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Imidacloprid Causes DNA Damage in Fish: Clastogenesis as a Mechanism of Genotoxicity

Fernando G. Iturburu[1] · María F. Simoniello[2] · Sandra Medici[3] · Ana M. Panzeri[4] · Mirta L. Menone[1]

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Abstract
Neonicotinoids are one of the most widely used insecticides in the world. DNA damage is considered an early biological effect which could lead to reproductive and carcinogenic effects. The present study aimed to evaluate DNA damage and bases oxidation as a mechanism of genotoxicity, on the freshwater fish *Australoheros facetus* acutely exposed to imidacloprid (IMI). The Comet assay with the nuclease ENDO III enzyme was performed for detecting pyrimidine bases oxidation using blood samples. Micronucleus and other nuclear abnormalities frequencies were also quantified. A significant increase of damage index at 100 and 1000 µg/L IMI was detected; while ENDO III score increased from 1 to 1000 µg/L IMI; varying both in a linear concentration-response manner. MN frequency increased in fish exposed to 1000 µg/L IMI. These results show that short-term exposures to environmentally relevant concentrations of IMI could affect the genetic integrity of fishes through oxidative damage.

Keywords Neonicotinoid · Fishes · DNA fragmentation · Micronucleus

Human population is mostly densely concentrated around water sources, particularly around rivers, mouth of estuaries and sheltered bays, being the focus of intensive human activities. Human activities are able to modify the aquatic environment through removal of biomass and habitats and via the addition of contaminants (Díaz-Cruz and Barceló 2015). In intensively cultivated regions, streams are severely affected by the input of agrochemicals such as pesticides (for example insecticides, herbicides or fungicides) and nutrients (Jergentz et al. 2005). Neonicotinoids are nowadays one of the most widely used insecticides in the world. They act systemically, travelling through plant tissues and protecting all parts of the crop, and are widely applied as seed dressings (Goulson 2013). The widespread use of these insecticides in agriculture results firstly in contamination of the soil of the treated crops, and secondly in the transfer of residues to the aquatic environment (Sánchez-Bayo et al. 2016). The first neonicotinoid insecticide available for use was imidacloprid (IMI), which was introduced to the agrochemical market in 1991 by Bayer (Jeschke et al. 2011). Since its introduction, IMI use had been applied for a wide type of uses including crop protection, veterinary care and garden use (Elbert et al. 2008; Simon-Delso et al. 2015). DNA damage is considered an early biological effect which could disturb biological structures and functions and lead to a genotoxic syndrome related with reproductive and carcinogenic problems (Anderson et al. 1994). Micronucleus test has been demonstrated to be a sensitive model to evaluate genotoxic compounds in fish under controlled conditions (Bolognesi et al. 2006) while the Comet assay (CA) with an extra-step involving DNA lesion-specific repair enzymes revealed to be useful for an effective assessment of genotoxic hazard, increasing sensitivity and limiting the risk of false negative results (Guilherme et al. 2012).

✉ Mirta L. Menone
mirta.menone@gmail.com

[1] Laboratorio de Ecotoxicología, Instituto de Investigaciones Marinas y Costeras (IIMyC)- UNMDP, CONICET, Facultad Cs. Ex. y Nat., Funes 3350, 7600 Mar del Plata, Buenos Aires, Argentina

[2] Cátedra de Toxicología, Farmacología y Bioquímica Legal, Facultad de Bioquímica y Ciencias Biológicas, UNL, Ciudad Universitaria, Paraje El Pozo. C.C. 242, 3000 Santa Fe, Argentina

[3] Fares Taie Instituto de Análisis, Magallanes 3019, 7600 Mar del Plata, Argentina

[4] Laboratorio de Genética, Instituto de Investigaciones Marinas y Costeras (IIMyC)- UNMDP, CONICET, Facultad Cs. Ex. y Nat., Funes 3250, 7600 Mar del Plata, Buenos Aires, Argentina

The present study aimed to evaluate DNA damage and bases oxidation as a mechanism of genotoxicity, on the freshwater fish *Australoheros facetus* acutely exposed to environmentally relevant concentrations of IMI.

