Chapter 5

Genotoxicity of the Neonicotinoid Insecticide Poncho (Clothianidin) on CD1 Mice Based on Alkaline Comet and Micronucleus Assays

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Abstract

Poncho is a commercial formulation of neonicotinoid insecticides and a new agrochemical in Mexico, and it has the active ingredient clothianidin. The genotoxic effects of this commercial formulation of clothianidin on CD1 male mice were analyzed using micronucleus and comet alkaline assays. Three concentrations of clothianidin (20, 40, and 80 mg/kg/body weight) as well as negative (deionized water) and positive controls (cyclophosphamide 40 mg/g/body weight) were intraperitoneally injected into groups of mice every 3 days for 21 days. Peripheral blood samples were drawn from the caudal vein and divided to carry out the comet alkaline and micronuclei assays. DNA damage was evaluated using three genotoxicity parameters: the comet frequency, the tail length, and the moment from 100 nuclei. Additionally, the micronuclei frequency was quantified in 2000 peripheral blood erythrocytes using Giemsa stain. The results of the comet assay showed that the neonicotinoid insecticide Poncho leads to a significant increase in these three genotoxic parameters and in micronuclei frequency in the peripheral blood erythrocytes of mice treated with either concentration as compared with negative controls. At 80 mg/kg/body weight of Poncho, higher micronuclei frequencies and many more DNA strand breaks were observed compared with the negative controls. This study demonstrates that the commercial neonicotinoid insecticide Poncho induces genotoxic effects in CD1 male mice.

Keywords: clothianidin neonicotinoid insecticide, DNA strand breaks, micronuclei frequency, peripheral blood cells

1. Introduction

The neonicotinoid pesticides are nicotine synthetic chemical compounds used in agriculture, veterinary, and human medicine. These pesticides are represented by three groups: nitromethylenes (nithiazine and nitenpyram), nitroguanidines (imidacloprid, thiamethoxam, dinotefuran, and clothianidin), and cyanoamidines (thiacloprid and acetamiprid) [1].

Diverse commercial formulations of neonicotinoid insecticides have been registered in Mexico, such as Jade (imidacloprid), Calypso (thiacloprid), and Poncho (clothianidin). Poncho® is one of the most widely used to treat maize seeds because of its broad-spectrum insecticide activity and relatively small risk to nontarget organisms and the environment. these pesticides have less persistence in the environment due to their low toxicity, no bioaccumulation in biological tissues, and high efficacy to control pests in agricultural crops, they are a replacement for pyrethroids, organophosphates, and carbamate pesticides [2].

Neonicotinoid insecticides act selectively on central nervous system as agonists of the nicotinic acetylcholine receptor (nAChR) in both invertebrates and vertebrates [3]. They are classified to be moderately toxic (category II) and slightly toxic (category III) to mammalian. Reviews of the safety of neonicotinoid insecticides have been performed by several regulatory agencies, and their authors have concluded that there are no indications of any human health effects [4].

Epidemiological studies have suggested a correlation between exposure to pesticides and increase the incidence of diseases and cancer. However, the toxic effects by exposure to pesticides depend on diverse factors, including time and exposure pathways, pesticide type, metabolism, diet, state of antioxidants, and general health [5]. It is important to evaluate the genotoxic actions of these new agricultural pesticides to increase the available toxicological data and to promote regular use that does not pollute the environment and leave pesticide residue in water and food sources where it is a possible risk to human health and the health of other organisms. Exposure to pesticides has also been associated with an increased incidence of functional alterations in the nervous [6], respiratory [7], reproductive [8], and immune systems [9].

