# Genotoxicity of the herbicide imazethapyr in mammalian cells by oxidative DNA damage evaluation using the Endo III and FPG alkaline comet assays

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# Genotoxicity of the herbicide imazethapyr in mammalian cells by oxidative DNA damage evaluation using the Endo III and FPG alkaline comet assays

Sonia Soloneski[1,2] · Celeste Ruiz de Arcaute[1,2] · Noelia Nikoloff[1,2] · Marcelo L. Larramendy[1,2]

**Abstract** We evaluated the role of oxidative stress in the genotoxic damage induced by imazethapyr (IMZT) and its formulation Pivot® in mammalian CHO-K1 cell line. Using the alkaline comet assay, we observed that a concentration of 0.1 μg/mL of IMZT or Pivot® was able to induce DNA damage by increasing the frequency of damaged nucleoids. To test whether the DNA lesions were caused by oxidative stress, the DNA repair enzymes endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), which convert base damage to strand breaks, were used. Our results demonstrate that after treatment of CHO-K1 cells with the pure active ingredient as well as the commercial formulation Pivot®, an increase in DNA strand breaks was observed after incubation of both Endo III and Fpg enzymes, indicating that both compounds induce DNA damage involving both pyrimidine and purine-based oxidations, at least in CHO-K1 cells. Our findings confirm the genotoxic potential of IMZT and suggest that this herbicide formulation must be employed with great caution, especially not only for exposed occupational workers but also for other living species.

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## Abbreviations

| | |
|---|---|
| Endo III | Endonuclease III |
| Fpg | Formamidopyrimidine DNA glycosylase |
| GDI | Genetic damage index |
| IMZT | imazethapyr |

## Introduction

The imidazolinones constitute a class of agrochemicals that are used worldwide as a selective pre- or postemergence herbicides for commercially important crops, including soybean, alfalfa, wheat, and barley, among others. This group of herbicides consists of six enantiomers and their methyl derivatives, i.e., imazapyr, imazethapyr (IMZT), imazapic, imazamox, imazaquin, and imazamethabenz-methyl (Lao and Gan 2006; Lin et al. 2007). Imidazolinones can be considered as one the most widely used agrochemicals in over 200 countries worldwide. Whereas they have demonstrated to be potent and highly selective for plants, they are nontoxic for animals (USEPA 2002). Imidazolinones are able to control weeds via inhibition of a plant enzyme. This inhibition causes a disruption in protein synthesis that, in turn, leads to interference in DNA synthesis and cell growth and eventually to weed death. The acetohydroxyacid synthase (EC 2.2.1.6.), formally classified as acetolactate synthase (E.C. 4.1.3.18), is the target enzyme of this group. In plants, this enzyme catalyzes the first step in the biosynthesis of the three branched-chain aliphatic amino acids valine, isoleucine, and leucine (Breccia et al. 2013; Sala et al. 2008; Zhou et al. 2010). However, since acetohydroxyacid synthase does not occur in animals, which rely on plants for these essential amino acids, imidazolinone herbicides, generally, have very low toxicity in mammals (Krieger 2001).

IMZT [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1*H*-2-yl) nicotinic acid] is employed as a selective herbicide to control grasses and broadleaved weeds including barnyardgrass, crabgrass, cocklebur, panicums, pigweeds, nightshade, mustard, smartweed, velvetleaf, jimsonweed, foxtails, seedling johnsongrass, lambsquarters, morning glory, and others in a variety of crop and noncrop situations (http://sitem.herts.ac.uk). It is employed particularly as an alternative herbicide for treatment of weeds in glyphosate-resistant soybean crops (USEPA 1989). The herbicide has a low solubility coefficient (1400 mg/L) having then high affinity with water (Johnson et al. 2000; Senseman 2007). Furthermore, the California Department of Pesticide Regulation (2009) has classified this herbicide as a potential ground water contaminant.

IMZT has been classified as a slightly toxic compound (class III) by the US EPA (1989, 2002) and as unlikely hazardous by the WHO (www.pesticideinfo.org). The herbicide has also been reported as a harmful irritant for the respiratory track, skin, and eyes as well as classified as a dangerous compound for the environment by the European Union (http://sitem.herts.ac.uk). Koutros et al. (2009, 2016) reported a significantly increased risk of bladder and colon cancer after IMZT exposure among 20,646 applicators they surveyed.

