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°C/109 mg/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.

Received: April 29, 2011; Accepted: July 13, 2011.

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. johnstonei* 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-
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 (\%NAp/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 (Bao-hong *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 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 CaCl₂, 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. 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 = \sum x N_i + N_1 x 1 + N_2 x 2 + N_3 x 3 + N_4 x 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\textsubscript{Ap/N}) for each experiment.

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

$$\text{AU}_\text{R} = \text{AU}_\text{T} - \frac{\%\text{N}_\text{Ap/N} \times \text{AU}_\text{T}}{100}$$

where AU\textsubscript{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 \textit{et al.} (1988) to detect DNA strand breakage in \textit{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 \textit{E. johnstonei} blood cells by the standard Comet procedure (Singh \textit{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\textsubscript{5 min} = 295 ± 11, AU\textsubscript{10 min} = 326 ± 9, AU\textsubscript{15 min} = 330 ± 11 and AU\textsubscript{30 min} = 361 ± 5).

Estimation of DNA strand breakage in \textit{E. johnstonei} blood cells

Table 1 shows the DNA strand breakage induced by BLM and 4NQO in \textit{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 ≤ 0.05; respectively). DNA strand breakage increased significantly (p ≤ 0.05) from a concentration of 4.7 \(\mu\text{g/mL}\) of BLM and 1.9 \(\mu\text{M}\) of 4NQO. The %N\textsubscript{Ap/N} in \textit{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 \textit{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.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>AU&lt;sub&gt;T&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;R&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;T&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;R&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;T&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;R&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;T&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;R&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;T&lt;/sub&gt;</th>
<th>AU&lt;sub&gt;R&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>79 ± 30</td>
<td>79 ± 30</td>
<td>100 ± 2</td>
<td>100 ± 2</td>
<td>91 ± 16</td>
<td>91 ± 16</td>
<td>95 ± 4</td>
<td>95 ± 4</td>
<td>80 ± 1</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>BLM (4.7 μg/mL)</td>
<td>94 ± 12</td>
<td>93 ± 12</td>
<td>102 ± 1</td>
<td>102 ± 1</td>
<td>115 ± 2</td>
<td>115 ± 2</td>
<td>134 ± 26</td>
<td>134 ± 26</td>
<td>116 ± 11</td>
<td>116 ± 11</td>
</tr>
<tr>
<td>4NQO (1.9 μM)</td>
<td>92 ± 8</td>
<td>91 ± 8</td>
<td>112 ± 8</td>
<td>112 ± 8</td>
<td>117 ± 4</td>
<td>114 ± 7</td>
<td>142 ± 1</td>
<td>142 ± 1</td>
<td>142 ± 9</td>
<td>142 ± 9</td>
</tr>
<tr>
<td>4NQO (7.5 μM)</td>
<td>67 ± 57</td>
<td>67 ± 57</td>
<td>116 ± 8</td>
<td>116 ± 8</td>
<td>139 ± 8</td>
<td>139 ± 8</td>
<td>179 ± 6</td>
<td>179 ± 6</td>
<td>184 ± 17</td>
<td>184 ± 17</td>
</tr>
<tr>
<td>4NQO (15.0 μM)</td>
<td>101 ± 10</td>
<td>100 ± 10</td>
<td>122 ± 4</td>
<td>122 ± 4</td>
<td>146 ± 11</td>
<td>146 ± 11</td>
<td>181 ± 2</td>
<td>181 ± 2</td>
<td>238 ± 0</td>
<td>238 ± 0</td>
</tr>
<tr>
<td>4NQO (30.0 μM)</td>
<td>97 ± 2</td>
<td>96 ± 2</td>
<td>133 ± 5</td>
<td>133 ± 5</td>
<td>153 ± 9</td>
<td>153 ± 9</td>
<td>193 ± 3</td>
<td>193 ± 3</td>
<td>270 ± 5</td>
<td>270 ± 5</td>
</tr>
<tr>
<td>4NQO (60.0 μM)</td>
<td>102 ± 3</td>
<td>102 ± 3</td>
<td>128 ± 3</td>
<td>128 ± 3</td>
<td>162 ± 9</td>
<td>162 ± 9</td>
<td>196 ± 3</td>
<td>196 ± 3</td>
<td>308 ± 22</td>
<td>308 ± 22</td>
</tr>
</tbody>
</table>

