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Cytogenetic damage in peripheral blood cultures of *Chaetophractus villosus* exposed *in vivo* to a glyphosate formulation (Roundup)

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ABSTRACT

Different concentrations of a glyphosate formulation, Roundup® Full II (66.2% glyphosate) were tested in culture peripheral blood of armadillo *Chaetophractus villosus* with cytogenetic biomarkers like mitotic index (MI), chromosomal aberrations (CA), sister chromatid exchange (SCE) and cell proliferation kinetics (CPK) by means of replication index. Adults animals of both sexes were exposed to RU at four concentrations ranging from 0.026 mL RU solution to 0.379 mL RU daily in oral treatment with the same volume (0.2 mL) during 7 days. We analyzed the induced damage at different times considering T0 as control value, one (T1), seven (T7) and 30 days (T30). One day after, only the higher concentration shows MI significant differences ($p < 0.05$), at T7 the frequency increases and at T30 it decreases reaching T0 values. The analysis of CA frequencies shows that only 0.106 mL RU/day exhibit significant differences *vs* T0 values. A great variability is expressed in the values of standard deviation (SD) and in the wide confidence intervals of the media. One day after treatments (T1) all four concentrations shows significant differences in SCE *vs* T0 values. Replication Index (RI) does not show significant differences. The dose-response behavior was not observed in either CA or SCE. The consistency of the findings obtained with the same biomarkers *in vitro* support the idea of expanding studies in order to characterize the risk doses for these mammals.

1. Introduction

The advance of the agricultural frontier has put at risk the biodiversity of different regions of Argentina and worldwide because of the significant increase in the use of agrochemicals (Ronco et al., 2016). Although today the spectrum of chemical agents in use and therefore under study has expanded, characterizations of the unwanted effects of herbicides mainly refers to Glyphosate (GLI) and its different commercial formulations (Bolognesi et al., 1997). Different mixtures and formulations based on GLI with different adjuvants were used from the beginning; being Roundup (RR or Ready Roundup) the formulation most widely used worldwide (Carrasco et al., 2012). Experimental data revealed that several agrochemicals exhibit genotoxic properties, therefore biological monitoring provides a useful tool to estimate the potential genetic damage associated with the exposure to them.

There are several reports in the literature about the toxic and genotoxic potential effects of GLI. Some of them refer to GLI, the active ingredient, but others to the formulations including Roundup. Initially a large proportion of studies were referred to the safety of these agrochemicals and comprised from cellular to organism levels including humans (Vigfusson and Vyse, 1980; Williams et al., 2000; Donadío De Gandolfi et al., 2009). Afterwards, different studies gathered evidence about the role of the commercial formulations of glyphosate isopropylamine salt in the induction of cytotoxic and genotoxic damage (Cox, 1998; Grisolia, 2002; Tsui and Chu, 2003; Çavas and Könen, 2007; Gasnier et al., 2009; Clair et al., 2012). In the 1990s the US Environmental Protection Agency (USEPA) classified GLI as a compound category E indicating "evidence of no carcinogenicity for humans" and the Us Forest Service (2010) reported that "glyphosate has no adverse effects in humans". In a recent report EPA's Office of Pesticide Programs established that for cancer descriptors, the available data and weight-of-evidence clearly do not support the descriptors “carcinogenic to humans”, “likely to be carcinogenic to humans”, or “inadequate information to assess carcinogenic potential” (USEPA, 2016). For the

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"suggestive evidence of carcinogenic potential" descriptor, considerations could be looked at in isolation; however, following a thorough integrative weight-of-evidence evaluation of the available data, the database would not support this cancer descriptor. The strongest support is for "not likely to be carcinogenic to humans" at doses relevant to human health risk assessment.

At the same time USEPA´s report established that the evaluation was focused on studies on the active ingredient glyphosate and that additional research could be performed to determine whether other components influence the toxicity of glyphosate formulations, given these identified data gaps.

On the other hand, recently the GLI was reclassified by the International Agency for Research of Cancer (IARC, 2015) as probably carcinogenic to humans (Group 2A). This IARC review highlights the need to continue studies of the effects of GLI in areas where its use has expanded because of its deleterious consequences for both human health and biodiversity (Mesnage et al., 2014; Séralini et al., 2014; Larramendy, 2017). However, the debate about the safety of agrochemicals still exists today (Williams et al., 2000; IARC, 2015; USEPA, 2016; Tarazona et al., 2017). The Roundup, like other commercial agrochemicals, contains different interactive compounds. The animals, plants and humans are exposed to formulations and not to glyphosate and excipients separately. In this context the genotoxic study of complex mixtures and not pure glyphosate becomes important.

