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Original Research Paper

Chronic Effects of Cadmium on Gonad Differentiation of the Spot Frog (*Pelophylax nigromaculata*) Larvae

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ABSTRACT

Cadmium (Cd), one of the most common endocrine disruptors (EDCs), plays an important role in sex differentiation by blocking receptors of sex steroid hormones in amphibians. To analyse the influence of Cd on gonadal sex differentiation of spot frog (*Pelophylax nigromaculata*) larvae, the fertilized eggs were exposed to Cd (100, 10, 1 and 0.1 µg/L) in water until complete metamorphosis. We observed sex ratio and gonadal condition, detected the expression and location of estrogen receptor (ER) and metallothioneins (MTs) on the gonad cells by immunohistochemical method. Some intersex gonads (the same gonad has testicular and ovarian-like elements) in Cd treatment at 10, 1 and 0.1 µg/L were observed. Compared to control, female/male ratios at Cd treatment levels (1 and 0.1 µg/L) were significantly different ($p< 0.05$). ER and MTs were positively expressed in the cytoplasm and nuclei of gonad, and the expression of ER and MTs did not show a monotonic linear relationship in the Cd treatment. In the relatively lower concentration (10, 1 and 0.1 µg/L), ER and MTs expressions were stronger, while it appeared to be weak immunoreactivity in higher concentration (100 µg/L). The expression and location of ER and MTs on the gonad cells and sex ratios were affected by Cd, indicating that Cd has estrogenic effect on *P. nigromaculata*.

INTRODUCTION

With rapid development of modern agriculture and industry, a number of endocrine-disrupting contaminants (EDCs) have been concerned during the past few decades (Fu et al. 2013). Cadmium (Cd) is one of most common EDCs found naturally in aquatic systems such as rivers, lakes, marine and oceans, existing in cadmium sulphate ($CdSO_4$), cadmium oxide (CdO) and cadmium chloride ($CdCl_2$) state. Cd can accumulate in the organisms for half-life of 15-30 years and time-consuming accumulation in reproductive organs, such as the ovaries and testis (Angenard et al. 2010). Recently, Cd has been reported to be released to the environment by smelter operations, the plastics or batteries use and mining operations (Zadorozhnaja et al. 2000).

Many studies have described that Cd has various effects on reproductive endocrinology (Henson & Chedrese 2004, Das & Mukherjee 2013), but definitive conclusions are different based on the treated concentration and animals (Mackenzie et al. 2003). In mammals, a number of studies have provided strong evidence that Cd at very low concentrations has a potent estrogenic activity through its interaction with estrogen receptor (ER) in vivo (Johnson et al. 2003) and in vitro (Garcia-Morales et al. 1994, Stoica et al. 2000). In teleosts, there is inconclusive evidence on the estrogenic properties of Cd. Some studies have indicated that there is the estrogenic activity in the embryos of male medaka (*Oryzias latipes*) exposed Cd with low concentrations (Foran et al. 2002), while others have suggested that there is absence of estrogenic activity in winter flounder (*Pleuronectes americanus*) (Pereira et al. 1993) and rainbow trout (*Oncorhynchus mykiss*) (Le Guevel et al. 2000). For amphibians, the reproductive system development can be sensitive to Cd in water, because of their permeable skin, their embryos hatch and larval development dependent on water for important life cycle stages (Alford et al. 2001). Several field studies suggested environmental estrogens can cause similar gonadal abnormalities in the wild (Hayes et al. 2002) However, present studies in the laboratory have opposite results. For example, Flament et al. (2003) demonstrated that Cd has no direct effect on sex determination-differentiation of amphibian *Pleurodeles waltl*. Sharma & Patino (2009, 2010) also confirmed that Cd does not affect the population sex ratios and testicular histology at 1, 8, 85 µg/L, and Cd does not have strong estrogenic in *Xenopus tadpoles*. However, the different adaptability of species to Cd should be taken into account, and the effects of Cd should be studied on the gonad differentiation of other amphibians under controlled conditions.

