Effect of Exposure to Bisphenol A and 17ß-estradiol on the Sex Differentiation in Zebrafish (*Danio rerio*)

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Received September 1, 2004
Accepted March 3, 2005

Abstract

Drastichová J., Z.Svobodová, M.Groenland, R. Dobšíková, V. Žlábek, D. Weissová, M. Szotkowská: Effect of Exposure to Bisphenol A and 17ß-estradiol on the Sex Differentiation in Zebrafish *(Danio rerio).* Acta Vet. Brno 74, 2005: 287-291.

The effects of bisphenol A and 17ß-estradiol on sex differentiation were investigated in the zebrafish, *Danio rerio*. The 20-day-old fry with undifferentiated gonads were fed with food containing bisphenol A at the dose of 500, 1000, 2000 mg·kg^{-1} and 17ß-estradiol at the dose of 20 mg·kg^{-1} diet for 45 days. Sex ratio and gonadal development after the chemical treatment were determined by histological examination of gonads. In the control group, the sex ratio of fry was 1:1 (female:male), i.e. 50% of females and 50% of males appeared. The sex of fry fed with 17ß-estradiol at the dose of 20 mg·kg^{-1} diet was all females. Feminization of the fry was induced by exogenic 17ß-estradiol. The sex of fry fed with bisphenol A at the dose of 500, 1000, 2000 mg·kg^{-1} diet was 1.4:1 ($p = 0.31$), 3.8:1 ($p = 0.01$) and 11.5:1 ($p < 0.01$). Bisphenol A induced feminization of the fry at the two highest doses tested.

Endocrine disrupters, 17ß-estradiol, fish, sex ratio

Assessment of fish reproductive performance is increasingly used to evaluate the impact of environmental disturbances (Bjerselius et al. 2001). Concern for the successful development and reproduction of fish population has been addressed to endocrine disrupting chemicals (EDCs). These are, broadly defined, natural and man-made agents present in the environment that interfere in some ways with normal endocrine function (Arcand-Hoy and Benson 1998). The ability to interfere with the endocrine system is found in several classes of environmental chemicals such as organochlorine pesticides and its metabolites, polychlorinated biphenyls, dioxin-like chemicals, bisphenolic compounds, alkylphenolic chemicals, some fungicides, phtalate plasticisers and antifouling paints (Tyler et al. 1998).

Endocrine-disrupting compounds have the potential to perturb sensitive hormone pathways that regulate reproductive functions. Developmental and reproductive toxicity may occur during larval development or the juvenile or adult stage. Exposure at an early life stage may lead to alterations in key developmental processes (e.g. sexual differentiation), as well as increased susceptibility to chemical insult as adults. Exposure at maturity could also disrupt normal reproductive parameters (Arcand-Hoy and Benson 1998).

Bisphenol A (BPA) is a commonly used name for 2,2-(4,4-dihydroxydiphenyl) propan. BPA is an industrially important compound used in many plastic applications, for example, plastic pipes, epoxy resins, and coating, and is discharged into the environment at manufacturing plants throughout the world (Staples et al. 1998). In 1993 the annual worldwide production of BPA reached 640 000 tons, out of which an estimate 0.017% (109

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tons) were released into the environment (Benjonathan and Steinmetz 1998; Staples et al. 1998). The environmental distribution has been estimated to be 32% in sediments, 43% in water, 24% in soil and 3.5 * 10^{-5}% in air (TemaNord, 1996). Besides mixing within the water column, BPA is subject to biodegradation, adsorption to suspended solids and sediments, and possibly photodegradation (Staples et al. 1998). Concentrations of bisphenol A in surface waters have been reported to be, in the most severe cases, as high as 17 200 $\mu g \cdot l^{-1}$ in leachates from hazardous waste landfill sites (Yamamoto et al. 2001), but usually concentrations have been around or below 1 $\mu g \cdot l^{-1}$ (Belfoid et al. 2002, Fromme et al. 2002).

BPA has an estrogenic potency which has been demonstrated both *in vitro* and *in vivo* (Soto et al. 1995; Coldham et al. 1997; Kwak et al. 2001). Endocrine disrupters are effective at sublethal concentrations (Stahlschmidt-Allner et al. 1997). It is, therefore, not possible to detect effects of such compounds using existing standard tests with endpoints such as mortality. Great effort has been, therefore, put into this area to develop methods for testing endocrine disrupters containing new endpoints such as gonadal development and vitellogenin induction (Andersen et al. 2003).

