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Effects of Bisphenol A on Sex Differentiation and Gonadal Development of Medaka, *Oryzias latipes*

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A study on the effects of bisphenol A (BPA) on sex differentiation and gonadal development in medaka, *Oryzias latipes*, was investigated by histological examination. The fish were exposed to aqueous solutions of BPA at nominal concentrations of 50, 100, and 200 μg/L from newly-hatched larvae stage to 70 d. The ovaries of female fish were composed of oocytes at the chromatin nucleolus and peri-nucleolus stages at 20 d after the exposure. The testes contained a number of spermatogonia and spermatocytes at 30 d. In the process of sex differentiation, gonadal development was not different in all experimental groups until 30 d after the exposure. At 70 d after the exposure, however, advanced development of oocytes in the ovary and inhibition of spermatogenesis in the testis were observed in the BPA-treated groups compared to the non-treated controls. More females than males were identified in the 50 and 100 μg/L BPA-treated groups, in comparison to the 200 μg/L BPA-treated group and non-treated controls. Medaka exposed to 200 μg/L BPA were bigger compared to other experimental groups. The present study suggests that BPA may lead to problems in either mating or sexual behavior due to the difference in growth and disparity of sexual maturation between male and female fish.

During the past several years, environmental endocrine disruptors have been a concern in scientific, popular, and political fields. European Commission (1996) defined endocrine disruptor as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function, while a potential endocrine disruptor is a substance that possesses properties expected to lead to endocrine disruption in an intact organism.

A variety of synthetic compounds have been shown to mimic estrogenic hormones. Among these compounds, bisphenol A (BPA) has been identified as an endocrine disrupting chemical (Olea et al., 1996). BPA is widely used as primary products of poly-carbonated plastics and epoxy resins. Polycarbonates are used in drink packs and earthen vessels and epoxy resins are used in the coating of cans, bottles, and water pipes (Brotons et al., 1995). BPA released to the environment is generally dissolved in water and may be partly adsorbed to the soil and sediment (Staples et al., 1998). BPA has been reported to exert estrogenic activity in mammalian species (Milligan et al., 1998). Evaluation of reproductive organ of the male offspring of female Wistar rats exposed to BPA has been reported (Dimond et al., 1998). The environmental toxicity of BPA has been actively researched, and those results were summed up and reviewed by Staples et al. (1998). However, only a few numbers of reports concerning the effects of BPA on sex differentiation and gonadal development in fish are available (Yokota et al., 2000; Metcalfe et al., 2001).

Medaka (*Oryzias latipes*) is an ideal test organism for a study on the estrogenic effects of endocrine disruptors (Metcalfe et al., 1999), because it is a differentiated gonochoristic fish and has no spontaneous intersex (Yamamoto, 1969). Therefore, the present study was conducted to evaluate sex differentiation and gonadal development of songsari exposed to BPA from newly hatched larvae to 70 d.

Materials and Methods

Chemical

The technical grade BPA (> 99.9% purity) used in this experiment was obtained from Aldrich Chemical Co. (Milwaukee, USA). A BPA stock solution of 100 mg/mL was prepared by dissolving BPA in acetone.

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Fish

Medaka used in this study were collected from a branch stream of the Seomjin River and transported to Marine Research Institute of Cheju National University (Korea). Breeding fish were maintained in re-circulating Jeju Island ground water over 3 months. During breeding, the fish (total length, 2.6±0.6 cm; body weight 0.21±0.1 g) were controlled to a light:dark photoperiod of 16:8 h. The fish were fed with an adequate amount of commercial diet (Ewha oils & Fat Industry Co. Ltd., Korea) and newly-hatched brine shrimp twice daily. Water temperature of the breeding aquaria ranged from 22 to 24℃. Some water plants were put in the aquaria for the spawned eggs to attach. Only the fertilized eggs were incubated in a 10 L glass container and checked for hatching.

Experimental conditions

Newly hatched larvae of medaka were exposed to BPA at sub-lethal concentrations in a static renewal system for 70 d after the hatching. The BPA treatment was performed in a 1 L glass aquarium filled with 900 mL of filtered Jeju Island ground water. The quality parameters of the ground water were pH 8.1 and COD 0.8 mg/L. According to the methods described by Gray and Metcalfe (1997), the aqueous solutions of BPA were renewed every 72 h for the first month and every 48 h thereafter. There were five experimental groups, each with 30 fish, duplicated (total n = 300) at the start of the experiment. Nominal BPA concentrations of 50, 100, and 200 μg/L were maintained by adding appropriate volumes (4.5, 9.0, and 18.0 μl) of BPA stock solution to the water in the aquaria. In the acetone control, acetone (18.0 μl) alone was added, and in the control, only the pure ground water was supplied. The fish were maintained in a ratio of light and dark photoperiod of 16:8 h and fed a commercial diet three times daily for the duration of the experiment.