The spot frog (*P. nigromaculata*) has a widespread distribution across most of East Asian countries. In recent years, environment has been polluted widely by Cd in China which influences the habitats of animals including *P. nigroma culata*. The primary objective is to determine the effects of

chronic Cd exposure on gonad differentiation, sex ratio, and the expression and location of metallothioneins (MTs) and estrogen receptor (ER) on gonad cells of *P. nigromaculata*.

MATERIALS AND METHODS

Treatment: $CdCl_2.2.5H_2O$ (molecular weight: 228.35, purity: 99.99%, Sigma-Aldrich Company production, the Cas number: 10108-64-2) was prepared into 1 mg/L Cd stock solution with deionized water. In March, 2011, the three couples of *P. nigromaculata* adults were collected from the suburb of Anqing city in China, and matched to spawn in the laboratory. After fertilization, the fertilized eggs were exposed to different concentrations of Cd (0.1, 1, 10 and 100 µg/L), and control was also set. The fertilized eggs were put into the 20 ×15 cm plastic round basin firstly. After the tadpoles reach Gosner 26 (GS 26) (Gosner 1960), they were transferred to the tank (60 × 40 × 35 cm) which held 4 litres dechlorinated tap water. There were 20 larvae in each tank, and each treatment was repeated three times. The tap water was aerated for 3 days before being used. The atmosphere and water temperature was checked every day. During the treatment, water was renewed with treated water every 48 hours, and the tanks were rotated regularly around the room to get out of the location effects. After water renewal, the tadpoles were prohibited against eating for half past a day. When the larvae come to the forelimb emergence, they were provided with a floating sponge to allow them to leave the medium during metamorphosis, and the number of animals was observed every 12 hours until metamorphosis. During the treatment, the animals fed a diet of boiled lettuce ad libitum every day until metamorphosis. The larvae were daily checked for metamorphose and mortality, and dead tadpoles were discarded. The research was approved by the local government, and the animals were handled in accordance with Animal Care and Use Committee guidelines.

The experiments were conducted in a natural photoperiod with light coming through the laboratory windows (approximately 13h light/11h dark). We used the PC300 waterproof portable meter (Clean, USA) to monitor the water conductance and salinity, and GDYS-201M multi parameter water quality analyser (Little Swan, China) to measure the ammonia and dissolved oxygen of tap water. The experimental water pH was 7.04-7.69, NH_3-N was 0.16-0.25 mg/L, hardness was 252–256 mg $CaCO_3$/L, and dissolved oxygen was 6.8-7.3 mg/L. Quality controls included a certified Cd solution and reagent blanks. We collected the water samples from the aquaria randomly to analyse the concentration of Cd during the experiment in order to verify the concentration as the primary exposure route by an Atomic Absorption Spectrophotometer (Perkin Elmer Pinaacle 900).

Gonadal morphology and histology: Each animal to reach metamorphosis (GS 46) was anaesthetized in 300 µg/L MS-222, given an ID number. They were dissected and distinguished gonad under an Olympus SZX16 stereomicroscope and photoed by the DP72 digital sight camera (Olympus America Ltd., USA), the number of the female and male individual was recorded to calculate the sex ratio. Then all gonads were fixed in 4% paraformaldehyde for 24h, put into alcohol and xylene, and then were embedded in paraffin. The slices (6 µm thick) were stained with haematoxylin and eosin. We used a microscope (Olympus BX51T-PHD-J11) and CMOS to observe and photo the gonad, including abnormalities, such as the presence of oocytes in testes, mixed sex and intersex. The image-plus software (Media Cybernetics, USA) was used to analyse the result of immuno-histochemistry.

