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Genotoxic effects of vinclozolin on the aquatic insect *Chironomus riparius (Diptera, Chironomidae)*☆

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ABSTRACT

Vinclozolin (Vz) is a pollutant found in aquatic environments whose antiandrogenic effects in reproduction are well known in mammals. Although its reproductive effects have been less studied in invertebrates, other effects, including genotoxicity, have been described. Therefore, in this work, we studied the genotoxic effects of Vz in the freshwater benthic invertebrate *Chironomus riparius.* DNA damage was evaluated with the comet assay (tail area, olive moment, tail moment and % DNA in tail), and the transcriptional levels of different genes involved in DNA repair (*ATM, NLK* and *XRCC1*) and apoptosis (*DECAY*) were measured by RT-PCR. Fourth instar larvae of *C. riparius*, were exposed to Vz for 24 h at 20 and 200 μg/L. The Vz exposures affected the DNA integrity in this organism, since a dose-response relationship occurred, with DNA strand breaks significantly increased with increased dose for tail area, olive moment and tail moment parameters. Additionally, the lower concentration of Vz produced a significant induction of the transcripts of three genes under study (*ATM*, *NLK* and *XRCC1*) showing the activation of the cellular repair mechanism. In contrast, the expression of these genes with the highest concentration were downregulated, indicating failure of the cellular repair mechanism, which would explain the higher DNA damage. These data report for the first time the alterations of Vz on gene transcription of an insect and confirm the potential genotoxicity of this compound on freshwater invertebrates.

1. Introduction

In the last two decades, we have seen growing scientific concern and public debate over the possible deleterious effects in humans and wildlife that can result from exposure to substances that interfere with the endocrine system. These substances are termed endocrine disruptor chemicals (EDCs). Recently, chemicals that alter androgen signaling have gained more attention, particularly antiandrogenic compounds such as vinclozolin (Vz). Vinclozolin is a dicarboximide fungicide widely used for the control of diseases in fruits, vegetables, and ornamental plants (Anway et al., 2006), and it has been described by the European Chemicals Agency ECHA as a substance that may damage fertility and disturb embryonic development, is toxic to aquatic life with long-lasting effects, and is suspected of causing cancer and allergic skin reactions. Vinclozolin possesses antiandrogenic activity in mammals and fish (Kavlock and Cummings, 2005; Kiparissis et al., 2003), and has been detected in surface waters at concentrations of 0.1–2.4 μg/L (El-Shahat et al., 2003).

The toxic potential of Vz has been studied in numerous species, mostly vertebrates, and has been shown to adversely affect a wide range of cellular components and processes (Flick et al., 2017; Hoffmann and Kloas, 2016; Jedeon et al., 2016). However, its molecular mechanisms of action have still not been fully characterized despite a variety of studies on its endocrine toxic effects. EDCs are reactive chemicals that exhibit different mechanisms of toxicity, and damage to genetic material may contribute toward the overall toxic impact of a particular chemical. However, although they have a diverse chemical nature, there is still little information about the capability of endocrine active compounds to interact with DNA, particularly in freshwater invertebrates that are poorly represented

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in previous studies (Martínez-Paz et al., 2013). Accordingly, Hagger et al. (2006) demonstrated a clear association between DNA damage and imposex induced by an EDC in the mollusk *Nucella lapillus*.

Genotoxicity is considered to be one of the most important toxicity parameters in chemical testing and environmental risk assessment. Breaks produced in DNA due to exposure to different compounds can become non-repaired lesions, and as such, have been employed as biomarkers of genotoxicity. Most studies that analyze the genotoxic effects caused by Vz are in human cells: it causes sister chromatid exchanges, structural aberrations and enhanced MN formation caused by BaP (Kevekordes et al., 1996; Lioi et al., 1998; Wu et al., 2005) and bovine lymphocyte cells (Lioi et al., 1998), and usually use the micronucleus test (MN). Almost nothing is known about the genotoxicity of Vz in invertebrates, only MN induction in embryos of the freshwater snail *Physa acuta* at non-environmentally relevant concentrations has been reported (Sánchez-Argüello et al., 2012). Genotoxic effects involve DNA damage, which can be analyzed by cytological methods, such as the comet assay, and determining the expression of genes related to DNA repair could help to clarify the mechanisms that cause this damage.

The comet assay has many advantages compared to other cytological genotoxic evaluation methods: it allows the qualitative and quantitative detection of low levels of DNA damage in any type of eukaryotic cell; it is a quick technique and can be performed with a low number of cells. In aquatic environments, most comet assays have been performed using *in vitro* systems, while many others have focused on invertebrates, particularly using filter feeding organisms like marine (Châtel et al., 2012; Kolarević et al., 2016) and freshwater (Frenzilli et al., 2009) bivalves. It is important to note that the comet assay measures direct and indirect DNA damage, and can be used to estimate the extent of DNA breaks, which itself reflects the number of damaged cells and the severity of damage. Thus, it is necessary to also account for mitochondrial or membrane damage that can cause extensive DNA fragmentation via apoptosis or necrosis (de Lapuente et al., 2015).

