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Evaluation of imazethapyr-induced DNA oxidative damage by alkaline Endo III- and Fpg-modified single-cell gel electrophoresis assay in *Hypsiboas pulchellus* tadpoles (Anura, Hylidae)

Juan Manuel Pérez-Iglesias[a, c], Celeste Ruiz de Arcaute[a, c], Guillermo S. Natale[b, c], S. Soloneski[a, c], Marcelo L. Larramendy[a, c, *]

[a] Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 No 3, B1904AMA La Plata, Argentina
[b] Centro de Investigaciones del Medio Ambiente (CIMA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

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ABSTRACT

Imazethapyr (IMZT) is a selective postemergent herbicide with residual action. Available data analyzing its effects in aquatic vertebrates are scarce. In previous studies, we demonstrated that IMZT induces lesions into the DNA of *Hypsiboas pulchellus* tadpoles using the single-cell gel electrophoresis (SCGE) assay as a biomarker for genotoxicity. Currently, this assay can be modified by including incubation with lesion-specific endonucleases, *e.g.*, endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), which detect oxidized pyrimidine and purine bases, respectively. The aim of this study was to evaluate the role of oxidative stress in the genotoxic damage in circulating blood cells of *H. pulchellus* tadpoles exposed to the IMZT-based Pivot H® formulation (10.59% IMZT) at a concentration equivalent to 25% of the LC_{50} (96 h) value (0.39 mg/L IMZT) during 48 and 96 h. Our results demonstrate that the herbicide induces oxidative DNA damage on *H. pulchellus* tadpoles at purines bases but not at pyrimidines. Our findings represent the first evidence of oxidative damage caused by IMZT on anuran DNA using the alkaline restriction enzyme-modified SCGE assay.

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1. Introduction

Imazethapyr (IMZT) [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1H-2-yl) nicotinicacid] is a member of the imidazolinone herbicides used to control grasses, broadleaved weeds, and others in a variety of crops and noncrop situations (MacBean, 2012). IMZT has been classified as a slightly toxic compound (Class III) by the U.S. Environmental Protection Agency (USEPA, 1989), and the European Union (PPDB, 2014) has classified the herbicide as a dangerous compound for the environment and has reported IMZT as a harmful irritant for the respiratory track, skin, and eyes. Though very little is known about its toxicity in nontarget organisms. When IMZT was administered orally, low or moderate acute toxicity was reported in rats (USEPA, 1989). Using algae and aquatic invertebrates, low levels of toxicity have been reported. When aquatic plants were employed as targets, *e.g.*, *Lemna gibba*, high acute levels of toxicity were observed (Maki and Johnson, 1976; USEPA, 2000). Among terrestrial invertebrates, insects such as honey bees and annelids such as earth worms have been reported to have extremely high sensitivity and low sensitivity to IMZT, respectively (USEPA, 2000). So far, the levels of acute toxicity exerted by the herbicide have not been found to be acutely toxic for fish, including channel catfish, bluegill, and rainbow trout (Kegley et al., 2014, 2016; PPDB, 2014; USEPA, 2000). Nevertheless, Moraes et al. (2011) reported disorders in oxidative stress parameters in liver cells of the common carp *Cyprinus carpio* after exposure to both the active ingredient IMZT and to imazapic-based commercial herbicide formulations.

Overall, very little is known about the genotoxic information of IMZT. Genotoxic studies of IMZT are scarce and contradictory. Whereas IMZT did not induce chromosomal aberrations in rat bone marrow cells, both negative and positive results have been reported for CHO cells with and without metabolic activation, respectively (USEPA, j). Furthermore, several studies employing the conventional alkaline version of the comet assay have shown that IMZT is genotoxic to some nontarget organisms, *i.e.*, bacteria *Salmonella typhimurium* and *Escherichia coli* (USEPA, 1989) and plants such as *Allium cepa* (Liman et al., 2015) as well as mammalian exposed *in vitro* cells (Soloneski et al., 2017). Recently, we demonstrated for the first time that the herbicide jeopardizes anuran amphibian *Hypsiboas pulchellus* tadpoles by inducing micronuclei induction and DNA primary damage evaluated the SCGE assay (Pérez-Iglesias et al., 2015). We observed that IMZT is able to induce acute toxic and genotoxic effects on the species, including mortality as the end point for lethality and frequency of micronuclei and other nuclear abnor-

* Corresponding author at: Cátedra de Citología, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Calle 64 No 3, B1904AMA La Plata, Argentina.
Email address: marcelo.larramendy@gmail.com (M.L. Larramendy)

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malities as well as DNA single-strand breaks as end points for genotoxicity (Pérez-Iglesias et al., 2015).

