being our main objective the assessment of the genotoxic potential of pesticides and fertilizers. The majority of these products have been registered for use in the control of pests affecting agriculture, but also for the control of vectors that constitute a hazard for the human health.

In this work, we show the procedures established for each of the *in vivo* assays used for the genotoxic evaluation of pesticides and fertilizers, besides of the fundamental results obtained with the realization of these studies.

MATERIALS AND METHODS

Studies were accomplished according to the international principles of Good Laboratory Practice⁶, the principles of care and use of laboratory animals^{7,8} and the OECD Guidelines⁹.

Environmental conditions: Animals were housed in plastic cages Type II (mice) and Type IV (rats) and

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Table 1: Information about tested substances

Test substances	Classification	Active principle	Use	Doses
OleoNim 80	Organic agent	Azadirachtin (1600 mg L ⁻¹)	Insecticide	2000 mg kg ⁻¹
NeoNim 60	Organic agent	Azadirachtin (15000 mg L ⁻¹)	Insecticide	60 mg kg ⁻¹
Griselesf®	Biological agent	<i>Bacillus sphaericus</i> strain 2362	Larvicide	2.8 × 10 ⁸ CFU mL ⁻¹
Bactivec®	Biological agent	<i>Bacillus thuringiensis</i> var <i>israelensis</i>	Larvicide	2.5 × 10 ⁸ CFU mL ⁻¹
Gluticid	Organic agent	<i>Pseudomonas</i> sp. metabolites	Fertilizer	36 mg kg ⁻¹
Jasmonic acid	Organic agent	Jasmonic acid	Fertilizer	9.95 mg kg ⁻¹
Indolacetic acid	Organic agent	Indolacetic acid	Fertilizer	3 mg kg ⁻¹

CFU: Colony forming units

maintained at a temperature of 25°C (±2°C) and 60% relative humidity with a 12 h light and dark photoperiod. The animals were fed with EMO 1002 diet (ALYco®, CENPALAB) and water *ad libitum*.

Substances: The biological and organic agents were NeoNim 60, OleoNim 80, Bactivec, Griselesf, Gluticid, Indolacetic acid, Jasmonic acid (Table 1). The selection of doses was based on realistic exposures, using dose levels higher than expected.

Cyclophosphamide (CP, Fluka) at a dose of 40 mg kg⁻¹ b.w., used as the positive control, was prepared in distilled water and administered via intraperitoneal injection. The negative controls groups received distilled water or saline orally by gavage (except for negative control of Gluticid, whose vehicle is Ammonium Sulphate 8%). The volume of substances administered in all case was 1 mL.

In vivo bone marrow micronucleus assay: The MN assay was carried out following standard protocols, as recommended by Schmid in 1976², Krishna and Hayashi in 2000¹⁰ and OECD Guideline 474¹¹.

The studies were carried out with Cenp:NMRI mice of both sex, 8-12 weeks old, and an average body weight between 27-30 g. For each assay, it was established treated groups and two control groups, negative and positive.

All tested substances were administered via oral gavage. The treatment schedule was two administrations at 24 h intervals and samples were collected 24 h following the final treatment. The mice were killed by cervical dislocation. Two femurs were removed and the bone marrow cells were flushed out with fetal bovine serum (PAA) into a centrifuge tube. After serum excess was removed by centrifugation, two bone marrow smears per animal were prepared. The smears were allowed to dry and were subsequently fixed in methanol (5 min). 24 h later the smears from each animal were stained with 5% Giemsa solution (Finlay) for 15 min for microscopic evaluation.

The incidence (%) of micronucleated polychromatic erythrocytes (MNPCE) was calculated based on the observation of 2000 polychromatic erythrocytes (PCE) per animal (totally 20000 per group) to determine the

mutagenic properties of the products. To detect possible cytotoxic effects, the PCE:NCE (normochromatic erythrocytes) ratio in 1000 erythrocytes/animal was calculated¹².

All data were entered into a database using the Statistical Package Scientific System, SPSS for Windows¹³. The mean and standard deviation of % of micronucleated polychromatic erythrocytes (MNPCE) and the PCE:NCE (normochromatic erythrocytes) ratio for each group were calculated. Statistical significance was assessed at the p<0.05 level.

Chromosome aberration assay: The CA assay design was based on standard protocols described by Legator³ and by the OECD¹⁴.

The studies were carried out with Cenp:SPRD rats of both sex, with an average body weight between 150-160 g. For each assay, it was established treated groups and two control groups, negative and positive.