Immunohistochemistry: We chose male and intersex young frogs (Gs 46) in each group to examine the distribution and abundance of ER and MTs on the testis using immunohistochemistry assay. The gonads were fixed in 4% paraformaldehyde and the paraffin sections were made, and then mounted onto the slides coated with polylysine. We used the xylene to dewax the sections, descending alcohols to rehydrate. Then the sections were immersed in the endogenous peroxidase (1% H_2O_2 with MeOH and distilled water) to block for 5-10 min in order to destroy inactivated endogenous enzymes at room temperature, then washed 3 times each with distilled water. 0.01 M phosphate-buffered saline (PBS; pH 7.4) were used to incubate the sections until the water boiled (92-95°C) for 2-3 times for 10 min each, 0.1M PBS wash 1-2 time interval. Slides were prepared under the antigen repaired buffer for 5-10 min, heated to 97°C for 15 min, washed by PBS 2-3 times for 10 min each, put into 10% normal goat serum (Boster Inc. China) for 20 min, and then incubated with rabbit polyclonal to ER-a (1: 100 in PBS, Boster Inc.) or mouse monoclonal to MTs (1: 100, ZSGB-BIO Inc., China), 10% biotin and 0.3% Triton X for 24h in a moist room at 4°C. The sections were then washed with 0.1M PBS for three times for five min each, and the primary antibody incubations were performed for 30 min at 20-37°C with a biotinylated secondary antibody (Boster Inc.) in PBS. Then, the sections were again washed with PBS (3 times × 2 min), and horseradish peroxidase conjugate (Boster Inc.) was put into the sections at 37°C about 30 min. After rinsing with PBS (0.1 M, 3 times × 5 min), we used diaminobenzidine (0.25 mg/mL; Sigma) with 0.2% H_2O_2 for the coloration. The colour reaction was stopped by immersion in nano-pure H_2O (3 × 5 min), followed by haematoxylin stain and dehydration in a graded ethanol series. The tissue was cleared with xylene and the slides were covered with coverslips and per mount.

Immunoreactive cell counting: The value of immunohistochemical score (IHS) was obtained via the estimation of the staining intensity (negative expressed in 0; weak in 1; moderate in 2, and strong in 3) and the percentage of immunoreactive cells (0 represents 0%, 1 represents 1-10%, 2 represents 11-50%, 3 represents 51-80%, 4 represents 81-100%) using image-pro plus analysis-based scoring systems (Media Cybernetics image Co., USA). When there are obviously differences in staining intensity between foci and there is a multiple focal immune activity, the average values of the most intense staining and the minimum intense were calculated. IHS intensity was recorded by multiplying the staining intensity score (0-3) and quantity score (0-4). The IHS score is used 0-12 to represent the intensity, 0 indicates negative; 1-4 indicates weak; 5-8 indicates moderate; and 9-12 indicates strong immunoreactivity (Soslow et al. 2000).

Analysis: To determine if Cd alter sex ratio, we used a G-test to examine differences among the treatment. We used the Statistica 6.0 (StatSoft, Tulsa, USA) to analyse data. One-Way variance analysis was used to determine differences between Cd treated group and control group. Values are presented as mean ± standard error (SE), and the statistical significance is a *P*-value less than 0.05 ($P < 0.05$).

RESULTS

Gonadal gross morphology, histology and sex ratios: Testis of the normal male *P. nigromaculata* was shorter, tubular, smooth, lacked pigmentation and had spermatogenic cells, while ovaries in untreated females were longer, elongated, and had melanin pigmentation, a segmented appearance, and oocytes. The percentage of female, male and intersex had a significant difference between the Cd treatments and the control (G-test, $P < 0.05$). Approximately 3.4%, 5% and 3.4% of animals observed in Cd treatment at 0.1, 1 and 10 μg/L, respectively, were classified as intersex (the same gonad has testicular and ovarian-like elements) (Fig. 1). Female/male ratios in *P. nigromaculata* had no significant difference between the two Cd treatments (1.10 ± 0.12 at 10 μg/L; 1.11 ± 0.14 at 100 μg/L) and the control (1.01 ± 0.09) ($P > 0.05$); while a significant difference was observed between the other two Cd treatments (2.17 ± 0.19 at 0.1 μg/L; 1.89 ± 0.11 at 1μg/L) and the control ($P < 0.05$, Fig. 2).