The single cell gel electrophoresis (SCGE) bioassay, also called the comet assay, is one of the most widely used methods to detect the genotoxic capability of xenobiotics both *in vivo* and *in vitro* since it is simple, fast, specific, and sensitive. The methodology in its alkaline or neutral version, detects a variety of DNA lesions at the single-cell level, including both single- and double-strand breaks as well as alkali-labile lesions (Azqueta and Collins, 2013; Collins et al., 2014). However, when nucleoids are digested with lesion-specific endonucleases, restriction enzymes will induce DNA breaks at the damage sites they recognize, and thus the breaks can be measured by the comet assay. Thus, different types of DNA lesions can be detected by using different lesion-specific enzymes. To date, the endonucleases most commonly used in this way are the bacterial enzymes endonuclease III (Endo III, also known as Nth) and formamidopyrimidine DNA-glycosylase (Fpg). Endo III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol (Azqueta and Collins, 2013; Azqueta et al., 2014). The glycosylase Fpg recognizes and removes a several oxidized purines from damaged DNA such as 8-oxo-7,8-dihydroguanine (8-oxo-Gua), 2,6 diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 4,6-diamino-5-formamidopyridine (FapyAde). The AP lyase activity of the Fpg leaves an AP sites which can be detectable by the comet assay (Azqueta and Collins, 2013; Azqueta et al., 2014). Thus, the modified methodology has be recommended for use as a sensitive biomarker to measure oxidative DNA damage in genotoxicity studies (Collins and Azqueta, 2012; Collins et al., 1996; Kushwaha et al., 2011; Wong et al., 2005).

In a previous study using the alkaline version of the SCGE assay, we demonstrated that Pivot H®, an IMZT-based herbicide commercial formulation, induces genetic damage in blood cells of *Hypsiboas pulchellus* tadpoles (Pérez-Iglesias et al., 2015). To assess the role of oxidative DNA damage in IMZT-induced genotoxicity, in the current study we used two restriction enzymes, namely, Endo III and Fpg, in combination with the SCGE assay in circulating blood cells of *H. pulchellus* tadpoles exposed *in vivo* to Pivot H® (10.59% IMZT).

2. Materials and methods

2.1. Chemicals

Pivot H® (10.59% IMZT, CAS 81335-77-5) was purchased from BASF Argentina S.A. $K_2Cr_2O_7$ [$Cr_{(VI)}$] (CAS 7778-50-9) was obtained from Merck KGaA (Darmstadt, Germany) and hydrogen peroxide (H_2O_2, CAS 7722-84-1) was purchased from Merck KGaA (Darmstadt, Germany). Endo III and Fpg were purchased from New England Biolabs® Inc. (Ipswich, MA). All other chemicals and solvents of analytical grade for the comet assay were purchased from Sigma Chemical Co.

2.2. Quality control

Determination of the concentration levels of IMZT in the test solutions was performed by QV Chem Laboratory (La Plata, Buenos Aires, Argentina) according to U.S. Geological Survey Report 01-4134 (Furlong et al., 2011). IMZT levels were analyzed by high performance liquid chromatography. Active ingredient samples from quadruplicated test solutions (0.39 mg/L) correspond to values obtained immediately after preparation (0 h) and 24 h thereafter. The detection limit for IMZT was 0.5 μg/L.

2.3. Test organisms

H. pulchellus is an anuran arboreal amphibian species from the Hylidae family. This species has an extensive distribution in the Neotropical America and is an abundant species in the Pampasic region of Argentina (Cei, 1980). Its natural habitats are subtropical or tropical dry lowland grasslands, subtropical or tropical seasonally wet or flooded lowland grasslands, intermittent fresh water lakes, intermittent fresh water marshes, and pasturelands (Kwet et al., 2004). This species lays its eggs in masses attached to the submerged stems of aquatic plants, and it is easy to handle and acclimate to laboratory conditions as previously stressed (Pérez-Iglesias et al., 2014, 2015; Ruiz de Arcaute et al., 2014).

