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Research Article

Estimates of DNA damage by the comet assay in the direct-developing frog *Eleutherodactylus johnstonei* (Anura, Eleutherodactylidae)

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Abstract

The aim of this study was to use the Comet assay to assess genetic damage in the direct-developing frog *Eleutherodactylus johnstonei*. A DNA diffusion assay was used to evaluate the effectiveness of alkaline, enzymatic and alkaline/enzymatic treatments for lysing *E. johnstonei* blood cells and to determine the amount of DNA strand breakage associated with apoptosis and necrosis. Cell sensitivity to the mutagens bleomycin (BLM) and 4-nitroquinoline-1-oxide (4NQO) was also assessed using the Comet assay, as was the assay reproducibility. Alkaline treatment did not lyse the cytoplasmic and nuclear membranes of *E. johnstonei* blood cells, whereas enzymatic digestion with proteinase K (40 μg/mL) yielded naked nuclei. The contribution of apoptosis and necrosis (assessed by the DNA diffusion assay) to DNA damage was estimated to range from 0% to 8%. BLM and 4NQO induced DNA damage in *E. johnstonei* blood cells at different concentrations and exposure times. Dose-effect curves with both mutagens were highly reproducible and showed consistently low coefficients of variation (CV ≤ 10%). The results are discussed with regard to the potential use of the modified Comet assay for assessing the exposure of *E. johnstonei* to herbicides in ecotoxicological studies.

Key words: bleomycin, Comet assay, DNA diffusion assay, *Eleutherodactylus johnstonei*, 4-nitroquinoline-1-oxide.

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Introduction

Amphibians are useful biological indicators because their sensitive skins and occurrence in aquatic and terrestrial habitats makes them vulnerable to environmental change (Blaustein and Wake, 1990; Lips, 1998). Studies using frogs as models to measure the environmental impact of xenobiotics have used mainly larval phases of biphasic frog species such as *Anaxyrus americanus*, *Hyla versicolor, Lithobates catesbeianus*, *Lithobates clamitans* and *Lithobates pipiens* (Berrill *et al.*, 1994; Relyea, 2004a,b). Most Neotropical frogs have direct development and reproduce on land (Hedges *et al.*, 2008). However, few studies have assessed the usefulness of these species as environmental bioindicators.

Eleutherodactylus johnstonei (Anura: Eleutherodactylidae) is a direct-developing frog (Hedges *et al.*, 2008) from the Lesser Antilles with recently established populations in several Caribbean islands (Kaiser, 1997), as well as in Central and South America (Ortega *et al.*, 2001; Kaiser *et al.*, 2002). As a consequence of its widespread distribution and certain life history features (reproductive flexibility and high environmental adaptability) (Ortega *et al.*, 2005), *E. jonhstonei* has been considered a successful invasive species (Rödder, 2009). These attributes also suggest that this species could be a useful model for evaluating the genotoxicological impact of environmental xenobiotics such as pesticides.

DNA damage by environmental xenobiotics is frequently assessed by single cell gel electrophoresis (SCGE) or the Comet assay (Singh *et al.*, 1988), which detects DNA strand breakage and alkali-labile sites by measuring the migration of DNA from immobilized individual cell nuclei. In this assay, the cells are embedded in agarose gel on microscopic slides, lysed and then electrophoresed under alkaline condition. Cells with damaged DNA show increased migration of DNA fragments from the nucleus and the length of the migration indicates the amount of DNA strand breakage; the latter can be estimated by manual and computerized image scoring procedures (Kumaravel *et al.*, 2009). The technique is highly sensitive for detecting DNA damage in any eukaryotic cell type and requires only a few cells. The minimal technical requirements for doing this as-

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say *in vitro* and *in vivo* are well established (Cotelle and Férard, 1999; Tice *et al.*, 2000; Hartmann *et al.*, 2003). The Comet assay is sufficiently sensitive for detecting DNA damage in frogs (Dhawan *et al.*, 2009).

