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The Ascidian Embryo Teratogenicity assay in *Ciona intestinalis* as a new teratological screening to test the mixture effect of the co-exposure to ethanol and fluconazole

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ABSTRACT

The aim of this work was to evaluate the Ascidian Embryo Teratogenicity assay (AET) as new alternative invertebrate model to test the developmental effects of the co-exposure to ethanol and fluconazole. *Ciona intestinalis* embryos were exposed to the azolic fungicide fluconazole, (FLUCO, 7.8–250 μM), to ethanol (Eth, 0.01–0.5%) and to their mixture (0.01% Eth + FLUCO 7.8–250 μM) from neurula to larval stage. At the end of the exposure period, larvae were morphologically evaluated and benchmark analysis performed by using the PROAST modelling software. Both compounds were teratogenic in a concentration-related manner, particularly affecting the pigmented organs. The co-exposure to Eth enhanced the effects of FLUCO, the additive hypothesis was not rejected by the modelling. The results demonstrated that AET could be considered a good vertebrate-free alternative model for toxicological investigation in embryos.

1. Introduction

Various animal models have been used to study teratogenic effects on embryos (Randall et al., 1977; Brown et al., 1979; Streissguth et al., 1980; Sulik et al., 1981; Cartwright and Smith, 1995). Despite the use of the *in vitro* culture of mammalian embryos (Brown et al., 1979) is probably the best experimental system for studying direct teratogenic effects on embryos, many difficulties and inconveniences involved with the technical complexity of culturing mammalian embryos still remain. Moreover, the use of vertebrates in scientific procedures is subjected to law and guidelines that limit the number of animals that can be employed.

Recently, ascidian embryos have been proposed as an excellent alternative experimental system for investigating the mechanisms underlying the development of chordates, and therefore of vertebrates (Passamaneck and Di Gregorio, 2005; Sasakura et al., 2012). Ascidians are widespread marine sessile, filter-feeding chordate organisms belonging to the Subphylum *Urochordata*, which has been recognized as the sister group of vertebrates (Delsuc et al., 2006).

Ascidian embryos display striking similarities to vertebrate ones as they develop through a swimming, tadpole like larva, which represents a simple prototype of the chordate body plan (Passamaneck and Di Gregorio, 2005), comprising a hollow neural tube lying dorsal to a rod-like notochord (Satoh, 1994). The central nervous system is composed by a sensory vesicle and a visceral ganglion, localized in the trunk, and by a nerve cord localized in the tail. The sensory vesicle contains two pigmented sensory organs, the otolith and the ocellus. Gametes can be obtained by *in vitro* fertilization. Moreover, ascidian embryos develop quickly, taking ~18 h from fertilization to the development of a free-swimming larva at 18 °C, allowing observing the effects of treatments within a day. All these characteristics offer several advantages for toxicological studies. The tadpole larva of *Ciona intestinalis*, one of the most studied species, consists of only ~2600 cells. Moreover, the release of its sequenced genome, which consists of ~16.000 genes (Dehal, 2002), has favoured the possibility to study toxicant effects on gene expression. Particularly, ascidian *Ciona intestinalis* larvae are used as model organisms for marine pollution monitoring (Bellas et al., 2004), in developmental and evolutionary biology (Satoh, 1995; Di Gregorio and Levine, 1998; Simmen et al., 1998; Corbo et al., 2001; Dehal, 2002), cardiac development (Davidson, 2007), central nervous system regeneration (Dahlberg et al., 2009), endocrine disruptor toxicity (Cima et al., 1996; Cangialosi et al., 2013) and for applying embryo-toxicity tests related to xenobiotic exposure (Cima et al., 1996; Pennati et al., 2006; Groppelli et al., 2007; Zega et al., 2009;

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Matsushima et al., 2013).

The aim of this work to use *Ciona intestinalis* as new invertebrate alternative teratological screening test (AET, Ascidian Embryo Teratogenicity assay) to evaluate the effects of the co-exposure to fluconazole, an azole compound, and ethanol.

Evaluating the toxicity of mixtures of multiple chemicals is one of the major objectives of today's toxicology since humans and all other organisms are exposed to multi-component chemical mixtures. The huge number of combinations of chemicals, the paucity of efficient test strategies for the risk assessment of mixtures, and the increasing societal need to reduce animal testing make the study of mixtures a very complex issue (EPA, 2002).

Azole compounds, classified into triazoles and imidazoles, are widely used as antifungal agents in human and veterinary pharmaceuticals, and some are also prescribed in cancer therapy (Kahle et al., 2008; Zarn et al., 2002). Their biological activity is based on the inhibition of fungal lanosterol-14R-demethylase (a cytochrome P-450 enzyme, encoded by the CYP51 gene) (Vanden Bossche, 1985; Zarn et al., 2002). Some authors, by *in vitro* and *in vivo* teratological studies in rat, as well as in patients with acute promyelocytic leukemia, also demonstrated the inhibitory potency of azole derivatives on CYP26, a P-450 enzyme that mediates the catabolism of retinoic acid (RA) (Van Wauwe et al., 1990; Schwartz et al., 1995; Vanier et al., 2003).

