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Chaetophractus villosus as a sentinel organism: Baseline values of mitotic index, chromosome aberrations and sister chromatid exchanges

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ABSTRACT

Sentinel species are useful tools for studying the deleterious effects of xenobiotics on wildlife. The large hairy armadillo (*Chaetophractus villosus*) is the most abundant and widely distributed mammal in Argentina. It is a long-lived, omnivorous, burrowing species, with fairly restricted home ranges. To evaluate the level of spontaneous genetic damage in this mammal, we determined the baseline values of several genotoxicity biomarkers. The study included 20 *C. villosus* adults of both sexes from eight pristine localities within its geographic distribution range. Genotoxicity analysis was performed on 72-h lymphocyte cultures, using mitomycin C as positive control. We obtained the baseline values of mitotic index ($MI = 10.52 \pm 0.30$ metaphases/total cells, $n = 20$), chromosome aberrations ($CA = 0.13 \pm 0.22$, $n = 20$), sister chromatid exchanges ($SCE = 6.55 \pm 0.26$, $n = 6$) and replication index ($RI = 1.66$, $n = 6$). MI and CA did not show significant differences ($P > 0.05$) among localities or between sexes. No significant differences in MI, CA, SCE, and RI ($P > 0.05$) were found between values from the pristine localities and historical data. There were significant differences in CA, SCE, and RI ($P < 0.05$) between lymphocyte cultures from pristine localities and those exposed to mitomycin C. We propose the large hairy armadillo as a sentinel organism for environmental biomonitoring of genotoxic chemicals due to its abundance, easy manipulation, well-known biology, the fact that it is usually exposed to different mixtures and concentrations of environmental contaminants, and the baseline values of genetic damage characterized by MI, CA, SCE and RI as biomarkers.

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1. Introduction

Chemical contamination of the environment, particularly due to long-term effects of low-level chronic exposures, has been associated with the decline or disappearance of animal populations [1,2] and adverse effects on human health [3–5]. Environmental contaminants can affect many vertebrate groups, including rodents and amphibians [6–9]. Contaminants may have chronic effects [10] such as immune suppression, endocrine disruption, reproductive inhibition or failure, teratogenesis, carcinogenesis and cellular and molecular alterations, including DNA damage and cell death [8,9,11–13].

Contaminants in the environment may be from natural or anthropogenic sources and may be of physical, chemical, or biological origin [14]. In Argentina, intensive agricultural practices have led to environmental changes [15]; the increasing use of transgenic crops is associated with the application of environmentally toxic agrochemicals for pest control [16].

Sentinel species are useful tools for studying the interactions between organisms and contaminants, acting as early alarm signals of the effects of xenobiotics on wildlife [17]. A suitable sentinel species should have the following properties: ubiquitous distribution; abundance; ease of manipulation; long life; tolerance to

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Table 1
Locality, geographic coordinates and sex of the specimens of *C. villosus* analyzed.

Specimens	Locality	Latitude	Longitude	Sex
1	Monteverde	35°47′S	59°99′O	F
2	Monteverde	35°47′S	59°99′O	F
3	Monteverde	35°47′S	59°99′O	F
4	Monteverde	35°47′S	59°99′O	M
5	Bahía Blanca	38°63′S	62°25′O	F
6	Bahía Blanca	38°63′S	62°25′O	M
7	Gral. Madariaga	36°99′S	57°15′O	F
8	Gral. Madariaga	36°99′S	57°15′O	M
9	Pellegrini	36°16′S	63°16′O	M
10	Pellegrini	36°16′S	63°16′O	F
11	Loma Verde	35°25′S	58°40′O	F
12	Loma Verde	35°25′S	58°40′O	M
13	Loma Verde	35°25′S	58°40′O	M
14	Navarro	35°00′S	59°27′O	F
15	Navarro	35°00′S	59°27′O	M
16	Saladillo	35°63′S	59°78′O	F
17	Saladillo	35°63′S	59°78′O	M
18	Santo Domingo	30°48′S	65°06′O	F
19	Santo Domingo	30°48′S	65°06′O	F
20	Santo Domingo	30°48′S	65°06′O	M

F: female; M: male.

the bioaccumulation of toxic substances; well-characterized life-history traits [18,19]. Many species endemic to America, *e.g.*, common carp, broad-snouted caiman, and aquatic macrophytes, are being used in studies of environmental contamination and ecological risk assessment [17,20,21]. Despite their importance in the ecosystem, mammals are often excluded from these studies. Among these, xenarthrans may include sentinel species for biomonitoring, since they are long-lived, omnivorous and burrowing animals, with fairly restricted home ranges [22]. However, studies of genetic toxicology in this group are lacking.

