Bull Environ Contam Toxicol (2015) 94:695–700
DOI 10.1007/s00128-015-1503-y

Effects of (Anti) Androgenic Endocrine Disruptors (DEHP and Butachlor) on Immunoglobulin M (IgM) and Leukocytes Counts of Male Rainbow Trout (*Oncorhynchus mykiss*)

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Received: 12 August 2014 / Accepted: 14 February 2015 / Published online: 24 February 2015

Abstract The effect of two anti-androgenic endocrine disrupting compounds, i.e. the plasticizer di (2-ethylhexyl) phthalate (DEHP) and herbicide butachlor, were evaluated for their effects on immunoglobulin M (IgM) and leukocytes in male rainbow trout. Also, plasma testosterone (T) concentration was measured to confirm their anti-androgenic effects. In the first experiment, trout were treated with 50 mg/kg (body weight) DEHP intraperitoneally, and in the second one, fish were exposed to 0.39 mg/L butachlor for 10 days. The results showed that T concentrations and white blood cells were significantly lower in fish exposed to either DEHP or butachlor compared to control fish ($p < 0.05$). Fish showed significantly elevated neutrophil levels and decreased lymphocyte levels in the butachlor ($p < 0.05$); however, no significant difference was observed in lymphocyte and neutrophils values in the DEHP treatment ($p > 0.05$). In addition, no significant differences were found in IgM, eosinophil and monocyte parameters in either DEHP or butachlor treatments ($p > 0.05$). These results confirmed that leukocytes counts can be considered as a novel marker of immunotoxicity triggered by (anti) androgenic endocrine disruptors.

Keywords IgM · Androgens · Leukocyte · Immunotoxicity

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During the past decade, many works have revealed that sex steroids modulate the immune system in addition to the reproductive function (Tait et al. 2008; Gilliver 2010), confirmed by the presence of sex steroid receptors in immune tissues viz. spleen, liver and anterior kidney leukocytes (Lynn et al. 2008; Shved et al. 2009; Slater et al. 1995; Todo et al. 1999). Also, relatively strong steroid-immune interactions have been reported in many teleosts, birds and mammals (Robertson 1961; Slater et al. 1995; Law et al. 2001; Roberts et al. 2004; Viney et al. 2005; Ros et al. 2006; Kurtz et al. 2007; Miles et al. 2007). Moreover, many reports show that androgens have anti-proliferative effect on lymphocytes (Slater and Schreck 1997; Saha et al. 2004; Ros et al. 2006) and suppress antibody secretion (Hou et al. 1999; Saha et al. 2004).

In the aquatic environment, many chemicals such as pesticides and industrial chemicals are described as endocrine disrupting contaminants (EDCs) due to their interference with the hormonal regulation in fish. Such chemicals can affect the immune system at the organ or the response level (Chapin et al. 1997; Misumi et al. 2009; Jin et al. 2010; Tellez-Bañuelos et al. 2010). The various effects of EDCs on the immune system in fish have been reviewed by Milla et al. (2011). Therefore, immune parameters in fish could be a useful tool for identification of environmental pollution in aquatic ecosystems (Weeks et al. 1992; Wester et al. 1994).

DEHP and butachlor are two anti-androgenic EDCs (Jobling et al. 1995; Tu et al. 2013) that may potentially cause immunotoxicity in fish. Di (2-ethylhexyl) phthalate (DEHP) is commonly used, as a plasticizer to the production of food packaging plastics, infant toys, electrical devices and medical equipment, such as tubing, blood bags and dialysis equipment (Heudorf et al. 2007; Hernández-Díaz et al. 2009). This compound has been reported in the

environment at concentrations up to 100 µg/L in water surface and 200 mg/kg in sediment (Petrovic et al. 2001; Fromme et al. 2002). It has been reported in fish tissue at a range of 1–2.6 mg/kg (European Commission 2003).