The main aim of this work was to assess the usefulness of the Comet assay for detecting DNA damage in *E. johnstonei*. The efficiency of alkaline, enzymatic and alkaline/enzymatic treatments for lysing *E. johnstonei* blood cells was also assessed using a DNA diffusion assay. Since positive Comet results do not necessarily reflect genotoxicity because DNA strand breakage may be associated with cellular apoptosis and necrosis, we used the DNA diffusion assay (Singh, 2000a) to determine the percentage of DNA strand breakage associated with apoptosis and necrosis ($\%N_{Ap/N}$) and thereby estimate the proportion of DNA strand breakage that was unrelated to apoptosis and necrosis. Cell sensitivity to the mutagens bleomycin (BLM) and 4-nitroquinoline-1-oxide (4NQO) was examined based on DNA strand breakage detected with the Comet assay (Baohong *et al.*, 2005; Kumaravel and Jha, 2006); the reproducibility of the assay in this species was also addressed.

Materials and Methods

Chemicals

Bleomycin (BML), dimethyl sulfoxide (DMSO), phosphotungstic acid, molecular grade and low gel temperature (LGT) agaroses, 4-nitroquinoline-1-oxide (4-NQO), silver nitrate and sodium N-lauryl sarcosine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). RNase- and DNase-free proteinase K and PK buffer were obtained from Promega Co. (Madison, WI, USA). Glycerol was purchased from Merck (Darmstadt, Germany). The other reagents and solvents were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Capture and maintenance of frogs

Eleutherodactylus johnstonei specimens were captured at several sites in the Bucaramanga metropolitan area (Santander, Colombia). Research and collecting permission was given by the Corporación Regional para la Defensa de la Meseta de Bucaramanga (File PC-0014-2008, Resolution 001368). Specimen sex was determined based on species sexual dimorphism and male calling. The frogs were maintained in glass terrariums at 24 ± 2 °C on a 12 h light/dark photoperiod, in conditions (vegetation, air circulation, humidity, illumination, etc.) that simulated the wild habitat. The frogs were fed flies, crickets, spiders, ants and mosquitoes that were captured on the campus of the Universidad Industrial de Santander (Bucaramanga, Colombia).

Blood sampling, cell counts and exposure to mutagens

Blood obtained by cardiac puncture of cold-anesthetized frogs was collected in heparinized Eppendorf tubes and placed on ice until assayed. Blood cells were counted in a Neubauer counting chamber and then diluted in 0.9% (w/v) NaCl solution to a cell density of 3.55×10^6 cells/mL. Aliquots of blood cells were treated with BLM (0.6-152.0 μg/mL) or 4NQO (1.9-60.0 μM) for 2, 4, 6, 10 and 12 h (selected on the basis of preliminary experiments). Treatments were done at 6 ± 2 °C to minimize basal DNA strand breakage. A negative control (0.9% NaCl solution) was always included in each assay. The experiments were done at least three times.

Estimation of DNA strand breakage in *E. johnstonei* blood cells

DNA strand breakage in *E. johnstonei* blood cells was initially assayed by using the alkaline Comet assay, as described by Singh *et al.* (1988) but with silver staining. Subsequently, DNA strand breakage was detected by the Comet assay as follows: *E. johnstonei* blood cells were centrifuged (10,000 rpm) and the pellet suspended in proteinase K solution (20 μL) prepared in PK buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8) at 40 μg/mL (concentration determined empirically). Aliquots (20 μL) of the cell suspension were mixed with 75 μL of 1% low melting point agarose and the mixture spread on slides containing a layer of 1.3% molecular grade agarose. The slides were covered with coverslips and incubated at 6 ± 2 °C for the agarose to solidify. After enzymatic lysis and agarose polymerization, the coverslips were removed and the slides were placed in a Comet assay tank (Cleaver Scientific Ltd, UK) containing cold alkaline electrophoresis buffer (0.3 N NaOH, 1 mM EDTA, pH 13) for 25 min. Electrophoresis was done at 25 V and current adjusted to 300 mA. The slides were routinely exposed to this current in the dark at 6 ± 2 °C for 30 min. After electrophoresis, the slides were placed in a staining tray and covered with a neutralizing buffer (0.4 M Tris-HCl, pH 7.5) in the dark for 5 min. Silver staining was done as indicated by Díaz *et al.* (2009).