Some investigations have demonstrated that clothianidin-based insecticides induce *in vitro* DNA damage in human peripheral lymphocytes [10] and affect the reproduction of rats [11] and birds [12], as well as DNA fragmentation in germinal cells and the inhibition or delay of embryonic development [13]. Because clothianidin represents a health risk and information on its *in vivo* genotoxic effects is scarce, the present study aimed to investigate the DNA damage caused by this agrochemical in the blood peripheral cells of CD1 mice through alkaline comet and micronuclei tests. The comet assay is an early effect biomarker that detects DNA single-

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and double-strand breaks, or apoptosis between others, induced by environmental genotoxins both *in vitro* [16] and *in vivo* [17].The micronucleus assay is one of the most widely used *in vivo* screening tests in genotoxicity testing [18]. Classically, a micronucleus is defined as a small extranuclear chromatin body originating from a centric fragment or a whole chromosome lost from the metaphase plate. Therefore, frequencies of micronuclei have been considered reliable biomarkers of both chromosome breakage and chromosome loss [18, 19]. Thus, a major strength of the assay is its ability to detect the effects of both clastogens [19] and aneugens [19].

2. Materials and methods

2.1. Preparation of the neonicotinoid insecticide dilutions

The neonicotinoid insecticide Poncho was donated by Bayer CropScience (México) for the genotoxicity assays. Poncho® (*E*-1-(2-Chloro-1, 3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine; RSCO-INAC-103K-301-342-048; flowable suspension: clothianidin, 600 g a.i/L) was diluted with deionized water; cyclophosphamide was also diluted in deionized water. The concentrations of clothianidin used were selected from our preliminary *in vivo* studies in mice using micronuclei and alkaline comet assays.

2.2. Animals

Two- to three-month-old CD1 male mice (~30–35 g body weight) were acclimatized for 2 weeks before the start of the treatments. The mice were housed in polypropylene cages with sawdust bedding at a controlled temperature of 20 ± 2°C and a humidity of 50 ± 10% with a 12-h light:12-h dark cycle. They were given a standard diet and water ad libitum in the Faculty of Sciences Biotery at University National Autonomous of Mexico. The animals were treated and housed in accordance with the Guide for the Care and Use of Laboratory Animals.

2.3. Experimental design

Twenty animals were divided into five groups of four animals each and intraperitoneally injected with 20, 40, or 60 mg/kg weight body of Poncho every 3 days for 21 days. A positive control group was intraperitoneally injected with 40 mg/kg weight body of cyclophosphamide, and a negative control group was injected with deionized water under the same conditions. Peripheral blood samples were obtained from the caudal vein of each mouse before treatment and every third day during treatment and used to carry out the alkaline comet and micronucleus assays. Additionally, body weight and food consumption were monitored before and during the treatments.

2.4. Alkaline comet assay

The alkaline comet assay was performed as previously described [14, 15]. Briefly, blood peripheral cells (1 μL) were mixed with 200 μL of low melting point agarose (0.5%) at 37°C, and 100 μL was placed on a frosted slide (Fisher) with normal melting point agarose (1%) and

covered with a coverslip. Two gels were made for each animal. The gels were solidified for 5 min at 4°C after they were immersed in a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) at 4°C for 1 h. Unwinding the DNA was realized in cold alkaline buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min. Electrophoresis was carried out at 25 V and 300 mA for 20 min. The gels were washed with neutralization buffer (0.4 M Tris, pH 7.5) and fixed with cold absolute methanol for 10 min. The gels were coded and stained with 70 μL of GelRed (10%) and observed in fluorescent microscope (Axiostar Plus Carl Zeiss) with an excitation filter (515–560 nm) and a barrier filter (590 nm) at 40× magnification using the Comet IV software. Three genotoxic parameters were quantified: (a) frequency of comets (nuclei with DNA damage), (b) length of tail (DNA fragmentation), and (c) tail moment in 100 consecutive nuclei (Figure 1)[20].

