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Toxicity and non-harmful effects of the soya isoflavones, genistein and daidzein, in embryos of the zebrafish, *Danio rerio*

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ABSTRACT

Based on the assumed oestrogenic and apoptotic properties of soya isoflavones (genistein, daidzein), and following the current OECD test-guidelines and principle of 3Rs, we have studied the potential toxicity of phytochemicals on the zebrafish embryos test (ZFET). For this purpose, zebrafish embryos at 2–3 h post-fertilisation (hpf) were exposed to both soya isoflavones (from 1.25 mg/L to 20 mg/L) and assayed until 96 hpf. Lethal and sub-lethal endpoints (mortality, hatching rates and malformations) were estimated in the ZFET, which was expanded to potential gene expression markers, determining the lowest observed effect (and transcriptional) concentrations (LOEC, LOTEC), and the no-observable effect (and transcriptional) concentrations (NOEC, NOTEC). The results revealed that genistein is more toxic (LC50–96 hpf: 4.41 mg/L) than daidzein (over 65.15 mg/L). Both isoflavones up-regulated the oestrogen (*esrrb*) and death receptors (*fas*) and *cyp1a* transcript levels. Most thyroid transcript signals were up-regulated by genistein (except for thyroid peroxidase/*tpo*), and the hatching enzyme (*he1a1*) was exclusively up-regulated by daidzein (from 1.25 mg/L onwards). The ZFET proved suitable for assessing toxicant effects of both isoflavones and potential disruptions (i.e. oestrogenic, apoptotic, thyroid, enzymatic) during the embryogenesis and the endotrophic larval period.

1. Introduction

The zebrafish, *Danio rerio*, is a small tropical freshwater teleost of the family Cyprinidae, and a well-established animal model for many different biomedical and animal research fields (Mayden et al., 2007). Since molecular sequencing and mapping have been well characterised in both humans and zebrafish, both genomes can be compared (www.ensembl.org, *D. rerio* genome release Zv9; Howe et al., 2013). Due to its very well known embryogenesis, optical transparency, small size, easy and affordable cultivation and reproduction, and high fecundity and short life cycle, all developmental life stages of the zebrafish have been used in many different research topics, such as development, environmental control, sex-differentiation, reproduction and endocrine disruption, nutrition, metabolism, eco-toxicology, etc. (Kimmel et al., 1995; Braunbeck et al., 2005, 2015; Hill et al., 2005; Gavaia et al., 2006; Lawrence, 2007; Segner, 2009; Goldstone et al., 2010; Dai et al., 2014; Bensimon-Brito et al., 2016; Nowosad et al., 2017; Ribas et al., 2017; among others).

Recently, the Organisation for Economic Cooperation and Development (OECD, 2013) has formalised a version of the zebrafish embryos toxicity test (FET) as the test guideline-TG236. This zebrafish embryo toxicity test (FET or ZFET) has been widely used to analyse the acute toxicity and effects of different natural and synthetic contaminants, including several flavonoids (Strähle et al., 2012; Williams et al., 2014; Braunbeck et al., 2015; Schreiber et al., 2018). The estimation of the lethal concentration (LC50), the lowest and the no-observed effect concentrations (LOEC and NOEC), and other similar toxicity parameters calculated at transcriptional levels (LOTEC and NOTEC) have been applied on different ecotoxicogenomic approaches for understanding molecular mechanisms of environmental chemical toxicity, using zebrafish embryos and endotrophic larvae as organism model (Nagel, 2002; Braunbeck et al., 2005, 2015; Hill et al., 2005; Lammer et al., 2009; Weil et al., 2009; Embry et al., 2010; Bugel et al., 2016; Glaberman et al., 2017; Schüttler et al., 2017).