Different species have been proposed as biomonitors of environmental contamination in order to characterize the deleterious effects of agrochemicals and to contribute to handle the different formulations in areas where various potentially toxic agents are applied. These biomonitors allow to analyze the biological consequences of a given exposure or even detect the occurrence of an exposure (when it is unnoticed or considered harmless) by providing new tools for individual or environmental control (Beeby, 2001; Talent et al., 2002; Embry et al., 2010; Amaral et al., 2012a, 2012b; Poletta et al., 2008, 2011; Burlibaşa and Gavrilă, 2011; Schaumburg et al., 2012). Studies of induced damage in experimental models are abundant in fish and amphibians, fewer in birds, and scarce in non-rodent mammals. In our laboratory, we have worked for several years in the characterization of *Chaetophractus villosus* (Xenarthra) performing genetic and cytogenetic research (Rossi et al., 2014, 2016) and studies on morphological, hormonal and seasonal reproductive parameters (Cetica et al., 2005; Luaces et al., 2011a, 2011b, 2012, 2014).

In this context and taking into account the superposition of the natural geographical distribution of this species with the agricultural frontier in Argentina, we undertook studies of GLI genotoxicity in this organism to characterize potential deleterious effects with the aim of use it as a sentinel organism in its natural distribution range. Initially, baseline values of chromosome aberrations (CA) and sister chromatide exchanges (SCE) were established in adult individuals from pristine areas by means of an *in vitro* design in culture of peripheral blood lymphocytes (Rossi et al., 2016)

Since there are data gaps between the formulations applied in the field and the active principle (GLI) we decided to use Roundup (RU) in our experiments because it is one of the most commonly utilized in our country and worldwide. In the first place, we studied the effects of different concentrations of RU *in vitro* on lymphocyte cultures of animals from areas free of exposure to agrochemicals (Luaces et al., 2017). Then these experiments were the basis for implementing the experimental design *in vivo* that is presented here. The potential *in vivo* genotoxic effects of RU in adult specimens of *C. villosus* were evaluated by the following biomarkers: mitotic index (MI), frequencies of CA, SCE and cell proliferation kinetics (CPK).

2. Materials and methods

2.1. Chemicals

Roundup® Full II formulation (66.2% glyphosate) was used. RU is a liquid water soluble herbicide, containing glyphosate potassium salt [N-(phosphonomethyl) glycine monopotassium salt, C3H7KNO5P] as its active ingredient (a.i.) (CAS No. 70901-12-1). Roundup® is a registered trademark of Monsanto Company. For lymphocyte culture, RPMI-1640 medium (Gibco, USA), fetal calf serum (Bioser, Argentina), antibiotics (penicillin and streptomycin, Sigma-Aldrich, USA), and phytohemagglutinin (PHA-M, Gibco, USA) were used. The analysis of CPK, the RI characterization and the SCE studies were performed with bromodeoxyuridine (BrdU, Sigma-Aldrich, USA) and Hoechst 33258 (Sigma-Aldrich, USA). May-Grünwald solution (Eosin–methylene blue solution, Merck, Argentina) was applied with Giemsa stain (Biopack, Argentina) for histological staining of blood cells.

2.2. Animals

A total of 12 adults (8 males and 4 females) of *C. villosus* were captured in Monteverde, Buenos Aires, Argentina (35°47'S, 59°99'W) in their natural geographic distribution, an area free of farming and urban activities which belongs to the natural distribution of this species, as shown in previous studies (Rossi et al., 2014). The area was selected to ensure that the animals had not been environmentally exposed to any xenobiotic since no activity associated with contamination risks is carried out there.

The animals were classified as adults taking into account that the weight was more than 3 kg, and in male specimens confirmed by sperm production. The average weight of male and female animals was 3.60 ± 0.34 kg and 3.34 ± 0.27 kg, respectively. All animals were identified by indelible numeration in the head. The procedures for the collection of blood samples and for housing and handling of animals in surgery were according to the guidelines of the Canadian Council on Animal Care (1993). The experimental procedures were approved by the Ethics Committees of the Universidad de Morón (CICUAL-UM; Acta HCS N° 607 21/12/2015) Prov. Buenos Aires, Argentina (PID 15003-16).