Among azoles, fluconazole (FLUCO), a bis-triazole derivative, is a clinically used fungicides commonly dosed for treating a variety of mycoses and infections (Debruyne, 1997). The use of FLUCO for prophylaxis and treatment of mycotic infections is also widespread among pregnant women. It is known that triazole derivatives closely mimic the morphological effects induced by excess of RA in mammals (Menegola et al., 2004, 2003) and in frogs (Groppelli et al., 2005). FLUCO causes specific teratogenic effects at the level of the branchial apparatus in mouse (Tiboni, 1993) and in rat embryos (Menegola et al., 2004), that are very similar to those obtained after RA exposure. The observed malformations are dose and stage dependent and consist in the perturbation of the hindbrain and of the branchial region from which craniofacial structures originate.

Teratogenesis caused by the maternal consumption of ethanol (Eth) has become a major concern to investigators in the field of health sciences given the widespread use and abuse of this substance also during pregnancy (Nakatsuji, 1983). Jones and Smith (1973) used the term "foetal alcohol syndrome" (FAS) to describe characteristic malformations observed in offspring of chronic alcoholic mothers: microcephaly, flat midface with short palpebral fissures, low nasal bridge with short nose and long smooth or flat phyltrum (de Sanctis et al., 2011; Joya et al., 2012; Memo et al., 2013). In studies on postimplantation rat embryos exposed *in vitro* to Eth, the reported malformations are mostly neural tube defects, rotation and cardiac abnormalities and hypoplasia of the first branchial arch (Giavini et al., 1992; Deltour et al., 1996; Duester, 1998; Kot-Leibovich and Fainsod, 2009).

Considering the increasing consumption of ethanol also in pregnant women, the co-exposure of ethanol and FLUCO and their possible interactions are matter of concern.

Since *Ciona intestinalis* larvae are sensible to the teratogenic action of exogenous RA (Nagatomo and Fujiwara, 2003) and azole compounds, we decide to use the AET to evaluate the existence of mixture effect by co-exposure to FLUCO and Eth.

2. Materials and methods

2.1. Animals and treatments

Adults of *C. intestinalis* were collected by the fishing service of the Roscoff Biological Station (France). Animals were maintained in aquaria filled with artificial seawater (Instant Ocean, salinity 32‰) at 16 °C and provided with circulation system as well as mechanical, chemical and biological filters. Constant light condition was preferred to promote gamete production.

Gametes collected from dissected gonoducts of at least three adults were transferred in Petri glass dishes containing Artificial Sea Water with Hepes (ASWH; pH 8) for *in vitro* cross-fertilization and maintained at 16 °C (Hotta et al., 2007). Seven hours post fertilization (hpf), early neurula stage embryos were collected for treatments. They were exposed for 15 h to increasing concentrations of Eth (0.01, 0.05, 0.1, 0.25, 0.5% corresponding to 1.7, 8.5, 17, 42.5, 85 mM), to increasing concentrations of FLUCO (0, 7.8, 15.75, 31.5, 250 μM) and to mixtures of 0.01% Eth + FLUCO (0, 7.8, 15.75, 31.5, 250 μM) dissolved in 10 mL of ASWH, without renewing the solution. FLUCO concentrations were chosen based on previous works (Groppelli et al., 2007) plus a high dose as positive control. Control embryos were maintained in ASWH. All chemicals were of reagent grade. FLUCO and Eth absolute were purchased from Sigma, Italy. FLUCO stock solution (250 μM) was prepared in ASWH to reach the final treatment concentrations. All solutions were freshly prepared.

Approximately 100 embryos, randomized between those derived from the three adults, were used for each treatment and each treatment was replicated three times ($n \approx 300$ per experimental group). When controls reached swimming larva stage (22 hpf), all specimens were fixed with 4% paraformaldehyde in PBS. After fixation, larvae of each experimental group were counted and morphologically examined under a dissecting microscope. For each experimental group, the percentage of dead and malformed larvae was recorded. Larval abnormalities were classified in four malformed phenotypes:

- trunk abnormalities, in which trunk appeared round in shape and the anterior side was flat, due to impairment of adhesive papillae development (Fig. 1C and D);
- pigmented organ abnormalities, in which otolith and ocellus appeared fused in a single spot and/or displaced on the dorsal portion of the sensory vesicle (Fig. 1E and F). In the samples displaying sensory vesicle protrusion on the trunk dorsal side, the pigmented organs were exposed to the surface;
- tail abnormalities, in which the larval tail appeared coiled, flexed or reduced in length (Fig. 1G);
- severe malformations, in which plurimalformed larvae were characterized by absence of sensory vesicle cavity, presence of a short, bent tail, round trunk with not elongated papillae. This group includes also larvae that failed the hatching event (Fig. 1H).

2.2. Statistical analysis

We used generalized linear models (GLM) to test the significance of differences in the incidence of malformations between each experimental group. The number of individuals with/without malformations per each experimental group was the dependent variable, while experimental groups were considered as fixed factors. GLMs showed overdispersion, as residual deviance was larger than the residual degrees of freedom, therefore we used a quasi-binomial error structure, and we tested significance using a F-test (Crawley, 2007). If substance exposures were significant, we performed Tukey's *post-hoc* tests (significant at $P < 0.05$) using the multcomp package in R (Hothorn et al., 2008), in order to identify specific effects of each concentration on larvae development.

2.3. Benchmark approach

Benchmark approach was applied only on data regarding target structures (pigmented organs) both for Eth and for FLUCO. Data on pigmented organ abnormalities were modelled by using PROAST 62.3 software in order to characterize the single dose response curves, obtain the relative potency factor (RPF) of Eth versus FLUCO and express Eth concentrations in FLUCO equivalents. Dose-additivity hypothesis was finally evaluated using the best model with covariates (Hill model, m3-