Plant ingredients and, mainly, soya beans and derived products are increasingly being used, not only for human diets and therapeutic uses of terrestrial vertebrates, but also to a greater extent for aquatic organisms (Ingham et al., 2004; Patisaul and Jefferson, 2010; Rearick et al., 2014; DiMaggio et al., 2016; Hussain and Green, 2017; Rietjens et al., 2017; Xiao et al., 2018). Indeed, a sustainable utilisation of vegetable (e.g. soya compounds) is progressively expanding for the production of aquafeeds (Francis et al., 2001; Gatlin et al., 2007; Gu et al.,

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2015; Li et al., 2016). The exposure of fish to different xenobiotic and phytochemicals (i.e. flavonoids, non-flavonoids) can also occur through contaminated waste, surface waters and coastal areas, derived from different anthropogenic activities, showing very variable levels, i.e. from nanomolar range until mg/L (Kiparissis et al., 2001; Spengler et al., 2001; Liu et al., 2010; Rocha et al., 2013; Rearick et al., 2014; Ribeiro et al., 2016). Several biological and physiological effects, and endocrine disrupting responses in different marine and freshwater fish species exposed to several flavonoids have also been extensively reported, including zebrafish (Pelissero et al., 1991a, 1991b; Bennetau-Pelissero et al., 2001; Kiparissis et al., 2003; Gatlin et al., 2007; Sassi-Messai et al., 2009; Schiller et al., 2014; Pinto et al., 2016; Sarasquete et al., 2017, 2018). Because of their structural similarity with natural oestrogens (i.e. 17 β-oestradiol), soya isoflavones possess oestrogenic (and anti-) properties (i.e. binding affinities for the oestrogen-related receptors, ESRRα, ESRRβ). These flavonoids also induce apoptotic effects via extrinsic and intrinsic pathways (i.e. death receptors, caspases, survivin, etc.), through oestrogen-independent pathways, by inhibition of protein tyrosine kinases -PTKs- (Akiyama et al., 1987; Miyahara et al., 2003; Kim et al., 2009; Patisaul and Adewale, 2009; Sassi-Messai et al., 2009; Schiller et al., 2013a, 2013b; Wang et al., 2013; Rietjens et al., 2017). Besides, flavonoids have also been related with cell proliferation and sex-differentiation processes, chromatin remodelling-DNA repair and epigenetic processes. In all of these processes participate several transcription factors, such as bromodomain and extra-terminal (BET) proteins, e.g. BRDT (Delcuve et al., 2009; Airoldi et al., 2010; Patisaul and Jefferson, 2010; Taniguchi, 2016; Úbeda-Manzanaro et al., 2016). Furthermore, isoflavones can affect to the thyroid hormone biosynthesis, metabolism and transport, what can alter the thyroid homeostasis (Chang and Doerge, 2000; Patisaul and Jefferson, 2010; Thienpont et al., 2011; Wang et al., 2013; Rietjens et al., 2017; Sarasquete et al., 2017). Additionally, reduced fertilisation rates, decreases of hatching percentages, and reproductive disruptions, are some of the most common toxic and endocrine responses induced by endogenous and exogenous factors, including hormones, xenobiotics, phytochemicals, etc. (Arukwe and Goksøyr, 2003; Segner et al., 2003; Kim et al., 2009; Sassi-Messai et al., 2009; Schiller et al., 2014; Luzio et al., 2016).

On the other hand, cytochrome P450 monooxygenases (CYPs) are haeme-containing mixed-function oxidases which participate in the synthesis and metabolism of hydrophobic endogenous compounds (steroids, sterols, fatty acids, retinoids, etc.), and several CYPs (i.e. CYP1A) play an important role in the metabolism and clearance of many xenobiotics, including phytochemicals (Whitlock, 1999; Cajaraville et al., 2000; Sarasquete and Segner, 2000; Hodek et al., 2002a, 2002b; Goldstone et al., 2010; Ronis, 2016). The majority of the natural flavonoids (i.e. isoflavones) are AhR antagonists, but some phytochemicals are AhR agonists, and weak inducers of CYP1A (Denison and Nagy, 2003; Harper et al., 2006; Ito et al., 2007; Amakura et al., 2008; Bugel et al., 2016; Sarasquete et al., 2017).