All organisms used for this study were collected from a temporary and unpolluted pond away from agricultural areas, in the vicinity of La Plata City (35°10′S,57°51′W; Buenos Aires Province, Argentina), at late cleavage stage (GS) 9 according to Gosner's (Gosner, 1960) classification. Hatchlings were transported to the laboratory and then acclimatized to a 16/8 h light/dark cycles in aquaria at 25 °C with dechlorinated tap water with artificial aeration. The physical and chemical parameters of the water were as follows: temperature, 25.0±1 °C; pH 8.0±0.1; dissolved oxygen, 6.3±0.3 mg/L; conductivity, 663±15.0 μS/cm; hardness, 181±35.0 mg/L $CaCO_3$. Commercially available fish food (Tetra Min®, TetraWerke, Germany) as a food source was supplied twice a week until individuals reached development stage 36 (GS36; range 35–37) according to Gosner (1960). Afterwards, individuals were randomly deposited in test chambers according to the experimental design. Hatches were collected with the permission of the Flora and Fauna Direction from the Buenos Aires Province (Buenos Aires, Argentina; code 22500-22339/13), and experimental procedures were approved by the Ethical Committee of the National University of La Plata (code 11/N754).

2.4. Experimental design

In vivo exposure to 0.39 mg/L IMZT was performed for 48 and 96 h according to the procedures described in detail elsewhere for *H. pulchellus* tadpoles (Pérez-Iglesias et al., 2014, 2015; Ruiz de Arcaute et al., 2014). Briefly, experiments were performed using five GS36 tadpoles for each experimental point, maintained in a 500 mL glass container and exposed in acute bioassay to concentration of IMZT equivalent to 25% of the corresponding LC50 (96 h) value. All test solutions were prepared immediately before use and replaced every 24 h. Tadpoles were not fed throughout the experiment. Experiments were performed in quadruplicate and run simultaneously. *H. pulchellus* tadpoles were sacrificed according to the American Society of Ichthyologists and Herpetologists criteria (ASIH, 2004). Blood samples for comet assay were obtained at 48 and 96 h after initial treatment. For positive controls, aliquots (15 μL) of blood cells were obtained, and the cells were immersed in low melting point agarose. After solidification of the second agarose layer, the coverslips were removed, and slides were treated with 50 μM H_2O_2, for 5 min, at 4 °C.

2.5. Enzyme-modified alkaline single cell gel electrophoresis assay

The endonuclease-modified comet assay described by Collins et al. (1996) and Guilherme et al. (2012) was employed with minor modifications. Briefly, immediately after lysis, the slides were washed three times in an enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH 8.0) for 5 min each at room temperature. Then they were drained and exposed to Endo III or Fpg diluted 1:1000 or 1:3000, respectively, following recommendation of the enzymes supplier. Briefly, slides were incubated with 50 μL of Endo III (0.5 U) or Fpg (0.13 U) as suggested elsewhere (Collins, 2004; Collins et al., 1993). Control cells were treated with 50 μL of the corresponding enzyme buffer. After incubation for 30 or 45 min at 37 °C under a humid atmosphere for Endo III- or Fpg-exposed samples, respectively, the slides were processed following conventional alkaline SCGE protocol as described previously (Soloneski et al., 2016). Control cells were treated with 50 μL of the corresponding enzyme buffer. After the enzyme restriction exposure, coverslips were removed and the slides placed on a horizontal electrophoresis unit with an electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH) for 10 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 40 min at 25 V and 250 mA. All the steps listed above were performed under yellow light or in the dark to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5) and stained with 4′,6-diamino-2-phenylindole (Vectashield Mounting Medium H1200; Vector Laboratories, Burlingame, CA). Slides were examined under an Olympus BX50 fluorescence photomicroscope