DNA damage was expressed as arbitrary units based on the classification of comets into five categories (0-4) proposed by Collins *et al.* (1997). The total amount of DNA strand breakage was expressed in total arbitrary units (AU_T) defined as: $AU_T = N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4$, where N_i is the number of nuclei scored in each category (Collins, 2002). One hundred cells per slide and two slides per blood sample were analyzed using a Nikon Eclipse E200 microscope and the results from at least three independent experiments were averaged to obtain the AU_T for each treatment.

For the DNA diffusion assay (Singh, 2000a), the cells were processed in a similar manner to the Comet assay, except that the nuclei were not subjected to electrophoresis. Nuclei with a diameter > 3 times the mean nuclear diameter were considered apoptotic/necrotic (Nigro *et al.*, 2002). The total number of nuclei and the number of apoptotic/ne-

crotic nuclei in each field were counted (minimum of 100 fields per slide) and the latter then expressed as a percentage of the former. As in the Comet assay, two slides per blood sample were analyzed and the results of at least three independent experiments were averaged to obtain the percentage of apoptotic/necrotic nuclei ($\%N_{Ap/N}$) for each experiment.

Based on the AU_T and $\%N_{Ap/N}$ estimates, the proportion of remaining DNA strand breakages was calculated (in arbitrary units) as:

$$AU_R = AU_T - \frac{\%N_{AP/N} \times AU_T}{100}$$

where AU_R corresponds to non-apoptotic/necrotic DNA strand breakages.

Statistical analysis

In all cases, the data passed the Kolmogorov-Smirnov and F-maximum tests for normality and variance homogeneity, respectively, so that parametric tests were used in subsequent data analyses. When a significant F-value was obtained in one-way analysis of variance (ANOVA) the groups were subsequently compared with Students *t*-test. Product-moment (Pearson) correlation analysis was used to examine the relationship between mutagen doses and the average estimates of DNA damage. A value of $p < 0.05$ indicated significance. All statistical analyses were done with STATISTICA V.6 software (StatSoft Inc).

Results

Cellular lysis assays

In the present work, we used the Comet assay described by Singh *et al.* (1988) to detect DNA strand breakage in *E. johnstonei* blood cells. These cells were not lysed by alkaline conditions and naked nuclei were not obtained. Although cells varied in their sensitivity to silver staining (Figure 1), neither the pH (between 10 and 13) of the incubation solution nor the length of incubation (2 h and 24 h) had any effect on the susceptibility to lysis (data not shown).

Since naked nuclei could not be obtained from *E. johnstonei* blood cells by the standard Comet procedure (Singh *et al.*, 1988), we compared the ability of alkaline, enzymatic and alkaline/enzymatic treatments to produce these nuclei, as assessed by the DNA diffusion assay (Singh, 2000a). Treatments that included enzymatic lysis (Figure 2B,C) were effective in producing naked nuclei from blood cells, in contrast to lysis by alkaline treatment (Figure 2A). Combined alkaline/enzymatic treatment (Figure 2B) was more aggressive to nuclear stability than enzymatic treatment (Figure 2C), as shown by the nuclear diameter. The Comet assay showed that alkaline/enzymatic treatment produced DNA damage after a very short exposure to alkaline lysis ($AU_{5\ min} = 295 \pm 11$, $AU_{10\ min} = 323 \pm 7$, $AU_{15\ min} = 326 \pm 9$, $AU_{30\ min} = 330 \pm 11$ and $AU_{45\ min} = 361 \pm 5$). In this assay, the negative control had a mean arbitrary unit value ($AU_{0\ min}$) of 38 ± 5. In contrast to the enzymatic treatment at 37 °C overnight (Figure 2C), the nuclei were still intact after treatment at 6 ± 2 °C during agarose solidification (Figure 2D). Hence, subsequent experiments involving cell lysis were done using only enzymatic treatment at 6 ± 2 °C. Figure 3 shows images of the Comet categories established for *E. johnstonei* blood cells.