Sampling

During the experimental period of 70 d, 34-50 fish were prepared for histological examination in each experimental groups. At 10 and 20 d after the BPA exposure, 5 fish were examined in all experimental groups. At 30 d, 30 fish were examined in all experimental groups, except for the control group. Only 16 fish were sampled in the control group because 14 fish died as an interruption of aeration in one aquarium of the control group at 30 d. At 70 d after the exposure, 8-10 fish were examined because 1 and 2 fish died in BPA 100 μg/L treatment and control groups, respectively.

Histology

The fish were placed in tissue bottles and fixed in Bouins fixative. The fish then were embedded whole in paraffin and sectioned (5 μm) with a microtome. The sections were stained using Hansen's hematoxylin and 0.5% eosin and examined under a light microscope. Sagittal sections of the gonads were viewed with a microscope monitoring system. Calculations of the proportion of the sagittal section area of the gonad occupied by each germ cell type were made. The classification of the developmental stages of oogenesis was according to Yamamoto and Yoshioka (1964) and spermatogenesis was according to Grier (1976). The sex of few fish could not be determined because the gonad was not sectioned properly during histological preparation (i.e., unknown sex).

Growth

At 10, 20 and 30 d after the exposure, 10-30 fish were anaesthetized with tricaine methane sulfonate (MS-222) and examined for the total length. At 70 d, 8-10 fish were examined for total length and body weight in each experimental group. Total of 297 fish were examined for the growth effect of BPA. The total weight and length of the fish were measured to the accuracy of 0.01 g using an electronic balance and to 0.1 mm with the dissecting microscope, respectively.

Statistics

The experimental data on growth (mean total length and body weight) in five groups were analyzed by one-way analysis of variance (ANOVA) and then by Duncans multiple tests. The data on sex ratio were distinguished from the gonads were analyzed by chi-square analysis. Differences were considered to be significant at the $P \leq 0.05$ level.

Results

Sex differentiation

The mean body length of newly-hatched larvae was 4.4 mm. The larvae still possessed the yolk. In sagittal section, the myotome, the notochord and the gut could be recognized. The germinal strand was located between the myotome and the gut. Gonia cells were visible in the posterior region of the primitive gonad. The gonia cells were nearly ovoid in shape and the average diameter of these cells was 8 μm. The ovaries of female songsari at 20 d after the exposure were composed of the oocytes at the chromatin nucleolus and peri-nucleolus stages in all experimental groups. The average diameter of the oocytes at the chromatin nucleolus stage was 19 μm, and that of the oocytes at the peri-nucleolus stage was 25 μm. The testis of the male fish at this time contained gonia cells alone in all experimental groups. The gonia cells were nearly round or ovoid and the average diameter of these cells was 7-8 μm. At 30 d after the exposure, the ovaries of the female fish were composed of the oocytes at the