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damage was quantified by the length of DNA migration, which was visually determined in 100 randomly selected and nonoverlapping cells (Azqueta et al., 2011; Collins, 2004; Collins et al., 1995; Kobayashi et al., 1995). DNA damage was classified in five classes (0-I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage), as suggested previously (Cavaş and Könen, 2007). Data are expressed as the mean number of damaged cells (sum of Classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was calculated for each test compound following Pitarque et al. (1999) using the formula GDI = [I (I) + 2 (II) + 3 (III) + 4 (IV)/*N* (0–IV)], where 0–IV represent the nucleoid type, and N_0–N_{IV} represent the total number of nucleoids scored. Afterward, oxidative DNA damage (OD) was obtained for each treatment using the formula OD = [(%GDI buffer + %GDI enzyme + %GDI herbicide) − (%GDI buffer + %GDI herbicide)], as indicated previously (Collins and Azqueta, 2012; Domijan et al., 2006; Mikloš et al., 2009; Soloneski et al., 2016).

2.6. Statistical analysis

To estimate the effects of the treatments (herbicide exposure, negative and positive control) on each group (with and without restriction enzymes), an one-way analysis of variance with Tukey's test was performed (Zar, 2010) using the GDI as a variable. To compare OD produced by IMZT in each enzyme (respect buffer enzyme), a *t*-test for the difference of means with equal variances was performed; previously, a test of homogeneity of variances was performed (Zar, 2010). The level of significance chosen was $\alpha = 0.05$ for all tests, unless indicated otherwise.

3. Results

Results of chemical analyses showed no significant changes ($P > 0.05$) in the concentration of the toxicant in treatments during the 24 h interval renewals of the testing solutions (concentration range, 98±5% recovery).

Data from the Endo III- and Fpg-modified SCGE assay obtained in circulating blood cells of *H. pulchellus* tadpoles exposed *in vivo* to 0.39 mg/L IMZT for 48 and 96 h are presented in Table 1, and the levels of net oxidative DNA damage are depicted in Fig. 1. Verification of the ability of Endo III and Fpg to recognize oxidized bases in our test system was repeated incubating cells with 50 μM H_2O_2 and employed as positive control. H_2O_2 treatment induced an enhancement in the frequency of damaged cells, the GDI, and OD values in enzyme buffer treated-cells exposed to Endo III ($p < 0.01$) and Fpg ($p < 0.001$) (Table 1; Fig. 1).

The treatment with Fpg produced a significant increase in DNA damage as well as of the GDI in tadpoles blood cells exposed to the IMZT-based herbicide formulation Pivot H® compared with cells without treatment with the enzyme at both times of exposure (enzyme buffer-treated cells; $p < 0.05$; Table 1; Fig. 1). Particularly, differences in DNA damage in tadpoles exposed to Pivot H® are due to an increase on type III and IV nucleoids after 48 y 96 h, respectively ($p < 0.05$; Table 1) when comparing cells treated with Fpg-enzyme with cells without treatment with the enzyme. On the other hand, post-treatment with Endo III did not induce any difference in the level of DNA damage and GDI in cells exposed to Pivot H® compared to enzyme buffer treated-cells ($p > 0.05$; Table 1; Fig. 1).

4. Discussion

The common tree frog, also called the Montevideo tree frog, *H. pulchellus*, is an arboreal anuran species in the family Hylidae. The species was recently reported as threatened by agricultural water pollution (specifically pesticide runoff) in the central inner part of Argentina (Kwet et al., 2004). Previous studies have stressed that the tested hylidae frog tadpoles can be considered suitable reference organisms in the risk assessment of lethal and sublethal effects induced by several emerging pollutants, including agrochemicals. Among them, the chemotherapeutic cyclophosphamide (Lajmanovich et al., 2005), the herbicide glufosinate ammonium (Peltzer et al., 2013), the insecticides fenitrothion (Junges et al., 2010), cypermethrin (Agostini et al., 2010), endosulfan (Agostini et al., 2013; Lajmanovich et al., 2005), imidacloprid (Ruiz de Arcaute et al., 2014), and the imidacloprid-based insecticide formulation Glacoxan Imida® (Pérez-Iglesias et al., 2014) can be included.