All tested substances were administered via oral gavage. The treatment schedule was one unique administration; and samples were collected 24 h following the treatment. To arrest mitosis, colchicine (4 mg kg⁻¹ b.w., Fluka) was injected intraperitoneally, 2 h before the animals were sacrificed by cervical dislocation. The femurs were stripped proximally and the bone marrow was aspirated in 1 mL of phosphate buffer solution (37°C). The suspension was centrifuged for 10 min at 1000 rpm and the bone marrow pellet was resuspended in KCl 0.75 mol L⁻¹ at 37 °C for 20 min and then fixed in cold methanol: acetic acid (3:1) for 15 min at room temperature. The treatment with fixative was repeated two times. Then, the cells were spread on glass slides and air-dried. The slides were stained with Giemsa (10%) for 8 min.

Fifty well spread metaphases per animal (totally 500 metaphases per group) were examined with a 100x magnification for the occurrence of structural and numerical chromosome aberrations. Total chromosome aberrations were used to compare with the negative control groups. All data were entered into a database using the Statistical Package Scientific System, SPSS for Windows¹³. The mean and standard deviation of total chromosome aberrations for each group were calculated. Statistical significance was assessed at the p<0.05 level.

Table 2: Results of micronucleus test in mice

Compounds	PCE:NCE (Mean±Standard Deviation)		% MNPCE (Mean±Standard Deviation)	
	Female (n=5)	Male (n=5)	Female (n=5)	Male (n=5)
Negative control	1.31±0.01a	1.26±0.23a	0.35±0.09a	0.33±0.02a
Negative control (for Gluticid)	1.32±0.30	1.00±0.12	0.24±0.16	0.36±0.26
Positive control	0.83±0.18a*	0.83±0.16a*	4.50±3.48a*	3.32±2.04a*
OleoNim 80	1.55±0.35	1.61±0.51	0.40±0.28	0.32±0.18
NeoNim 60	1.21±0.14	1.22±0.17	0.36±0.33	0.40±0.20
Griselesf®	1.41±0.36	1.46±0.13	0.24±0.33	0.20±0.24
Bactivec®	1.46±0.17	1.61±0.42	0.12±0.18	0.08±0.11
Gluticid	0.91±0.09**	1.18±0.32	0.52±0.26**	0.40±0.28
Jasmonic acid	1.15±0.26	1.06±0.12	0.44±0.26	0.68±0.88
Indolacetic acid	1.13±0.17	1.23±0.13	0.20±0.24	0.16±0.26

*Values were calculated based on different positive controls of the laboratory, *significantly different from the negative control ($p < 0.05$), **significantly different from his negative control ($p < 0.05$)

Table 3: Results of Chromosome aberrations test in rats

Substances	Total CA (Mean±Standard Deviation) (n=10)
Negative control	1.94±0.83a
Negative control (for Gluticid)	2.62±2.46
Positive control	39.66±11.39a*
OleoNim 80	3.00±2.16
NeoNim 60	1.68±2.61
Griselesf®	3.60±2.63
Bactivec®	4.60±2.12
Gluticid	3.36±2.56
Jasmonic acid	2.07±1.70
Indolacetic acid	3.34±2.07

*Values were calculated based on different positive controls of the laboratory
CA: Chromosome aberrations, *significantly different from the negative control ($p < 0.05$)

RESULTS

Table 2 shows the MN test results obtained for female and male NMRI mice treated with the different biological and organic agents: the number of MNPCE for each animal and means, for untreated controls and treated animals. None of the experiments showed statistically significant increase in the average number of MNPCE at the tested dose as compared with the negative control, except for the Gluticid in females. On the other hand, treatment with CP 40 mg kg⁻¹ b.w. led to a significant elevated frequency ($p < 0.05$) of MNPCE in all the experiments.

Table 2 also shows the ratio between the average numbers of PCE, in relation to NCE in 1000 randomly cells analyzed for each animal. The PCE:NCE ratio was not significantly different from the negative control group, except for the Gluticid in females, indicating that the other products do not show cytotoxic properties in NMRI mouse bone marrow cells at the tested dose.

Table 3 shows data on the induction of CA (total chromosome aberrations per group) in bone marrow cells of rats following *in vivo* exposure to the tested products. None of the products induced statistically significant increase in the average number of chromosomal aberrations ($p > 0.05$) when compared with the negative control. As expected, treatment with

CP 40 mg kg⁻¹ b.w. led to a significant increase of total chromosome aberrations ($p < 0.05$).