ER and MTs expression in gonad: ER expressed positively in both cytoplasm and nucleus of germ cells in male young frogs. The colour of positive expression is from light yellow to brown, and the positive reaction production showed particle aggregation, while the negative control did not have positive reaction. In the intersex gonads, the ER expression became stronger, distributed mainly in the oocytes (Fig. 3). Although ER expressed in the cells in the Cd treatment, it did not show monotonic trend between the expression and treated concentration. ER expression was more obvious in 1 μg/L treatment ($P < 0.05$) (Fig. 4).

Cd treatments have positive expression in varying degrees of MTs in cytoplasmic and nucleus with granular and brown, while the negative control did not have positive reaction. The expression of MTs in the cells showed inverted U in the treatment. MTs expression was more obvious in 0.1 and 1μg/L treatment ($P < 0.05$), and weak reactivity in 100 μg/L (Fig. 5).

DISCUSSION

The juvenile frog of *P. nigromaculata* completes its gonadal differentiation until complete metamorphosis (GS 46), and the gonads differentiation of amphibian is very sensitive to EDCs during the larval development (Uller & Helanterä 2011). We observed exposure to Cd leading the sex ratio to female-biased sex ratios and the gonad to intersex gonad, which indicates that Cd has a certain hormone effect. The feminization and intersex gonads appearance may relate to steroidogenesis inhibition (Walsh et al. 2000). Some studies have shown that Cd can increase the transcription level of the progesterone receptor (PR) gene, but decrease the level of ER mRNA (Garcia-Morales et al. 1994, Stoica et al. 2000) by the high affinity to the receptor and blocking estradiol binding in vivo (Stoica et al. 2000, Martin et al. 2003). It suggests that the interactions between Cd and receptors of steroid hormones may impair gonadogenesis differentiation and development. To our knowledge, the malformations have been reported in some amphibian larvae exposed to the exogenous estrogens or xenoestrogens following exposure to exogenous estrogens or xenoestrogens (Hayes et al. 2002). However, some studies have observed that population sex ratios and testicular histology are not affected by Cd (1, 8 and 85 mg/L Cd) in *Xenopus* tadpoles (Sharma & Patiño 2009, 2010). We presume the different influence of EDCs on sex ratios, and testicular histology is related to the species, treated concentration and exposed periods. For example, Hogan et al. (2008) exposed northern leopard frog tadpoles (*Rana pipiens*) to synthetic estrogen in five distinct periods, and observed that the tadpoles show a sex ratio biased to female, but the sex ratios and intersex gonads appearance are different during five distinct exposed periods. Mackenzie et al. (2003) indicated that gonadal differentiation and development is easy to be altered when exposed to EDCs in amphibians, but leopard frogs (*R. pipiens*) are more susceptible than wood frogs (*R. sylvatic*a) to development of sex reversal and intersex gonads. Considering the sensitivity of the amphibians, many studies continue to believe that EDCs can lead amphibians gonadal abnormalities and feminization (Reeder et al. 2005, McCoy et al. 2008).