One of the fish suggested as a test animal is the zebrafish, *Danio rerio*. In this species, males pass through a stage of juvenile hermafroditism. Approximately 10 days post hatch the differentiation of the gonads begins and all fish, irrespective of their definitive sex, develop ovaries. At approximately day 23 post hatch the ovaries of approximately half of the fish start to transform into testes. This process is completed at approximately 40 days post hatch. In the remaining fish, the development and maturation of ovaries continue (Takahashi 1977). Due to the lability of sex differentiation in fish, exposure to endocrine disrupters during certain critical periods of early development can lead to sex reversal (Andersen et al. 2003).

In this study, zebrafish (*D. rerio*) was used to evaluate the estrogenic effects of bisphenol A and 17ß-estradiol presented in a diet on fish. Sex ratio and gonadal development of fry were investigated by histological methods. We tried to verify the hypothesis that bisphenol A and 17ß-estradiol presented not only in water but also in food can influence the fish organism.

Materials and Methods

The experiment on zebrafish was carried out in a semistatic test. Fish were divided into five groups: one control, three bisphenol treated and one estradiol treated group. Juvenile zebrafish (*Danio rerio*), 20 days post hatch (dph), were placed in two 15 litre glass aquaria (filled with 10 litre of water) for each test group in the number of 25 individuals in each aquarium, i.e. 50 individuals per group. The bath was changed three times a week. Basic physical and chemical indices of water used in the test were as follows: pH 7.67 - 7.98, $ANC_{4.5}$ (alkalinity) 3.5 - 3.8 $mmol \cdot l^{-1}$, COD_{Mn} 0.9 - 2.4 $mg \cdot l^{-1}$, BOD_5 1.07 - 2.38 $mg \cdot l^{-1}$, NH_4^+ 0 $mg \cdot l^{-1}$, NO_3^- 22.68 - 29.23 $mg \cdot l^{-1}$, NO_2^- 0 $mg \cdot l^{-1}$. During the test the water temperature varied between 23.2-24.9 °C. Water was continuously aerated. Oxygen saturation of water was above 60% (ranging from 79.1 to 90.5 %). Fish were kept on a photoperiod of 12:12 h.

Fish were fed three times a day with experimental diet. The test substances bisphenol A and 17ß-estradiol were dissolved in 96 % ethanol. The ethanol solutions were mixed with brine shrimp eggs (*Artemia salina,* Sanders Brine Shrimp Company Inc.) decapsulated by the method of Adámková (1999). Resulting concentration of bisphenol A was 500 $mg \cdot kg^{-1}$, 1000 $mg \cdot kg^{-1}$ and 2000 $mg \cdot kg^{-1}$ of feed. Resulting concentration of estradiol was 20 $mg \cdot kg^{-1}$ of feed. The diet of control fish was treated with ethanol only. After thorough mixing, the ethanol was evaporated from the feed. Fish were fed approximately 2% of their body weight daily.

The experiment was terminated after 45 days at the fish age of 65 dph, 220.85 ± 43.78 mg with mean body weight, and 21.4 ± 1.74 mm mean body length. The sex was determined by histological method at 46 to 50 individuals from each group. Fish were fixed in 10% formalin, embedded in paraffin. The sex was confirmed by light microscopic evaluation of haematoxylin-eosin stained sections.

The Fisher's test was used to examine differences in sex ratio between the control group and exposure groups.

Results

The sex of fry in the zebrafish was identified by the morphology of gonads. Table 1 shows the sex ratio and the gonadal development of zebrafish. In the control group, the sex ratio of fry was 1:1 (female:male), i.e. 50% of females and 50% of males appeared. In the 17ß-

estradiol group, the sex ratio was 1:0, i.e. 100% of females appeared. The fish in this group showed an apparent alteration in sex ratio, which was highly statistically significant ($p < 0.001$) from the control group. In the bisphenol A groups, the sex ratio was 1.4:1 (58% of females) in 500 mg·kg^{-1} diet, 3.8:1 (79% of females) in 1000 mg·kg^{-1} diet and 11.5:1 (92% of females) in 2000 mg·kg^{-1} diet. There was no significant difference ($p = 0.31$) in the group fed with bisphenol A at the dose 500 mg·kg^{-1} diet compared with the control. But there was a significant difference in the groups fed with bisphenol A at the dose 1000 mg·kg^{-1} diet ($p = 0.01$) and at the dose 2000 mg·kg^{-1} diet ($p < 0.01$) compared with the control. No intersex individuals were found in any group tested.

In all groups, there were fish that could not be sex-determined. Occurrence of differentiated or undifferentiated gonadal tissue was not found in these animals, and they may have still been juvenile.