We recently employed *H. pulchellus* as a target species to evaluate the acute lethal and sublethal effects of the herbicide imazethapyr-based commercial formulation Pivot H® (Pérez-Iglesias et al., 2015). Whereas mortality was used as the end point for lethality, frequency of micronuclei and other nuclear abnormalities as well as DNA single-strand breaks evaluated by the SCGE assay were employed to test genotoxicity. Behavioral, growth, developmental, and morphological abnormalities were also employed as sublethal end points. Mortality studies revealed equivalent LC_{50} (96 h) values of 1.49 and 1.55 mg/L IMZT for Gosner stages 25 and 36, respectively. Behavioral changes, *i.e.*, irregular swimming and immobility, as well as a decreased frequency of keratodonts were observed. The herbicide increased the frequency of micronuclei in circulating erythrocytes of tadpoles exposed for 48 h to 1.17 mg/L IMZT. However, regardless of the concentration of the herbicide assayed, an enhanced frequency of micronuclei was observed in tadpoles exposed for 96 h within the 0.39–1.17 mg/L IMZT range. Our results also demonstrated that the herbicide was able to induce other nuclear abnormalities, *i.e.*, blebbed and notched nuclei, only when tadpoles were exposed for 96 h. In addition, we observed that exposure to IMZT within the 0.39–1.17 mg/L range increased the genetic damage index in treatments lasting for both 48 and 96 h (Pérez-Iglesias et al., 2015). To the best of our knowledge, this study represents the first evidence of acute lethal and sublethal effects exerted by IMZT on amphibians and highlights the ability of this herbicide to jeopardize exposed nontarget living species, at least *H. pulchellus* tadpoles (Pérez-Iglesias et al., 2015).

Despite the battery of end points assessed on *H. pulchellus* in our previous study (Pérez-Iglesias et al., 2015), further questions regarding the type of genetic damage that this compound is able to introduce into DNA molecules are still unanswered, since this aspect has yet to be covered. Our current results clearly demonstrate exposure to 0.39 mg/L IMZT is able to introduce damage into the DNA of circulating blood cells of *H. pulchellus* tadpoles. To elucidate a possible mechanism of IMZT-induced DNA damage, we employed two restriction enzymes in the current study, namely, Endo III and Fpg, in combination with the SCGE assay, to reveal the presence of oxidized pyrimidines and purines, respectively, as a result of herbicide-induced oxidative stress in circulating blood cells of *H. pulchellus* tadpoles exposed *in vivo* to Pivot H®.

Oxidative DNA damage can be measured to assess the genotoxic/carcinogenic potential of environmental chemicals. A direct assessment of oxidative damage may provide important information about the molecular effects of oxidative stress on DNA (Collins et al., 1996). Currently employed methods to measure oxidative DNA damage have certain limitations, such as artifactual DNA oxidation during sample isolation and DNA hydrolysis during high-performance liquid chromatography with electrochemical detection, gas chromatography–mass spectrometry, or HPLC–tandem mass spectrometry analyses, and DNA oxidation assays with antibodies are only semiquantitative and thus may produce misleading results (Collins, 2004; Gedik and Collins, 2005; Halliwell, 2000). In contrast, the SCGE assay is one of the most promising methods for detecting the genotoxic potential of chemicals because it is simple, fast, specific, and sensitive. Furthermore, the assay requires only small samples and can directly quantify the amount of oxidative DNA damage (Collins, 2004; Tice et al., 2000). Furthermore, lesion-specific endonucleases, such as Fpg or Endo III, also known as Nth, can recognize specific oxidatively damaged bases and create additional breaks to aid in the detection of oxidative DNA damage in a modified SCGE assay (Collins, 2004; Collins and Azqueta, 2012; Smith et al., 2006). Fpg specifically recognizes the number of oxidized purine bases and other ring-opened purines, whereas Endo III recognizes oxidized pyrimidines (Collins, 2004; Collins and Azqueta, 2012; Smith et al., 2006). We could emphasize the importance of using the modified SCGE technique to assess oxidative damage introduced into the DNA of amphibians as a consequence of pesticide exposure. Our observations reveal that treatment with both Endo III and Fpg buffers induce an enhancement in the frequency of DNA damage

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Table 1

Analysis of DNA damage measured by modified comet assay in *Hypsiboas pulchellus* tadpoles exposed *in vivo* to the imazethapyr-based herbicide formulation Pivot H®.Please do note that the whole last column of this table, named OD, is totally missing. Please do use the attached doccument for addressing this mistake.