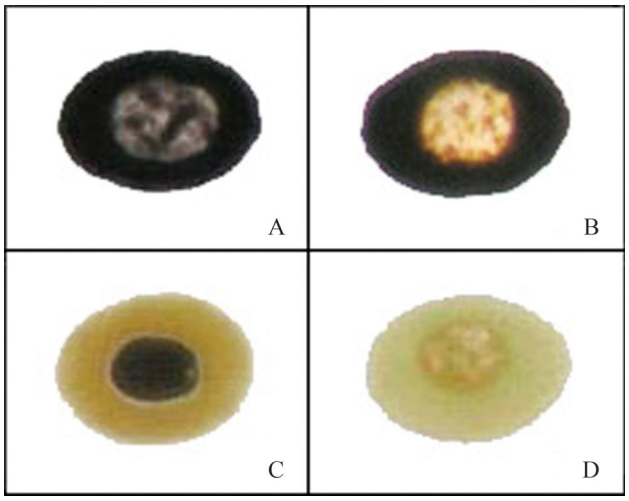

Figure 1 - Differential response of *E. johnstonei* blood cells to silver staining during the alkaline lysis Comet assay: (A) Cells totally stained, (B) Cells with only cytoplasm stained, (C) Cells with only nuclei stained and (D) Unstained cells.

Estimation of DNA strand breakage in *E. johnstonei* blood cells

Table 1 shows the DNA strand breakage induced by BLM and 4NQO in *E. johnstonei* blood cells at different doses and incubation times. A marked dose-response relationship was observed for both doses and incubation times. Dose-response curves to BLM and 4NQO after a 12 h exposure (Table 2) showed marked correlations ($R = 0.83$ and 0.90, $p \leq 0.05$; respectively). DNA strand breakage increased significantly ($p \leq 0.05$) from a concentration of 4.7 μg/mL of BLM and 1.9 μM of 4NQO. The $\%N_{Ap/N}$ in *E. johnstonei* ranged from 0% to 8% (mean: 2.8%). The assay reproducibility under the conditions in this model was consistently high, with coefficients of variation ≤ 10%.

Discussion

In this work, we used a modified Comet assay to detect DNA strand breakage in the direct-developing frog *E. johnstonei*. Previous studies of DNA damage in frogs used alkaline treatment to lyse the cells prior to analysis by the Comet assay (Table 3). Alkaline conditions are generally sufficient to cause cellular lysis in all frog species.

Eleutherodactylus johnstonei blood cells were resistant to tolerant to alkaline treatment in the standard procedure (Singh *et al.*, 1988). This finding suggests that *E. johnstonei* contains alkali-resistant but proteinase K-sensitive proteins that stabilize and/or protect the nuclei. The Comet assay with enzymatic (proteinase K) lysis has been used to assess DNA intactness in mammalian sperm cells (Baumgartner *et al.*, 2009), which have highly compact nuclear DNA (Ward and Coffey, 1991). Proteinase K digests proteins associated with nuclei and eliminates DNA-protein bonds generated by some xenobiotics, action that facilitates the electrophoretic migration of damaged DNA (Merk *et al.*, 2000; Singh, 2000b).