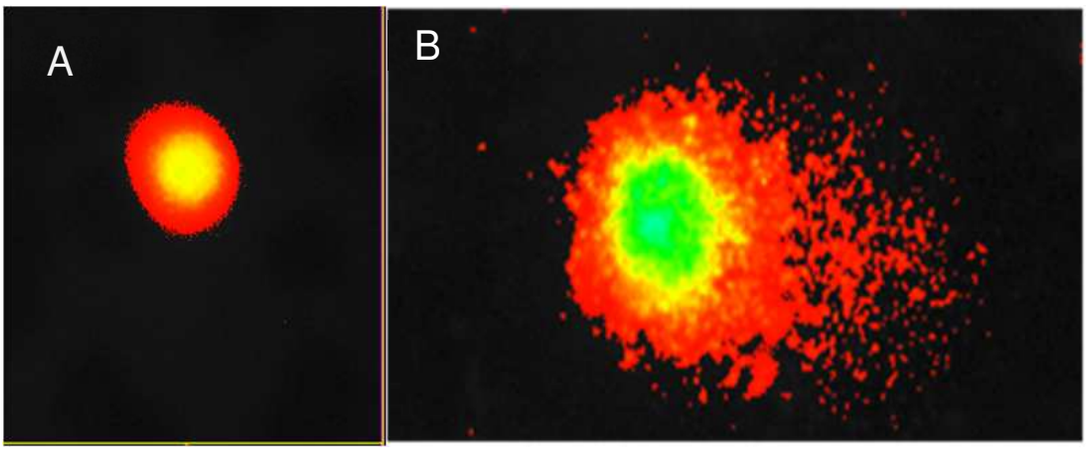

Figure 1. Peripheral blood cell from CD1 male mice. A: Nuclei without DNA damage (without comet) and B: Nuclei with DNA damage (with comet).

2.5. Micronuclei test

Two microliters of peripheral blood was used for blood smears (two for each animal), air-dried, and immediately fixed in absolute methanol and stained with Giemsa (10%) in Sorensen's solution for 6 min. The frequency of micronuclei (MN) was estimated by analyzing 2000 peripheral blood erythrocytes from each animal (1000 per slide) in each group by microscopy at 1000× magnification. The criteria for the identification of micronuclei were based on a standard procedure, and the analysis was restricted to micronuclei in the vicinity of the main nuclei (Figure 2) [18, 19,20].

2.6. Statistical analysis

Comet frequencies, tail lengths, tail moments, and frequencies of micronuclei are reported as the mean ± the standard error of the mean (SEM) obtained for one treatment group. One-way analysis of variance (ANOVA), Student *t* test, and Bonferroni test were used to determine significant differences between the treated groups and the negative and positive control groups using GraphPad Prism. The results were considered statistically significant at $P < 0.05$.

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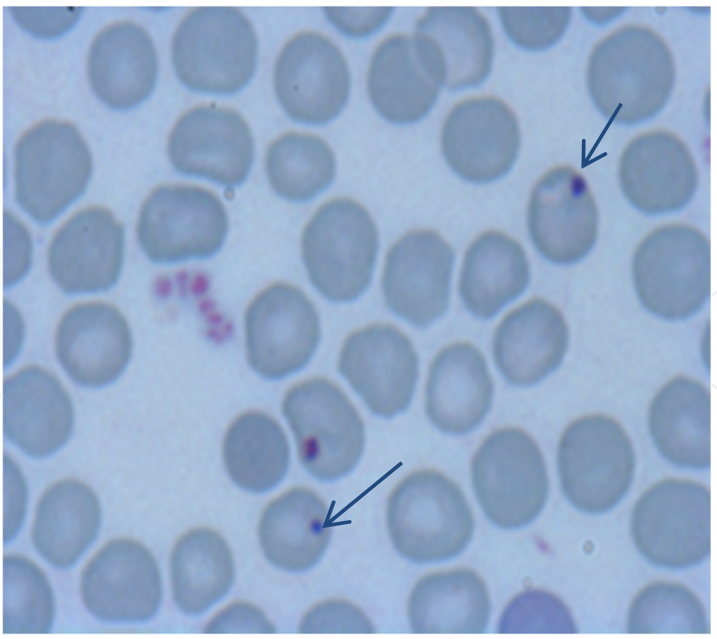

Figure 2. Peripheral blood erythrocytes with micronucleus from CD1 male mice after exposition with Poncho neonicotinoid insecticide.