Chaetophractus villosus, the large hairy armadillo, is endemic to South America, where it occupies a broad range of environments. Its distribution extends from Gran Chaco of Bolivia, Paraguay and Argentina (18°20′S) to southernmost Patagonia, including Tierra del Fuego province (Argentina) and the Magallanes Region (Chile) (55°15′S) [23]. Currently, *C. villosus* is found throughout Argentina (Fig. 1), where it is the most abundant armadillo species. In this country, its distribution overlaps with intensive agricultural and/or highly contaminated areas due to the rapid expansion of the agricultural frontier [16,24].

The presence of xenobiotics, herbicides, and pesticides may cause genetic alterations with life-threatening physiological consequences for wildlife populations [25,26]. Several short-term techniques are applied as genotoxicity biomarkers in wild species because of their sensitivity to detect chromosomal and DNA damage [27,28].

Chromosome aberrations, micronuclei, and sister chromatid exchanges are the most frequently used cytogenetic endpoints in hazard identification assays [29,30]. We have determined baseline values of these parameters in *C. villosus*.

2. Materials and methods

Twenty adults of *C. villosus* (ten females and ten males, Fig. 1a) were captured from eight pristine localities within the species' geographic range during 2014 (see Table 1 and Fig. 1) [31,32]. Individuals were sexed by measuring the anal-genital distance. Blood samples were taken between the first and second or the second and third rings of the tail using sterile and heparinized 21-gauge needles [33]. Animals were released at the capture site after processing. Animal handling conformed to the standards of the International Council for Laboratory Animal Science.

Two whole blood samples per animal were cultured for 72 h at 34 °C in 10 mL of RPMI 1640 medium supplemented with L-glutamine (Gibco, Grand Island, NY, USA) [34], 10% fetal calf serum (BIOSER, Argentina), antibiotics (250 U/mL penicillin and 250 mg/mL streptomycin, Sigma–Aldrich, St. Louis, MO, USA), and phytohemagglutinin M (PHA, Sigma–Aldrich, 2% v/v). Cell viability was measured using trypan blue and only samples with >95% viable cells were cultured; otherwise, cell concentration was adjusted. Cell mixture was placed on a hemocytometer (Neubauer chamber) for counting leukocytes, while the lymphocytes were counted from blood smears. The temperature of the lymphocyte culture was set at 34 °C because the body temperature of *C. villosus* ranges between 33 and 36 °C [35]. All samples had a cell viability of about 800,000 lymphocytes/mL, indicating appropriate conditions for the application of this technique in the studied species [35]. Bromodeoxyuridine (BrdU, 1 mg/mL, Sigma–Aldrich) was added 24 h after culture initiation, followed by treatment with colchicine (Sigma–Aldrich), 0.1 μg/mL, for 1 h before cell harvest.

For each locality, positive control values were obtained by adding mitomycin C (MMC, Sigma–Aldrich), 0.03 μg/mL, to a third lymphocyte culture after a 48-h incubation period. Chromosome preparations were then obtained according to standard cytogenetic methods [36]. To characterize SCE and cell proliferation kinetics, slides were stained with 3% Giemsa in tap water or using the fluorescence-plus-Giemsa (FPG) technique [37].

The MI was calculated by the number of metaphases in at least 2000 lymphocyte nuclei and CA by the number of structural rearrangements in 200 metaphases, with results expressed as percentages. Baseline values of MI and CA obtained from the pristine localities were compared with historical data to detect variations over time.

The analysis of SCE was carried out on six randomly chosen adults (three females and three males), based on previous studies [38,39]. SCE was counted in 60 metaphases per individual and centromeric exchanges were excluded from total counts.

Cell proliferation kinetics was determined by SCE analysis, considering cells in first mitosis (M_1; chromosomes with both chromatids dark-stained), second mitosis (M_2; each chromosome with one dark-stained chromatid) and third and/or subsequent mitosis (M_3; chromosomes with both chromatids light-stained or with one dark-stained and one light-stained chromatid). The cell replicative index (RI) was calculated from counts of at least 200 metaphases per individual, according to the following equation: RI = 1 (% of cells in M_1) + 2 (% of cells in M_2) + 3 (% of cells in M_3)/100 [40].