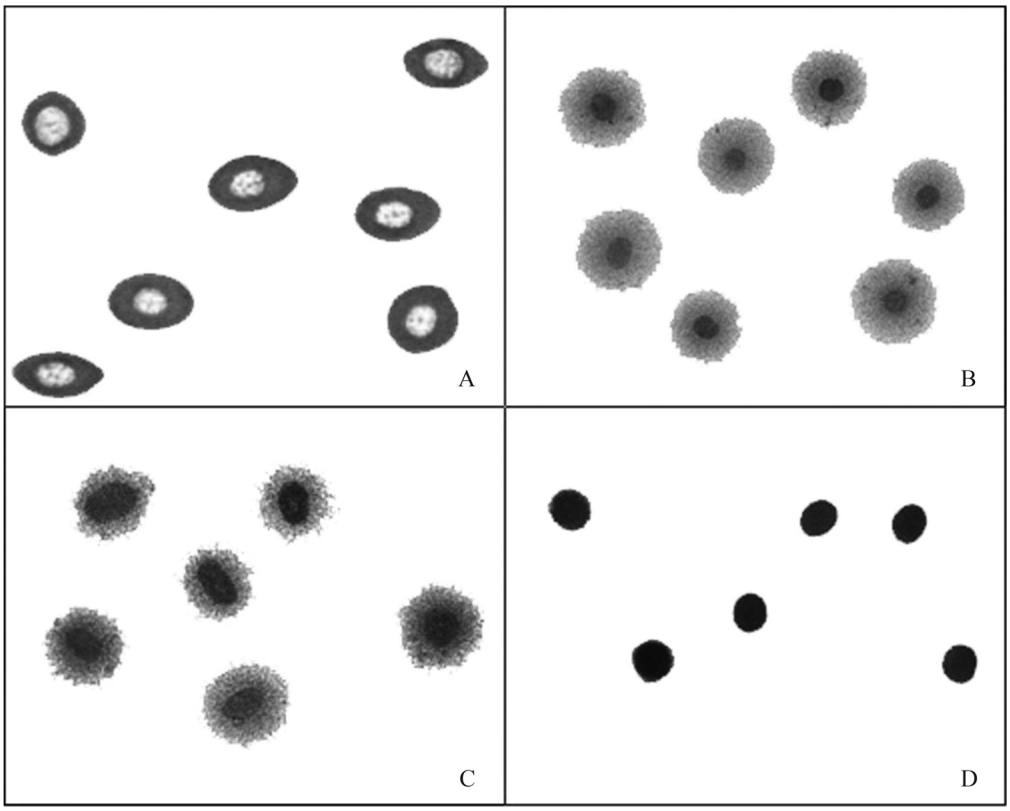

Figure 2 - DNA diffusion assay images from *E. johnstonei* blood cells after: (A) alkaline lysis, (B) alkaline/enzymatic (40 μg/mL proteinase K) lysis at 37 °C overnight, (C) enzymatic (40 μg/mL proteinase K) lysis at 37 °C overnight, and (D) enzymatic (40 μg/mL proteinase K) lysis at 6 ± 2 °C during agarose solidification (10 min).

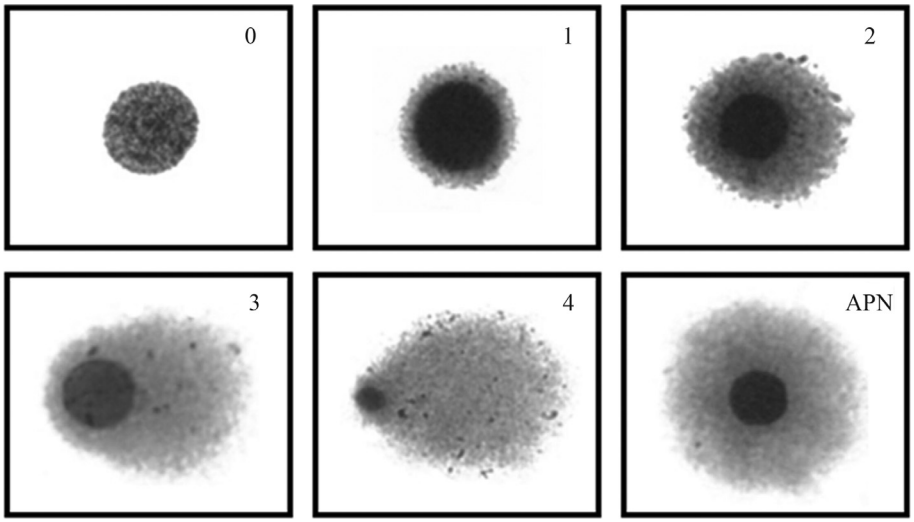

Figure 3 - Visual comet classification into five categories (0-4) proposed by Collins *et al.* (1997). Images of comets (from *E. johnstonei* blood cells) stained with silver nitrate. APN: Apoptotic/necrotic nuclei detected by the DNA diffusion assay.

Table 1 - Estimates of DNA damage in *E. johnstonei* blood cells exposed to BLM and 4NQO for different times.