Compound	Exposure time (h)	Treatment	No. animals analyzed	No. of nucleoids analyzed	Proportion of damaged nucleoids (%)[a]				% of damaged nucleoids (II + III + IV)	GDI±SE[b,c]
					Type 0 + I	Type II	Type III	Type IV		
Control	48	Negative	10	1128	70.90	15.47	12.06	1.57	28.74	1.13±0.13
		Buffer Endo III	9	1049	59.10	24.12	15.44	1.34	40.57	1.42±0.18
		Endo III	8	927	57.39	35.50[###]	12.62	1.25	43.06	1.49±0.09[#]
		Buffer Fpg	10	959	60.58	23.67	13.24	2.50	40.14	1.48±0.08[#]
		Fpg	10	1051	54.81[#]	25.02[#]	18.08	2.09	44.89[#]	1.55±0.04[##]
Pivot H®		0.39 mg/L	9	914	46.61	38.18	14.00	1.20	52.62	1.54±0.12[**]
		Buffer Endo III	8	806	47.52	37.84	13.65	0.99	52.44	1.63±0.05
		Endo III	9	941	45.17	37.62	16.26	0.95	54.62	1.66±0.08
		Buffer Fpg	9	988	50.51	30.77	17.41	1.32	48.83	1.62±0.11
		Fpg	9	983	29.91[#,&]	40.18	27.06[#,&]	2.85	69.94[#,&]	1.98±0.12[#,&]
Control	96	Negative	9	972	73.42	17.72	7.38	1.48	26.23	1.12±0.07
		Buffer Endo III	9	929	70.49	25.55	7.11	1.33	29.55	1.17±0.08
		Endo III	9	995	67.64	26.13[#]	5.53	0.70	31.85	1.19±0.08
		Buffer Fpg	9	984	55.89[##]	31.81[##]	10.36	1.93	44.31[##]	1.49±0.07[##]
		Fpg	9	1006	49.20[###]	42.44[###]	12.22	1.88	50.64[###]	1.57±0.10[###]
Pivot H®		0.39 mg/L	10	1105	50.95	31.95	15.29	1.81	49.55	1.56±0.14[**]
		Buffer Endo III	9	1039	50.79	36.84	10.31	2.06	48.77	1.52±0.08
		Endo III	10	1102	49.37	33.03	14.25	3.36	50.67	1.64±0.13
		Buffer Fpg	9	991	42.38	35.12	19.27	3.23	57.17	1.78±0.12
		Fpg	9	930	30.97[#,&]	36.77	24.52	7.74[##,&]	69.22[#,&]	2.08±0.08[##,&]
Positive control[e]		50 μM	10	1000	29.94	38.62	29.61	1.84	72.14	2.00±0.09[***]
		Buffer Endo III	10	1160	29.18	32.29	19.51	9.01	65.56	1.93±0.16
		Endo III	10	993	15.11[#]	29.47[#]	41.81	13.60[#]	79.32[#,&&]	2.53±0.14[#,&&]
		Buffer Fpg	10	1161	35.25	35.46	22.40	6.89	64.37	1.99±0.10
		Fpg	10	991	12.62[##]	15.48[##]	29.35	42.56[###]	86.07[##,&&&]	3.05±0.17[###,&&&]

*P < 0.05.

[d]Oxidative DNA damage (OD).

[a] I–IV indicate grades of DNA damage as mean values of pooled data from three independent experiments.

[b] Results are presented as mean values of pooled data from three independent experiments ±S.E. of the mean.

[c] Genetic damage index (GDI).

[e] Hydrogen peroxide (H_2O_2, 50 μM) was used as positive control.

[**] P < 0.01 compared with negative control value.

[***] P < 0.001 compared with negative control value.

[#] P < 0.05 compared with respective group control.

[##] P < 0.01 compared with respective group control.

[###] P < 0.001 compared with respective group control.

[&] P < 0.05 compared with respective buffer-enzyme.

[&&] P < 0.01 compared with respective buffer-enzyme.

[&&&] P < 0.001 compared with respective buffer-enzyme.