DISCUSSION

Most of the regulatory agencies require for the pesticide registration, the assessment of a wide variety of potential human health and environmental effects associated to the use of the product. These tests evaluate whether a pesticide has the potential to cause harmful effects on humans, wildlife, fish, and plants, including endangered species and nontarget organisms, as well as, a possible contamination of water. Potential human risks range from short-term toxicity to long-term effects such as cancer and reproductive system disorders¹⁵.

The determination of the mutagenic behavior of pesticides requires tests of high sensitivity and specificity. A tiered strategy is often used in order to obtain reproducible and biologically relevant data. Stage 1, *in vitro*, uses bacterial gene mutation assays, assays measuring clastogenicity and aneugenicity, and assays measuring the induction of gene mutations in cultured mammalian cells. Stage 1 can detect most mutagenic hazards. Stage 2, *in vivo* testing in somatic cells of rodents, is required to determine whether *in vitro* positives are reproduced *in vivo* and to detect activity only produced in intact animals. In most cases, *in vivo* assessment is based on the micronucleus assay in rodent bone marrow. Stage 3, *in vivo* germ-cell testing, is rarely required for pesticides that have been shown to be mutagenic in somatic cells *in vivo*¹⁶.

The products were administered by gavage, since this is the most common route of human exposure. It is probably most appropriate for predicting effects in humans to utilize a route of exposure that most resembles that anticipated or known to be the route of human exposure¹⁷. Besides, the gastrointestinal tract is one of the most important sites where exogenous substances are absorbed¹⁸.

Chromosomal damage was implicated as the major cause for the appearance of micronuclei at the last stage

of mitosis and as an indicator of genotoxic insult to the nuclei¹⁹. Currently, rodent bone marrow chromosomal aberration and micronucleus assay are present in the guidelines from different countries and organizations. In the chromosomal aberrations test, none of the products showed genotoxic damage.

The MN assay is based on the frequency of MN, structures that originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division^{11,20}. The formation of MN is considered to be an effective biomarker of diseases and processes associated with the induction of DNA damage. The micronucleus assay of the Gluticid evidence a significative increase of the % MNPCE in females when compared to negative control group. A significant diminish of the PCE/NCE relation was also observed in this sex. These results allowed to state that Gluticid is a causative agent of genotoxic and citotoxic effects in somatic cells of mice at the tested dose. Taken into account that active principle of Gluticid is a mixture of *Pseudomona sp.* metabolites, further studies are needed to determine which of the components is the causative agent.

Negative results obtained with Bactivec® and Griselesf® are in accordance to the active ingredient of these products, *Bacillus thuringiensis* (Bt) and *Bacillus sphaericus* (Bsp), respectively. The U.S. EPA has determined that the numerous toxicology studies conducted with Bt microbial products show no adverse effects and it has concluded that these products are not toxic or pathogenic to humans^{21,22}. Our results agree with those obtained by Grisolia²³, which also tested *B.t.i.* in Swiss mice obtaining no bone marrow suppression²³.

Safety on Bsp formulations has been extensively tested, so EPA concludes that there is a reasonable certainty that no harm will result from an aggregate exposure to people, including infants and children, to residues of *Bacillus sphaericus*. The Agency has stated that there is reliable data to support the conclusion that Bsp is practically non-toxic to mammals, including infants and children and thus, a margin of exposure (safety) approach is not needed to protect adults or infants and children²⁴.

Jasmonic acid and its methyl esters are ubiquitous in plants. They have hormone properties, help regulating plant growth and development and they seem to participate in leaf senescence and in the defense mechanism against fungi²⁵. Indolacetic acid has influence in the vegetal growing, because of its regulation properties. Its main effect is performed at the root taking, performance increase and germination improvement²⁶.

Azadirachtin is the active ingredient of NeoNim 60 y OleoNim 80. Technical azadirachtin has been evaluated

for the potential to cause gene mutations in the *Salmonella typhimurium* strains at any dose (235, 50, 500, 5,000 micrograms/plate) with or without S-9 activation. The study was negative²⁷. The Ames test was negative with or without metabolic activation for the formulated product Azatin-EC. The UDS and Mouse Lymphoma studies were also negative²⁸.

No chromosome aberrations induction and increase of micronucleus formation were detected in six of the products. In the case of Gluticid, was found to be a causative agent of genotoxic and citotoxic effects in somatic cells of female mice at the tested dose. It could be concluded that the two assays included in our battery test has proved to be useful to obtain the genotoxic profile of tested products, allowing this results a more safety utilization of the products.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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