ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY 38 (2014) 672–683

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/etap

Detection of immunotoxic effects of estrogenic and androgenic endocrine disrupting compounds using splenic immune cells of the female three-spined stickleback, *Gasterosteus aculeatus* (L.)

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ARTICLE INFO

Article history:
Received 13 June 2014
Received in revised form
25 July 2014
Accepted 5 August 2014
Available online 13 August 2014

Keywords:
Endocrine disruptors
Sex-steroid hormone
Respiratory burst
Necrosis
Apoptosis
Phagocytosis activity

ABSTRACT

Today, the list of endocrine disrupting compounds (EDCs) in freshwater and marine environments that mimic or block endogenous hormones is expanding at an alarming rate. As immune and reproductive systems may interact in a bidirectional way, some authors proposed the immune capacities as attractive markers to evaluate the hormonal potential of environmental samples. Thus, the present work proposed to gain more knowledge on direct biological effects of natural and EDCs on female fish splenic leucocyte non-specific immune activities by using *ex vivo* assays. After determining the optimal required conditions to analyze splenic immune responses, seven different EDCs were tested *ex vivo* at 0.01, 1 and 100 nM over 12 h on the leucocyte functions of female three-spined stickleback, *Gasterosteus aculeatus*. In summary, we found that natural hormones acted as immunostimulants, whilst EDCs were immunosuppressive.

Abbreviations: EDCs, endocrine disrupting compounds; 11KT, 11-ketotestosterone; E2, 17β–estradiol; EE2, 17α–ethinylestradiol; BPA, bisphenol A; NP, 4-*n*-nonylphenol; TB, trenbolone acetate; MT, 17α–methyltestosterone; L15, Leibovitz 15 medium; DMSO, dimethylsulfoxide; PI, Propidium Iodide; ROS, reactive oxygen species; H_2DCF-DA, 2'–7'–dichlorofluorescin diacetate; MFI, mean fluorescence intensity.

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http://dx.doi.org/10.1016/j.etap.2014.08.002
1382-6689/

1. Introduction

An endocrine disruptor compound ((EDC) is defined as an exogenous substance that alters the functioning of the endocrine system and consequently causes adverse health effects on an intact organism, or its progeny, or (sub-) populations. Due to their potential hazard on aquatic wildlife (Canesi et al., 2007; Casanova-Nakayama et al., 2011) and their expanding detection in freshwater (Peck et al., 2004; Pal et al., 2010) and marine (Braga et al., 2005; Pinto et al., 2005) environments, research on the effects of EDCs in the past few decades has grown rapidly. In this context, fish are considered as good indicators to assess the toxicity of endocrine disrupting chemicals (Jin et al., 2010). Also, numerous studies address the impact of EDCs on certain parameters of the reproductive system of fish at the molecular (*e.g.* brain aromatase gene expression), biochemical (*e.g.* biotransformation enzymes, plasma vitellogenin) and histological (*e.g.* sexual differentiation, gonad maturation, fecundity, fertility, intersex) levels. (Hinfray et al., 2006; Kallivretaki et al., 2006; Cheshenko et al., 2008).

Despite the fact that immune and reproductive systems may interact in a bidirectional way (Ansar Ahmed, 2000; Engelsma et al., 2002), endocrine disruption effects on the fish immune system have received limited attention and more concern has been made on estrogenic compounds (Jin et al., 2010; Casanova-Nakayama et al., 2011). Recently, some studies demonstrated that environmental estrogens may also affect the immune system of aquatic wildlife (Canesi et al., 2007; Casanova-Nakayama et al., 2011). For example, Liney et al. (2006) found that estrogen-active effluents changed the structure and function of the reproductive system of the roach, *Rutilus rutilus*, at higher concentration than those who impaired the immune function.

