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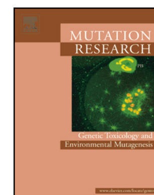

Spontaneous genetic damage in the tegu lizard (*Tupinambis merianae*): The effect of age

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ABSTRACT

Several studies indicate that certain factors such as age, sex or nutritional status among others, may affect the level of DNA damage, both induced and spontaneous, so it is very important to consider them for a more accurate interpretation of the findings. The aim of this study was to analyze the influence of age, sex, and nest of origin on spontaneous genetic damage of *Tupinambis merianae* determined by the comet assay (CA) and the micronucleus (MN) test, in order to improve reference data for future in vivo studies of xenobiotics exposure in this species. Sixty-five tegu lizards of three different ages: newborns (NB), juveniles (JUV) and adults (AD), both sexes and from different nests of origin were used. Blood samples were collected from the caudal vein of all animals and the MN test and CA were applied on peripheral blood erythrocytes to determine basal frequency of MN (BFMN) and basal damage index (BDI). The comparison between age groups showed statistically significant differences in the BFMN and BDI ($p < 0.05$). NB animals showed significantly higher BDI values in relation to JUV and AD ($p < 0.016$), but no statistically differences were found between the latter two. NB showed lower BFMN respect to other age groups, being statistically significant only when compared to AD ($p < 0.016$). BFMN or BDI showed no statistically significant differences between sexes or nests of origin ($p > 0.05$). A weak negative relationship was found only between BFMN and weight of NB tegu lizard ($p = 0.014$; $R^2 = 0.245$). Basal values of genetic damage obtained with both biomarkers in the tegu lizard evidenced that age is an intrinsic factor that should be taken into account to avoid misunderstanding of the results in future biomonitoring studies.
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1. Introduction

The study of biomarkers of chronic genotoxicity in sentinel species can facilitate detection of adverse effects in natural environments [1]. Toxicological research on lizard species has increased, but toxicity data on reptiles remains relatively sparse [2,3]. The Tegu lizard (*Tupinambis merianae*) has several attributes that make it attractive for the biomonitoring of natural environments: longevity, wide geographic distribution, plasticity to live in natural or anthropic habitats, and the fact that is a generalist predator. Also, as an ectotherm, its lower metabolic rate may retard the detoxication of xenobiotics.

The genetic material suffers injuries from both exogenous agents and the products of endogenous processes (e.g., reactive oxygen species), leading to macromolecular alterations and subsequent mutations [4,5]. DNA damage levels may be affected by factors such as age, sex, and nutritional status [6–9]. The effect of age has been evaluated in mammals [5,7,10,11], including humans [12], but it has been little studied in wild animal populations. In reptiles, another important biological factor is the “clutch effect”, that is, the genetic influence of the parents on the hatchlings associated with the incubation environment (e.g., temperature and humidity) [13]. Animals from different nests may respond differently to subsequent environmental conditions [14]. Knowledge of the factors that influence spontaneous DNA damage provides context for interpreting the genotoxic effects of xenobiotics [15]. The micronucleus test (MN) and the comet assay (CA) are sensitive short-term tests that allow one to measure different genotoxicity endpoints in

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particular cells [5,16], without prior knowledge of karyotype or cell turnover rate [17,18], parameters required by some other assays. Previously, we demonstrated the utility and sensitivity of the CA and MN tests in peripheral blood erythrocytes of the tegu lizard, and we proposed this native species as a sentinel organism for monitoring genotoxicity in their natural environments in Argentina [19]. Here, we have analyzed the influences of age, sex, and nest of origin on spontaneous genetic damage of *T. merianae*, as determined by the CA and MN tests, to provide reference data for future studies with this species.

2. Materials and methods

2.1. Animals

This study was approved by the Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (No. 05-11, Santa Fe, Argentina) and all animals were treated in accordance with the Reference Ethical Framework for Biomedical Research: Ethical Principles for Research with Laboratory, Farm, and Wild Animals [20], using non-invasive techniques of blood collection and minimizing stress and suffering by suitable management methods.