Materials and Methods

Australoheros facetus is a South American cichlid fish which inhabits freshwater bodies, mainly in the basin Paraná and del Plata, and shallow lakes in the Pampa's region (Casciotta et al. 2005). Juvenile specimens were obtained from non-anthropized lagoons of the General Pueyrredón municipality by net fishing. Fish were acclimatized for 2 months to laboratory condition in 140 L tanks (12 h:12 h, light: dark photo-period; 15 ± 1°C water temperature; pH 8.5). This acclimatization time allowed fish to grow and reach the assay size. For the CA experiment fish of mean total length 6.78 ± 0.44 cm; while for the cytogenetic biomarkers assay fish of mean total length 7.07 ± 0.35 cm were used.

Fish were exposed to IMI (*N*-{1-[(6-chloro-3-pyridyl) methyl]-4, 5-dihydroimidazol-2-yl} nitramide) analytical standard, which was obtained from Sigma-Aldrich (CAS no. 138261-41-3). This insecticide has high water solubility (610 mg/L at 20°C) and an octanol–water partitioning coefficient (log Kow) of 0.57 (IUPAC 2017). IMI stock solution was prepared in MilliQ water. The appropriate exposure medium was prepared by diluting different amounts of the stock solution in tap water immediately before exposure. Water samples were directly injected in the UPLC-MS/MS (Waters Xevo TQS-microTM) to quantify the IMI concentration in water. The separation of the samples was carried out using a precolumn from WatersTM (4 × 2.00 mm) and a UHPLC C18 column (2 × 100 mm, particle size 1.7 µm). The mobile phase consisted of solution C (water 95%, methanol 5%, formic acid 10 mM, HCOONH4 10 mM) and solution D (metanol 100%, formic acid 10 mM, HCOONH4 10 mM) at a flux of 0.3 mL/min. A gradient with the following conditions was performed: 0–1 min 100% C, 1–15 min from 100% C to 100% D and maintained until 17 min. Column temperature was maintained at 45°C with an injection volume of 10 µL. The retention time for IMI was 5.5 min. The limit of detection was 0.5 ng/mL and the limit of quantification was 1 ng/mL. Concentrations of 1.04 ± 0.13, 9.70 ± 0.27, 75.07 ± 0.23 and 810.81 ± 16.51 µg/L IMI corresponding to nominal concentrations of 1, 10, 100 and 1000 µg/L IMI were measured. These concentrations included from environmental concentrations (Van Dijk et al. 2013; Anderson et al. 2015) to a one order of magnitude below lethal concentration of the species (Iturburu et al. 2017).

Two assays were performed, one for CA and other for cytogenetic biomarkers. Both blocks were performed in aquaria with tap water (temperature 16 ± 1°C), illumination with fluorescent lamps with 12:12 h light: dark periods, and pH 8.5. Six fish per treatment (n = 6) were exposed to 0 (negative control, Co-), 1, 10, 75 and 810 µg/L during 48 h. In the block for CA, an in vitro positive control (Co+) was performed from blood of non-exposed fish by adding 50 µM H_2O_2 (1700 µg/L) to the slide, during 10 min. In the block for cytogenetic biomarkers no positive control was necessary to perform because previous studies registered significant induction of micronucleus (MN) and other nuclear abnormalities (NA) in *A. facetus* exposed to 50 mg/L of methyl methanesulfonate (Iturburu et al. 2017). Fish were transferred to exposure aquaria 48 h prior starting the assays. Twenty-four hours before the assay, leftover food and feces were removed to prevent IMI adsorption during the assay. The fish were starved during the assay. Control fishes suffered the same manipulation than IMI exposed fish. The assays were carried out in static conditions because IMI stability in aqueous solution was already analyzed and reported, at least for a period of 48 h (Iturburu et al. 2017). No mortality was recorded during the assays. After exposure, fish were removed from the tanks and blood was extracted by cardiac puncture, with heparinized syringes. Blood extraction and fish euthanasia were performed according to the protocols approved by the Animal Ethical Committee at the National University of Mar del Plata (CICUAL/UNMDP, OCA 146/15).