Butachlor [2-chloro-2, 6-diethyl-N-(butoxymethyl)-acetanilide] is a common herbicide to control weeds in rice fields (Yu et al. 2003). The LC_{50} (96 h) of butachlor in fishes has reported to range from 0.14 to 0.52 mg/L (Tomlin 1994). Many adverse effects of this herbicide on fish species have been reported (Lasheidani et al. 2008; Guo et al. 2010; Tu et al. 2013). Tu et al. (2013) reported that butachlor caused endocrine disruption, developmental toxicity and immune toxicity in the zebrafish (*Danio rerio*) embryo.

This study was conducted to investigate the effects of DEHP and butachlor on plasma immunoglobulin M (IgM), leukocyte counts and plasma testosterone (T) level in male rainbow trout (*O. mykiss*).

Materials and Methods

In total, 90 male rainbow trout (with mean body weight and length of 410 ± 10.5 g 30 ± 3.5 cm, respectively) were obtained from a local farm (Mazandaran Province, North of Iran). Fish were kept in two well-aerated 1000 L tanks for acclimatization to laboratory condition (pH 7.9 ± 0.1, hardness 180 mg/L (as $CaCO_3$) and total alkalinity 150 mg/L) for 10 days. The water was supplied from dechlorinated tap water that had been filtered by cationic resin. Ten fish without any exposure were sampled as an initial control. Eighty remaining fish were randomly introduced into four 1000 L tanks, each containing 20 fish.

The effects of butachlor and DEHP were investigated in two independent experiments. The applied dosages of chemical compounds were determined based on previous studies on male fish (Cravedi and Perdu-Durand 2002; Lasheidani et al. 2008; Uren-Webster et al. 2010). The duration of both experiments was 10 days. The fish were not fed during the experiment.

Two tanks were used for the DEHP experiment, including Group A as control and group B as treatment. On days 1 and 5 of the experiment, fish from group A and B were anesthetized with MS-222 (100 mg/L) prior to intraperitoneal injection with either 500 µL of olive oil (Group A) or 500 µL of olive oil containing DEHP (Group B). The concentration of DEHP in olive oil was adjusted such that each fish received a dose of 50 mg/kg DEHP body weight. DEHP was obtained from Merck (Germany).

In the second experiment, fish in Group D were exposed to 0.39 mg/L butachlor in water for 10 days while fish in Group C served as the control. The exposure concentration was chosen to be 75 % of the reported LC_{50} (96 h) in rainbow trout (0.52 mg/L, Tomlin 1994). Butachlor was obtained from Herbicides Production Company (Saveh, Iran), and was reported to be of 60 % purity. To confirm exposure, the butachlor concentrations were measured using HPLC analysis as described in Junghans et al. (2003) and Del Buano et al. (2005). During the experiment, the dead fish were counted and removed. Water was renewed daily containing the experiment concentration of butachlor in group D.

Water physiochemical characteristics were monitored twice daily (before and after water exchange) and water quality was maintained as follows. The water temperature (°C), pH, oxygen (mg/L), ammonia (ng/L) and nitrite (µg/L) levels were 16.3 ± 0.9, 7.9 ± 0.1, 7.5–9, 23 ± 2.3 and 33.4 ± 0.6, respectively. Also, the photoperiod was held at 12:12 (L:D).

On days 1, 5, 10 of experiment, fish specimens were sampled from each group (n = 15) and anesthetized immediately using MS-222 (100 mg/L). The blood samples were taken from the caudal vasculature using a heparinized syringe and divided into two aliquots. The first aliquot was transferred to a 2 mL heparinized tube containing 0.01 mL of sodium heparin solution (5000 I.U) (Rotexmedica, Germany) to measure leukocytes, and other one in the same heparinized tube was quickly centrifuged (5000 rpm, 10 min at 4°C), and the obtained plasma samples were stored at −20°C until subsequent analysis.

In addition, the blood smears were prepared using Giemsa staining. The smears were air-dried, fixed by 96 % ethanol for 30 min and stained with Giemsa for 30 min. The smears were evaluated for leukocyte differential count under a compound microscope based on Klontz (1994).

Plasma T concentration was measured using a gamma counter and Immunotech radioimmunoassay (RIA) kit (Beckman Coulter, Villepinte, France). Plasma immunoglobulin M was measured by turbidimetric immunoassay kit obtained from Biosystems S.A. (Barcelona, Spain). After blood was sampled, fish were returned to their tanks. All data were expressed as mean ± SE. Data analyses were performed using the SPSS package (SPSS 1998, Armonk, New York, USA), by one-way ANOVA, with significance determined at the $p < 0.05$ level.