Newborn (NB; 1 day old; 24 animals), juvenile (JUV; 1 year old; 21 animals) and adult (AD; more than 4 years old, 20 animals; 10 males and 10 females) tegu lizards were used for this study. All lizards were weighed (Electronic Compact Scale, TH 5000, precision 0.1–1 g) and snout-vent length was measured (SVL, precision 0.1 cm). Adult lizards were obtained from the breeding stock in captivity in the "*Proyecto Iguana*" (PI, Lizard Project, Santa Fe, Argentina). Sex determination was performed through the scale mound method applied by Hall [21] and Yanosky and Mercolli [22]. (A white scale group elevated to form a little mound on the ventral dark surface, lateral to the vent, in adult males, allows differentiation from females. We were also able to distinguish the males by hypertrophy of the jaw muscles, which makes the neck appear as wide as the body [21]). The NB and JUV animals came from eggs collected in the Managed Natural Reserve "El Fisco" (30°11′26″S, 61°0′27″ O), located in Santa Fe Province, Argentina, corresponding to a Protected Natural Area (Law 12.930, 2008), where the tegu lizard occurs and no farming or other activity that may cause pollution is allowed, minimizing possible exposure of the eggs to xenobiotics prior to collection. Eggs were artificially incubated as part of the ranching program "*PI*" under controlled conditions of temperature (29–31.5 °C) and relative humidity (<20%). JUV lizards were maintained under controlled conditions from hatching until the time of study and were fed ad lib. three times per week, alternating with cleaning days. The food consisted of a mixture of 50% minced chicken heads/50% dry pellets and a variety of fruits (apple, banana), vegetables (pumpkin, carrot), and chicken eggs. The NB animals were maintained under controlled conditions for 24 h and then bled. NB and JUV lizards cannot be sexed due to lack of differentiation of the mound when animals are below 20 cm SVL [22].

2.2. Blood samples

Peripheral blood samples were obtained from the caudal vein as described by Olson et al. [23], with heparinized syringes and 21 G × 1″ needles for AD (1000 μl) and JUV (50 μl) lizards, and 25 G × 5/8″ needles for NB lizards (25 μl). For each sample, the MN and CA tests were applied on peripheral blood erythrocytes [19].

2.3. Micronucleus test (MN)

The MN test was performed according to Schaumburg et al. [19]. For each animal, two blood smears were made on clean glass slides. Slides were coded for 'blind' analysis and then examined under the Olympus C×21 microscope (total magnification 1000×). For identification of MN, we used the criteria of Schmid [24]. Erythrocytes (1000) were analyzed for each animal of all ages, recording the basal frequency of MN as follows: BFMN = number of erythrocytes with MN per 1000 erythrocytes counted.

2.4. Comet assay (CA)

The technique was based on the protocol of Schaumburg et al. [19]. All slides were coded for blind analysis, stained with ethidium bromide (EB) (2 μg/ml), and 100 randomly selected comet images (50 from each replicate) were analyzed per animal under a fluorescence microscope (Leica DMLB) at total magnification = 400. The baseline damage index per animal was reported: $BDI = 1n_1 + 2n_2 + 3n_3 + 4n_4$, where n_{1-4} are the numbers of cells in each damage category [19,25].

2.5. Statistical analysis

The statistical analysis was conducted with the software SPSS 17.0 for Windows. Mean and standard error (SE) were calculated from data of all animals. Since the data did not show normality or homogeneity or variance, BFMN and BDI were analyzed by non-parametric tests. We used the Kruskal–Wallis test, followed by the Mann–Whitney test, for comparison of BFMN and BDI values between age groups and the nest of origin. We applied the Bonferroni correction according to the number of analyses by pairs carried out, so that a value $p < 0.016$ was considered statistically significant. We then used the Student *t*-test for comparison of BFMN and BDI between males and females (only in the case of adults). Linear regressions were carried out to analyze the relation between BFMN and BDI with morphometric parameters (weight and SVL) of all animals. The value $p < 0.05$ was considered statistically significant.

3. Results

Table A.1 presents the average morphometric parameters and genotoxicity measurements for the groups of animals. (Individual animal results are shown in Table A.2.)