The CA was performed using erythrocytes from blood samples, following the methodology of Singh et al. (1988) with the modifications carried out by Collins et al. (1996) to detect bases oxidation. For *A. facetus*, the nuclease ENDO III enzyme was used to detect pyrimidine bases oxidation. Briefly, after blood extraction, the sample was diluted in PBS solution (1:40). This same cell suspension used in the comet assay was mixed with fluorescent with DNA binding dye-mix working solution of 100 µg/mL acridine orange and 100 µg/mL ethidium bromide (prepared in Ca^{2+} and Mg^{2+} free PBS) and examined under a fluorescent microscope (400X). A total of 200 cells were counted per sample and the percentage of viable cells was determined by fluorescent DNA- binding dyes. The standard Alkaline CA procedure originally described by Singh et al. (1988) was used with modifications. Briefly, for each fish, four slides were performed: two were treated with the enzyme and two with the buffer solution alone. An aliquot of cell solutions was mixed with 1% low melting point (LMP) agarose, and this mixture was added to slides previously coated with 1% normal melting point (NMP) agarose. After LMP layer gelification, the slides were submerged in fresh cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 10% DMSO and 0,1% Tritón-X, pH 10), and left at 4°C overnight. After lysis process, slides were washed and excess liquid dabbed off with tissue. Slides were incubated with 50 µL of ENDO III enzyme (1/3000 dilution) or its respective buffer solution

(40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8.0 with KOH). All slides were incubated at 37°C for 30 min and at the end of the incubation period, coverslips were removed and slides were placed in the electrophoresis tank to continue with the Comet assay (Poletta et al. 2016). DNA undwinding was performed in freshly made alkaline solution (300 mM NaOH, 1 mM EDTA; pH > 13) during 10 min, electrophoresed 10 min, at 24 V, 300 mA (0,70 V/cm). All of the steps were carried out under conditions of minimal illumination and low temperature (on ice). Later, the slides were neutralized (0.4M Tris–HCl, pH 7.5) and dehydrated with ethanol. Finally, slides were coded for 'blind' analysis, stained with DAPI (Sigma Aldrich, 20 µg/mL) and comet images were obtained from each sample under fluorescent microscopy. One hundred randomly selected cells (50 from each of two replicated slides) were scored.

For each fish two slides were prepared placing one drop of blood and performing the smear technique. The smears were fixed in absolut methanol 15 min, and they were allowed to dry at room temperature. Once dry, the smears were stained with 15% Giemsa solution (Merck) during 15 min. To establish the MN and NA frequencies, 2000 erythrocytes were counted by sample using an optic microscope (Olympus CX31) with a magnification of ×1000. The smears were evaluated in a random and blind review of only one observer. Only cells with intact nuclear and cell membranes and with erythrocyte morphology were included. Fish erythrocytes have a rounded-ovoid well defined nucleus, and unlike other blood cells, when erythrocytes are stained with Giemsa solution there is a pronounced colour difference between the nucleus and the cytoplasm. It was identified as MN each non-refractory cytoplasmic particle, with ovoid shape and the same stain pattern than the nucleus, and well defined borders.

The frequency of MN was quantified on 1000 erythrocytes, averaging two smears by fish. To characterize the NA, we adopted the criterion of Bolognesi et al. (2006), grouping lobed and blebbed nuclei in only one category called buds. The NA were grouped in buds (nucleus with an evagination in its membrane with presence of chromatin) and notched (nucleus with a notch in the membrane with absence of chromatin). The frequencies of each category of NA were calculated as the number of NA every 1000 erythrocytes, averaging both smears.

DNA damage index (DI) score in CA was obtained from cells visually classified into five classes (Simoniello et al. 2009). The classification was performed according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4). Damage index was calculated as: DI = n1 + 2.n2 + 3.n3 + 4.n4, where n1, n2, n3 and n4 are the number of cells in each class of damage, respectively. Also ENDO III score were calculated as the substraction of DI values obtained with the ENDO III enzyme, minus the DI obtained without the enzyme. Net enzyme-sensitive sites are then the measure of the oxidized bases concerned (Collins 2009). Regarding cytogenetic biomarkers, both MN and NA frequencies were calculated as number per 1000 cells. Data are expressed as mean ± standard deviation. Normality and homoscedasticity of variances were verified by D'agostino & Pearson´s and Bartlett's tests, respectively. To test differences among concentrations, one-way ANOVA was applied (followed by a post hoc Dunnett test) if assumptions were satisfied. If not, the non- parametric Kruskal–Wallis (plus a post hoc Dunn test) test was applied. Student´s T test was performed to compare DI and ENDO sites in Co- vs. Co+ (Zar 1999). Linear regression was tested (log [IMI] vs. biomarker) to test if genotoxic biomarkers respond linearly in a concentration- response manner. In all tests, the significance level was 5%.