Standart cages with a floor area of 0.50 m^2 (1 × 0.5 m^2) were employed to house individuals. Cages were provided with softwood shavings as bedding and cleaned twice a week. The room temperature was maintained at 21 ± 2 °C and the light was controlled over the experimental design by a time switch to provide 12 h light (08:00–20:00) alternating with darkness. Commercial food premium for dogs with corn oil, fruits and vegetable and water were given *ad libitum* (Ferrari et al., 1998)

2.3. Experimental design and treatments

The animals were randomly divided into four groups (n = 3, two males and one female); they were given a subchronic exposure to RU for 7 days and afterwards we analyzed the possible remaining effects after 30 days post exposure.

Group 1: received 0.260 mL of a solution of Roundup® Full II (66.2% glyphosate), (RU).

Group 2: received 0.053 mL of a solution of Roundup® Full II (66.2% glyphosate), (RU).

Group 3: received 0.106 mL of a solution of Roundup® Full II (66.2% glyphosate), (RU).

Group 4: received 0.379 mL of a solution of Roundup® Full II (66.2% glyphosate), (RU).

Each group received the RU solution diluted in 'ultrapure' water Mili-Q in a daily oral treatment with the same volume (0.2 mL) during 7 days. The RU doses were selected according to the concentration of GLI found in water after agricultural practices (Peruzzo et al., 2008). Within

each of the four groups, the basal values (controls) were determined without RU exposition at the beginning of experimental design (T0).

2.4. Blood collection

Peripheral Blood samples were taken from the space between the first and second (or the second and third) ring of the tail using a sterile and heparinized 21-gauge needle (Luaces et al., 2011b). The extractions were performed 24 h before the first oral treatment with RU (group 1 or control group), or 1 day and 7days after the first RU exposition (in groups 2–5). In addition, an extraction was performed in all treated groups, 30 days after the last exposition of RU to evaluate the post treatment conditions.

2.5. Lymphocyte culture

Neubauer counting chamber, and blood smears stained with May Gründwald-Giemsa, were used to determine the number of lymphocytes per milliliter of blood in each sample, in order to determine the volume of blood required to produce test samples containing 800,000 lymphocytes/mL. Two cultures for each individual with BrdU 0.1 mL (1 mg/mL) were performed to analyze SCE, AC and CPK. Blood samples were cultivated for 72 h at 34 °C according previous studies (Rossi et al., 2014) in a final volume of 10 mL of RPMI 1640 medium supplemented with L-glutamine, 10% fetal calf serum, antibiotics (250 U/mL penicillin and 250 mg/mL streptomycin), and 0.2 mL of phytohaemagglutinin.

Two hours before cell harvest, colchicine (Sigma-Aldrich) was added at a concentration of 1 μg/mL. After centrifugation for 5 min at 600 rpm, cells were suspended in 0.075 M KCl and incubated at 37° for 20 min, then after another centrifugation the supernatant was discarded and cells were fixed in methanol: acetic acid (3:1). Metaphases obtained by the standard cytogenetic air-dry method (Evans et al., 1964) were controlled in slides and stained according to Hoescht technique to study CA, SCE and CPK (Perry and Wolff, 1974)

2.6. Measurements of Mitotic index, chromosome aberrations, sister chromatid exchanges and cell proliferation kinetics

Mitotic index inhibition was scored as the number of metaphases per at least 1000 nuclei. The frequency of CA was calculated as the number of cells with structural chromosomal aberrations in 100 metaphases. All slides were coded for blind analysis and gaps were excluded in the calculation of chromosomal aberrations frequencies. Both values (MI and CA) were expressed as percentages. CPK was determined considering first mitosis (M_1) (both chromatids dark-stained), second mitosis (M_2) (one chromatid of each chromosome dark-stained) and third and/or subsequent mitosis (M_3) (a proportion of chromosomes with both chromatids light-stained or mixed). The RI was established in at least 100 metaphases per individual and it was calculated as follows:

$$RI = [1(\%\text{of cells in } M_1) + 2(\%\text{of cells in } M_2) + 3(\%\text{of cells in } M_3)]/100$$

SCE frequency was determined in M_2. Centromeric exchanges were not included in the total SCE counts. SCE was analyzed in 60 metaphases per individual. Metaphases were photographed using a Leitz DMRB microscope and a Leica DFC 300 FX digital camera (Leica Microsystems).