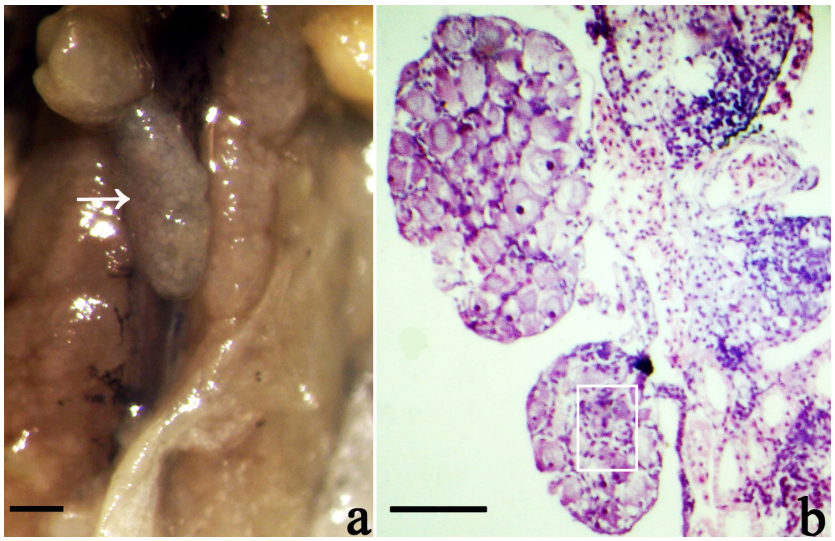

Fig. 1: Intersex gonads in young frog *P. nigromaculata* treated with Cd.a: Anatomy of intersex gonad; →: gonad, bar = 5 mm; b: Microstructure, bar = 50 μm

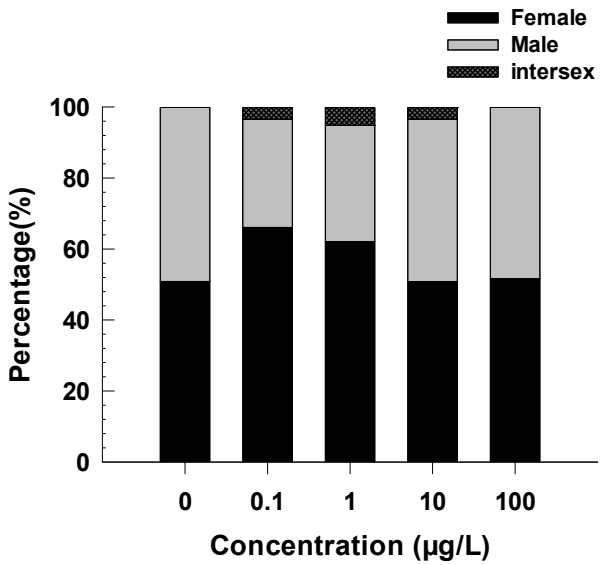

Fig. 2: Sex ratio in young frog *P. nigromaculata* treated with Cd.

Interestingly, exposure to low concentration Cd (0.1, 1 and 10 μg/L) results in female-biased sex ratios, abnormal intersex gonads, while high concentration Cd (100 μg/L) does not. The monotonous change in sex ratios of *P. nigromaculata* is in accord with the monotonic change of ER and MTs. A certain dose of Cd can decrease the level of ER protein and ER mRNA and suppress ER gene transcription (Le Guevel et al. 2000), so the hormone effects of Cd can be effectively assessed by detecting the changes of ER in gonadal cells (Lutz & Kloas 1999). In this experiment, the expression of ER did not exhibit monotonic trend, though ER express can be observed at all gonadal cells in Cd treatment (Fig. 3). At the same time, the expression of MTs is also non-monotonic. MTs have cysteine-rich proteins and low molecular weight, which have strong capability to bind metal ions (Savva & Li 1999, Loumbourdis et al. 2007). MTs play an important role in copper and zinc homeostasis, detoxification (Klaassen et al. 2009). The detoxification of MTs on amphibians has been reported, such as *Duttaphrynus melanostictus* (Shuhaimi-Othman et al. 2012), *Rana ridibunda* (Loumbourdis et al. 2007), *Pleurodeles waltl* (Mounaji et al. 2002), *Rana catesbeiana* (Cooper & Fortin 2010) and two neotenic salamanders (*Proteus anguinus* and *Necturus maculosus*) (Dobrovoljc et al. 2003). The experimental results indicated that the MTs expression in the cell of *P. nigromaculata* did not linearly increase monotonously with the Cd concentration increasing.