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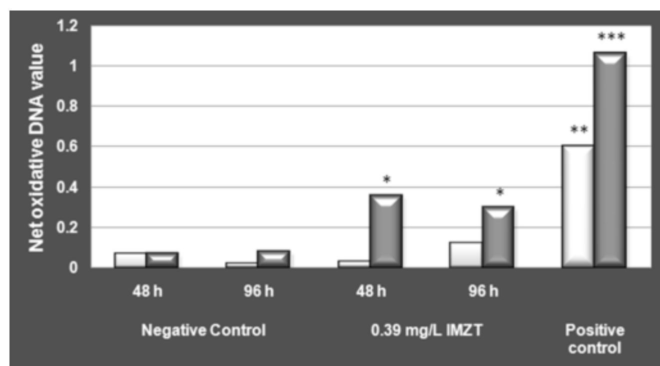

Fig. 1. Imazethapyr-based commercial formulation herbicide Pivot H®-induced DNA damage measured by modified comet assay using Endo III (light gray bars) and Fpg (dark gray bars) enzymes in circulating blood cells of *Hypsiboas pulchellus* tadpoles exposed *in vivo*. The net oxidative DNA damage was calculated as the difference between the score obtained after incubation with the respective enzyme or with the buffer. Hydrogen peroxide (50 μM) was used as positive control, respectively. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; significant differences with respect to control values.

duce lesions into cellular DNA, and thus increasing the length of the nucleoids (Collins and Azqueta, 2012; Demir et al., 2014; Soloneski et al., 2016, 2017). Furthermore, our results demonstrate that the modified SCGE assay using Fpg, but not Endo III, turned out to be highly sensitive and effective in detecting genetic oxidative damage introduced into the DNA of circulating blood cells of *H. pulchellus* tadpoles by the herbicide IMZT-based commercial formulation Pivot H® at purine bases, but not pyrimidines. So far, only Calevro et al. (1998) have analyzed the extent of oxidative DNA damage employing the Fpg-modified alkaline comet assay in amphibians, using primary brain cell cultures from the newt *Pleurodeles waltl* exposed to the cadmium(II). Although the authors demonstrated that cadmium(II) is not able to induce oxidative DNA base modifications in larval brain cells, they show that the agent has the capability to generate DNA strand breaks and to interfere with the repair of xenobiotic-induced oxidative damage (Calevro et al., 1998). Thus, to the best of our knowledge, our current findings represent the first evidence of DNA oxidative damage caused by exposure to a xenobiotic, such as the IMZT-based commercial herbicide formulation Pivot H®, revealed using the restriction enzyme-modified alkaline SCGE assay not only for the Neotropical anuran species *H. pulchellus*, but for amphibians worldwide.

Finally, our observations corroborate that the IMZT-based herbicide formulation Pivot H® exerts genotoxic effects (Liman et al., 2015; Pérez-Iglesias et al., 2015; USEPA, 1989) and acts as an oxidizing agent, as suggested previously by Moraes et al. (2011), on nontarget organisms such as *C. carpio*. As remarked by Collins (2004), this modified SCGE technique can be used in combination with biosensors for contamination of the environment with genotoxins, at least in aquatic vertebrates.

Although the IMZT treatment in this study includes only one concentration, it represents a relatively high end of the threshold value of 14 μg/L IMZT found in the surface water of the Azul River basin (Buenos Aires, Argentina) reported by Peluso et al. (2008), even considering the recommended application field ratios of 100–150 g a.i./ha reported for Argentina (Bindraban et al., 2009; CASAFE, 2011). It should be mentioned that the IMZT concentrations found in Argentinean crop production areas is nearly 7.6 times higher than the highest concentration reported for surface water in United States (Mattice et al., 2011) or even 51.8 and 40.0 times higher than the highest concentration reported for Brazilian drinking and surface waters, respectively (Souza Caldas et al., 2011). Thus, the concentration of IMZT employed in this investigation would be expected to be almost improbable in the environment, perhaps observed only when specific events occurred (*e.g.*, direct application, drainage into ditches, or accidental discharge). Although, we cannot rule out that amphibian populations and also occupationally exposed human workers could be exposed accidentally to these agrochemicals at this range of concentrations. However, our observations highlight that the methodology employed in our study can be used as a sensitive and informative biosensor for contamination of the environment with genotoxins for aquatic vertebrates, at least *H. pulchellus* tadpoles.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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