3. Results

3.1. DNA damage induced by Poncho neonicotinoid insecticide in peripheral blood cells of CD1 mice

Table 1 shows the mean values of the comet frequencies, tail lengths, and tail moments measured from the peripheral blood cells of CD1 mice after the intraperitoneal injection of Poncho (20, 40, and 80 mg/kg body weight every 3 days) over 21 days. The genotoxicity results indicate that after the second administration of 20 mg/kg body weight of Poncho (at 6 days), there was no statistically significant effect on these three genotoxic parameters in the blood peripheral cells compared with either the negative control group at 6 days or basal levels in pretreatment mice. However, after the third administration with 20 mg/kg body weight of Poncho, the mean values of these genotoxic parameters were significantly greater than those of the negative control group and the pretreatment mice. This increase in induced DNA damage in the blood cells remained throughout 21 days of treatment.

After the second administration of 40 mg/kg body weight (at 6 days), the mean values of the three genotoxic parameters were significantly higher in the blood cells of the treated CD1 mice than in the negative control group or in the pretreatment mice. Furthermore, after the third intraperitoneal administration, the mean comet frequency, tail length, and tail moment were greatly increased compared with the negative control group and the pretreatment mice. A higher frequency of comets with greater tails occurred after sixth administrations (at 15 days), and apoptotic nuclei were observed after the seventh administration (results not shown).

Concentration of Poncho mg/Kg	Animals (n)	Genotoxic parameters means ± SEM	Repeted exposition day 0	3	(after days) 6	9	12	15	18	21
20	4	frequency of comets	4.0 ± 1.9	6.0± 0.8	4.0± 1.0	12.0 ±1.8*	16.0 ± 2.1*	24.0 ± 1.6**	36.0 ± 2.4**	44.0 ± 1.0**
		Tail lenght	16.33 ± 0.9	16.77± 0.9	18.22 ± 0.9	34.00 ± 0.9**	36.44± 2.4**	40.67± 2.9**	44.31± 2.2**	54.22 ± 2.2**
		Tail moment	0.36 ± 1.2	0.39 ± 0.4	0.56± 0.6	2.11 ± 0.9**	2.44± 2.9**	2.99 ± 2.0**	3.33± 2.0**	3.88 ± 2.0**
40	4	frequency of comets	4.0 ± 1.1	6.0± 1.2	18.00±1.0*	34.0 ± 0.6*	46.0 ± 2.0*	52.0 ± 2.2**	42.0 ± 1.3**	40.0 ± 1.9**
		Tail lenght	18.44 ± 0.9	21.08± 1.3	36.22±1.3**	48.00 ± 2.9**	36.44± 0.9**	60.67± 2.4**	44.31± 2.2**	54.22 ± 2.2**
		Tail moment	0.36 ± 0.9	0.88 ± 1.1	2.96 ± 1.9**	2.61 ± 1.4**	2.44± 1.2**	3.69 ± 1.6**	3.33± 1.0**	3.28 ± 1.2**
80	4	frequency of comets	4.0 ± 1.9	16.0 ± 0.8*	36.0± 1.0**	50.0 ± 0.6**	71.0± 1.4**	64.0± 1.2**	50.0 ± 0.9**	54.0 ± 2.1**
		Tail lenght	12.22 ± 0.9	36.77 ± 2.1*	43.22 ± 0.9**	66.90 ± 1.9**	86.48 ± 2.2**	62.90± 2.4**	64.66± 2.4**	52.82 ± 1.8**
		Tail moment	0.64 ± 1.4	3.12 ± 2.0*	3.99 ± 0.9**	5.66 ± 1.5**	6.88 ± 2.7**	4.99 ± 1.9**	5.12± 2.9**	4.67 ± 2.9**
Negative Control Group	4	frequency of comets	6.0± 1.9	2.0± 0.8	4.0± 1.0	6.0± 0.6	6.0 ± 07	8.0 ± 1.0	6.0 ± 1.0	4.0± 2.0
		Tail lenght	14.33 ± 0.8	22.77± 2.4	23.11 ± 2.4	18.40 ± 2.1	16.24± 0.9	20.99± 1.4	14.31± 0.8	22.33 ± 1.4
		Tail moment	0.52 ± 1.2	0.77 ± 0.8	0.83 ± 1.0	0.99± 1.2	1.44± 0.8	0.99 ± 0.5	0.86± 0.9	0.78 ± 1.6
CP Positive control	4	frequency of comets	4.0 ± 1.2	10.0 ± 0.8*	18.0 ± 1.0**	24.0 ± 0.6**	30.0 ± 0.9**	56.0 ± 1.4**	44.0 ± 2.7**	34.0 ± 2.3**
		Tail lenght	18.48 ± 1.6	26.77± 2.2*	33.22 ± 2.4**	38.00 ± 1.2**	42.44± 2.2**	70.67± 2.3**	44.31± 0.9**	34.82 ± 1.8**
		Tail moment	0.72 ± 1.2	1.99 ± 1.9*	3.66 ± 2.3**	3.88 ± 2.1**	5.44± 2.0**	4.56 ± 2.0**	3.99± 1.2**	3.24 ± 0.4**