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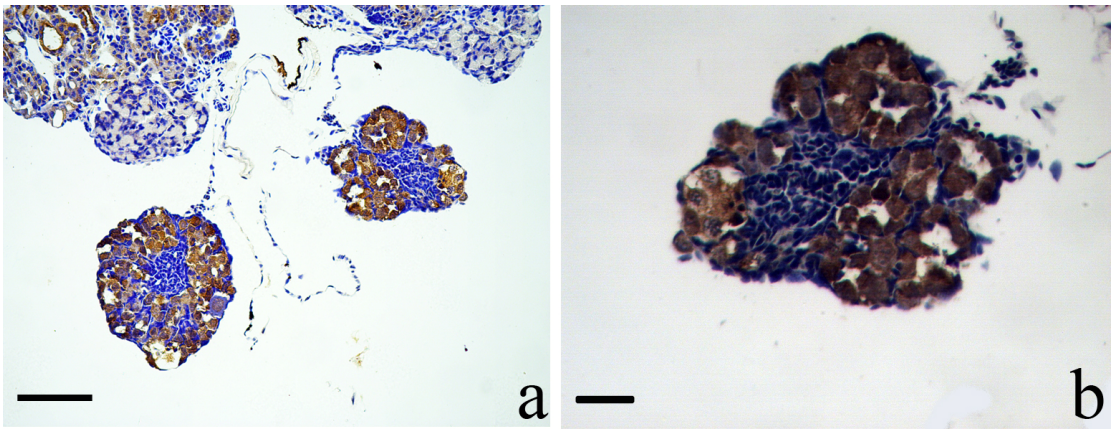

Fig. 3: The ER immunohistochemistry expression of intersex gonada: Microstructure of intersex gonad, bar = 25 μm; b: intersex gonad, bar = 50 μm

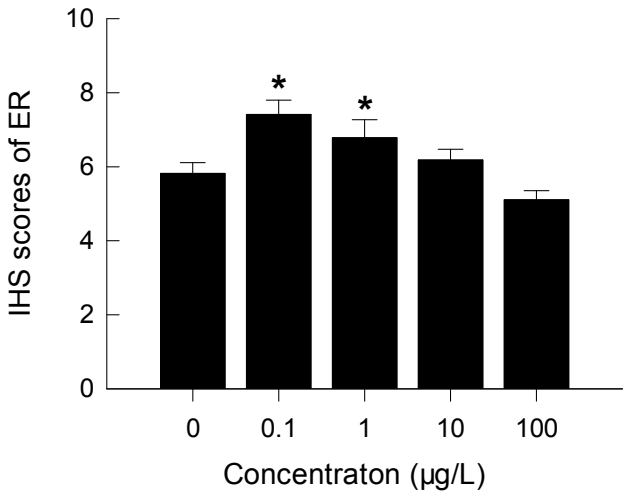

Fig. 4: Relative value of ER in the cells of young frog *P. nigromaculata* (Mean ± *SE*) *Indicates that there was a significant difference between Cd treated group and control group (P<0.05).

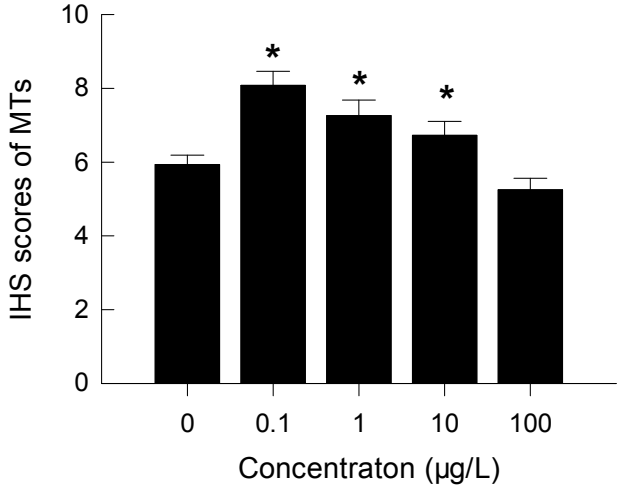

Fig. 5: Relative value of MTs in the cells of young frog *P. nigromaculata* (Mean ± *SE*) *Indicates that there was a significant difference between Cd treated group and control group (P<0.05).

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