2.7. Statistical analysis

The data for males and females were analyzed together since in previous work and no differences were found between the sexes (Luaces et al., 2017). Differences in CA frequencies between treated samples and the negative controls (without RU) were analyzed using the Kruskal-Wallis test by ranks (with significance set at $p < 0.05$), followed by the Kruskal-Wallis all-pairwise comparison test for contrasts between treatments. SCE values comparisons were made using one-way

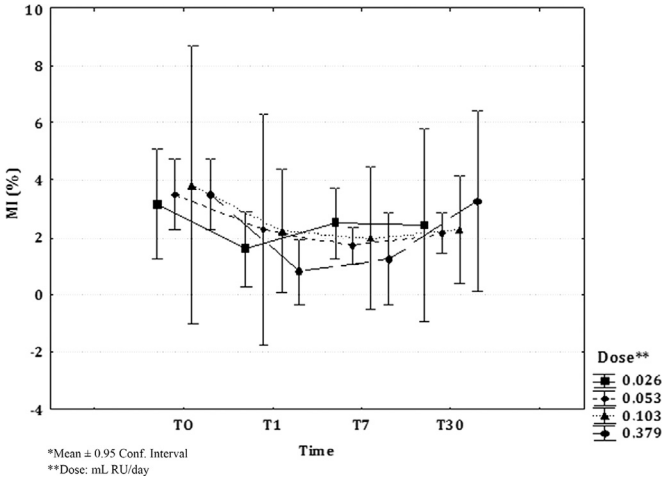

Fig. 1. Variation in mitotic index (MI, %)* with different concentrations of RU** at time 0, 1, 7 and 30 days after treatments.

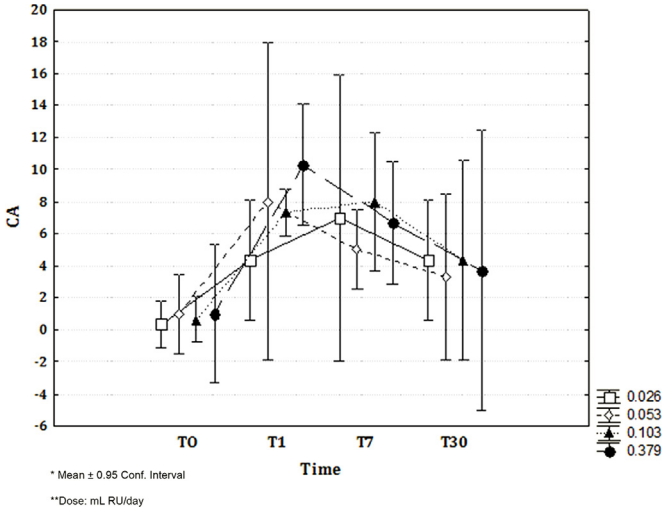

Fig. 2. Variations in the frequency of chromosomal aberrations (CA)* with different concentrations of RU** at times 0, 1, 7 and 30 days after treatments.

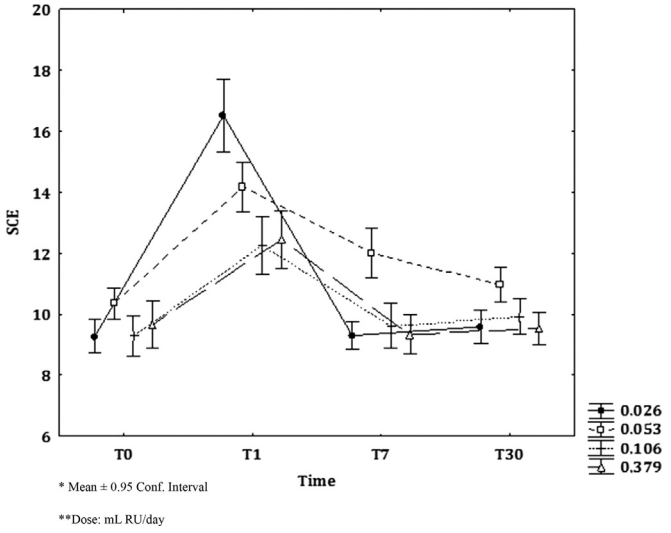

Fig. 3. Variations in sister chromatide exchange (SCE)* frequencies at different times after treatments with RU**.