Table 1. Sex ratio of zebrafish (65 days post hatch) exposed to bisphenol A and 17ß-estradiol

Group	n	females (%)	males (%)	sex ratio (female: male)	sex determined (%)	probability
Negative control	50	50	50	1:1	84	
Bisphenol A (500 mg·kg^{-1})	47	58	42	1.4:1	81	
Bisphenol A (1000 mg·kg^{-1})	46	79	21	3.8:1	80	$p = 0.01$
Bisphenol A (2000 mg·kg^{-1})	50	92	8	11.5:1	85	$p < 0.01$
17ß-estradiol (20 mg·kg^{-1})	46	100	0	1:0	85	$p < 0.001$

Discussion

One major purpose of the present investigation was to determine impact of bisphenol A and 17ß-estradiol on sex differentiation in juvenile zebrafish. Previous studies have shown that the most critical period for phenotypic sex determination occurs before and after hatching (Pandian and Sheela 1995). In the present study, zebrafish fed with a diet contaminated with bisphenol A at the dose of 1000 and 2000 mg·kg^{-1} diet showed skewed sex ratio after 45 days of exposure to the experimental diet. The sex ratio of zebrafish fry was significantly different compared with the control group ($p = 0.01$ and $p < 0.01$). Bisphenol A induced feminization of the fry at these two highest concentrations.

BPA is classified as slightly to moderately toxic to fish (Staples et al. 1998). However, current knowledge on bisphenol A toxicity for the early life stages of aquatic species, especially fish, is limited to very few studies. Lee et al. (2003) investigated the estrogenic effect of bisfenol A on the sex differentiation of Korean rockfish (*Sebastes schlegeli*). They fed the 51-day-old fry with food containing bisphenol A at the dose of 0.05, 0.5, 5, 50 and 100 mg·kg^{-1} for 29 days. After investigation of the sex ratio and gonadal development they observed no significant difference compared with the control fish. Their results are in accordance with our examination of zebrafish, because we did not observe estrogenic effect of bisphenol A at a concentration of 500 mg·kg^{-1} on the sex differentiation compared with the control group. Shioda and Wakabayashi (2000) observed a significant decrease in the number of eggs and hatchings in medaka (*Oryzias latipes*) exposed to bisphenol A at a concentration of 10 µmol·l^{-1}. Pastva et al. (2001) observed transient embryonic deformities in medaka (*Oryzias latipes*) embryos exposed to bisphenol A at a concentration of 200 µg·l^{-1}. Honkanen et al. (2004) observed morphological and histological changes in salmon yolk-sac fry exposed to bisphenol A at the concentration of 100 and 1000 µg·l^{-1}.

Bisphenol A also persists in sediments. Fromme et al. (2002) measured BPA at the concentrations from 0.01 to 0.19 mg·kg^{-1} in sediments from riverbeds of 35 waterways in

Germany. Concentrations of BPA up to 50 ng g^{-1} have been reported for sediments from the Masan Bay, Korea (Khim et al. 1999). Belfroid et al. (2002) measured BPA in the liver and muscle of bream (*Abramis brama*) and flounder (*Platichthys flesus*) at selected freshwater and marine locations in the Netherlands. An interesting point is that BPA was shown to be present even in flounder that lived at locations where no BPA was observed in the surface water. One explanation is that this bottom dwelling species feed primarily on benthic fauna, which might have accumulated BPA that had been absorbed to the sediment (Belfroid et al. 2002).

Presented results verified the hypothesis, that 17ß-estradiol and bisphenol A (in high concentrations) in food can influence a sex differentiation in juvenile zebrafish.

Vliv bisfenolu A a 17ß-estradiolu na diferenciaci pohlaví u dánia pruhovaného (*Danio rerio*)

Vliv bisfenolu A a 17ß-estradiolu na diferenciaci pohlaví byl zhodnocen u dánia pruhovaného, *Danio rerio*. 20-ti denní plůdek s nediferencovanými gonádami byl krmen dietou obsahující bisfenol A a 17ß-estradiol v koncentraci 500, 1000, 2000 mg·kg^{-1} a 20 mg·kg^{-1} po dobu 45 dnů. Poměr pohlaví a vývoj gonád byl určen na základě histologického vyšetření. V kontrolní skupině byl poměr pohlaví 1:1, tzn. 50 % samic a 50 % samců. Pohlaví u plůdku krmeného krmivem obsahujícím 17ß-estradiol v dávce 20 mg·kg^{-1} bylo 100 % samičí. Feminizace plůdku byla způsobena exogenním 17ß-estradiolem. Poměr pohlaví u plůdku krmeného krmivem obsahujícím bisfenol A v dávce 500, 1000, 2000 mg·kg^{-1} bylo 1.4:1 ($p = 0.31$), 3.8:1 ($p = 0.01$) a 11.5:1 ($p < 0.01$). Bisfenol A způsobil feminizaci plůdku ve dvou nejvyšších testovaných koncentracích.

Acknowledgements

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic (MSM project No. 6215712402).

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