Statistically significant differences were found between the age groups for both BFMN and BDI ($p < 0.016$). We observed an age-dependent increase in BFMN. Newborn animals showed a lower BFMN but a statistically significant difference was found only when compared to adults ($p < 0.016$). In contrast, BDI decreased with age. Newborn animals had significantly higher BDI values compared to either juveniles or adults ($p < 0.016$); no significant difference was observed between the latter groups. No significant clutch effect on DNA damage was seen and there was no difference between male and female adults. A weak negative correlation was found only between BFMN and weight of newborns ($p = 0.014$; $R^2 = 0.245$). No relation was found between BDI and morphometric parameters of the animals, for any of the age groups.

4. Discussion

The effects of potential confounding factors should be reported in biomonitoring studies [26], especially when sentinel organisms from different (e.g., contaminated vs reference) sites are compared [27]. Literature evidence on the role of animal age in genotoxicity biomonitoring studies is inconsistent. Aging is accompanied by functional impairments resulting from endogenous and exogenous damage. A pronounced age-dependent increase of cytogenetic damage is generally found for MN frequencies, whereas inconsistent results have been reported for other genotoxicity endpoints [12,28].

In previous studies in reptiles, Poletta et al. [14] found no statistically significant differences in BFMN or DI between 4-month and 10-month old juvenile *Caiman latirostris*. The authors noted that the difference in age between these two groups was not large enough to have an influence on basal DNA damage of such a long-lived species. In mammals, Zúñiga González et al. [7,29] observed a greater frequency of MN in newborns and young compared to older animals (in *Rattus norvegicus*, *Canis familiaris*, *Giraffa camelopardalis*, *Odocoileus virginianus*, *Oryctolagus canniculus*). In another study, the authors investigated the variation of the MN frequency with age in the gray squirrel (*Sciurus aureogaster*) and found higher values in newborns than in adults [30]. A similar trend was observed in mice: erythrocyte BFMN was significantly higher in newborn than in adults; but in contrast, the rate of BDI in leukocytes increased with age [5]. These authors suggested that the reticuloendothelial system, as it matures, may "retire" cells with spontaneous MN from the circulation. Also, peripheral blood receives micronucleated erythrocytes from several hematopoietic organs (liver, bone marrow, and spleen) in newborn mice [5]. In contrast, our results show an increase in BFMN with age, with significant difference only between newborns and adults, although the difference between juveniles and adults ($p = 0.017$) was close to the critical value ($p = 0.016$) for statistical significance. Some authors suggest that an increase in MN frequency with age is due to a combination of factors, including the cumulative effect of acquired mutations in genes involved in DNA repair, errors in chromosome segregation mechanistic, and/or a deficiency in cell cycle checkpoints [15,30,31].

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Other authors showed that not only do mutations accumulate, but the rate of production also increases with age [32]. This would explain the decrease in the resistance of cells to genomic damage induced by endogenous and exogenous stressors, associated with age [5].

In contrast to the BFMN, our results for the CA showed a decrease of BDI with age. This difference may be due to the fact that the assays measure different endpoints: the MN test detects loss of "laggard" whole chromosomes (aneugenic events) or acentric fragments that deviate from the original chromosome (clastogenic events), while the CA measures strand breaks, cross-linking and alkali-labile sites, which can subsequently be removed by DNA repair systems [33]. The higher values of DI observed in newborns may be explained by immaturity of the repair mechanisms specific to the types of damage measured by the CA in young animals [34,35], lesions that might be correctly repaired in adults, reducing the background DNA damage. Collins et al. [36] and Brendler-Schwaab et al. [37] stated that the effects detected by the CA depend on the kind of primary DNA damage and the time point of analysis.

The use of the tegu lizard as a biological model has many advantages: the availability of samples from captive specimens in the "*PI*" [19] and the presence of nucleated erythrocytes in the peripheral blood (which avoids sacrifice of animals and the complex procedures associated with cell culture [14]). The animals used in this study were healthy: no evidence of disease was detected by experts in reptile management. In the case of neonates and juveniles, no effect of genotoxic agents is expected to have occurred during the embryonic period or the first months of life, as both the eggs and neonates were maintained under controlled conditions in the laboratory. So, we may assume that differences between groups in the BFMN and BDI reported reflect variations in genetic susceptibility of animals of different ages, an important factor to be considered in future studies.