Results and Discussion

The DI significantly increased in blood exposed to H_2O_2 in vitro ($p < 0.05$). For this positive control, DI had a score 60% higher than the negative control. Regarding IMI treatments, exposures to 100 and 1000 µg/L showed a significant increase of DI with respect to the negative control ($p < 0.0001$) (Table 1). Linear regression test showed a positive linear concentration- response with a high fit goodness ($R^2 = 0.99$) for DI in fish exposed to growing concentration of IMI (Fig. 1). In vivo studies of IMI genotoxicity with terrestrial vertebrates have been focused mainly in model rodents. These works pointed to determine DNA damage in blood components or in spermatogonial tissues, finding DNA damage from 8 mg/kg/day for long term exposure (Bal et al. 2012) to 170 mg for a single exposure (Arslan et al. 2015). The lowest observed effect concentrations (LOECs) for amphibians showed a

Table 1 Damage Index and ENDO Sites (ENDO III) Scores expressed as mean ± standard deviation (SD) in erythrocytes of the freshwater fish *Australoheros facetus* (n = 6) exposed to imidacloprid

Chemical	Concentration (µg/L)	DI	ENDO III score
Negative control (Co−)	0	137 ± 4	14 ± 3
H_2O_2 (Co+)	1700 (≈ 50 µM)	222 ± 2	47 ± 3*
Imidacloprid	1	149 ± 5	84 ± 7*
	10	176 ± 6	91 ± 2*
	75	208 ± 8*	61 ± 6*
	810	231 ± 4*	59 ± 6*

DI damage index. Asterisk (*) indicates significant difference from control (Co−) (p value < 0.05)

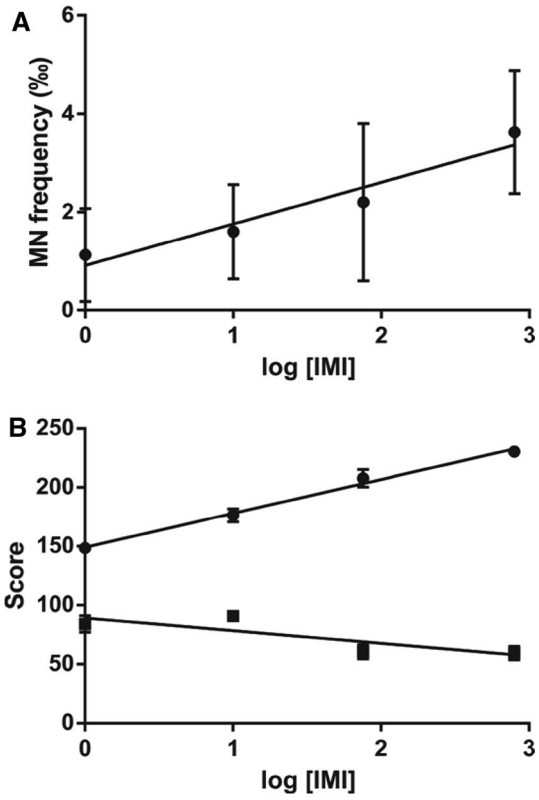

Fig. 1 Linear regression between logarithm of imidacloprid (IMI) concentration and **A** micronucleus (MN) frequency filled circle = MN frequency; mean ± standard deviation (SD); and **B** damage index (DI) scores in the freshwater fish *Australoheros facetus* exposed to IMI, filled circle = DI for comet assay without ENDO III enzyme (ENDO III Score), filled squared = ENDO III Score; mean ± standard deviation (SD)