Treatments	Leukocyte DNA damage after exposure to mutagens for the indicated times									
	2 h (3)*		4 h (3)		6 h (3)		10 h (3)		12 h (3)	
	AU_T	AU_R	AU_T	AU_R	AU_T	AU_R	AU_T	AU_R	AU_T	AU_R
NC	42 ± 2	41 ± 2	41 ± 0	40 ± 0	70 ± 12	68 ± 12	73 ± 8	71 ± 8	72 ± 9	69 ± 9
BLM (4.7 µg/mL)	78 ± 2	77 ± 2	81 ± 6	79 ± 6	114 ± 12	111 ± 12	115 ± 16	112 ± 16	159 ± 13	151 ± 13
BLM (9.5 µg/mL)	82 ± 2	81 ± 2	92 ± 2	90 ± 2	164 ± 16	159 ± 16	149 ± 10	145 ± 10	183 ± 12	174 ± 12
BLM (19.0 µg/mL)	89 ± 7	88 ± 7	110 ± 3	108 ± 3	174 ± 14	169 ± 14	170 ± 9	166 ± 9	219 ± 7	208 ± 7
BLM (38.0 µg/mL)	99 ± 3	98 ± 3	123 ± 4	120 ± 4	172 ± 9	167 ± 9	179 ± 16	174 ± 16	289 ± 13	275 ± 13
BLM (76.0 µg/mL)	101 ± 5	100 ± 5	139 ± 26	137 ± 26	159 ± 16	154 ± 16	209 ± 7	204 ± 7	293 ± 1	279 ± 1
BLM (152.0 µg/mL)	110 ± 3	108 ± 3	164 ± 15	161 ± 15	181 ± 7	176 ± 7	256 ± 6	250 ± 6	332 ± 8	315 ± 8
NC	79 ± 30	79 ± 30	100 ± 2	100 ± 2	91 ± 16	91 ± 16	95 ± 4	95 ± 4	80 ± 1	80 ± 1
4NQO (1.9 µM)	94 ± 12	93 ± 12	102 ± 1	102 ± 1	115 ± 2	115 ± 2	134 ± 26	134 ± 26	116 ± 11	116 ± 11
4NQO (3.7 µM)	92 ± 8	91 ± 8	112 ± 8	112 ± 8	117 ± 4	117 ± 4	142 ± 1	142 ± 1	142 ± 9	142 ± 9
4NQO (7.5 µM)	67 ± 57	67 ± 57	116 ± 8	116 ± 8	139 ± 8	139 ± 8	179 ± 6	179 ± 6	184 ± 17	184 ± 17
4NQO (15.0 µM)	101 ± 10	100 ± 10	122 ± 4	122 ± 4	146 ± 11	146 ± 11	181 ± 2	181 ± 2	238 ± 0	238 ± 0
4NQO (30.0 µM)	97 ± 2	96 ± 2	133 ± 5	133 ± 5	153 ± 9	153 ± 9	193 ± 0	193 ± 0	270 ± 5	270 ± 5
4NQO (60.0 µM)	102 ± 3	102 ± 3	128 ± 3	128 ± 3	162 ± 9	162 ± 9	196 ± 3	196 ± 3	308 ± 22	308 ± 22

The values are the mean ± SEM from at least three independent experiments with two replicate slides in each. *The total number of male frogs used per experiment with each mutagen. AU - arbitrary units, AU_T - total DNA damage measured with the Comet assay, AU_R - the remaining non-apoptotic/necrotic DNA damage, BLM - bleomycin, 4NQO - 4-nitroquinoline-1-oxide and NC - negative control (0.9% NaCl solution). The $\%N_{Ap/N}$ (see Materials and Methods) was the percentage of apoptotic/necrotic nuclei counted in 100 slide fields, and ranged from 0% to 8%.

Table 2 - Dose-response relationships between BLM and 4NQO concentrations and the estimated DNA damage. An exposure time of 12 h was used in all experiments.