Slides were coded for blind analysis and gaps were excluded from the calculation of CA frequencies.

Metaphases were photographed using a Leitz DMRB microscope equipped with a Leica DFC 300 FX digital camera (Leica Microsystems, Wetzlar, Germany).

Data distribution was evaluated using histograms and the Shapiro–Wilks test was used to test for normality. A nested analysis of variance (ANOVA) was used to determine the effects of the three independent variables (locality, individual and sex) on MI, with "individual" being nested within "locality". A generalized linear model (GLM) with a binomial distribution and logit-link function was used to analyze CA.

Variables with normal distribution were analyzed by the Student's *t*-test, while variables with non-normal distribution were analyzed with the Mann–Whitney test. The Student's *t*-test was used to test statistical significance for differences in MI between values from pristine localities and historical data, and for differences in MI, SCE and RI between values from pristine localities and the positive control. The Mann–Whitney test was used for statistical analysis of CA. Mean ± standard deviation (SD) were calculated for all the variables and $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using R version 3.1.2 [41].

3. Results

The 20 *C. villosus* individuals sampled from the pristine localities had a karyotype $2n = 60$ with 58 autosomes and an XX/XY sex chromosome system, in accordance with previous studies on this species.

Table 2 shows mean values of MI and CA of the armadillos from the pristine localities. The baseline values for MI and CA were 10.52 ± 0.30% and 0.13 ± 0.22%, respectively. No significant differences in MI or CA were found among individuals from the same or different localities and between sexes ($P > 0.05$ in all cases). These MI and CA values of the armadillos from pristine localities were compared with historical data (MI = 10.51 ± 1.08% and CA = 0.13 ± 0.22%) obtained from 20 free-living *C. villosus* individuals sampled across the species' distribution range between 1990 and 2013 (data not shown). No significant differences in MI or CA were observed between values from pristine localities and historical values ($P > 0.05$).

A significant increase in DNA damage was detected in cultures exposed to MMC (CA = 16.25 ± 2.07%) compared with values from pristine localities ($P < 0.05$). Chromatid breaks were the only type of CA represented in both lymphocyte cultures from pristine localities and those exposed to MMC (Fig. 1b). With regard to the cell proliferation kinetics, the frequencies of M_1, M_2, and M_3 cells were 41.2%, 51.1% and 7.7%, respectively (Fig. 1b–e). Data of RI and SCE of