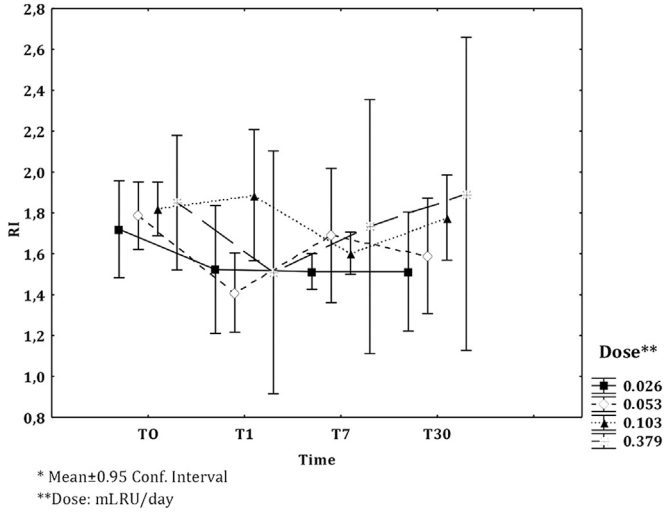

Fig. 4. Variations in Replication Index (RI)* throughout the experiments.

ANOVA (with significance set at $p < 0.05$) and the post hoc Bonferroni test for the comparison of media.

The differences are illustrated by graphs representing the mean ± 0.95 confidence intervals (see Results: Figs. 1–4).

3. Results

All the specimens of *C. villosus* were exposed to RU during seven days following the schedule experimental exposed in Materials and Methods and none of the genotoxic effect biomarkers analyzed differed significantly between sexes. Table 1 illustrates the results obtained for mitotic index (MI) and chromosomal aberrations (CA) at different times and with different doses of RU during the experiment. The variations in MI throughout the experiments are represented in Fig. 1. There are no differences among the concentrations at time 0 (T0). One day after (T1), only the higher concentration shows significant differences with basal values ($p < 0.05$), after 7 days (T7) the frequency increases ($p < 0.05$) and thirty days after (T30) it reaches the control values (T0).

Regarding CA (Fig. 2), at the beginning of the treatments (*T0*) there were no significant differences among the animals before they received all four RU concentrations. One day after, (*T1*) the frequencies of CA rise with all concentrations when compared with T0 values although there is a great variability that is expressed in the values of standard deviation (SD) and in the wide confidence intervals of the media (Table 1 and Fig. 2). As a consequence, there are no significant differences with the two lower concentrations, although with the 0.106 and 0.379 mL RU/day treatments there are significant differences ($p < 0.05$) when compared with basal levels. When compared with CA frequencies at *T0*, *T1* and T30, only 0.106 mL RU/day give significant differences with *T0*. Thirty days after treatments (*T30*) CA media values tested are lower than those obtained for *T1* and *T7*. They also present a great variability, no clear dose response effect and no significant differences are found (Table 1 and Fig. 2). Regarding the kind of CA induced, most of them are chromatid breaks, being chromosome breaks the second one in frequency (Table 1).

The use of BrdU in the peripheral blood culture of *C. villosus* (see Section 2) allows for analyzing CA in the first cycle and *SCE* in the second cycle as well as the characteristics of their *CPK*. Table 2 shows the frequencies of *SCE* and *RI* as well as the percent of cells in metaphase from the first three cycles (M_1, M_2, M_3) after *T*1, *T7* and *T*30 from the beginning of treatments (*T0*).

One day after treatments (T1) all four concentrations give significant differences in SCE frequencies when compared with control values (T0) (Table 2 and Fig. 3). *T7* and *T30* afterwards, in all frequencies are in range with those of T0 except in treatments with 0.053 mg RU/day. In this case there are significant differences relative to controls at T7 ($p < 0.05$) and even though at T30 the frequency diminishes and does not show significant differences with that of T0, it is significantly higher when compared with the other concentrations. Replication Index do not show significant differences due to the great variability observed that is expressed in the wide confidence limits of the media. Neither there is a clear dose response, although with 0.053 mg RU/day at T1 a significant diminution is observed (Table 2 and Fig. 4).

Table 1
Chromosomal aberrations (CA) and mitotic index (MI, %) after treatments of *C. villosus* with Roundup (RU).