5. Conclusions

Our results demonstrate that age is an intrinsic factor that influences spontaneous genetic damage in the tegu lizard, while sex and nest of origin seem to have no substantial effects. We observed different responses for MN test and CA among age groups of lizards, which may be explained by the types of damage that each biomarker detects. Age is a factor that should be taken into account in future biomonitoring studies on this species.

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Appendix A. Appendix A

See Tables A.1–A.2

Table A.1
Morphometric parameters and basal values (mean ± SE) of MN and DI in peripheral blood erythrocytes.

	N	Nests of origin	SVL (cm)	Weight (g)	BFMN	BDI
NB	24	3	8.52 ± 0.05	17.31 ± 0.31	0.16 ± 0.07*	146.2 ± 4.2*
JUV	21	3	18.37 ± 0.38	263.71 ± 18.57	0.29 ± 0.12	104.5 ± 0.65
AD	20	NK[b]	42.15 ± 0.93	3714 ± 311.20	0.95 ± 0.27	103.9 ± 0.97

N—number of animals per age group; SVL—snout-vent length; NK[b]—not known; BFMN—basal frequency of micronucleus; BDI—basal damage index.
* Significantly different with respect to the other age groups ($p < 0.05$).

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Table A.2
Individual results for each parameter measured in the 65 animals used in this study.

N^a	Age	BFMN	BDI	SVL (cm)	Weight (gr)
1	NB	0	156	9	17.5
2	NB	0	156	8.7	16.9
3	NB	0	142	8.4	17.8
4	NB	0	142	8.3	17
5	NB	0	164	8.5	17.7
6	NB	1	160	8.5	17.5
7	NB	0	154	8.5	16.9
8	NB	0	114	8.6	18.2
9	NB	0	140	8.9	20.2
10	NB	0	140	8.5	19.3
11	NB	0	122	8.8	18.1
12	NB	0	184	8.8	20.1
13	NB	0	167	8	15.9
14	NB	0	132	8.6	19.2
15	NB	0	172	8.6	18.7
16	NB	0	134	8.3	17.1
17	NB	0	110	8.3	15.5
18	NB	0	112	8.5	16.4
19	NB	0	145	8.8	17.6
20	NB	1	162	8.4	15
21	NB	1	145	8.3	15.7
22	NB	0	169	8.5	15.5
23	NB	1	119	8.2	14.4
24	NB	0	168	8.6	17.2
25	JUV	1	103	16	135
26	JUV	0	103	16.6	186
27	JUV	1	101	16.2	169
28	JUV	0	104	21	400
29	JUV	0	104	20	336
30	JUV	2	109	20	329
31	JUV	0	106	17.5	213
32	JUV	1	102	20.3	352
33	JUV	0	104	17.2	175
34	JUV	1	112	20.3	350
35	JUV	0	104	17	213
36	JUV	0	102	18	282
37	JUV	0	106	17	191
38	JUV	0	108	16	125
39	JUV	0	100	17	260
40	JUV	0	105	20.5	330
41	JUV	0	106	18	285
42	JUV	0	104	20.8	390
43	JUV	0	101	20.2	348
44	JUV	0	108	18	199
45	JUV	0	102	18.1	270
46	AD	5	116	46	4700
47	AD	1	110	40	3510
48	AD	1	102	47	4440
49	AD	2	111	44	3840
50	AD	1	100	40.5	3490
51	AD	0	101	47	4350
52	AD	2	102	40.5	3340
53	AD	1	104	51.5	8000
54	AD	0	100	48	5530
55	AD	0	105	46.5	5420
56	AD	0	102	43.5	3180
57	AD	2	108	37.5	2190
58	AD	0	104	39	2930
59	AD	1	100	39	2650
60	AD	0	102	40	3180
61	AD	1	104	38	2500
62	AD	1	100	39.5	3000
63	AD	1	102	39	2930
64	AD	0	100	37.5	2300
65	AD	0	104	39	2800

N^a—number of animal; age of animals: NB (newborn), JUV (juvenile), AD (adult; BFMN—baseline frequency of micronucleus; BDI—baseline damage index; SVL—snout–vent length.

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