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Histological observation on unique phenotypes of malformation induced in *Xenopus tropicalis* larvae by tributyltin

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Abstract

Tributyltin (TBT), a biocide used in antifouling paints, has shown strong teratogenic effects on *Xenopus tropicalis* embryos at environmentally relevant concentrations. *X. tropicalis* embryos were exposed to 50, 100 and 200 ng/L tributyltin chloride for 72 hr. The histological changes were further observed on abnormal eyes, enlarged trunks, enlarged proctodaeums and absence of fins induced by TBT. The lens and the retinal layers of abnormal eyes were slightly or barely differentiated, and that the pigment epithelium was neither continuous nor smooth. The abdomens were full of undifferentiated gut tissue with yolk-rich inclusions in the tadpoles with enlarged trunks. The proctodaeums formed a bump-like or columnar structure. The mass of yolk-rich cells occupied the lumen, blocked the opening and even turned inside out of the proctodaeum. Both the ventral and dorsal fins in trunks and tails became narrow or even disappeared totally. Our results suggest that great changes of histology took place corresponding to the unique phenotypes. The gut tissue was poorly differentiated, which led to the failed elongation of the guts and subsequently the enlarged trunks. The enlarged proctodaeums were due to the undifferentiation of inner layer, the expansion of outer epidermal part and the absence of fins around them. In brief, the histological observations provided insights into the reason of the unique external malformations in some degree.

Key words: *Xenopus tropicalis*; tributyltin; teratogenicity; histology; proctodaeum; fin
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Introduction

It is a trend to use embryo toxicity test as an animal alternative method in hazard and risk assessment of chemicals and scientific research in toxicology (Embry et al., 2010). Besides the fish embryo toxicity test, the frog embryo teratogenesis assay-*Xenopus* (FETAX) is a useful method to test the developmental toxicity of chemicals (ASTM, 1998; Hoke and Ankley, 2005). The main endpoints include mortality, malformation, and growth inhibition in FETAX. The specific phenotypes of malformations, however, receive little attention (Baba et al., 2009).

Tributyltin (TBT) has been widely used as a biocide in antifouling paints since the 1960s and been found in freshwater worldwide (Champ, 2000). For example, the concentrations of TBT were up to 33.4 and 68.9 ng Sn/L in two sites of Three Gorges Reservoir (Gao et al., 2006), 37.6 ng Sn/L in Yunnan Dianchi Lake, and 425.3 ng Sn/L in Huangpu River of China (Jiang et al., 2001). TBT is considered to be the most toxic substance ever introduced into the marine environment (Alzieu, 1996). It is also known as an endocrine disruptor which promotes adverse effects from snails to mammals (Shimasaki et al., 2003; Shi et al., 2005; Grün et al., 2006; Decherf et al., 2010).

In our previous study, we found that unique malformations are induced in *Xenopus tropicalis* embryos by TBT at environmentally relevant concentrations after 24, 36 and 48 hr of exposure (Guo et al., 2010). The most obvious alterations were abnormal eyes, enlarged proctodaeums, narrow fins and skin hypopigmention. The reasons leading to the morphological malformations, however, are not well known.

Based on the findings of morphological malformations above, we exposed embryos of *X. tropicalis* to 50, 100 and 200 ng/L tributyltin chloride (TBTCl) for 72 hr, and the histological changes were observed on the main phenotypes of malformations. Our aim was to determine the histological abnormalities induced by TBT and to reveal the internal relationship between histological changes and the morphological malformations.

1 Materials and methods

1.1 Chemicals

TBTCl (purity 97%), 3-amino-benzoic acid ethyl ester (MS-222), and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (USA). All other chemicals

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used in this study were analytical grade.

1.2 Exposure experiments

The husbandry of *X. tropicalis* adults and breeding were performed as described previously (Guo et al., 2010). The exposure experiments were referred to the FETAX with some modifications as described (ASTM, 1998; Guo et al., 2010). Fifty embryos with jelly coats at stage 10 were put into each acid-washed glass Petri dish (12 cm diameter) with FETAX medium, DMSO solution or TBTCl solution (Nieuwkop and Faber, 1994). The dishes were incubated at (26 ± 0.5)°C for 24 hr in dark to avoid the photodecomposition of TBTCl. The dead embryos were removed, and the media were renewed at 24 hr intervals. After 72 hr of exposure, the surviving tadpoles were anaesthetized with 100 mg/L MS-222 immediately. The tadpoles were then fixed with Bouin's solution for 24 hr, washed with tap water, and preserved with 70% ethanol.