Results and Discussion

The water quality of control group and treatments did not change during the experiment due to daily renewing of the water.

Also, during the experiments, a single fish died from control group A during injection, and two fish died that were exposed to butachlor. In group A fish died during injection. Measured concentrations of butachlor in the water in Group D ranged from 0.37 to 0.39 mg/L.

The alterations in plasma T of treated fish during the experiment are shown in Fig. 1a. The plasma T at the beginning of the experiment in the initial control group was 1.18 ± 0.6 ng/mL and increased over 10 days to 4.33 ± 0.61 and 3.76 ± 0.94 ng/mL in the group A (control of DEHP) and C (control of butachlor), respectively. At the end of the experiment, the plasma T in DEHP and butachlor treatments were 1.09 ± 0.1 and 1.14 ± 0.78 ng/mL, respectively, significantly lower than those of control groups ($p < 0.05$).

The recent studies have shown that exposure of fish to a variety of EDCs such as butachlor and DEHP suppresses the testosterone and subsequently, reproductive success (Thibaut and Porte 2004; Uren-Webster et al. 2010; Crago and Klaper 2012; Chang et al. 2013). Similarly, the present study showed that plasma T in fish treated with butachlor or DEHP was lower than controls. Chang et al. (2013) suggested that butachlor inhibits the gonadal steroidogenesis. Furthermore, EDCs can competitively bind to steroid-binding proteins (Tollefsen 2002). Therefore, inhibition of gonadal steroidogenesis and binding to steroid-binding proteins might be the reasons why butachlor resulted in lower plasma T in male rainbow trout.

Crago and Klaper (2012) suggested that lower plasma T of the male fathead minnow exposed to a mixture of DEHP and linuron may be linked to increasing steroid catabolism. Therefore, similar reason i.e. increasing T catabolism may be resulted lower plasma T in male rainbow trout affected by DEHP. However, the suggestion of Crago and Klaper (2012) was based on mRNA expression profile, which can be different from that of the individual chemical component in both the expressed genes and level of expression (Filby et al. 2007; Finne et al. 2007). Furthermore, exposure to higher concentrations of DEHP is caused disrupting spermatogenesis in zebrafish and mammals by increasing peroxisome proliferation via PPAR signaling pathway (Onorato et al. 2008; Uren-Webster et al. 2010).

Regarding IgM levels, no significant difference was found between DEHP or butachlor treatments and control groups ($p > 0.05$). However, there were slight decreases in IgM levels in controls over the experiment while there were no changes in treated groups (Fig. 1b).

The results of leukocyte differential counts are shown in Fig. 2a–e. The exposure of male rainbow trout to butachlor led to significant decreases ($p < 0.05$) in total WBC counts of 24.9, 23.8 and 45.7 % on days 1, 5, and 10, respectively, in comparison to that of the control group. Also, the total WBC counts in DEHP treatment decreased significantly ($p < 0.05$) by 26.1 and 30.9 % on days 5 and 10, respectively, compared to the control group (Fig. 2a).

The lymphocytes were significantly reduced in the fish exposed to butachlor ($p < 0.05$) with a decrease of 25.6 % at the end of experiment; however, the level of neutrophils was 28.4 % higher than that of the control ($p < 0.05$). The values for lymphocytes and neutrophils did not show significant differences ($p > 0.05$) between DEHP treatment and control (Fig. 2b, c).Also, no significant effects were observed in eosinophil or monocyte counts with DEHP and butachlor treatment over 10 d ($p < 0.05$).