Genotoxic and cytotoxic studies of IMZT are scarce and contradictory. It has been generally reported to be nonmutagenic for the bacterial reverse mutation assay in *Salmonella typhimurium* and *Escherichia coli* with and without S9 metabolic activation (Magdaleno et al. 2015). Furthermore, similar negative results have been observed for both the wing somatic mutation and recombination test (SMART) of *Drosophila melanogaster* and the Chinese hamster ovary cell/hypoxanthine-guanine phosphoribosyl-transferase (CHO/HGPRT) assay, regardless of the presence of microsomal activation fraction S9 (Fragiorge et al. 2008; USEPA 2002). 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 1989). On the other hand, IMZT is highly toxic to nontarget organisms such as aquatic and terrestrial plants. A marked growth inhibition was observed in the green microalga *Pseudokirchneriella subcapitata* after exposure to a commercial formulation of IMZT (Magdaleno et al. 2015). Furthermore, the herbicide was able to induce both cytotoxicity and chromosomal anomalies including, e.g., spindle disturbances, c-metaphases, chromatin bridges, micronuclei (MNs), and sticky, lagging, and scattered chromosomes on *Triticum durum* and *Vicia faba* root meristematic cells (El-Nahas 2000; Rad et al. 2011). Whereas Liman et al. (2015) reported that pure IMZT induced a marked cytotoxicity estimated by both a root growth inhibition and a reduction of the mitotic index in *Allium cepa* meristematic cells, Magdaleno et al. (2015) demonstrated that the IMZT-based commercial formulation Verosil® exerted such cytotoxic effect by accumulating in the onion meristematic

cells at the prophase stage of the mitosis. In addition, in the latter cellular system, IMZT caused an increase in the frequency of chromosomal aberrations as well as the induction of DNA single-strand breaks evaluated by the comet assay (Liman et al. 2015). Similarly, after exposure to a commercial IMZT preparation, an increase in the frequency of MNs was reported in *A. cepa* (Magdaleno et al. 2015). Qian et al. (2015) examined the IMZT toxicity in roots of *Arabidopsis thaliana* using a molecular approach. They suggested that IMZT inhibits the synthesis of branched chain amino acids (BCAAs; valine, leucine, and isoleucine) not only by strongly increasing BCAA catabolism, affecting then the pattern of growth on root, shoot, and leaves of *A. thaliana* (Qian et al. 2015). In *Lactuca sativa*, Magdaleno et al. (2015) reported IMZT cytotoxicity employing the root elongation assay. Finally, we recently demonstrated that the herbicide IMZT exerts mortality and genotoxicity in Montevideo tree frog *Hypsiboas pulchellus* tadpoles (Pérez-Iglesias et al. 2015). We observed that the IMZT-based formulation Pivot H® increases the frequency of micronucleated erythrocytes and other nuclear abnormalities, e.g., blebbed nuclei and notched nuclei. Furthermore, the induction of primary DNA lesions estimated by the comet assay on circulating blood cells from tadpoles exposed in vivo under laboratory conditions to IMZT was also demonstrated (Pérez-Iglesias et al. 2015). This latter study represents the first evidence of acute lethal and sublethal effects exerted by IMZT on amphibians. However, at the light of this information, the possibility that whether the herbicide could be considered as a direct genotoxicant or a pro-genotoxicant is still not elucidated.

The comet assay is one of the most widely used methods to detect the genotoxic capability of xenobiotics both in vivo and in vitro because 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. Accordingly, different lesions can be detected upon the use of 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 et al. 2013, 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 apurinic/apyrimidinic lyase activity of the Fpg leaves an apurinic/apyrimidinic site which can be detectable by the comet assay (Azqueta et al. 2013, 2014).

In recent years, IMZT has become one of the most used herbicides in Argentina, both alone and in combination with the nonselective herbicide glyphosate in soybean production, to improve the control of many annual grass and broadleaf weeds species (CASAFE 2013). Despite its generalized use all over the world, the precise cellular mechanisms by which IMZT exerts its toxic effect are not yet fully understood. In the present study, CHO-K1 cells were employed to characterize the mode of action underlying the genotoxic potency exerted by the herbicide IMZT as an active ingredient and one of the commercial IMZT-based herbicide formulations most commonly used in Argentina, Pivot® (10.59% IMZT), by estimation of single-strand breaks and alkali-labile lesions introduced into cellular DNA by the alkaline comet assay. Furthermore, the study also aimed to unravel whether IMZT can also induce DNA lesions by other mechanisms, such as oxidative stress, by employing the Endo III and Fpg alkaline comet assay.