1.3 Observations and measurements of tadpoles

Tadpoles were observed with an Olympus SZX16 dissecting microscope (Japan), and images were taken with an Olympus DP25 camera (Japan). The whole body length, snout-vent length, trunk width and tail fin width were measured in 15 tadpoles (if available) of each replicate using computer-assisted image analysis (iSee V3.873). The trunk width and the tail fin width were measured in the middle of the trunks and the tails, respectively.

1.4 Histology

To determine the internal changes of the malformed organs, 25–30 tadpoles were randomly selected from each group for series histological sectioning. The tadpoles were wax embedded and transversely sectioned (6 μm) through the whole body. The slides were then stained with Hematoxylin-Eosin (HE) and observed with a Nikon 801 microscope (Japan).

1.5 Statistical analysis

Statistical analysis was conducted with the aid of SPSS 13.0 software. Each dish of 50 embryos was considered a replicate, and there were 4 replicates per group (n = 4). Dish-to-dish variation was handled using one-way ANOVA, followed by Dunnett's test.

2 Results

No significant differences were observed between FETAX medium and DMSO controls, and the statistical significance was analyzed between the treatment and FETAX medium control groups. Only 2.5% tadpoles showed loss of pigment of eyes with small eyes or bent tails in the control groups (Table 1). In TBT treatment groups, however, all the surviving tadpoles showed notable malformations. The most characteristic phenotypes were abnormal eyes, enlarged trunks, enlarged proctodaeums and absence of fins (Table 1).

2.1 Abnormal eyes

The normal eyes were round and black (Fig. 1a). The subphenotypes of abnormal eyes included the exerted lens, loss of pigment, and variations in size (Fig. 1d, g, j). Histological observations showed that the lens, the retinal layers, and the pigment epithelium were well developed in normal eyes (Fig. 1b, c). In the abnormal eyes, the lens and the retinal layers were slightly or barely differentiated (Fig. 1e, f, h, i), and the pigment epithelium was neither continuous nor smooth. The degree of histological changes was concentration-dependent.

Table 1 Effects of tributyltin (TBT) on survival, growth and morphology in *Xenopus tropicalis* tadpole

Index	Concentrations of tributyltin chloride			
	0 ng/L	50 ng/L	100 ng/L	200 ng/L
Survival (%)	97.0±2.2	65.5±5.7***	30.5±5.7***	17.0±2.2***
Whole body length (mm)	5.36±0.16	4.74±0.24**	3.67±0.03***	3.28±0.05***
Snout-vent length (mm)	1.98±0.07	1.82±0.09	1.42±0.07***	1.74±0.04**
Trunk width (mm)	0.86±0.01	0.85±0.01	0.70±0.03***	0.90±0.05
Trunk width/snout-vent length	0.43±0.02	0.47±0.02	0.49±0.04	0.58±0.02***
Tail length (mm)	3.38±0.18	2.92±0.17	2.25±0.05***	1.74±0.04***
Tail length/snout-vent length	1.71±0.14	1.61±0.07	1.59±0.11	1.00±0.04***
Tail fin width (mm)	0.39±0.03	0.22±0.02***	0.09±0.01***	0.03±0.01***
Tail fin width/tail length	0.11±0.01	0.08±0.01***	0.04±0.01***	0.02±0.01***
Teratogenic phenotypes (%)				
Big eye	0.0±0.0	8.0±2.5***	20.0±3.7***	42.0±3.7***
Small eye	1.5±0.9	11.0±2.2***	21.5±4.8***	27.5±4.3***
Exerted lens	0.0±0.0	0.0±0.0	6.0±2.4***	17.5±4.3***
Loss of pigment of eye	2.5±0.9	27.0±5.9***	34.0±3.7***	41.5±4.3***
Enlarged trunk	0.0±0.0	11.0±2.2***	100±0.0***	100±0.0***
Enlarged proctodaeum	0.0±0.0	100±0.0***	100±0.0***	100±0.0***
Narrow fin	0.0±0.0	100±0.0***	76.5±5.2***	18.0±3.7***
Absence of fin	0.0±0.0	0.0±0.0	23.5±5.2***	82.0±3.7***
Bent notochord	0.0±0.0	11.0±2.2***	18.5±3.8***	29.5±4.3***
Bent tail	1.5±0.9	15.5±3.0***	41.5±4.3***	58.5±4.3***
Skin hypopigmentation	0.0±0.0	11.5±2.2***	19.5±3.0***	29.0±3.6***
Total malformation	2.5±0.9	100±0.0***	100±0.0***	100±0.0***

Each value represents the mean ± SD of four replicates.
*** One-way ANOVA was used with $p < 0.001$; ** $p < 0.05$ compared to the FETAX medium control group.