The communication between endocrine disrupters and immune system was mediated by hormones and cytokines (Ahmed 2000; Lutton and Callard 2006). Suzuki et al. (1997) stated that androgens and plasma IgM have a negative correlation during the reproductive cycle in rainbow trout. However, our results did not confirm such a negative correlation. Tellez-Bañuelos et al. (2010) showed that the serum levels of IgM in juvenile Nile tilapia (*Oreochromis niloticus*) increase by short in vivo exposure

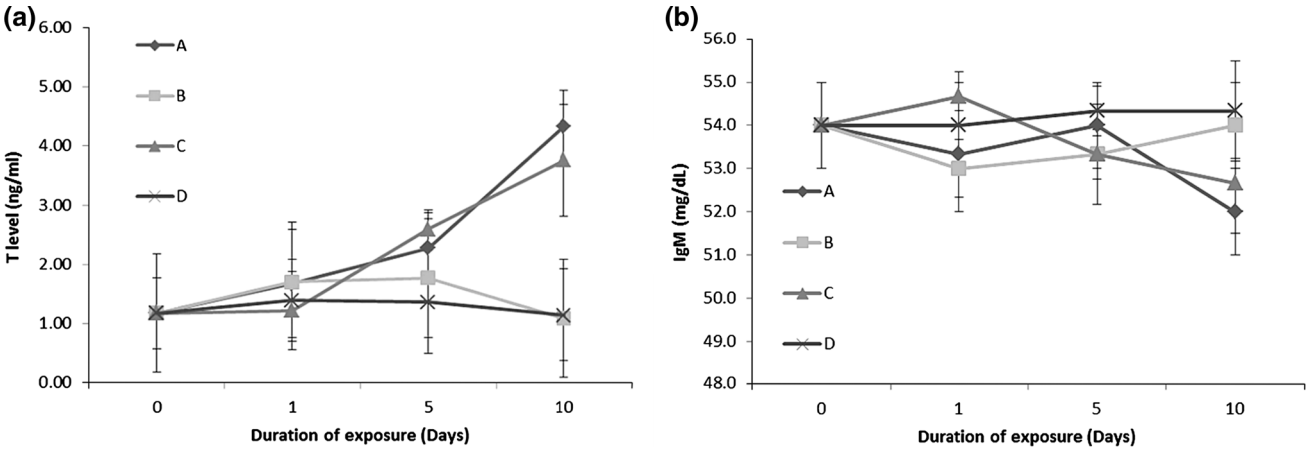

Fig. 1 Plasma testosterone (T) levels (**a**), Plasma IgM levels (**b**) in male rainbow trout affected by butachlor exposure and DEHP (*A* Fish injected with olive oil as carrier, *B* fish injected with olive oil containing DEHP, *C* fish without butachlor exposure (control) and *D* fish with butachlor exposure)