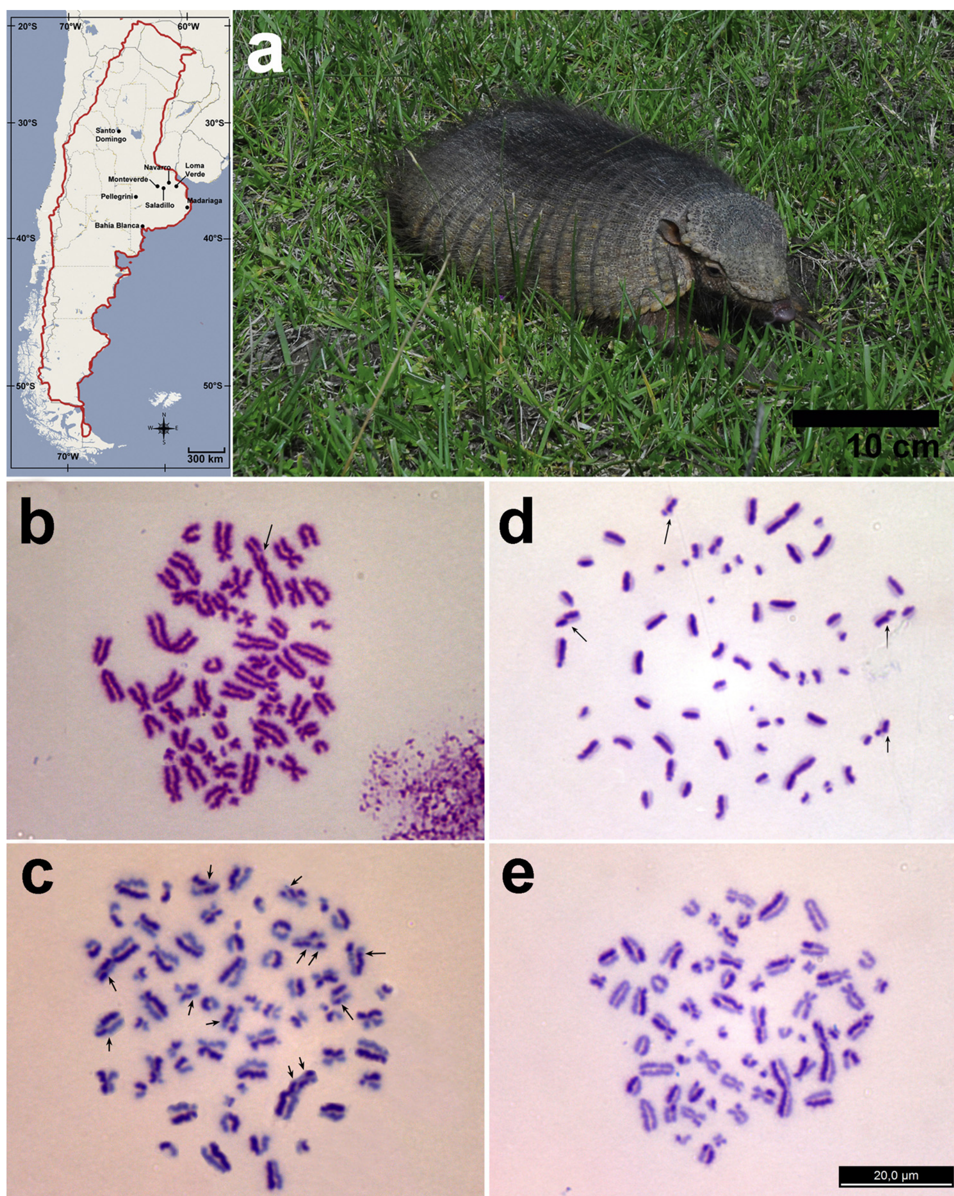

Fig. 1. Map showing the geographic distribution of *C. villosus* and the eight pristine localities sampled. (a) A male of *C. villosus* adult specimen. (b–e) Chromosomic aberrations (CA) and sister chromatid exchange (SCE) analysis in *C. villosus* . (b) M_1 showing chromatid break (arrow) in a male of *C. villosus* metaphase, ($2n = 60$, XY). (c) M_1 of a male *C. villosus* showing sister chromatid exchange (arrows) basal value. (d) M_2 of a male *C. villosus* exposed to mitomycin C showing sister chromatid exchange (arrows). (e) M_3 of a male *C. villosus*.

Table 2
Mitotic index (MI) and chromosome aberrations (CA) of the analyzed specimens of *C. villosus*.

Specimens	Localities	N° of cells examined	MI (%) (mean ± SD)	CA (%) (mean ± SD)[a]	N° of cells examined (MMC)	CA (%) (mean ± SD)[a] (MMC)
1–4	Monteverde	10392	10.54 ± 0.26	0.13 ± 0.23	2247	16.22 ± 2.18
5,6	Bahía Blanca	5168	10.21 ± 0.17	0.13 ± 0.25	2128	17.06 ± 3.09
7,8	Gral. Madariaga	5056	10.45 ± 0.43	0.13 ± 0.25	2159	14.85 ± 1.60
9,10	Pellegrini	5005	10.60 ± 0.26	0.13 ± 0.25	2198	17.66 ± 2.66
11-13	Loma Verde	7694	10.72 ± 0.39	0.17 ± 0.26	2095	15.55 ± 1.87
14,15	Navarro	5051	10.52 ± 0.25	0.13 ± 0.25	2093	16.36 ± 1.58
16,17	Saladillo	5346	10.43 ± 0.28	0.13 ± 0.25	2323	17.44 ± 0.81
18-20	Santo Domingo	8087	10.56 ± 0.29	0.08 ± 0.20	2063	14.90 ± 4.09
Mean ± SD		–	10.52 ± 0.23	0.13 ± 0.22	–	16.25 ± 2.07[*]

MMC: mitomycin C.
No significant differences in baseline values of MI and CA were found among individuals from the same or different localities ($P > 0.05$ in all cases).
N°: number.
SD: standard deviation.
[a] CA counted in 200 metaphases.
[*] Statistically different from values obtained from pristine localities ($P < 0.05$, Mann–Whitney–Wilcoxon test).