Treatment	Leukocyte DNA damage after exposure to mutagen								CV (%)
	Exp. 1 (3)		Exp. 2 (3)		Exp. 3 (3)		Mean ± SEM		
	AU_T	AU_R	AU_T	AU_R	AU_T	AU_R	AU_T	AU_R	
NC	72 ± 9	69 ± 9	87 ± 8	80 ± 8	65 ± 6	62 ± 6	75 ± 9	70 ± 9	10
BLM (4.7 µg/mL)	159 ± 13 *	151 ± 13 *	142 ± 16 *	131 ± 16 *	145 ± 10 *	139 ± 10 *	149 ± 7	140 ± 7	6
BLM (9.5 µg/mL)	183 ± 12 *	174 ± 12 *	171 ± 16 *	157 ± 16 *	183 ± 10 *	176 ± 10 *	179 ± 6	169 ± 6	4
BLM (19.0 µg/mL)	219 ± 7 *	208 ± 7 *	220 ± 4 *	202 ± 4 *	240 ± 13 *	231 ± 13 *	226 ± 9	213 ± 9	5
BLM (38.0 µg/mL)	289 ± 13 *	275 ± 13 *	265 ± 10 *	244 ± 10 *	276 ± 14 *	265 ± 14 *	277 ± 10	261 ± 10	4
BLM (76.0 µg/mL)	293 ± 1 *	279 ± 1 *	299 ± 3 *	275 ± 3 *	296 ± 19 *	285 ± 19 *	296 ± 2	279 ± 2	1
BLM (152.0 µg/mL)	332 ± 8 *	315 ± 8 *	338 ± 9 *	311 ± 9 *	343 ± 3 *	330 ± 3 *	338 ± 4	318 ± 4	2
							r = 0.83 ($p \leq 0.05$)		
NC	78 ± 7	78 ± 7	80 ± 1	80 ± 1	93 ± 6	93 ± 6	84 ± 7	84 ± 7	10
4NQO (1.9 µM)	133 ± 6 *	133 ± 6 *	116 ± 11 *	116 ± 11 *	129 ± 3 *	129 ± 3 *	126 ± 7	126 ± 7	7
4NQO (3.7 µM)	147 ± 2 *	147 ± 2 *	142 ± 9 *	142 ± 9 *	155 ± 6 *	155 ± 6 *	148 ± 5	148 ± 5	4
4NQO (7.5 µM)	176 ± 16 *	176 ± 16 *	184 ± 17 *	184 ± 17 *	190 ± 3 *	190 ± 3 *	183 ± 6	183 ± 6	4
4NQO (15.0 µM)	227 ± 9 *	227 ± 9 *	238 ± 0 *	238 ± 0 *	222 ± 11 *	222 ± 11 *	229 ± 7	229 ± 7	4
4NQO (30.0 µM)	261 ± 2 *	261 ± 2 *	270 ± 5 *	270 ± 5 *	257 ± 5 *	257 ± 5 *	263 ± 5	263 ± 5	3
4NQO (60.0 µM)	316 ± 8 *	316 ± 8 *	308 ± 22 *	308 ± 22 *	299 ± 21 *	299 ± 21 *	308 ± 7	308 ± 7	3
							r = 0.90 ($p \leq 0.05$)		

The values are the mean ± SEM from three independent experiments with two replicate slides in each. The total number of male frogs used per experiment with each mutagen is shown in parentheses. The average values from the three experiments are shown. AU - arbitrary units, AU_T - total DNA damage measured with the Comet assay, AU_R - the remaining non-apoptotic/necrotic DNA damage, BLM - bleomycin, CV - coefficient of variation (%), 4NQO - 4-nitroquinoline-1-oxide, NC - negative control (0.9% NaCl solution) and r - Pearson correlation coefficient. The $\%N_{Ap/N}$ (see Materials and Methods) was the percentage of apoptotic/necrotic nuclei counted in 100 slide fields, and ranged from 0% to 8%. *$p < 0.05$ compared to the negative control (NC) (ANOVA followed by Student's *t*-test).

Table 3 - Cellular lysis conditions used in different studies to detect DNA damage by the Comet assay in frogs.