genotoxic effect on tadpoles in a range of 8–30 mg/L (Feng et al. 2004; Ruíz De Arcaute et al. 2014), concentrations certainly higher than those studied in the present work. Few data on fish are available such as in the cichlid fish Nile tilapia (*Orechromis niloticus*) exposed during 96 h (Ansoar-Rodríguez et al. 2015) and in other fishes but at higher concentrations, 300 µg/L in a 7 days-exposure for zebrafish (*Danio rerio*) (Ge et al. 2015) and 43 mg/L in a 6 days- exposure for *Misgurnus anguillicaudatus* (Xia et al. 2016).

In the case of ENDO III score, H_2O_2 treatment increased the score more than three times with respect to the negative control ($p < 0.0001$). All the IMI concentration treatments showed an increase of ENDO III score ($p < 0.0001$), ranging from 4.2 to 6.5 times (Table 1), demonstrating oxidative damage and representing an important step towards the understanding of IMI´s mechanism of genotoxicity. For ENDO III the scores showed a negative linear relation with the concentration, but the goodness of fit was not to strong ($R^2 = 0.68$) (Fig. 1).

The MN frequency varied from 0 to 2‰ in the negative control samples. MN frequency increased significantly when fish were exposed to 1000 µg/L IMI during 48 h ($p < 0.05$) (Table 2). The linear regression analysis showed a positive linear relation of MN frequency and the log [IMI] ($R^2 = 0.94$) (Fig. 1), in agreement with previous studies made in our laboratory with *A. facetus* (Iturburu et al. 2017).

Total NA, notched and buds frequencies had no significant differences with any of the IMI concentrations tested ($p > 0.05$), and no linear relationship was observed with any of these three biomarkers of nuclear abnormalities ($p > 0.05$). Nuclear evaginations categorized like "buds" did not reproduce the increase observed previously in *A. facetus* (Iturburu et al. 2017), probably because the mechanism responsible for the formation of NA has not been fully explained. In fact, some authors do not consider them as indicators of genotoxic damage but some other do (Ruíz De Arcaute et al. 2014). A positive correlation between the DNA oxidation biomarker 8-OHdG and NA in wild populations of fish exposed to chemical pollution was found, leading the authors to suggest that oxidative stress could be a mechanism involved in the formation of NA (Oliveira et al. 2010). Therefore, further studies will be necessary to clarify the findings of NA in *A. facetus*.

Although our previous analysis of size-classified MN suggested DNA fragmentation in fishes exposed to IMI (Iturburu et al. 2017), it is known that CA is a more sensitive biomarker than the MN frequency (Pinheiro-Araldi et al. 2015). This fact was confirmed in *A. facetus*, since a significant increase of DI values of CA was observed from an IMI concentration one order of magnitude lower than the concentration at which MN frequency increased. The

Table 2 Micronucleus (MN) and nuclear abnormalities (NA) frequencies expressed as mean ± standard deviation (SD) in erythrocytes of the freshwater fish *Australoheros facetus* (n = 6) exposed to imidacloprid

Treatment	N° of cells analyzed	MN (%)	Notched (%)	Buds (%)	Total NA (%)
Negative control (Co−)	10,000	1.1 ± 0.7	2.4 ± 1.0	1.8 ± 1.1	4.2 ± 1.8
1 µg/L IMI	8000	1.1 ± 0.9	3.3 ± 1.6	2.9 ± 2.3	6.1 ± 3.8
10 µg/L IMI	9000	1.6 ± 1.0	3.1 ± 1.6	1.3 ± 0.4	4.4 ± 2.0
75 µg/L IMI	11,000	1.8 ± 1.7	5.0 ± 2.1	2.3 ± 1.0	7.3 ± 2.6
810 µg/L IMI	8000	3.6 ± 1.2*	4.0 ± 1.1	1.8 ± 1.3	5.8 ± 1.2

*Significant difference from control (Co−)

analysis of comet and ENDO III sites assays allowed the characterization of the mechanism underling this effect, which is the oxidative damage specifically in pyrimidine bases. This study adds data to support the growing evidence on the negative environmental effects of neonicotinoids in non- target organisms.

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