In fish, phagocytosis, from chemotaxism to bacterial destruction as well as leucocyte mortality seem to be the main immune functions which are subject to hormonal impact. For instance, Slater and Schreck (1997) observed *in vitro* immunosuppressive effects of testosterone due to salmonid leucocytes death after direct hormonal action on leucocyte androgen receptors. On the contrary, in gilthead seabream (*Sparus aurata* L.), *ex vivo* exposure of head-kidney leucocytes to testosterone and 11-ketotestosterone (11KT) do not have any impact on head kidney leucocyte viability (Águila et al., 2013). This discrepancy underlines the toxic effect of androgens at higher concentrations (Slater and Schreck, 1997) and the destabilization of the immune function at lower concentrations (Águila et al., 2013). In fact, modification by both androgens of the expression of toll-like receptors in acidophilic granulocytes and in macrophages might regulate the sensitivity of phagocytes to pathogens, without leading to effects on leucocyte phagocytic and respiratory capacities (Águila et al., 2013). Concerning estrogen compounds, *in vivo* treatment with 17β-estradiol (E2) (Shelley et al., 2013) and *in vivo* and *in vitro* exposures to 17α-ethinylestradiol (EE2) (Cabas et al., 2012) alter the fish immune gene expression of pro-inflammatory molecules without leading to impacts on phagocytic capacity and respiratory burst. Moreover, as observed below, EE2 and E2 were able to alter in a dose-dependent manner the immune system of gilthead seabream (*S. aurata* L.) (Cabas et al., 2012) and goldfish (*Carassius auratus*) (Yin et al., 2007), respectively.

The aim of the present study was to evaluate the impacts of some estrogen and androgen on the immune function of female three-spined stickleback (*Gasterosteus aculeatus* L.). The stickleback is a well described model fish species used to assess the endocrine disruptor potential of EDCs due to the presence of both estrogenic (vitellogenin) and androgenic (spiggin) end-points (Katsiadaki et al., 2002; Hahlbeck et al., 2004; Jolly et al., 2006; Sanchez et al., 2008; Le Mer et al., 2013). Nevertheless, as showed by (Casanova-Nakayama et al., 2011), current data are too limited to inform conclusive ideas of the *in vivo* EDC impact mechanisms on fish immune function. Thus, after determining the optimal test conditions necessary to analyze fish splenic immune responses, we proposed here some *ex vivo* assays. These assays will increase knowledge on the direct biological effects of EDCs on fish non-specific immune activities, based on the phagocytic function (respiratory burst and phagocytosis activities) and cellular mortality (necrosis and apoptosis). Due to their roles in reproductive physiology, E2 and 11KT were chosen. E2 was the principal sex-steroid detected in the plasma of females whereas 11KT is usually more abundant in male fish (Gonçalves et al., 2010). In males, the 11KT tested concentrations are representative of plasmatic hormonal levels detected in the spawning period, with concentration ranging from 40 to 1300 nM. Outside of the breeding season concentrations are orders of magnitude lower (<7 nM) (Mayer et al., 1990; Páll et al., 2002; Ian et al., 2004). E2 plasmatic concentrations in females are clearly weakly modulated between sexual cycles. During the reproductive period, from May to July, the E2 concentration are around 3 nM, and are then lower hereafter (Björkblom et al., 2009). As female hormonal concentrations are weakly modulated, we chosen them as models for the assays The EE2, synthetic estrogen widely used as oral contraceptive, two industrial chemicals, bisphenol A (BPA) and 4-*n*-nonylphenol (NP) were studied due to their xenoestrogenic actions (Jones et al., 2000; Schultis and Metzger, 2004) especially on fish (Olsen et al., 2005; Torres-Duarte et al., 2012). In the same manner, two synthetic androgens, trenbolone acetate (TB) (Davis et al., 2000) and 17α-methyltestosterone (MT) (Hahlbeck et al., 2004), were also tested. Moreover, the chosen concentrations were around current environmental concentrations of EDC detected in sewage-treatment plants effluents, rivers and seawater (Desbrow et al., 1998; OSPAR, 2001). The spleen tissue was chosen as it is an important target for steroids (Casanova-Nakayama et al., 2011; Milla et al., 2011).

2. Materials and methods

During this project, all experiments were conducted in accordance with the Commission recommendation 2007/526/EC on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Moreover, all experimental protocols were approved by the Ethical Committee of the French National Institute of Industrial Environment and Risks (INERIS).