Genetic model*	Cell type	Buffer composition	Lysis conditions			References
			pH	Temperature	Time	
Anaxyrus americanus *Lithobates catesbeianus* *Lithobates clamitans*	Erythrocytes	Buffer 1: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 10% DMSO, 1% sodium sarcosinate, pH 10	alkaline	Room temperature	2 h	Ralph *et al.* (1996) Clements *et al.* (1997) Ralph and Petras (1998a)
Anaxyrus americanus *Lithobates clamitans* *Euphlyctis hexadactylus* *Lithobates pipiens*	Erythrocytes	†Buffer 2: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 10% DMSO, 1% sodium sarcosinate, 1% Triton X-100, pH 10	alkaline	Room temperature	2 h	Ralph and Petras (1997) Ralph and Petras (1998b) Rajaguru *et al.* (2001)
Fejervarya limnocharis *Pelophylax nigromaculata*	Erythrocytes	Buffer 2	alkaline	Room temperature	1 h	Feng *et al.* (2004) Liu *et al.* (2011)
Xenopus laevis *Silurana tropicalis*	Lymphocytes	Buffer 2	alkaline	On ice (0 °C)	30 min	Banner *et al.* (2006)
Pseudepidalea raddei	Hepatocytes	Buffer 2	alkaline	4 °C	1 h	Liu *et al.* (2006)
Bufo gargarizans *Pseudepidalea raddei*	Erythrocytes Hepatocytes	Buffer 2	alkaline	4 °C	2 h	Huang *et al.* (2007) Yin *et al.* (2008) Yin *et al.* (2009)
Pelophylax nigromaculata	Testicular cells	Buffer 3: 2.5 M NaCl, 10 mM Na_2EDTA, 10 mM Tris-HCl, 10% DMSO, 1% SDS, 1% Triton X-100, pH 10	alkaline	4 °C	2-4 h	Wang and Jia (2009)
Pelophylax lessonae *Xenopus laevis*	Erythrocytes	Buffer 4: 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 10% DMSO, 1% Triton X-100, pH 10	alkaline	4 °C	1 h	Maselli *et al.* (2010) Mouchet *et al.* (2006)
Eleutherodactylus johnstonei	Erythrocytes	Buffer 5: 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.04 mg/mL proteinase K, 0.8% LGT agarose, pH 8	neutral	Kept at 6 ± 2 °C for solidification	10 min	Present work

(*)Anuran species names follow Frost (2011). †First reported by Singh *et al.* (1988).

The intactness of sperm DNA is regularly analyzed with the Comet assay after alkaline and enzymatic treatments (Speit *et al.*, 2009). However, alkaline/enzymatic treatment was particularly aggressive to nuclear stability in *E. johnstonei* blood cells. For this reason, we used a neutral (pH 8) and single enzymatic digestion with proteinase K *in situ* in agarose gels; this procedure considerably reduced the assay costs and time. The temperature during cellular lysis is another critical variable that affects basal DNA damage in the Comet assay, as indicated in previous reports (Speit *et al.*, 1999; Banáth *et al.*, 2001). In *E. johnstonei* erythrocytes, enzymatic lysis at low temperature (6 ± 2 °C) was ideal for obtaining naked nuclei with low levels of basal DNA damage.

The results described here show that the Comet assay can provide a good estimation of DNA damage in *E. johnstonei.* The assay was reproducible and sensitive enough to detect DNA strand breakage in *E. johnstonei* blood cells. The basal DNA damage estimated for the species agreed with previously reported values (Collins *et al.*, 1997). In addition, the DNA damage observed here was poorly associated to apoptosis and necrosis, in contrast to the situation in humans (Tice *et al.*, 2000), sea lions (El-Zein *et al.*, 2006) and dolphins (Díaz *et al.*, 2009).

This study has shown the usefulness of amphibians as bio-indicators. A simultaneous study (Meza-Joya *et al.*, in preparation) in our laboratory examined the toxic and genotoxic effects of a glyphosate-based herbicide (Roundup®SL - Cosmoflux®411F) on *E. johnstonei*. The study again showed that the Comet assay was highly sensitive for detecting DNA damage induced by this herbicide. This finding suggests that the Comet assay is an accurate method for detecting DNA damage in *E. johnstonei* after exposure to environmental xenobiotics.

In conclusion, the alkaline Comet assay (Singh *et al.*, 1988) was inappropriate for measuring DNA strand breakage in *E. johnstonei*. Alkaline lysis can be replaced by enzymatic lysis (proteinase K), with good results. In contrast, combined alkaline/enzymatic treatment or long incubations (overnight) at 37 °C with proteinase K generate unstable nuclei and result in consistently elevated basal DNA damage. The contribution of apoptosis and necrosis to the overall DNA damage in *E. johnstonei* was negligible, as assessed by the Comet assay. The Comet assay is a repro-

ducible, sensitive method for detecting DNA strand breakage in *E. johnstonei*.

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