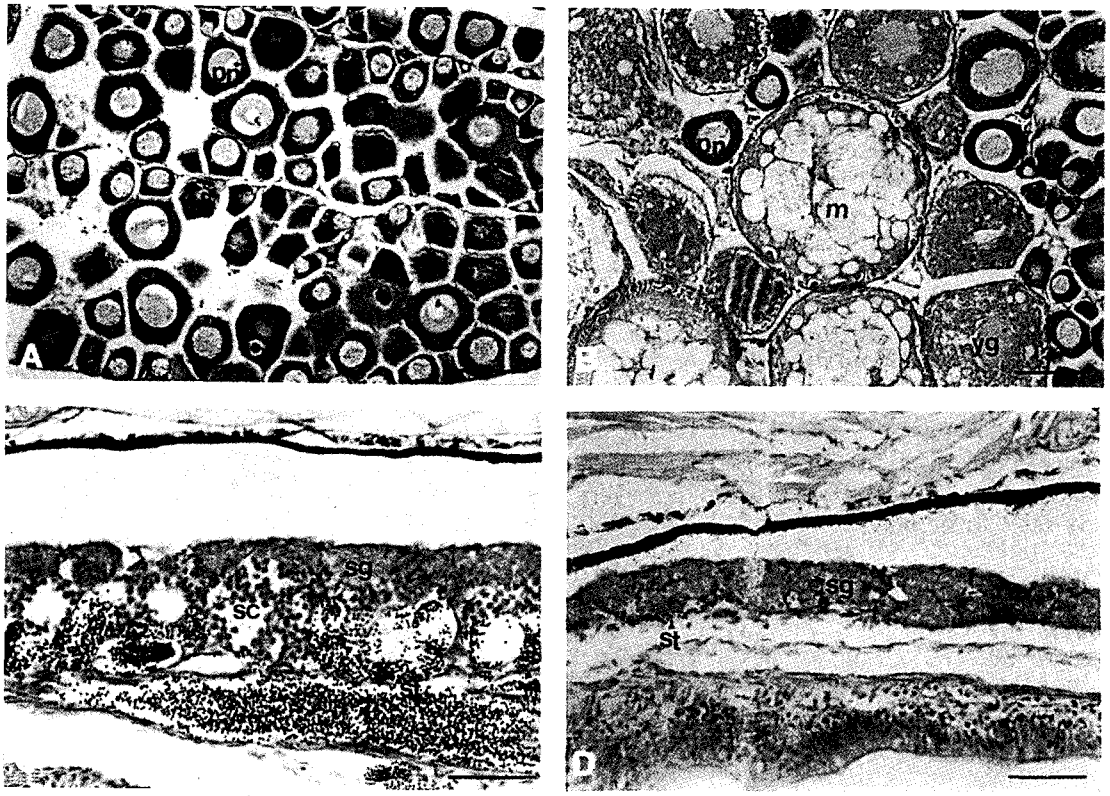

Fig. 1. Photomicrographs of the gonad of medaka, *Oryzias latipes*, exposed to bisphenol A from newly-hatched larvae to 70 days. A, Ovary of the control group. Scale bar = 50 μm. B, Ovary of BPA 200 μg/L-treated group. Scale bar = 50 μm. C, Testis of the control group. Scale bar = 25 μm. D, Testis of BPA 200 μg/L-treated group. m; mature oocyte, pn; peri-nucleolus oocyte, sc; spermatocyte, sg; spermatogonium, st; spermatid, Yg; yolk glouble oocyte. Scale bars = 25 μm.

chromatin nucleolus stage and peri-nucleolus stage and had an increased number of oocytes. In the testis of the male fish at this time, a number of spermatogonia and spermatocytes were observed in the testicular lobule. No difference of gonadal development in the process of the sex differentiation was observed in both control groups and experimental groups until 30 d after the BPA exposure.

Gonadal development

In the control groups, the ovaries of the female fish at 70 d after the exposure were composed of the oocytes at the chromatin nucleolus, peri-nucleolus and yolk vesicle stages (Fig. 1-A). The frequency of each developmental stage of the oocytes were 12.6±1.50%, 87.4±1.50% and 0.01±0.004% in the control group and 10.5±1.90%, 88.0±1.56% and 1.4±0.36% in the acetone control group, respectively. Otherwise in the BPA-treated groups, the ovaries at this time were composed of the oocytes at the chromatin nucleolus, peri-nucleolus, yolk vesicle, yolk globule and mature stages (Fig. 1-B). The frequency of each developmental stage of the oocytes was 8.3±0.23%, 88.6±2.33% and 3.1±2.55% in 50 μg/L BPA-treated group, 4.3±0.02%, 89.4±1.57%, 4.0±0.67% and 2.3±0.87% in 100 μg/L BPA-treated group and 3.9±0.14%, 80.4±1.09%, 8.8±2.33%, 4.9±1.81% and 1.9±1.48% in 200 μg/L BPA-treated group (Fig. 2).

The frequency of the spermatogenesis stage in the testis of male fish at 70 d after the exposure is shown in Fig. 3. The frequency of spermatogonium, spermatocyte and spermatid in the testis were 67.9±5.24%, 9.4±3.69% and 22.7±1.55% in the control group and

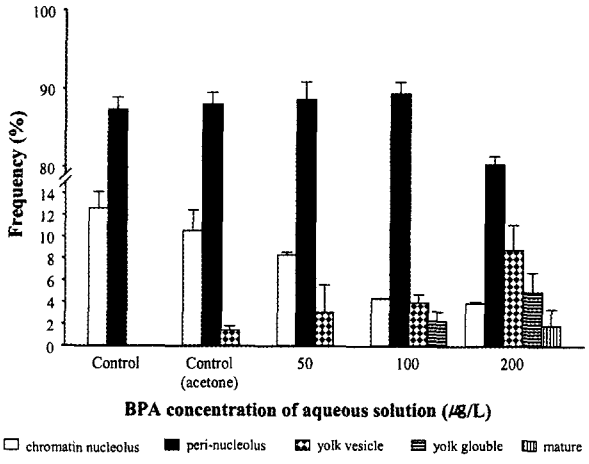

Fig. 2. Frequency of developmental stage of oocytes in the ovary of female medaka exposed to bisphenol A from newly hatched larvae to 70 days.