Genotoxic parameters means =100 nuclei for organism in each group

*Significant differences among treated groups by analysis of Student ´t° test p <0.05, and therefore the Bonferroni comparison test was applied, p<0.01 **

CP=40 mg/kg of cyclophosphamide

Table 1. DNA damage induced by Poncho neonicotinoid insecticide in peripheral blood cells of CD1 male mice

In CD1 mice treated with 80 mg/kg body weight of Poncho insecticide, the means of the three genotoxic parameters significantly increased after the first administration (at 3 days) and remained higher than those of the negative control group and the pretreatment mice throughout the treatment (Table 1). This high concentration of insecticide produced severe DNA damage in the blood peripheral cells after three treatments, with 70% of cells producing comets and larger tail lengths and tail moments than the negative control group or the pretreatment mice. However, the mean comet frequency, tail length, and tail moment were significantly decreased after 15 days of administration compared with the negative control group and the

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	Animals	**Treatment squeme**		**(after days)**					
Concentration	(n)	day 0	3	6	9	12	15	18	21
of Poncho		Frequency of micronuclei (means ± SEM)							
mg/Kg									
20	4	0.5 ± 0.91	0.7 8± 0.82	1.66 ± 0.70*	2.94 ± 0.82*	2.67 ± 0.39*	2.44 ± 0.66*	3.69 ± 1.04*	5.46 ± 1.04*
40	4	0.8 ± 0.86	0.92 ± 0.23	2.33 ± 0.42*	3.82 ± 0.44*	4.99 ± 0.99*	8.66 ± 0.79*	14.0 ± 1.09*	16.0 ± 1.02*
80	4	0.7 ± 0.33	0.74 ± 0.84	3.55 ± 0.48*	5.45 ± 0.62*	7.0 ± 0.49*	12.0 ± 0.99.*	18.0 ± 0.99*	24.0 ± 0.93*
Negative Control Group	4	0.8 ± 0.93	0.92 ± 0.80	0.57 ± 1.09	1.0± 0.69	0.78 ± 0.79	1.0 ± 0.88	1.0 ± 0.90	1.0± 0.76
positive Control Group CP	4	0.5 ± 0.29	4.96 ± 0.83*	5.88 ± 1.00*	7.99 ± 1.01*	9.78 ± 1.10*	13.09± 1.42*	13.0 ± 1.22*	14.0 ± 1.02*

n=2000 peripheral blood erythocytes forn organism in each group

*Significant differences among treated groups by analysis of Student °t° test were found the p <0.05, and therefore the Bonferroni comparison test was applied, p<0.01

CP= 40 mg/Kg of cyclophosphamide

Table 2. Micronuclei frequency in perypheral blood erythrocytes from CD1 male mice treated with Poncho neonicotinoid insecticide

pretreatment mice. Greater numbers of apoptotic nuclei were observed throughout the course of treatment (data not shown).

