TRIBUTYL Tin CAUSES MASCULINIZATION IN FISH

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Abstract—We examined the effect of tributyltin (TBT) on the sex differentiation process in genetically female Japanese flounder (Paralichthys olivaceus). The fish were fed an artificial diet containing tributyltin oxide (TBTO) at concentrations of 0.1 and 1.0 μg/g diet from 35 to 100 d after hatching, which includes the sex differentiation period. The ratio of sex-reversed males significantly increased to 25.7% of the flounder fed the 0.1 μg/g diet and to 31.1% of those fed the 1.0 μg/g diet compared with the control (2.2%). From morphological and histological examination of the fish in the TBT-treated groups, normal females had typical ovaries and sex-reversed males had typical testes. These results clearly demonstrated the masculinization of flounder exposed to TBTO. This is the first report of TBT inducing sex reversal in vertebrates.

Keywords—Tributyltin oxide Sex reversal Japanese flounder P450 aromatase Endocrine disruption

INTRODUCTION

Tributyltin (TBT) is a strong endocrine disruptor and is a highly hazardous chemical in the marine environment because of its strong masculinizing effect on mollusks, a condition known as imposex [1]. Although its use has been strictly regulated in some countries in the last decade, TBT still remains at hazardous levels, as indicated by continuing high imposex frequencies in mollusks [2,3].

The adverse effects of TBT in fish have been reported in reproduction [4,5], growth [6,7], and behavior [7]. The TBT inhibits the activity of cytochrome P4501A [8] and suppresses the immune system [9,10]. In our previous studies of cultured Japanese flounder and wild flatfish, we detected concentrations of TBT in the blood that were more than 10 times higher than the concentrations in muscle [11]. The high accumulation of TBT in blood was attributed to the binding of TBT to a specific protein in the blood [12]. Nirmala et al. [4] found a transgenerational effect in medaka larvae spawned from parents administered TBT, resulting in impaired swim-up function. Therefore, TBT is suspected to affect the development of fish. Impaired sexual development by TBT is well known in mollusks as imposex. Imposex causes development of a penis in females, and it is hypothetically attributed to TBT inhibiting aromatization [13]. However, no study has been performed on the effect of TBT on sex differentiation in fish. In the present study, we examined the effect of TBT on the sex differentiation process in fish, using only XX larvae of Japanese flounder, and demonstrated that TBT induced sex reversal of genetically female flounder into phenotypic males. This is the first report on TBT inducing sex reversal in vertebrates.

MATERIALS AND METHODS

Tributyltin oxide (TBTO) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). To prepare the diet, TBTO (0.1 or 1.0 μg/g diet) was dissolved in ethanol and mixed with an artificial fish food (Love Larva, Maruha, Tokyo, Japan). The prepared diet was then dried with an air blower.

Japanese flounder have an XX (female)/XY (male) sex determination mechanism [14]. To obtain progeny that were all genetically female, normal females were mated with sex-reversed, meiotic gynogenetic males, as described by Kitano et al. [15]. Briefly, a meiotic gynogenetic diploid was induced by cold-shock treatment (1°C for 30 min, 4 min after insemination) of eggs that had been inseminated by ultraviolet-irradiated (1,000 erg/mm², 1 min) sperm. All the resultant larvae were genetically XX. The phenotypic sex was then reversed to male by treatment with 17α-methyltestosterone. These sex-reversed phenotypic males were mated with normal females to produce all genetically female broods. The larvae (XX) were reared at 18°C in a 2,000-L tank until the beginning of the experiment.

At 35 d after hatching, the larvae (~0.04 g in body wt, 12 mm in total length) were randomly selected and transferred to 500-L tanks (150 larvae in each tank), each equipped with a filtered flow-through system using natural seawater maintained at 18°C. The larvae were fed the artificial diet containing TBTO (0.1 or 1.0 μg/g diet) ad libitum from 35 to 100 d after hatching, which includes the sex differentiation period [16,17]. Fish were maintained in three separate tanks corresponding to the treatment being received. The individual was considered the unit of replication in this study. Following the exposure period, fish were fed the control diet and maintained for 200 d at ambient temperature.

For the analysis of cytochrome P450 aromatase (P450arom) and elongation factor-1α (EF-1α) gene expression, total RNA was extracted using Isogen (Nippongene, Tokyo, Japan) from individual gonads of juveniles at 100 d after hatching. Gene expressions of P450arom and EF-1α were analyzed by reverse transcription-polymerase chain reaction [18]. The products were electrophoresed on a 2%
aggregates were significantly less than that in the control (p < 0.05; ANOVA). The mean total length of the fish in the TBT-treated groups was 25.9 ± 6.4 cm, and 31.1 ± 5.4 cm in the control group. This indicated no significant difference among treatment groups.

The survival rate of the fish in the control group was 42.3% at 100 d after hatching. Expression of the P450arom gene was slightly detectable in the gonads of only two out of four individuals in each TBT-treated group but was readily detected in the gonads of all examined control individuals (Fig. 2). Thus, P450arom gene expression was suppressed by administration of TBTO at 0.1 and 1.0 μg/g diet.

At 100 d after hatching, the TBT concentrations in fish from each group were 0.018 ± 0.004 μg/g in the 0.1-μg/g group (n = 5), 0.159 ± 0.017 μg/g in the 1.0-μg/g group (n = 5), and 0.007 ± 0.002 μg/g in the control group (n = 4). The concentrations of TBT in the 0.1-μg/g diet and 1.0 μg/g diet. In the control diet, TBT was detected at 0.02 μg/g. The concentrations of TBT in the bodies of fish in the 0.1- and 1.0-μg/g groups, respectively, were 2.6 and 23.0 times greater than those in the control.