With the present study, we aimed at providing substantiating evidence for the suitability of a zebrafish embryo-based transcriptomics approach to test the toxicity of two isoflavones, genistein/GEN and daidzein/DAID, analysing especially apoptotic and oestrogenic pathways and several signals of thyroidal disruption. For this purpose, we have estimated several acute toxicity parameters determined by means of LC50, as well as NOEC and LOEC values and their dependent lethal and sub-lethal effects on the fertilised eggs, embryos and lecithotrophic larvae of the zebrafish exposed to both soya isoflavones (GEN and DAID) at different concentrations. Percentages of eggs mortality, hatching rates and larval survival, as well as morphological alterations are analysed. In addition, the sensitivity of different gene expression patterns, to both isoflavones, it has also been studied and compared (NOTEC and LOTEC values). Thus, we have analysed differential ontogenetic baseline expression patterns, and the transcriptional variations of different genes (controls and isoflavone exposures), such as: hatching enzyme 1/*he1a1*, oestrogen-related receptor beta/*esrrb*, thyroid signalling cascade (thyroglobulin/*tg*, thyroid hormone receptor beta/*thrb*, thyroid peroxidase/*tpo*, thyroid stimulating hormone beta subunit/*tshb*, transthyretin/*ttr*, and iodothyronine deiodinases/*dio1*, *dio2*, *dio3b*), pro- and anti-apoptosis pathways (pro-apoptosis factors as the fas cell surface death receptor/*fas*, and the caspases/*casp6*; and the anti-apoptotic baculoviral IAP repeat containing 5/*birc5*), transcription factors such as bromodomain testis specific/*Brdt*, and the haemoprotein cytochrome P450 family 1 subfamily A/*cyp1a*.

2. Material and methods

2.1. Biological samples

Fertilised eggs of zebrafish, *Danio rerio* (genotype AB/Tübingen) were maintained at the Institute of Marine Sciences of Andalusia (ICMAN-CSIC, Puerto Real, Spain). The facilities of ICMAN-CSIC are in agreement with the European Convention for the Protection of Animals used for Experimental and Scientific purposes and they were approved for experimentation by the Ministry of Agriculture and Fisheries (REGA-ES110280000311) in accordance with current EU (Directive 2010/63) and Spanish legislation. The experimental procedure (project AGL2014-52906-R) was approved by the Spanish National Research Council (CSIC) Ethics Committee, and by dependent Spanish Competent Authority, Junta de Andalucía (no. 09-7-15-278, RD53/2013).

2.2. Toxicity tests

Genistein/GEN ($C_{15}H_{10}O_5$, LC Laboratories, MA, USA) and Daidzein/DAID ($C_{15}H_{10}O_4$, LC Laboratories, MA, USA) were dissolved in ethanol to make up 20 mM and 5 mM stock solutions, respectively, and then they were kept in darkness at 4 °C until they were used.

The zebrafish embryos-larvae tests (ZFET) have been performed according to the current guidelines (OECD, 2006, 2012, 2013). Fertilised eggs were randomly distributed into 500 mL glass beakers (25 eggs by container) in triplicate as control groups (with and without the carrier) and both isoflavone treatment groups (nominal concentrations of 1.25 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L and 20 mg/L), and they were incubated under semi-static conditions at around 25–28 °C with a photoperiod of 14:10 light:dark cycle, from 2 to 3 h post-fertilisation (hpf) until 96 hpf. All treatments were renewed daily with freshly prepared stock solution, and mortality and hatching rate were evaluated.

The LC50, NOEC, LOEC toxicity values for both isoflavones were estimated by using the Probit analysis. Statistical differences of surviving larvae between controls and treatments were analysed by ANOVA and Tukey's test post-hoc test. Differences were considered statistically significant at $p < 0.05$. Shaphiro-Wilk's test and Levene's test were used to check the normality of the data distribution and the homogeneity of the variances, respectively. The SPSS 23.0.0.0 software (IBM) was used to perform these analyses. For statistical evaluation, control and solvent-control were pooled, as they did not differ significantly with regard to survival. In addition, NOTEL and LOTEL values according to Weil et al. (2009) were also analysed for different gene expression patterns and variations induced by both isoflavones in embryos-larvae exposed from 2 to 3 hpf and during 24, 48, 72 and 96 hpf to different increasing concentrations of both isoflavones (from 1.25 mg/L until 20 mg/L), such as it was also performed for other different acute toxicity tests in early larval stages of other marine fish species (Ortiz-Delgado et al., 2018). Lethal and sublethal effects were evaluated as previously described (Nagel, 2002), and dead embryos were removed. In addition to coagulation of eggs, abnormal or no development of somites, absence of heartbeat, and lack of detachment of the tail bud from the yolk-sac were also assessed (Weil et al., 2009).