The values are the mean ± SEM from at least three independent experiments with two replicate slides in each. *The number of male frogs used per experiment with each mutagen. AU - arbitrary units, AU<sub>T</sub> - total DNA damage measured with the Comet assay, AU<sub>R</sub> - the remaining non-apoptotic/necrotic DNA damage, BLM - bleomycin, 4NQO - 4-nitroquinoline-1-oxide and NC - negative control (0.9% NaCl solution). The %N<sub>ap/n</sub>(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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exp. 1 (3)</th>
<th>Exp. 2 (3)</th>
<th>Exp. 3 (3)</th>
<th>Mean ± SEM</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>72 ± 9</td>
<td>69 ± 9</td>
<td>87 ± 8</td>
<td>80 ± 8</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>BLM (4.7 μg/mL)</td>
<td>159 ± 13 *</td>
<td>151 ± 13 *</td>
<td>142 ± 16 *</td>
<td>131 ± 16 *</td>
<td>145 ± 10 *</td>
</tr>
<tr>
<td>4NQO (1.9 μM)</td>
<td>183 ± 12 *</td>
<td>176 ± 12 *</td>
<td>176 ± 17 *</td>
<td>176 ± 16 *</td>
<td>183 ± 10 *</td>
</tr>
<tr>
<td>4NQO (7.5 μM)</td>
<td>219 ± 7 *</td>
<td>208 ± 7 *</td>
<td>220 ± 4 *</td>
<td>204 ± 4 *</td>
<td>240 ± 13 *</td>
</tr>
<tr>
<td>4NQO (15.0 μM)</td>
<td>289 ± 13 *</td>
<td>275 ± 13 *</td>
<td>265 ± 10 *</td>
<td>244 ± 10 *</td>
<td>276 ± 14 *</td>
</tr>
<tr>
<td>4NQO (30.0 μM)</td>
<td>293 ± 1 *</td>
<td>279 ± 1 *</td>
<td>299 ± 3 *</td>
<td>275 ± 3 *</td>
<td>296 ± 19 *</td>
</tr>
<tr>
<td>4NQO (60.0 μM)</td>
<td>332 ± 8 *</td>
<td>315 ± 8 *</td>
<td>338 ± 9 *</td>
<td>311 ± 9 *</td>
<td>343 ± 3 *</td>
</tr>
</tbody>
</table>

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<sub>T</sub> - total DNA damage measured with the Comet assay, AU<sub>R</sub> - 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<sub>ap/n</sub>(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).
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°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-

<table>
<thead>
<tr>
<th>Genetic model*</th>
<th>Cell type</th>
<th>Buffer composition</th>
<th>Lysis conditions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaxyrus americanus</em></td>
<td>Erythrocytes</td>
<td>Buffer 1: 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 10% DMSO, 1% sodium sarcosinate, pH 10</td>
<td>alkaline</td>
<td>Room temperature</td>
</tr>
<tr>
<td><em>Lithobates catesbeianus</em></td>
<td>Erythrocytes</td>
<td>Buffer 2: 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 10% DMSO, 1% sodium sarcosinate, 1% Triton X-100, pH 10</td>
<td>alkaline</td>
<td>Room temperature</td>
</tr>
<tr>
<td><em>Lithobates clamitans</em></td>
<td>Erythrocytes</td>
<td>Buffer 2: 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 10% DMSO, 1% sodium sarcosinate, 1% Triton X-100, pH 10</td>
<td>alkaline</td>
<td>Room temperature</td>
</tr>
<tr>
<td><em>Fejervarya limnocharis</em></td>
<td>Erythrocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>Room temperature</td>
</tr>
<tr>
<td><em>Pelophylax nigromaculata</em></td>
<td>Erythrocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>Room temperature</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>Lymphocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>On ice (0 °C)</td>
</tr>
<tr>
<td><em>Silurana tropicalis</em></td>
<td>Lymphocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Pseudepidalea raddei</em></td>
<td>Hepatocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Bufo gargarizans</em></td>
<td>Erythrocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Pseudepidalea raddei</em></td>
<td>Hepatocytes</td>
<td>Buffer 2</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Pelophylax nigromaculata</em></td>
<td>Testicular cells</td>
<td>Buffer 3: 2.5 M NaCl, 10 mM Na₂EDTA, 10 mM Tris-HCl, 10% DMSO, 1% SDS, 1% Triton X-100, pH 10</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Pelophylax lessonae</em></td>
<td>Erythrocytes</td>
<td>Buffer 4: 2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 10% DMSO, 1% Triton X-100, pH 10</td>
<td>alkaline</td>
<td>4 °C</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>Erythrocytes</td>
<td>Buffer 5: 50 mM Tris-HCl, 10 mM CaCl₂, 0.04 mg/mL proteinase K, 0.8% LGT agarose, pH 8</td>
<td>neutral</td>
<td>Kept at 6 ± 2 °C for solidification</td>
</tr>
<tr>
<td><em>Eleutherodactylus johnstonei</em></td>
<td>Erythrocytes</td>
<td>Buffer 5: 50 mM Tris-HCl, 10 mM CaCl₂, 0.04 mg/mL proteinase K, 0.8% LGT agarose, pH 8</td>
<td>neutral</td>
<td>Kept at 6 ± 2 °C for solidification</td>
</tr>
</tbody>
</table>

(*)Anuran species names follow Frost (2011). †First reported by Singh et al. (1988).
ducible, sensitive method for detecting DNA strand breakage in *E. johnstonei*.

**Acknowledgments**

The Corporación Regional para la Defensa de la Meseta de Bucaramanga (CDMB) provided the research and collecting permits. This work was supported by the Rectoría de Investigaciones y Extensión, Universidad Industrial de Santander, Colombia (Grant No. 5163).

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