## Material and methods

### Chemicals

Imazethapyr [5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-4,5-dihydroimidazol-1$H$-2-yl) nicotinic acid, Pestanal®, CAS 81335-77-5] was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) (Fig. 1). Pivot® (10.59% IMZT, excipients q.s.) was purchased from BASF Argentina S.A. (Buenos Aires, Argentina). Hydrogen peroxide ($H_2O_2$) was purchased from Merck KGaA (Darmstadt, Germany), and dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Endo III and Fpg were obtained from New England Biolabs® Inc. (Ipswich, MA). Bleomycin (BLM, Blocamycin®) was provided by Gador S.A. (Buenos Aires, Argentina). All other chemicals and solvents of analytical grade were obtained from Sigma-Aldrich Co.
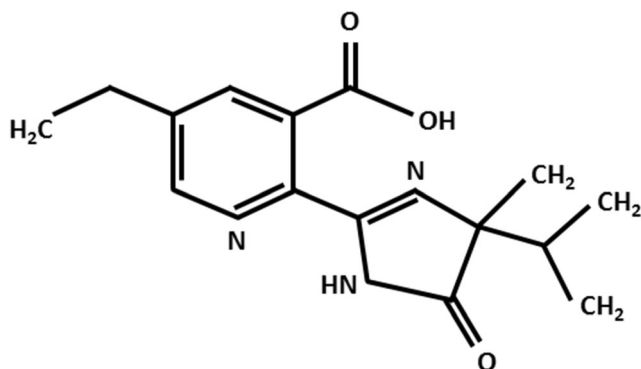


**Fig. 1** Chemical structure of imazethapyr

### Cell culture and chemical treatments

CHO-K1 (CCL-61; American Type Culture Collection, Rockville, MD, USA) cells were cultured in Ham's F-10 culture medium supplemented with 10% fetal calf serum, 100 U/penicillin, and 10 μg/ streptomycin (Gibco, Grand Island, NY, USA) at 37 °C in a 5% $CO_2$ atmosphere. The cells were plated in 35 mm Petri dishes (3 mL; $3.5 \times 10^5$ cells/mL). Cells were dosed of IMZT and with IMZT-normalized concentrations of Pivot®, respectively. The IMZT concentrations used were 0.1, 0.25, and 1.0 μg/mL employing a pulse treatment of 90 min for exposition. Prior to use, IMZT was dissolved in DMSO and diluted in culture medium, whereas Pivot® was diluted directly in culture medium. For all the treatments in all experiments, the final solvent concentration was <1%. Control groups, solvent, and positive controls (1.0 μg/mL BLM and 25 μM $H_2O_2$, 5 min, 4 °C) were run in parallel with herbicide-treated cultures. None of the treatments produced pH changes in the culture medium (range, 7.2–7.4). The same batches of culture medium, serum, and reagents were used throughout the study. Each experiment was repeated three times, and cultures were performed in duplicate for each experimental point. After herbicide treatment, the exposed cells were detached with a rubber-policeman, collected by centrifugation (1500 rpm, 5 min), and then gently resuspended in fresh culture medium at $1 \times 10^5$ cells/mL.

### Cell viability assay

Cell viability was determined at the end of the treatment period (90 min) using the ethidium bromide/acridine orange double staining methodology (McGahon et al. 1995; Montenegro et al. 2007; Nikoloff et al. 2013, 2014a, 2014b; Soloneski et al. 2016). Aliquots of the cell suspension (1 mL) were centrifuged (1500 rpm, 5 min), and the pellet was homogenized with 100 μL in fresh culture medium. Subsequently, in a 1:1 freshly prepared mixture of ethidium bromide (100 μg/mL) and acridine orange (100 μg/mL), 10 μL was mixed with the cell suspension (10 μL). Viability was analyzed using an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination. Acridine orange intercalates into the DNA giving it a green appearance. Ethidium bromide also intercalates into DNA, making it appear orange, but it is only taken up by nonviable cells. Viable cells appeared fluorescent green, whereas orange-stained nuclei indicated dead cells. At least 500 cells were counted per experimental point, and results are expressed as the percentage of viable cells among all cells. Cultures were duplicated for each experimental point, for at least three independent experiments.