Time[a]	Dose[b]	MI[c] (%)	CA[c,d]	CA[e]				
				B'	B"	CC	DC	A/P
0	0.026	3.17 ± 0.76	0.33 ± 0.58	1	–	–	–	–
	0.053	3.50 ± 0.50	1.00 ± 1.00	3	–	–	–	–
	0.106	3.83 ± 1.95	0.67 ± 0.58	2	–	–	–	–
	0.379	3.50 ± 0.50	1.00 ± 1.73	3	–	–	–	–
1	0.026	1.60 ± 0.53	4.33 ± 1.53	10	2	1	–	–
	0.053	2.27 ± 1.62	8.00 ± 4.00	17	4	–	–	3
	0.106	2.23 ± 0.87	7.30 ± 0.58***	17	4	–	–	1
	0.379	0.80 ± 0.46	10.33 ± 1.53***	25	4	–	1	1
7	0.026	2.50 ± 0.50	7.00 ± 3.61	17	3	–	–	1
	0.053	1.70 ± 0.26	5.00 ± 1.00	10	5	–	–	–
	0.106	2.00 ± 1.00	8.00 ± 1.73	16	7	–	–	1
	0.379	1.27 ± 1.27	6.67 ± 1.53	16	2	1	–	1
30	0.026	2.43 ± 1.36	4.33 ± 1.53	11	2	–	–	–
	0.053	2.17 ± 0.29	3.33 ± 2.08	9	1	–	–	–
	0.106	2.27 ± 0.75	4.33 ± 2.52	13	–	–	–	–
	0.379	3.27 ± 1.27	3.66 ± 3.51	10	1	–	–	–

*** Significant differences with respect to T0 ($p < 0.05$).
[a] Time is expressed in days.
[b] Dose: mL RU/day.
[c] Data are presented as mean ± standard deviation for n = 3.
[d] Chromosomal aberrations (CA)/100 cells ± standard deviation.
[e] Total number of CA in 300 cells scored (B': chromatid breaks; B": chromosome breaks; CC: complex aberrations; DC: dicentric chromosome; A/P: aneuploidy or polyploidy).

Table 2
Sister chromatid exchanges (SCE) and replication index (RI) after treatments of *C. villosus* with Roundup (RU).

Time[a]	Dose[b]	SCE ± SD[c]	M1(%)	M2(%)	M3(%)	RI ± SD[c]
0	0.026	9.27 ± 2.63	42.00	43.00	15.00	1.72 ± 0.09
	0.053	10.34 ± 2.46	41.00	39.33	19.67	1.79 ± 0.07
	0.106	9.28 ± 3.20	38.00	42.00	20.00	1.82 ± 0.05
	0.379	9.66 ± 3.75	36.33	42.33	21.33	1.85 ± 0.13
1	0.026	16.51 ± 5.75*	55.33	37.00	7.67	0.98 ± 0.12
	0.053	14.17 ± 3.89*	63.00	33.00	4.00	1.41 ± 0.08
	0.106	12.26 ± 4.53*	31.67	48.00	20.33	1.89 ± 0.13
	0.379	12.43 ± 4.54*	55.33	38.33	6.33	1.51 ± 0.24
7	0.026	9.30 ± 2.21	51.67	45.33	3.00	1.51 ± 0.03
	0.053	12.01 ± 3.95*	40.67	49.67	9.67	1.69 ± 0.13
	0.106	9.62 ± 3.56	47.00	45.67	7.33	1.60 ± 0.04
	0.379	9.33 ± 3.12	40.33	46.00	13.67	1.73 ± 0.25
30	0.026	9.59 ± 2.62	50.33	42.67	7.00	1.51 ± 0.12
	0.053	10.97 ± 2.79	49.00	42.00	9.00	1.59 ± 0.11
	0.106	9.91 ± 2.79	38.33	45.67	16.00	1.78 ± 0.08
	0.379	9.53 ± 2.49	28.33	54.00	17.67	1.89 ± 0.31

[a] Time is expressed in days.
[b] Dose: mL RU/day.
[c] Mean of SCE/60 cells ± standard deviation.
* Significant differences with respect to T0 ($p < 0.05$).

4. Discussion

Many genetic events may lead to genotoxicity regarding scientific and mechanistic aspects. The testing strategy should cover all the possible mechanisms of genotoxicity, *i.e.* gene mutation and chromosomal aberrations including both clastogenic and aneugenic effects. Besides, the assessment of genotoxicity represents an essential component of the safety assessment of all types of substances. Several *in vitro* tests are available at different stages of development and acceptance, yet they are not considered at present sufficient to fully replace animal tests needed to evaluate the safety of substances (Corvi and Madia, 2017). It is known that the most controversial aspects of GLI toxicity are related to its potential chronic toxicity, that is to say the effects that are produced by extended exposure to low concentrations of a chemical. These effects can be cumulative and are not observed immediately; on the contrary they are expressed in the medium or long term (Mañas et al., 2009a, 2009b).