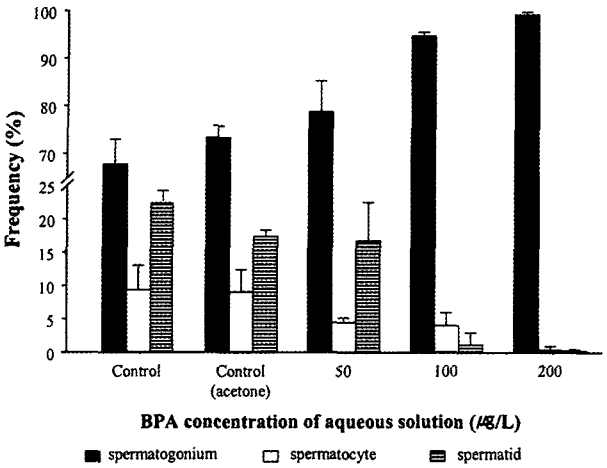

Fig. 3. *Frequency of developmental stage of spermatogenesis in the testis of male medaka exposed to bisphenol A from newly hatched larvae to 70 days.*

73.5±2.40%, 9.1±3.34% and 17.4±0.94% in the acetone control group. In the BPA-treated groups, the frequency of spermatocytes and spermatid was decreased (Fig. 1-C, D). The frequency of each stage was 78.8±6.5%, 4.5±0.72% and 16.7±5.77% in 50 μg/L BPA-treated group, 94.8±0.81%, 4.0±1.96% and 1.2±1.80% in 100 μg/L BPA-treated group, 99.3± 0.58%, 0.5±0.38% and 0.2±0.20% in 200 μg/L BPA-treated group.

Sex ratio and growth

Chi-square analysis indicated that sex ratios of female to male were 2:1 in 50 μg/L BPA and 100 μg/L BPA-treated groups (Table 1; $P > 0.05$). However, the sex ratios in the control groups and 200 μg/L BPA-treated groups were 1:1 ($P > 0.05$).

The mean total lengths of the fish at 10, 20, 30 and 70 d after the exposure are presented in Table 2. Duncans multiple test indicated that mean total length at 30 d after the exposure was greater for fish in 200 μg/L BPA-treated group in comparison to the control groups and other treatment groups. At 70 d after the exposure, the mean total length of the fish in 200 μg/L BPA-treated group was significantly higher than was noted in the control groups and other-treatment groups ($P < 0.05$). The mean body weight of the fish in this treated group was also significantly higher than the control group, but no difference was noted in the acetone control group and other BPA-treated groups.

Discussion

The developmental stage of oocyte in the ovary of female medaka at 70 d after the BPA exposure was yolk vesicle stage in the control groups and vitellogenic stage in the treatment groups. In sex differentiation of fishes, 17β-estradiol (E_2) advanced ovarian development in comparison with untreated females as observed in coho salmon, *Oncorhynchus kisutch* (Foyle, 1993) and in pejerrey, *Odontesthes bonariensis* (Strüssmann et al., 1996). In fish, E_2 stimulates vitellogenin synthesis of hepatocyte and plays a role in accumulation of vitellogenin within eggs (Specker and Sullivan, 1994). BPA similar to E_2 may contribute to accumulation of vitellogenin in oocyte of medaka.

In the present study, the testes of male medaka exposed to BPA showed several morphologic changes, including less developed testicular structure with lobules clearly separated from each other. Inhibition of spermatogenesis in the testis was observed in the BPA-treated groups compared to the control groups. This result indicates that BPA inhibits proliferation of spermatogonia in medaka. Nonylphenol and 17β-estradiol have severe effects on the testis and Sertoli cells in the eelpout, *Zoarces viviparous* (Christiansen et al., 1998 a, b). Estrogen inhibits the differentiation of Leydig cells, Sertoli cells and early formation of the spermatic duct in the European eel, *Anguilla anguilla* (Colombo and Grandi, 1995). In sexually developing fish, pronounced effects on vitellogenin synthesis caused by exposure to various estrogenic chemicals were accompanied by concomitant and significant decreases in testicular growth (Jobling et al., 1996).