Table 3
Cell cycle kinetics, replication index (RI) and frequency of sister chromatid exchange (SCE) of the analyzed specimens of *C. villosus.*

Specimens	Localities	M_1 (%)	M_2 (%)	M_3 (%)	N° of metaphases examined	RI	SCE/cell ± SD
3	Monteverde	37.86	50.49	11.65	206	1.74	6.56 ± 2.22
4		41.09	55.81	3.10	258	1.62	6.21 ± 2.60
11	Loma Verde	43.44	48.36	8.20	244	1.65	6.27 ± 3.79
12		39.45	50.46	10.09	218	1.71	6.69 ± 4.31
14	Navarro	40.87	52.17	6.96	230	1.66	6.72 ± 2.28
15		43.65	49.21	7.14	252	1.63	6.84 ± 2.60
Pristine localities values		41.19	51.14	7.67	–	1.66	6.54 ± 0.26
4 (MMC)	Monteverde	60.59	33.99	5.42	203	1.45	12.85 ± 2.32
12 (MMC)	Loma Verde	61.19	32.42	6.39	219	1.45	12.15 ± 2.71
14 (MMC)	Navarro	60.00	33.04	6.96	230	1.47	12.67 ± 3.21
Positive control values	60.58	33.13	6.29	–	1.46*	12.56 ± 0.36*	

MMC: mitomycin C.
N°: number.
SD: standard deviation.
* Statistically different from values obtained from pristine localities ($P < 0.05$, Student's *t*-test).

armadillo's lymphocyte cultures are shown in Table 3. The RI value from pristine localities (1.66) differed significantly ($P < 0.05$) from that of lymphocyte cultures exposed to MMC (1.46). The frequency of SCE from pristine localities was 6.55 ± 0.26 per cell (Table 3 and Fig. 1c), this value being significantly different ($P < 0.05$) from that of lymphocyte cultures exposed to MMC (SCE = 12.56 ± 0.36 per cell, Fig. 1d).

4. Discussion

Environmental contaminants may have detrimental effects on organisms, short-term and long-term. Thus, understanding their interaction with wildlife is of great importance [14]. In the last ten years, native sentinel organisms have become a new tool for monitoring potential biological effects of xenobiotics, with many species being identified as suitable candidates [20,42–45].

In this work, we have studied genotoxicity biomarkers in the armadillo *C. villosus*, the first species of Xenarthra to be examined in this manner. We selected this mammalian model because it exhibits characteristics that fit the definition of a sentinel species [18,19], particularly its wide distribution and longevity. It is difficult to make comparisons between this model and those reported in the literature because there are few genotoxicity studies in wild mammals and none in armadillos, although genotoxicity biomarkers have been used in other vertebrates, both in captivity [46] and in the wild [43,47–51].

Our research group has experience in cytogenetic [34,52], immunological [33], hormonal [53], developmental [54], and reproductive [55–57] aspects of this species. With regard to the cytogenetic aspect, we mainly focused on the karyotype and its variants and on the cytogenetic characterization using C—, G— and NOR— banding. The comet assay and micronucleus test are much more widely used as genotoxicity biomarkers [17] than the ones used in this study. However, our knowledge of cytogenetic and reproductive techniques in the armadillo model provides us with useful biomarkers to monitor the interaction between contaminants and this species.

Additionally, experience acquired during more than 20 years of fieldwork allows us to understand how agricultural expansion has led to environmental changes affecting the populations of *C. villosus* . This prompted us to standardize genotoxicity techniques for determining baseline values of MI, CA, and, for the first time, SCE and RI, to be used as biomarkers in *C. villosus*. The genetic health status of the armadillo can be characterized using peripheral blood or fibroblasts [58]. We used the former based on the fact that we are well-trained in armadillo handling [33,59] and on our background knowledge providing insight into the cytogenetic framework for the characterization of Xenarthra spp. [34,60]. The body temperature of *C. villosus* fluctuates between 33 and 36 °C [35], which is lower than that of other mammals, including humans. Therefore, we decided to set the temperature of *in vitro* cultures at 34 °C rather than the usual 37 °C [61] and obtained vigorous lymphocyte proliferation, confirming that it is the optimal temperature for their growth [34,60].

Prior to this study, free-ranging armadillos from some localities were found infected with filariae. As expected, they showed abnormal cell counts and hence the baseline values of all parasitized animals were excluded from the analyses.

In a previous study, we observed chromosome rearrangements in some *C. villosus* populations, such as pericentric inversion (localities of Jacinto Aráuz, General Madariaga and Pellegrini) and short arm deletion (locality of Loma Verde) [34], which were absent in the karyotype of the individuals from the eight pristine localities considered here.

