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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