The direct effect of GLI on early mechanisms of morphogenesis in vertebrates caused concern about its effects not only on the specific organisms considered targets but also on other species of the fauna and flora because the biodiversity in sowing and surrounding areas as well as human offspring in exposed populations could be affected (Mc Laughlin and Mineaub, 1995; Bolognesi et al., 1997; De Roos and Svec, 2005; Dimitrov et al., 2005; Mañas et al., 2009b; Paganelli et al., 2010; Alvarez-Moya et al., 2011). In this context, new studies with different biomarkers and experimental designs were developed analyzing GLI and its formulations, giving results that revealed induced damage *in vitro* and *in vivo* assays in different species of plants (Alvarez-Moya et al., 2014) and animals, such as fishes (Cavas and Konen, 2007; Cavalcante et al., 2008; Simoniello et al., 2008), amphibians (Cattaneo et al., 2011; Lajmanovich et al., 2013, 2015; Soloneski et al., 2016), reptiles (Poletta et al., 2008, 2009; López González et al., 2013; Schaumburg et al., 2016; Burella et al., 2017). In mammalian species a recent review reported a wide diversity of results (Kier and Kirkland, 2013; Kier, 2015). Studies on non-conventional experimental animals exposed to pesticides have been published during the current year (Zebral et al., 2017; Larramendy, 2017).

Chaetophractus villosus is a native mammal species useful to test the potential genotoxic effect of RU in natural populations because of its geographic distribution in Argentina and taking into account that the armadillo is a fossorial species, the oral exposure is considered the primary route of concern (USEPA, 2016). Previous work of Rossi et al. (2016) and Luaces et al. (2017) showed that the parameters studied here were good indicators in order to evaluate genotoxic damage. Then, the same tests and biomarkers previously analyzed *in vitro* (Luaces et al., 2017), CA and SCE, were applied. CA assays detect both structural and numerical aberrations. Structural CA are of two types: chromatid and chromosome and include breaks, deletions and rearrangement (OECD, 2015). Numerical CA generally results from the loss of an entire chromosome giving rise to aneuploid cells.

The study of the *in vitro* effect of the RU in peripheral blood cultures of adult specimens of both sexes from pristine zones as well as the baseline values of MI and CA show a coincidence in the type of chromosomal rearrangement detected in the *in vivo* studies reported here (Rossi et al., 2016; Luaces et al., 2017). Chromatid aberrations prevail in all the studies performed (Table 1, Fig. 2).

The basal levels of CA at T0 do not show statistical differences suggesting that there were no previous differential sensibility to RU in the treated specimens. Regarding the induction of CA, one day after the beginning of treatments only the higher dose gave significant differences ($p < 0.05$) and though at T1, and T7 the media values rise, the great variability observed does not allow to establish significant differences (see Table 1, Fig. 2). To the best of our knowledge, no description of CA in *C. villosus* of our wild fauna has been reported previously. In that sense, this one is an original contribution and the first register of CA, SCE and RI *in vivo* adult armadillos (Xenarthra) exposed to RU.

In this results reported here we detected differences in the MI at the end of exposure (T1) only with the highest concentration (Table 1 Fig. 1). There is a large variability among individuals, but it can be inferred that these concentrations would not act as cytostatic or cytotoxic since MI values are in the order of those previously observed in cytogenetic *in vitro* studies or in historical values for this Xenarthra species (Rossi et al., 2016; Luaces et al., 2017).

While there is limited evidence of genotoxic effects in some *in vitro* experiments, i*n vivo* effects are given more weight particularly when the same genetic endpoint was measured, which is consistent with current OECD guidance. Frequencies of CA analyzed *in vivo* here, shows a great variability that make it difficult to establish significant differences associated to genotoxic effect for most of the analyzed doses. These results are in line with the observations of Sivikova and Dianovsky (2006) who reported no statistically significant increases in CA in bovine lymphocytes treated with GLI (62% pure) following 24-h exposure. At the same time, Dimitrov et al. (2005) analyzed CA in bone marrow C57BL mice (males only) using water as vehicle for oral administration of RU and sampling at different times. They found negative results but significant decreases in MI at all doses and time points. Although they use a different cellular type, the results are in concordance with ours in culture of lymphocytes of *C. villosus* where no dose-response relation was observed in the induction of genotoxicity. The findings obtained 30 days (T30) after the end of treatments indicate that the initial frequencies (T0) are restored. It seems that the repair mechanisms in this mammal are efficient after the first day of exposure (T0) thus the data could allow to interpret a subchronic effect (Table 1 and Fig. 2).