In this study, more females than males were identified in the 50 μg/L and the 100 μg/L BPA-treated groups in comparison to the controls and the 200 μg/L BPA-treated group. The present histological analysis of 205 fish, however, resulted in uncovering one intersex individual. Fry of medaka, *Oryzias latipes*, exposed to

Table 1. Sex ratio identified from histological examination of medaka, *Oryzias latipes*, gonads which were exposed to bisphenol A from newly-hatched larvae to 70 days

Experimental groups	Days after exposure								Number			Sex ratio
	10		20		30		70		Female	Male	Unknown	Female : Male
	Female	Male	Female	Male	Female	Male	Female	Male				
Control	2	2	3	1	8	8	4	4	17	15	2	1.13:1*
Control (acetone)	1	4	3	2	14	16	6	4	24	26	0	0.92:1*
BPA 50 μg/L	2	3	3	2	21	9	7	3	33	17	0	1.94:1**
BPA 100 μg/L	2	2	3	1	20	9	5	4	30	16	3	1.88:1**
BPA 200 μg/L	2	2	3	1	12	17	6	4	24	23	3	1.04:1*

* Sex ratio of female to male analyzed by chi-square was 1:1 ($P > 0.05$).
** Sex ratio of female to male analyzed by chi-square was 2:1 ($P > 0.05$).

Table 2. Changes of mean total length and body weight of medaka, *Oryzias latipes* exposed to bisphenol A at 10, 20, 30 and 70 days after exposure

Experimental groups	Days after exposure								
	10		20		30		70		
	Length (cm)	n	Length (cm)	n	Length (cm)	n	Length (cm)	Weight (g)	n
Control	0.65 ± 0.04^{a}	10	0.83 ± 0.11^{a}	10	1.13 ± 0.14^{b}	30	1.69 ± 0.20^{b}	0.04 ± 0.02^{b}	8
Control (acetone)	0.66 ± 0.04^{a}	10	0.85 ± 0.04^{a}	10	1.15 ± 0.14^{b}	30	1.74 ± 0.22^{b}	0.06 ± 0.03^{ab}	10
BPA 50 μg/L	0.65 ± 0.04^{a}	10	0.85 ± 0.14^{a}	10	1.18 ± 0.17^{b}	30	1.76 ± 0.34^{b}	0.06 ± 0.02^{ab}	10
BPA 100 μg/L	0.65 ± 0.05^{a}	10	0.88 ± 0.12^{a}	10	1.19 ± 0.12^{b}	30	1.87 ± 0.29^{b}	0.06 ± 0.02^{ab}	9
BPA 200 μg/L	0.66 ± 0.05^{a}	10	0.90 ± 0.07^{a}	10	1.23 ± 0.14^{a}	30	2.01 ± 0.31^{a}	0.07 ± 0.03^{a}	10

Values in the same column followed by a different letter are significantly different ($P < 0.05$).

both 4.0 and 29.4 μg/L 17β-estradiol exhibited 53% testis-ova or presumptive hermaphroditism in approximately 40% female and 5% male in each dose group (Wester and Canton, 1986). Gray and Metcalfe (1997) reported that fry of medaka exposed to 100 μg/L *p*-nonylphenol (NP) induced both intersex state (i.e., testis-ova) in males as well as sex reversal (i.e., male to female), while exposure to a lower concentration of NP (50 μg/L) induced only testis-ova.

Medaka exposed to 200 μg/L BPA were found to become bigger than the fish of other experimental groups. Similarly, fish exposed to estrogenic compounds were bigger than the control fish (Gray and Metcalfe, 1997; Moon, 1999). Sharpe et al. (1995) found that rats exposed to estrogenic compounds became larger than the control rats. 17β-estradiol has a stimulatory effect upon growth in fish, possibly through stimulation of the release of pituitary growth hormone, which in turn may stimulate both protein and lipid synthesis (Malison et al., 1988).

On the basis of present study, BPA may lead to serious problems in either mating or sexual behavior due to difference in growth and disparity of sexual maturation between male and female fish. The mechanisms underlying the advancement of ovarian development and inhibition of testicular growth by BPA are unknown. However, ovarian and testicular development may be dependent on the concentration of BPA exposure.

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