### Comet assay (standard and modified version)

After treatment, the cells were processed for the standard comet assay methodology following the alkaline procedure

described by Singh et al. (1988) with minor modifications as reported elsewhere (Soloneski et al. 2016). After treatment, an aliquot of 50 μL of cell suspension were mixed with 120 μL of 0.5% low melting point agarose. The mixture was quickly dropped onto slides that had been previously coated with 1% normal melting agarose. Then, the slides were immersed in ice-cold freshly prepared lysis solution containing 1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na$_2$EDTA, 10 mM Tris, pH 10.0, 1% Triton X-100, and 10% DMSO and then lysed for 1 h at 4 °C in darkness. Subsequently, the slides were equilibrated in an electrophoresis buffer (1 mM Na$_2$EDTA, 300 mM NaOH) for 25 min at 4 °C to allow the cellular DNA to unwind, followed by electrophoresis in the same buffer and temperature for 30 min at 25 V and 250 mA (0.8 V/cm). At last, the slides were neutralized with 0.4 M Tris–HCl (pH 7.5) and stained with 4′-6′-diamino-2-phenylindole (DAPI; Vectashield mounting medium H1200; Vector Laboratories, Burlingame, CA). For slide analysis, an Olympus BX50 fluorescence photomicroscope equipped with an appropriate filter combination was employed. DNA damage was estimated by DNA migration length visually determined in randomly selected and nonoverlapping 100 cells (Azqueta et al. 2011; Collins et al. 1995; Collins 2004; Kobayashi et al. 1995). DNA damage was classified in five classes (0–I, undamaged; II, minimum damage; III, medium damage; IV, maximum damage) (Cavas and Konen 2007). Results are expressed as the mean number of damaged nucleoids (sum of classes II, III, and IV) and the mean comet score for each treatment group. The genetic damage index (GDI) was determined as GDI = [(I) + 2(II) + 3(III) + 4(IV)]/$N_{(I–IV)}$, where I–IV represent the nucleoid type and $N_I$–$N_{IV}$ represent the total number of nucleoids scored (Pitarque et al. 1999). Each experiment was repeated three times, and cultures were performed in duplicate for each experimental point. The modified version of the comet assay was performed following the methodology described by Collins et al. (1995, 1996). The cells were treated with normalized concentrations of 0.1 μg/mL IMZT or Pivot®, respectively, for 90 min as described previously ("Cell culture and chemical treatments" section). The concentration employed in this section was selected considering that 0.1 μg/mL of both compounds did induce significant DNA damage after 90 min of exposure in less than 50% of the nucleoids analyzed by the standard comet assay. 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 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 enzyme supplier. Briefly, slides were incubated with 50 μL Endo III (0.5 U) or Fpg (0.13 U) as suggested elsewhere (Collins et al. 1993; Collins 2004). Control cells were treated with a 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, slides were processed following the standard comet assay protocol. Analysis of the slides was performed as previously described in this section. Then, net oxidative DNA damage (OD) was obtained for each treatment using the formula OD = [(%GDI buffer + enzyme + herbicide) − (%GDI buffer + )], as indicated previously (Collins and Azqueta 2012; Demir et al. 2014; Domijan et al. 2006; Mikloš et al. 2009; Soloneski et al. 2016).

### Statistical analysis

Data were analyzed using Statistica 7.0. software. The proportion of damaged cells per replicate was calculated. Each proportion was angular transformed, and a one-way ANOVA with Dunnett's test was performed, whereas a one-way ANOVA with Tukey's test was performed for comparison between negative and solvent control data. ANOVA assumptions were corroborated with Barlett's test for homogeneity of variances and a $\chi^2$ test for normality. In cases which did not perform the assumptions of normality, a Kruskal–Wallis test was made. Differences in viability in treated and control cells were evaluated by $\chi^2$ test. To check for a dose-dependent response to the treatments, Spearman's rank order linear correlation analysis was also performed. To compare net OD produced by IMZT in each enzyme (with respect to buffer enzyme), a $t$ test for the difference of means with equal variances was performed. The chosen level of significance was 0.05 unless indicated otherwise.

### Results

Data of the alkaline comet assay obtained in CHO-K1 cells exposed during a short-pulse treatment of 90 min with different concentrations of IMZT and Pivot®, including the proportion of different nucleoids, GDI, and viability values, are presented in Table 1. Positive control induced an enhancement in the frequency of damaged cells as well as of the GDI compared to the control group ($P < 0.001$) by increasing the frequency of type II, III, and IV comets ($P < 0.001$). On the other hand, solvent control did not alter the frequency of damaged nucleoids compared with that of control cultures ($P > 0.05$). While different nucleoid damage categories are visualized in Fig. 2, mean frequencies of cells from each damage grade are depicted in Table 1. IMZT and Pivot® treatments increased the GDI values at all exposure concentrations ($0.01 > P < 0.001$). Statistical analyses demonstrated that the GDI increase induced by both IMZT and Pivot®, regardless of the concentration assayed, was due to an enhancement in the frequency of type II ($P < 0.001$) and type III comets ($P < 0.01$), but not type IV comets ($P > 0.05$), and a concomitant decrease of type I comets ($P < 0.01$).