Results obtained when *C. villosus* RU exposed samples are evaluated after *T7* show a great variability, all confidence intervals are superimposed, therefore no clear differences in concentration effects can be detected. Treatments with 0.053 mg RU/day are higher to those of T0, a possible explanation to these observations is that the animals used for this concentration have a different sensitivity or metabolism of the drug than those utilized for the other treatments. Additional studies with *in vivo* lymphocyte culture and post-exposure time analysis were not found in the literature. It is necessary to enlarge the sampling but it is a main problem of the *in vivo* experimental design when one works with wild specimens of medium or big size that at difficult to keep in laboratory.

Different reports of positive results for DNA damage endpoints indicate that GLI and its commercial formulations tend to elicit DNA damage effects at high or toxic dose levels, but the data suggest that this is due to cytotoxicity rather than to DNA interaction.

Some authors consider that GLI and typical commercial formulations are not genotoxic since this formulated activity is perhaps associated with the surfactants present in many commercial formulations (Rossi et al., 2016; Luaces et al., 2017). According to EPA (2016) most of the positive findings reported *in vivo* were seen at relatively high doses that are not relevant for human health risk assessment.

The sister chromatid exchange test is an assay that permits to evaluate the consequences of primary DNA damage and, although the mechanisms of action for the chemical induction are unclear, the assay gives information about the instability of the genome exposed to potential genotoxic agents. Positive SCE findings were reported in two works using bovine lymphocytes (Lioi et al., 1998a; Šiviková and Dianovský, 2006) and in two studies with human lymphocytes (Lioi et al., 1998b; Mesnage et al., 2014). Although Lioi et al. (1998a, 1998b) demonstrated differences at some doses in all these studies the induction did not show a clear dose response, that is also the case in the present *in vivo* work performed in *C. villosus*. In genotoxic studies it is not unusual that the some compounds do not show a dose-dependent effect in the condition of the experiment. The general reasons are that higher doses may exert toxic effects in the target cells, so more damaged cells die and the genotoxicity is not expressed at higher concentrations.

It is noteworthy that in the peripheral blood cultures studied here,

no cytotoxicity was observed with any of the tested doses when cells in M1 were studied to analyze CA and in M2 for SCE, neither when RI was characterized all throughout the experiments. In our experimental design only one day after the beginning of treatments (T1) all four concentrations show significant differences ($p < 0.05$) in SCE frequencies (Table 2 and Fig. 3). In all the *in vivo* studies in *C. villosus* the induction of CA as well as the SCE did not demonstrate a clear dose response. Similar conclusions are drawn when RI was analyzed (Table 2, Figs. 3 and 4). Sivikova and Dianovsky (2006) referred that the observed increases in SCEs did not show a clear concentration related effect across a 40-fold increase in the doses tested, therefore they could not confirm a dose response relationship.

Most probably the results obtained with RU exposed specimens of *C. villosus* regarding the analysis of CA and SCE frequencies are due to the high variability observed among individuals, although no differences were detected between sexes, a larger sample would show a greater uniformity of data. The consistency of the findings obtained by applying the same biomarkers *in vitro* (Luaces et al., 2017) and *in vivo* in spite they do not show a dose-response ratio, support the idea of expanding studies in order to characterize the risk doses for these mammals.

5. Conclusions

The findings presented here may help to inform discussions among government agencies with regard to different formulations of RU potential genotoxic effects in order to protect both public and environmental health. From a public health point of view, one of the main objectives of toxicology is to eliminate hazardous substances from our environment. In particular, genotoxicity assessment theoretically is aimed at eliminating genotoxic agents as soon as possible (Nesslany, 2017). From the point of view of conservation biology, protection protocols specific to preserve biodiversity have to be designed immediately with the purpose of eliminating soils or surface water contaminants in areas where herbicides or pesticides commercial formulations such as the RU are widely used on the farm agriculture, which is the case of Argentina and other American countries.

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Conflict of interest

The authors declare no conflicts of interest.

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