The genotoxicity biomarkers obtained from the pristine localities allow us to draw two important conclusions: first, the low variability in analyzed baseline values among the different pristine localities points to *C. villosus* as a possible sentinel species; second, the results obtained for the biomarkers fulfill the need to establish baseline values for the analysis of potential toxic or genotoxic exposure. The MI values of the pristine localities are in agreement with historical biomarker data, adding new information on this species. The basal MI values reported for humans (7.5 ± 0. 7%) [62] are similar to the basal values for the armadillo, reported here, whereas the values for bovines are lower (1.8 ± 2.0%) [63].

The cultures exposed to MMC showed a significantly lower number of metaphases than those of pristine localities, suggesting interference with the cell cycle. This indicates that MMC is a good positive control and, hence, MI inhibition may be considered a suitable genotoxicity biomarker in *C. villosus*. As in the case of MI, the baseline value of spontaneous structural chromosome rearrangements showed no significant differences among individuals from the same locality, reinforcing the idea that the variables chosen remained stable among individuals from different pristine localities during the study period. In addition, no statistically significant differences were observed between pristine and historical values, indicating low parameter variability. The frequency of CA in human lymphocytes exposed to MMC was 32.4 ± 4.9%, as compared to a baseline value of 1.0 ± 1.2% [64]. MMC is used as a positive control in cultured human lymphocytes because it induces DNA damage [29]. Our results reveal that it can also be used as a positive control for the lymphocytes of *C. villosus*.

Other *in vivo* studies were carried out in cultured lymphocytes of rabbits and monkeys after intraperitoneal injection of MMC [65,66]. In *C. villosus*, the induction of CA by MMC suggests that it can be used as a positive control for cultured lymphocytes because this system contains the necessary enzymes for its metabolism.

Genetic toxicology endpoints such as CAs and SCEs have also been used as biomarkers because they provide relevant information for early detection of the carcinogenic effect of exposure to xenobiotics [67]. Therefore, the analysis of CA in *C. villosus* as a sentinel species may be useful for assessing health risk associated with environmental contamination, which has a negative effect on living organisms, including humans.

In this study, we characterized the cell-cycle kinetics of *C. villosus* for the first time. The baseline frequency of SCE was similar to that of humans [29,68] and lower than that of Platyrrhini monkeys (5.6 ± 0.26 SCE/cell for *Saimiri boliviensis* and 4.2 ± 0.3 SCE/cell for *Alouatta caraya*) [69]. MMC increased SCE frequency of both *C. villosus* and humans, but it was raised to about double (from 6.55 ± 0.26 SCE/cell to 12.56 ± 0.36 SCE/cell) in the former and to five times (31.77 ± 7.24 SCE/cell) in the latter, compared to the baseline value [29]. These results indicate that SCE frequency is a good indicator of chromosomal damage in *C. villosus*, despite the higher sensitivity of humans to MMC. An increase in SCE frequency was also reported for other mammal species exposed to different mutagenic agents. In rats, SCE frequency also doubled (from 7.67 ± 2.91/cell to 22.00 ± 4.36/cell) after exposure to methyl methanesulfonate [70], while in bovines chloramphenicol caused a small but statistically significant effect on the basaline SCE frequency (6.37 ± 1.07/cell) [71].

The identification of suitable biomarkers and sentinels is gaining interest. Although all organisms respond to environmental pollution, some species are better indicators of habitat quality than others, because they are easier to capture and manipulate and show higher sensitivity to toxic effects.

Many animal groups have been identified as valuable sentinels for genotoxicity assessment of environmental pollutants, including birds [72,73], marine mammals [74,75] and aquatic organisms [76]. So far, only non-mammalian species endemic to Argentina have been proposed as sentinel organisms [17,20,42]. In comparison, *C. villosus* would be better suited for this role, since it occurs throughout the country over a broader range of terrestrial habitats. In addition, the armadillo has physiological and behavioral advantages over non-mammals. It is phylogenetically related to humans [54], sharing physiological systems and mechanisms of exposure, assimilation, and clearance of substances [77]. Therefore, the armadillo model may be more appropriate for assessing human risk. On the other hand, armadillos show burrowing behavior, causing them to come into contact with pollutants in deep soil layers and groundwater, which were transported from the surface by percolation and leaching [78]. Contaminants may be detected in areas well beyond the application site, depending on the physical and chemical characteristics of the soil. We encourage the use of the large hairy armadillo as a sentinel species for genotoxicity monitoring of environmental pollutants.

Conflict of interest

The authors declare no conflicts of interest.

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