Therefore, we have analyzed the transcriptional activity of the genes *ATM*, *DECAY*, *NLK*, and *XRCC1*, because of their putative relationship with genotoxic effects. ATM (ataxia-telangiectasia mutated) is a protein that is mediated in response to DNA double-strand breaks (DSB) and has a number of important roles in the cell (Guleria and Chandna, 2016; Iijima et al., 2008), while DECAY (death executioner caspase related to Apopain/Yama) is a key factor in canonical apoptotic signaling, since it shares homology with mammalian caspase 3 and caspase 7 (Dorstyn et al., 1999). NLK (NEMO-like kinase) was described as a tissue-specific factor that regulates the activity of several signal transduction pathways, including Wnt/β-catenin, Notch and Bmp (bone morphogenetic protein) signaling and it is the first member of a family of proteins known as NEMO-like kinases (NLK) (Ishitani and Ishitani, 2013). Recently, NLK has been related with DNA damage repair mechanisms because it is required for p53 activation in response to DNA damage (Zhang et al., 2014). Finally, X-ray repair cross complementing 1 (XRCC1) is a base excision repair (BER) enzyme involved in the core processes of single strand break repair, simulating endonuclease action. It also acts as a scaffold protein in the subsequent restoration of the site (Brem and Hall, 2005; Hanssen-Bauer et al., 2012).

Chironomus riparius is a species widely used in ecotoxicology because it can easily be cultured under laboratory conditions, has a relatively short life cycle, represents the species richest and ecologically one of the most important groups of invertebrates and has been selected as an organism for investigating the potential endocrine disrupting effects of chemicals and as a reference organism in standardized protocols (OECD, 2004a; OECD, 2004b; OECD, 2010; OECD, 2011). Exposure to some well-known endocrine disruptors, such as DEHP (bis(2-ethylhexyl)phthalate), cadmium, and some UV filters, produced changes in the activity of different genes, showing that these compounds greatly affect the expression of genes, particularly those related to hormonal pathways (Herrero et al., 2016, 2017; Ozáez et al., 2016). On the other hand, preliminary studies have been performed about the genotoxic effects of some compounds in this species and related species (Lee et al., 2009; Nair et al., 2011; Park and Choi, 2009) showing genotoxic effects on aquatic organisms.

In a previous study, we showed that Vz can interfere with genes related to the endocrine system, ecdysone metabolism, the detoxification mechanism, and the stress response (Aquilino et al., 2016). In order to enhance the previous study, the aim of the present study is to evaluate the genotoxic effects of Vz, and the possible mechanisms involved in this response at the molecular and cellular level in *C. riparius*. The comet assay was performed to investigate the level of DNA damage in terms of strand breaks and alkali labile sites, while real-time PCR was used to analyze the mRNA levels of several genes involved in the DNA damage response.

2. Material and methods

2.1. Animals

Fourth instar larvae of the midge *Chironomus riparius* were the experimental animals. Stock cultures were reared under standard laboratory conditions in the Laboratory of Biology and Environmental Toxicology (UNED) for several generations, according to OECD guidelines (OECD, 2004a; OECD, 2004b; OECD, 2010; OECD, 2011). Larvae were grown from egg masses in aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, and 0.025 mM KH_2PO_4) supplemented with nettle leaves, commercial fish food (TetraMint), and cellulose tissue in 6 L polyethylene tanks. Cultures were maintained under constant aeration at 20 °C and under standard light-dark periods (16L: 8D).

2.2. Treatments

For experimental treatments, fourth instar larvae were exposed to 20 and 200 μg/L of vinclozolin (Vz; CAS No. A 50471-44-8, purity ≥99%) from Sigma-Aldrich (Germany) diluted in 30 mL culture medium without sediment (glass vessels were used to carry out the exposures). Three different experiments of n = 13 per treatment for the genotoxicity assay, and n = 9 per treatment for the expression analysis. The duration of the treatment was 24 h for both assays. This exposure time was chosen considering the DT50 of Vz (50% degradation time or half-life) previously assessed in medium test (Sánchez-Argüello et al., 2012). Vz half-life under the laboratory conditions of our experiments was10.8 h. Assuming a first-order kinetic dissipation ($C=C_0e^{-kt}$; K = Ln2/DT50) the concentration at 24 h can be estimated as 4.2 and 42.8 μg/L for their respective nominal concentrations (20 and 200 μg/L). The present study consisted of short-term exposure and it was therefore not necessary to maintain nominal concentrations by daily injection of compound.

The untreated control larvae were exposed to the same concentration of solvent (acetone 0.1%).

2.3. Comet assay

The comet assay was performed following a modified version of the method of Singh et al. (1988). Thirteen larvae were homogenized over gauze and resuspended in 3 mL 1x PBS. The homogenate was centrifuged for 5 min at 1500 rpm at 4 °C, and resuspended in

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