3.2. Micronuclei frequency in peripheral blood erythrocytes from CD1 mice treated with Poncho insecticide

The micronuclei frequencies in the peripheral blood erythrocytes of CD1 male mice treated with the Poncho insecticide are shown in Table 2. No significant increase in micronuclei frequency was observed in the blood cells of the male mice after the first administration with 20 mg/kg body weight Poncho compared with either negative control or pretreatment mice. However, after the second administration, the frequency of micronuclei was significantly higher in the treated mice than in the negative controls or the pretreatment mice ($P < 0.05$). This significant induction of micronuclei was observed and remained constant throughout the treatment course.

At concentrations of 40 and 80 mg/kg body weight, no significant differences in micronuclei frequency compared with controls or pretreatment mice were observed after the first administration first ($P < 0.05$). However, after the second administration, both 40 and 80 mg/kg body weight of Poncho significantly increased the micronuclei frequency in treated mice compared with negative control or pretreatment mice ($P < 0.05$). The observed production of chromosomal damage was consistent in all of the groups after administration throughout the treatment course (Table 2).

Cyclophosphamide, which was used as a positive control, produced significant increases in DNA strand breaks and the frequency of micronuclei in the blood peripheral cells of treated CD1 male mice compared with the negative control and pretreatment mice. An increase in the percentage of micronuclei was observed after the second injection and throughout the treatment course (Tables 1 and 2).

4. Discussion

The present study was designed to evaluate DNA damage using alkaline comet assay and measurements of micronuclei frequency in CD1 male mice treated every 3 days over a 21-day period with low (20 mg/kg body weight) and high concentrations (40 and 80 mg/kg body weight) of the neonicotinoid insecticide Poncho (clothianidin). We found that clothianidin induced concentration- and time-dependent increases in DNA strand breaks and micronuclei frequency in the peripheral blood cells of CD1 mice. Our results are in agreement with data obtained from *in vivo* genotoxic studies performed with other neonicotinoid insecticides. In Wistar albino rats intraperitoneally injected with 50 or 100 mg/kg body weight of commercial acetamiprid and imidacloprid formulation, Confidor® and pure imidacloprid for 90 days induced a significant increase of frequency of micronuclei and chromosomal aberrations in the bone marrow cells [21]. Therefore, this increase in micronuclei frequency was observed at 300 mg/kg body weight of imidacloprid (Confidor®) in rat bone marrow cells [22]. At 0.2 or 0.5 mg/kg imidacloprid in dry soil for 14 days produced spermatic malformations in *Eisenia fetida*. The same species of nematode exposed to 0.05, 0.1, 0.2, or 0.5 mg/L imidacloprid for 2 h caused a significant increase in DNA damage in coelomocytes with relationship response doses [23, 24]. Also, an increase in the frequency of micronuclei and DNA strand breaks were observed in peripheral blood erythrocytes of *Rana N. Hallowell* exposed to 0.05, 0.1, 0.2, 0.5, 8, or 32 mg L^{-1} imidacloprid (pure compound) for 7 days [25].

When comparing the genotoxic action of the three tested concentrations of Poncho insecticide in CD1 male mice, we observed that at low concentrations (20 mg/kg body weight), DNA damage occurred after the third administration, and the genotoxic effect was constant throughout the treatment course. This increase was also observed at concentrations of 40 and 80 mg/kg body weight of insecticide, although these concentrations produced even higher DNA damage at a few time points during the treatment course. Additionally, we observed that the long-term administration of high concentrations (40 and 80 mg/kg body weight) of clothianidin produced an apoptotic response in the peripheral blood cells of this animal model. Perhaps this apoptotic response was associated with the increased induction of DNA damage in the blood cells, as well as a mechanism to eliminate damaged cells from the blood tissue. Another interesting observation in this study was that the increased DNA damage observed upon treatment with either concentration of Poncho insecticide injected every third day persisted for at least 48 h. These results indicate that the observed DNA damage was not repaired and was possibly caused by excessive reactive oxygen species (ROS) production or greater accumulation of the pesticide or intermediate metabolites that interfered with the

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repair mechanisms of the mice or interacted with DNA to produce the fragmentation visualized through micronuclei and comet formation.