**Table 1**  Analysis of DNA damage measured by comet assay in CHO-K1 cells exposed during 90 min to imazethapyr (IMZT) or Pivot®

| Compound | Concentration (µg/mL) | Proportion of damaged nuclei (%)[a] | | | | DNA damage (%)[b] | GDI | Viability (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | | Type I | Type II | Type III | Type IV | (II + III + IV) | | |
| Control | | 90.00 | 9.67 | 0.33 | 0.00 | 10.00 ± 1.52 | 1.07 ± 0.01 | 99.67 ± 0.33 |
| DMSO[c] | | 90.57 | 8.81 | 0.63 | 0.00 | 9.43 ± 2.16 | 1.10 ± 0.08 | 97.33 ± 0.67 |
| IMZT | 0.1 | 53.38*** | 41.44*** | 4.95** | 0.23 | 46.62 ± 1.04*** | 1.52 ± 0.01** | 99.33 ± 0.33 |
| | 0.25 | 42.46*** | 53.36*** | 3.71* | 0.46 | 57.54 ± 1.70*** | 1.62 ± 0.01*** | 99.33 ± 0.33 |
| | 1 | 46.25*** | 47.22*** | 5.33** | 1.21 | 53.75 ± 2.43*** | 1.62 ± 0.04*** | 99.00 ± 0.58 |
| Pivot® | 0.1 | 47.37*** | 46.05*** | 6.25** | 0.33 | 52.63 ± 3.43*** | 1.60 ± 0.03*** | 98.33 ± 0.67 |
| | 0.25 | 54.52*** | 39.68*** | 5.16** | 0.65 | 45.48 ± 2.17*** | 1.52 ± 0.06*** | 99.00 ± 0.58 |
| | 1 | 44.09*** | 47.92*** | 7.35** | 0.64 | 55.91 ± 1.71*** | 1.65 ± 0.02*** | 99.50 ± 0.29 |
| BLM[d] | | 12.25*** | 43.87*** | 25.64*** | 18.23*** | 87.75 ± 2.78*** | 2.50 ± 0.03*** | 99.00 ± 0.58 |

*GDI* genetic damage index

*P < 0.05; **P < 0.01; ***P < 0.001; values in regard to control values

[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] Dimethyl sulfoxide (DMSO, 0.05%) was used as solvent control for pure imazethapyr

[d] Bleomycin (0.1 µg/mL) was used as positive control

Results from the cell viability assays, which were run before the comet assay, are presented in Table 1. Regardless of the concentration, no significant alterations in cell viability were found in cultures treated with IMZT, Pivot®, DMSO, and BLM compared to control groups ($P > 0.05$).

The role of oxidative stress in the induction of DNA strand breaks by IMZT was further explored employing the Endo III- and Fpg-modified comet assay, and the results are presented in Table 2, whereas the levels of net OD are depicted in Fig. 3. $H_2O_2$ treatment (positive control) induced an enhancement in the GDI ($P < 0.001$) as well as the net OD values after treatment with both Endo III ($P < 0.001$) and Fpg ($P < 0.001$) compared to samples without enzymes (Table 2; Fig. 3). The employ of Endo III and Fpg showed significant differences compared to the enzyme buffer-treated cells ($P > 0.05$). DNA digested with both Endo III or Fpg produced a significant increase in the GDI in nucleoids exposed to 0.1 µg/mL of both

IMZT and the IMZT-based herbicide formulation Pivot® compared with enzyme buffer-treated cells ($P < 0.001$; Table 2; Fig. 3). Therefore, a significant enhancement in OD values was observed in those nucleoids exposed to IMZT ($P < 0.01$) and Pivot® ($P < 0.001$) after digestion with Endo III as well as in those nucleoids incubated with Fpg enzyme ($P < 0.001$), respectively. Finally, the increase of OD observed after incubation with Fpg was more evident than that of Endo III for both IMZT and Pivot®, although not reaching statistical significance ($P > 0.05$; Fig. 3).