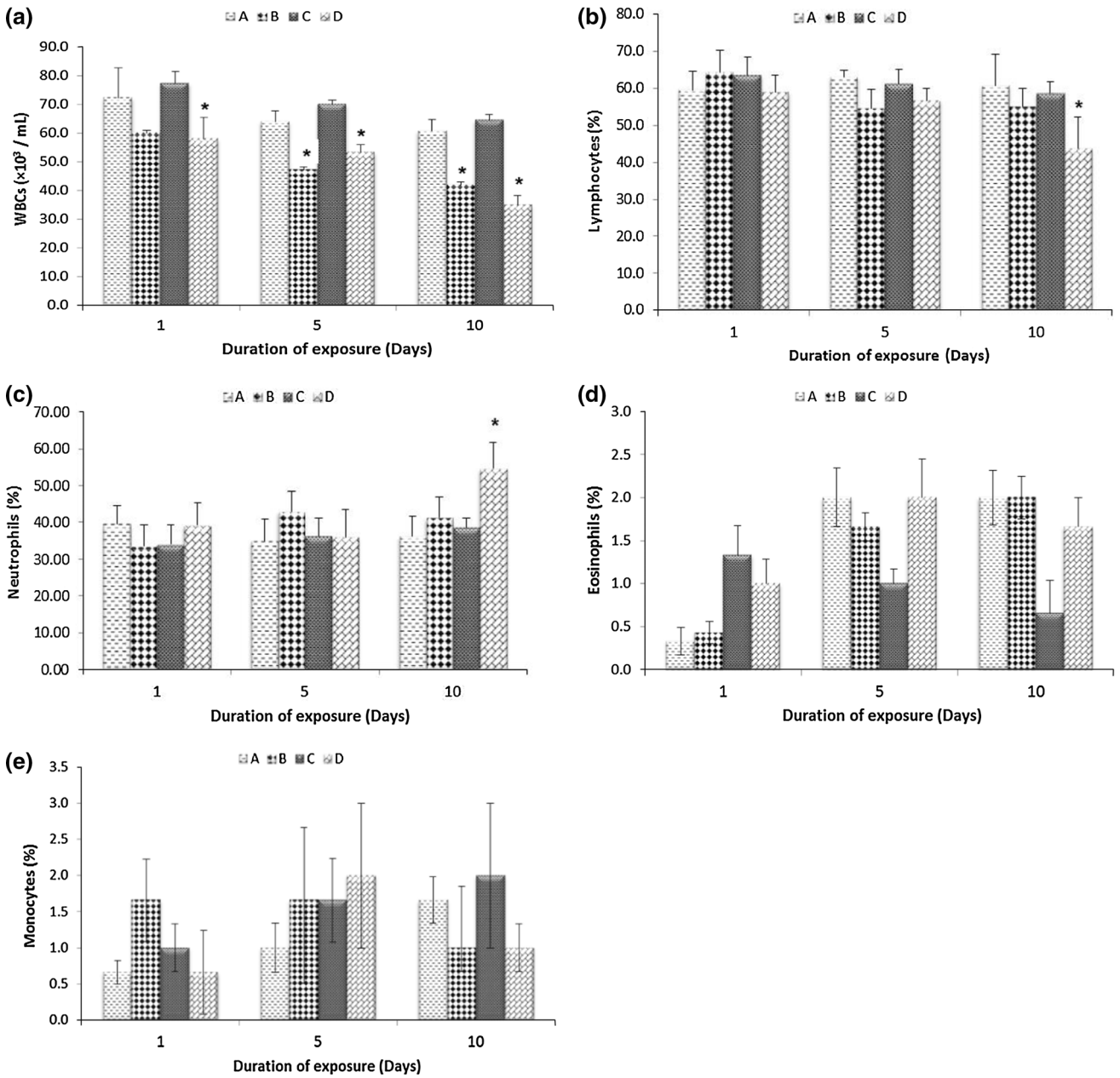

Fig. 2 Leukocyte differential count (**a** WBC; **b** lymphocyte; **c** neutrophils; **d** eosinophils; **e** monocytes) in male rainbow trout affected by butachlor exposure and DEHP (*A* Fish injected with olive oil, *B* fish injected with olive oil containing DEHP, *C* fish without butachlor exposure (control) and *D* fish with butachlor exposure). *Significant at $p < 0.05$

to a low concentration of the organochlorine pesticide endosulfan. Moreover, p,p′-DDE and lindane up-regulated the gene expression of IgM in gilthead seabream (*Sparus aurata L.*) (Cuesta et al. 2008). Different contaminants may display such contradictory immunological effects in different fish species.

The results also revealed a decrease in total leukocyte counts and lymphocytes in both treatments except for lymphocytes in DEHP treatment. The effects of butachlor and DEHP on WBC counts in the present study are in agreement with the evidences reviewed by Milla et al. (2011) that (anti) androgens target the leukocytes. The genes (such as Ucp2, Bcl2 and iNOS) related to reactive oxygen species (ROS) and nitrogen reactive free radical production were affected by some EDCs and their mixture in zebrafish (Jin et al. 2010). Hence, a reduction in total WBC counts and lymphocytes values could be caused by heavy oxidative stress and the balance of nitric oxide (NO) production, which could lead to immune cell deaths. Based on the results, butachlor exposure showed an enhancing

effect on neutrophil levels in the fish. This may have been due to the ability of butachlor to induce an immune response. The *IL-1b* gene, a neutrophil and macrophage activator gene, issignificantly induced by butachlor in embryonic zebrafish (Tu et al. 2013).

In conclusion, our findings suggest adverse effects of DEHP and butachlor on the immune system and T level in male rainbow trout, which may lead to an increase in disease susceptibility and incomplete or inappropriate development of the gonads. The results of the present study also confirm that leukocyte counts can be considered as a novel biomarker of immunotoxicity triggered by (anti) androgenic endocrine disrupters as proposed by Milla et al. (2011).

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