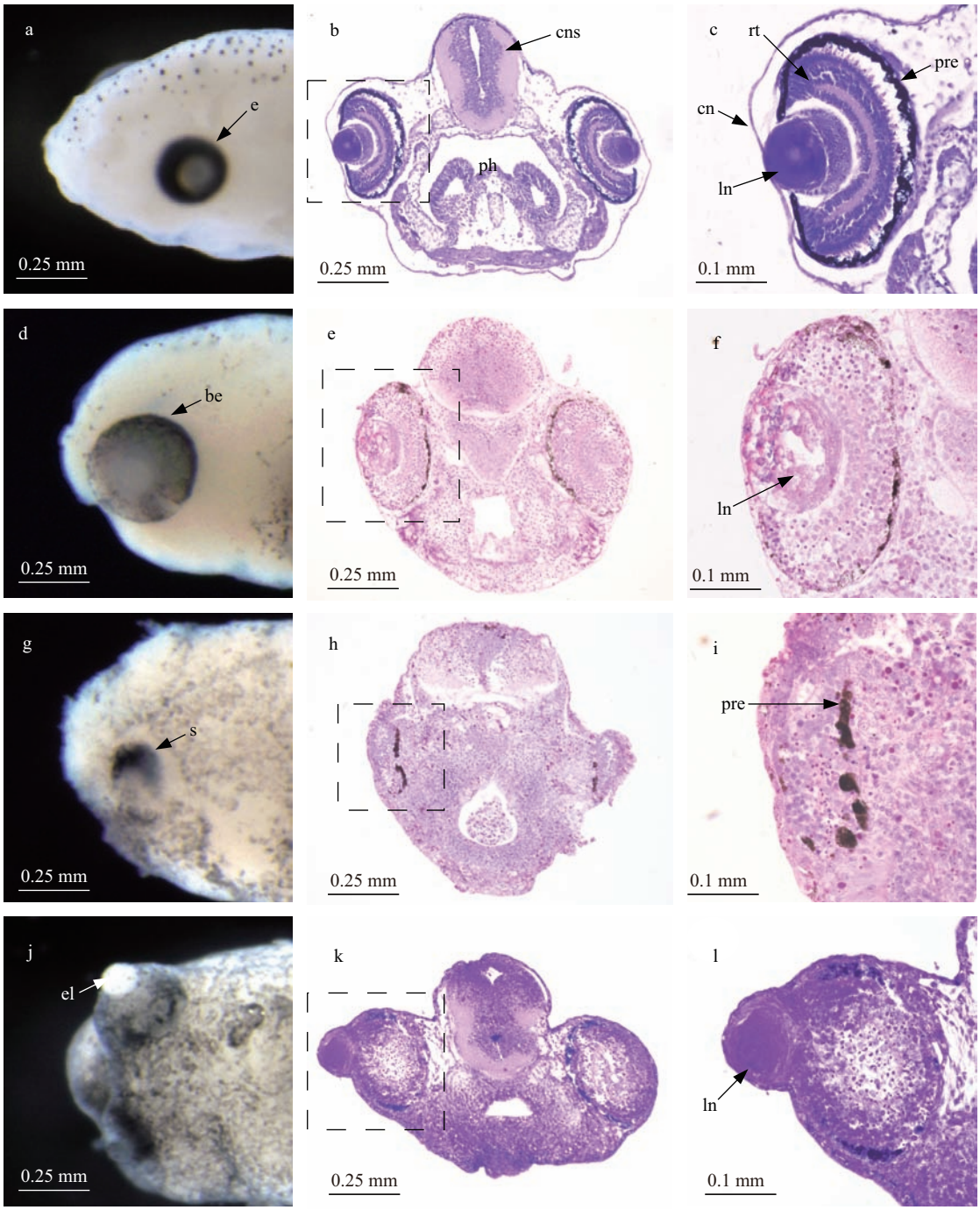

Fig. 1 Phenotypes and histological abnormalities (stained with HE) of eyes induced in *Xenopus tropicalis* tadpoles by tributyltin after 72 hr of exposure. The nominal concentrations of tributyltin chloride were 0 (a–c) and 200 ng/L (d–l). The boxed areas of b, e, h and k were magnified in c, f, i and l, respectively. be: big eye; cn: cornea; cns: central nervous system; e: eye; el: exerted lens; ln: lens; rt: retina; ph: pharynx; pre: pigmented retinal epithelium; se: small eyes.

2.2 Enlarged trunks

The snout-vent length (SVL) decreased in 100 and 200 ng/L TBTCl treatment groups, and the trunk width (TRW) only decreased in 100 ng/L TBTCl treatment group (Table 1). The proportion of TRW and SVL (TRW/SVL), however, increased in 200 ng/L TBTCl treatment group (Table 1). In the abdominal cavity of control tadpoles, the digestive tracts were complex and well curved (Fig. 2a). The gut tissue was greatly differentiated, and the structure of stomach, intestine, pancreas and liver could be clearly distinguished (Fig. 2b). In the posterior trunk, the epithelial cells of the distal large intestine formed a columnar epithelium (Fig. 2c).