## Discussion

Based on the results obtained in this study employing IMZT and Pivot®, it can be inferred that all tested concentrations of the herbicide were able to induce DNA damage at equivalent

**Fig. 2** Digitized comet images showing undamaged (*0-I*) and damaged nucleoids (*II–IV*) of IMZT-treated CHO-K1 cells. They represent classes *0–IV* as used for visual scoring (*0-I* undamaged, *II* minimum damage, *III* medium damage, *IV* maximum damage). Cells were stained with DAPI and capture at fluorescent microscope. Magnification: ×1000
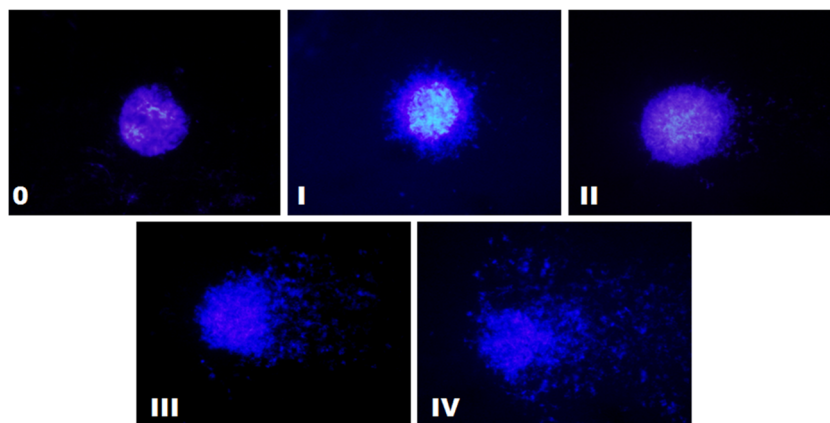
**Table 2** Analysis of DNA damage as measured by comet assay after treatment of CHO-K1 cells with 0.1 μg/mL imazethapyr (IMZT) or Pivot® and incubation with or without lesion-specific endonuclease Endo III and Fpg

| Compound | Treatment | Proportion of damaged nuclei (%)[a] | | | | DNA damage (%)[b] | GDI[b] | OD[b] |
|---|---|---|---|---|---|---|---|---|
| | | Type I | Type II | Type III | Type IV | (II + III + IV) | | |
| Control | | 88.27 | 10.26 | 1.17 | 0.30 | 11.73 ± 1.53 | 1.08 ± 1.04 | |
| | Buffer Endo III | 83.45 | 11.82 | 3.72 | 1.01 | 16.55 ± 6.33 | 1.22 ± 0.10 | |
| | Endo III | 78.21 | 10.26 | 10.57 | 0.96 | 21.79 ± 0.27 | 1.34 ± 0.01* | 0.12 ± 0.01 |
| | Buffer Fpg | 77.67 | 16.33 | 3.67 | 2.33 | 22.33 ± 5.84 | 1.31 ± 0.12* | |
| | Fpg | 63.64 | 30.84 | 2.92 | 2.60 | 36.36 ± 7.33 | 1.44 ± 0.10** | 0.13 ± 0.05 |
| DMSO[c] | | 87.67 | 11.33 | 1.00 | 0.00 | 12.33 ± 2.90 | 1.11 ± 0.03 | |
| | Buffer Endo III | 61.39 | 35.13 | 2.22 | 1.26 | 38.61 ± 5.04 | 1.42 ± 0.06 | |
| | Endo III | 63.38 | 31.08 | 3.38 | 2.16 | 36.62 ± 3.34 | 1.44 ± 0.04 | 0.02 ± 0.08 |
| | Buffer Fpg | 62.62 | 32.39 | 3.43 | 1.56 | 37.38 ± 2.85 | 1.41 ± 0.01 | |
| | Fpg | 63.66 | 30.03 | 5.71 | 0.60 | 36.34 ± 11.41 | 1.44 ± 0.17 | 0.03 ± 0.17 |
| IMZT | | 47.03 | 44.29 | 8.45 | 0.23 | 50.34 ± 3.10 | 1.56 ± 0.06*** | |
| | Buffer Endo III | 22.94 | 37.92 | 28.74 | 10.40 | 77.06 ± 1.62 | 2.27 ± 0.05*** | |
| | Endo III | 14.83 | 48.90 | 23.66 | 12.61 | 85.18 ± 1.35 | 2.34 ± 0.10*** | 0.07 ± 0.04## |
| | Buffer Fpg | 40.00 | 39.36 | 16.45 | 4.19 | 60.00 ± 3.16 | 1.85 ± 0.07* | |
| | Fpg | 25.81 | 31.94 | 13.54 | 28.71 | 74.19 ± 4.90 | 2.44 ± 0.20*** | 0.59 ± 0.24### |
| Pivot® | | 48.05 | 45.45 | 6.17 | 0.33 | 51.95 ± 2.70 | 1.57 ± 0.02*** | |
| | Buffer Endo III | 17.61 | 43.76 | 28.69 | 9.94 | 82.38 ± 1.07 | 2.31 ± 0.07*** | |
| | Endo III | 9.55 | 39.81 | 42.04 | 8.60 | 90.45 ± 3.23 | 2.50 ± 0.09*** | 0.19 ± 0.03### |
| | Buffer Fpg | 43.34 | 46.74 | 7.05 | 2.87 | 56.66 ± 3.71 | 1.69 ± 0.01* | |
| | Fpg | 20.63 | 57.50 | 11.25 | 10.62 | 79.38 ± 3.64 | 2.12 ± 0.14*** | 0.43 ± 0.15### |
| H₂O₂[d] | | 42.00 | 28.00 | 20.33 | 9.67 | 58.00 ± 1.15 | 1.99 ± 0.09*** | |
| | Buffer Endo III | 28.67 | 32.67 | 23.33 | 15.33 | 71.33 ± 7.69 | 2.25 ± 0.21*** | |
| | Endo III | 7.33 | 43.00 | 26.33 | 23.34 | 92.67 ± 2.33 | 2.66 ± 0.15*** | 0.41 ± 0.17### |
| | Buffer Fpg | 41.00 | 40.00 | 12.00 | 7.00 | 59.00 ± 4.04 | 1.85 ± 0.04*** | |
| | Fpg | 17.33 | 62.00 | 15.34 | 5.33 | 82.67 ± 5.36 | 2.09 ± 0.05*** | 0.24 ± 0.04### |