**DISCUSSION**

The survival rate of the fish in the control group was 42.3% from 35 to 100 d posthatch. In general, high mortality is observed in marine teleosts during early life stages. The survival rates of Japanese flounder in a previous study [16] were about 20% at 35 d after hatching and 10% at 100 d after hatching. Expression of the P450arom gene was slightly detectable in the gonads of only two out of four individuals in each TBT-treated group but was readily detected in the gonads of all examined control individuals (Fig. 2). Thus, P450arom gene expression was suppressed by administration of TBTO at 0.1 and 1.0 μg/g diet.

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**Testis in phenotypic males**

**Ovary in phenotypic females**

![Fig. 1. Gonadal histology of the ovary from a female and the testis from a sex-reversed male flounder in each group at 300 d after hatching. Ovaries and testes from the tributyltin-treated groups were histologically indistinguishable from those in the control.](image-url)
Tributyltin causes masculinization in fish

Fig. 2. P450arom gene expression in gonads of XX flounder exposed to tributyltin oxide at 100 d after hatching. Gene expression of elongation factor (EF)-1α and P450arom was analyzed with reverse transcription-polymerase chain reaction.

50% from 36 to 100 d posthatch. Thus, larval quality was level enough to conduct the experiment in the present study.

This study clearly demonstrated the masculinization of flounder exposed to TBTO. The proportion of sex-reversed males treated with TBTO significantly increased to 25.7% in the 0.1-µg/g group and to 31.1% in the 1.0-µg/g group compared with the control (2.2%). Concentrations of TBT in body tissues increased from 0.007 µg/g (control) to 0.018 (0.1-µg/g group) and 0.159 µg/g (1.0-µg/g group). From our results, the response of Japanese flounder to TBT may have an upper limit rather than being liner.

In the control, one fish was masculinized, although Kitano et al. [18] reported no masculinization of genetically female, untreated fish maintained for 300 d after hatching. The masculinization in the control might be attributed to the low but detectable concentration of TBT in body tissues (0.007 µg/g) and diet (0.02 µg/g) of the control fish. Iijima et al. [19] reported that TBT found in cultured freshwater fishes might be from fishmeal in the feed. Oshima et al. [11] detected 0.017 µg/g of TBT in muscle of cultured Japanese flounder. It is likely that Japanese flounder inhabiting polluted areas are continuously exposed to low levels of TBT via water or diet and thus are masculinized.

In this study, we observed considerable suppression of growth in the TBT-treated groups. The suppression of growth in fish by TBT was also observed in rainbow trout [6] and inland silverside [20]. Suppression on feeding or metabolic rates caused by TBT may lead to a lower growth rate in TBT-treated groups than in the control.

Our results imply a skewed sex ratio not only in flounder but also in other fish inhabiting TBT-polluted areas because the TBT concentrations in diet (0.1 or 1.0 µg/g diet) were at environmentally possible levels. Harino et al. [21] detected TBT in seawater at 0.074 µg/L, in bottom sediment at 0.64 µg/g, in plankton at 9.8 µg/g, and in mussels at 0.17 µg/g in Otsuchi Bay, Japan. In areas polluted with TBT, potent amounts of TBT might have been taken up by juvenile fish via seawater or diet; the TBT may then have accumulated in blood or tissues and affected the sex differentiation process, although there are no reports about masculinization of fish in Japanese inshore water.

Our findings suggest a possible link between the suppres-
sion of the P450arom gene and sex reversal of genetic females of fish. The expression of the P450arom gene was suppressed in the gonads of TBT-treated groups at 100 d after hatching. The masculinization of mollusks by TBT is hypothetically attributed to the inhibition of aromatization or sulfate conjugation [13]. The physiological mechanism of sex differentiation has not been clear in fish. However, Kitano et al. [18] showed that the treatment of genetically female flounder with aromatase inhibitor (fadrozole) or 17α-methyltestosterone caused suppression of P450arom gene expression in the gonads and induced sex reversal of genetic females into phenotypic males without intersex. These results support the hypothesis that the sex reversal of genetic females exposed to TBT might be due to suppression of the P450arom gene in the gonads.

Recently, the masculinizing effect of TBT has been observed in mammals. Ogata and coworkers reported that TBT chloride increased the anogenital distance in newborn female rats [22]. This suggests that TBT chloride can exert a masculinizing effect on newborn female mammals. They also report a decrease in the ventral prostate weight and concentration of 17β-estradiol in the serum of male rats administered TBT chloride at concentrations of 25 and 125 ppm in the diet for two generations. They conclude that this might be caused by the inhibition of aromatase by TBT [23]. Further, aromatase inhibition by TBT was also observed in human granulosa-like tumor cell line [24] and human placenta [25]. These results indirectly support our results.

Tributyltin has been detected in fish and shellfish collected from coastal [11,21,26], pelagic [26,27], and deep-sea areas [28]. In marine mammals, high concentrations of TBT have also been detected in cetaceans [29], seals [30], and sea otters [31]. Furthermore, detectable butyltin residues have been found in terrestrial mammals such as raccoon dogs [32]. Kannan et al. [33] reported the occurrence of TBT in human blood and suspected that it impaired the endocrine system. However, the effect of TBT on development in mammals is still unclear.

This is the first report of TBT inducing sex reversal in vertebrates. Our results clearly demonstrated the masculinization of flounder exposed to environmentally relevant concentrations of TBT. In wildlife, TBT is still detected at hazardous levels and has the potential to disrupt endocrine systems. Thus, further study is needed to elucidate the effects, mechanisms, and risks of TBT on endocrine disruption in a variety of animals.

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