The genotoxic and apoptotic actions induced *in vivo* by Clothianidin in peripheral blood cells may be related to the following factors: (a) the penetration or absorption of the pesticide in the body and the metabolic pathways that differentially produce intermediate metabolites, (b) the generation and overall levels of ROS or free radicals that subsequently inactivate detoxification mechanisms leading to increased oxidative stress, and (c) alterations in DNA damage repair mechanisms. Another probable explanation for the effects is the accumulation of the original molecule, which may produce DNA single-strand breaks and induce the formation of micronuclei in the blood tissue.

The biotransformation of clothianidin insecticide has been studied in rats [26] and mice [27, 28]. The *in vivo* metabolic pathways of clothianidin in the liver microsomes of rats have been described. The major metabolic reactions (Phase I metabolism) are oxidative demethylation by microsomal CYP-450 enzymes to form *N*-(2-chlorothiazol-5-ylmethyl)-*N*-nitroguanidine [28] During Phase II metabolism, it is metabolized mainly to *N*-methyl-*N*-nitroguanidine and 2-(methylthio) thiazole-5-carboxylic acid via glutathione conjugation and other guanidine derivatives [27, 28]. Perhaps, some of these metabolic products are involved in the genotoxicity of clothianidin in CD1 male mice.

In general, the involvement of ROS has been postulated as a possible mechanism of the genotoxicity and oxidative damage to cellular molecules such as lipids, proteins, and nucleic acids caused by neonicotinoid pesticides [29]. Rats treated with 32 mg/kg body weight of clothianidin by gavage for 90 days exhibited significantly increased sperm DNA fragmentation and apoptosis caused by oxidative stress [11, 12]. Similar effects were observed in the germinal cells of male and female quails exposed to 50 mg/kg body weight of clothianidin [13].

Poncho neonicotinoid insecticide is a genotoxic agent that is likely to be a source of free radicals or ROS in CD1 mice. ROSs, such as superoxide anions (O_2●), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH–), are highly reactive with DNA and produce DNA damage, including single- and double-strand breaks and micronuclei formation. The increased DNA damage detected in the blood peripheral cells of the CD1 mice in this study could be considered potential premutagenic lesions, which may increase the risk of cancer and contribute to autoimmune diseases and other alterations [30].

We used cyclophosphamide as a positive control because it is a potential genotoxic agent in experimental animals [31]. It is a DNA alkylating agent and a good inducer of oxidative stress. Cyclophosphamide is metabolized to 4-hydroxycyclophosphamide, aldophosphamide mustard, and acrolein by cytochrome P-450 enzymes, which are involved in the increase of oxidative stress, DNA damage, and apoptosis in mammalian cells and produce cytotoxicity in various target organs [32].

In our research, cyclophosphamide induced a significant increase in the three genotoxic parameters measured as well as an increase in the frequency of micronuclei in the peripheral blood cells of CD1 male mice. These results are in agreement with and reported in numerous

in vivo genotoxic studies of cyclophosphamide, which induced sister chromatid exchange, micronucleus, DNA strand breaks, and other genetic alterations [33].

The DNA damage observed in the blood tissue of CD1 male mice treated with both low and high concentrations of neonicotinoid insecticide Poncho suggests potential health risks to humans and animals. Furthermore, the data obtained with two genetic assays corroborate the *in vivo* genotoxic action of this neonicotinoid insecticide and support its regular use since this insecticide does not pollute the environment or leave pesticide residue in water and food sources where it is a possible risk to ecosystems.

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