*GDI* genetic damage index, *OD* net oxidative damage

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; when compared with respective control; ## $P < 0.01$; ### $P < 0.001$, indicate Endo III- and Fpg-sensitive sites compared with respect to buffer enzyme

[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] Dimethyl sulfoxide (DMSO, 0.05%) was used as solvent control for pure imazethapyr

[d] Hydrogen peroxide ($H_2O_2$, 25 μM) was used as positive control

levels, independently of the concentration assayed. The herbicide exposure increased DNA migration in CHO-K1 cellular nucleoids fourfold to fivefold compare to values of control cultures. The results verify previous findings depicting the genotoxic potential of IMZT through the induction of DNA damage evaluated by comet assay (Liman et al. 2015; Pérez-Iglesias et al. 2015). To our knowledge, this is the first report demonstrating the genotoxic potential of IMZT in mammalian cells in vitro, at least in CHO-K1 analyzed using the comet assay.

The adaptation of the alkaline comet assay to incorporate lesion-specific endonucleases increases its specificity and sensitivity through the recognition of DNA damaged bases and introduction of additional breaks (Collins et al. 2014). To elucidate a possible mechanism of IMZT-induced DNA damage, we included Endo III and Fpg incubation in the alkaline modified comet assay to reveal the presence of oxidized pyrimidines and purines, respectively, as a result of herbicide-induced oxidative stress in CHO-K1 cells. Our observations reveal that treatment with both Endo III and Fpg buffers induces an enhancement in the frequency of DNA damage revealed by the end point. This observation is in total agreement with previous reported indicating the ability of these enzyme buffers to introduce lesions into cellular DNA and thus increasing the length of the nucleoids (Collins and Azqueta 2012; Demir et al. 2014; Soloneski et al. 2016). Furthermore, the results we obtained also showed that DNA damage and, then, the net OD were increased in IMZT- and Pivot®-treated
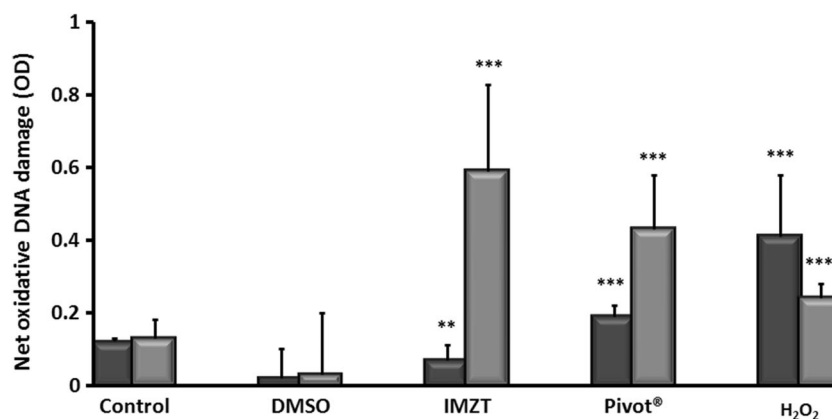
**Fig. 3** Effect of treatment with IMZT and the commercial formulation Pivot®. Induced DNA damage in CHO-K1 cells was measured by the modified comet assay using Endo III (*dark gray bars*) and Fpg (*light gray bars*) enzymes. Net oxidative DNA damage was calculated as the difference between the scores obtained before and after incubation with the respective enzyme or the buffer. Hydrogen peroxide (25 μM) and DMSO were used as positive and solvent controls, respectively. Data are expressed as means ± SD. **$P < 0.01$ and ***$P < 0.001$ indicate Endo III- and Fpg-sensitive sites compared with respect to buffer enzyme

nucleoids after incubation with Endo III and Fpg. The significant difference in DNA damage with and without Fpg treatment in the comet assay indicates that both IMZT and Pivot®, most provably by the IMZT present within the technical formulation, are able to induce oxidized purines in the DNA of exposed cells, which can include 8-oxo-Gua, FapyGua, FapyAde, as well as other ring-opened purines, as stated previously (Azqueta et al. 2013, 2014). The exact mechanism of DNA oxidation by IMZT is not clear, although it was recently reported that IMZT causes an increase in ROS generation and a drastic reduction in both antioxidant gene transcription and enzyme activity, which can lead to oxidative stress, in *A. thaliana* (Qian et al. 2015). They found that IMZT induced excessive ROS production, thus damaging the chloroplast membranes and impairing chlorophyll synthesis (Qian et al. 2015). Similarly, Moraes et al. (2011) reported that a commercial formulation of IMZT also caused oxidative stress in the fish *Cyprinus carpio* by increasing the activity of both AChE and catalase enzymes as well as inducing alterations in metabolic parameters that could be indicative of redox imbalances related to a possible oxidative stress situation. ROS overproduction also can originate multiple lesions into DNA, including single- and double-strand breaks, and cause deficiency in antioxidant cellular defense, apurinic site generation, and modified pyrimidines and purines, among others side effects, thus providing a link between oxidative DNA damage and numerous degenerative processes, including carcinogenesis (Wiseman and Halliwell 1996). Further studies are required to obtain better and more comprehensive knowledge of the possible mechanism(s) through which the herbicide IMZT affects DNA at the purine base level.

We also analyzed the ability of IMZT and Pivot® to induce oxidative stress-induced DNA damage by estimation of pyrimidine oxidation after incubation with Endo III. In the present study, an increase in Endo III-recognized break sites was induced in CHO-K1 cells following treatment with IMZT or with the formulated commercial product. The results also demonstrate, for the same treatment, that the number of strand breaks cleaved by Endo III was smaller than that cleaved by Fpg. Thus, it appears valid to claim from the current data that both compounds induce DNA damage associated with the oxidation of pyrimidines.

In conclusion, in the present investigation, we have demonstrated that IMZT causes an increase in DNA damage in in vitro mammalian cells. The most interesting result was, however, that the pure active ingredient IMZT and the IMZT-based commercial formulation Pivot® induce oxidative DNA damage by both purine and pyrimidine-based oxidations. Hence, the use of this herbicide should be controlled, since this compound is widely used in agricultural fields and households as active ingredient in several commercial formulated products.

Today, evaluation of the impacts of pesticides on human and living organisms is a clue concern of environmental policies in many countries worldwide. Furthermore, it is essential to strike a balance between pesticide usages minimizing the negative consequences induced by pesticide usage while maximizing their beneficial effects for productive crops. Human population from different categories are often implicated on potential genotoxic effect of many pesticides, and it should be considered with particular attention not only by occupational workers, which manipulates directly large amount of pesticides in agronomical practices, but also by people that consume contaminated crop products. Accordingly, a proper evaluation of genotoxic risk associated with the employ of the herbicide IMZT in agricultural practices is an important

activity in order to reduce the negative impact not only on human health but also on the environment.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflicts of interest.

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