In TBT treatment groups, the abdomens of the tadpoles were not transparent, and no definitive structure of guts was observed (Fig. 2d, g). The abdomens were full of undifferentiated gut tissue with yolk-rich inclusions. The intestines failed to elongate or to coil, only forming a simple tubular structure (Fig. 2e, f). In the tadpoles with severe malformations, the intestines were still yolk-rich

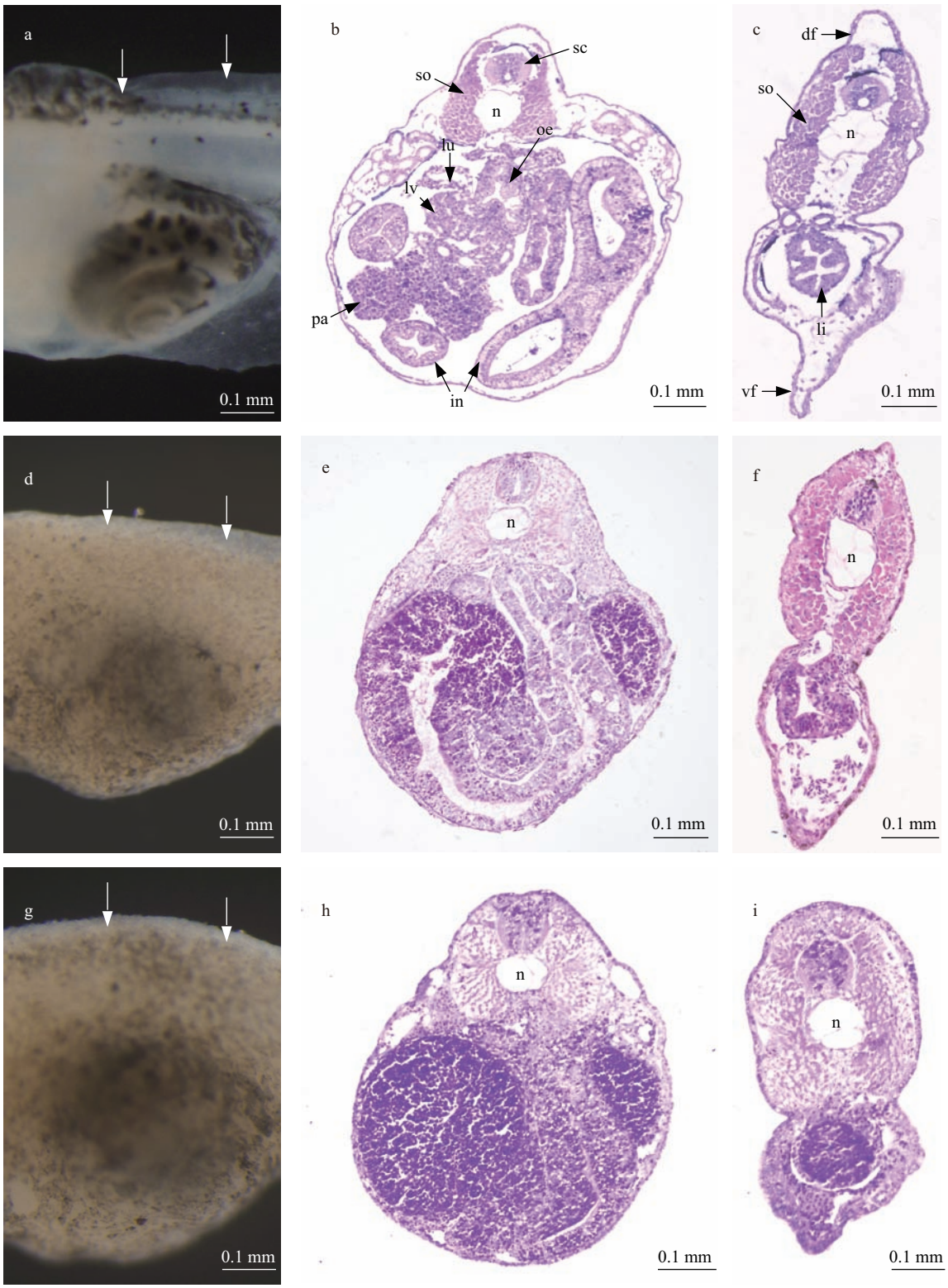

Fig. 2 Histological abnormalities (stained with HE) of trunks induced in *Xenopus tropicalis* tadpoles by tributyltin after 72 hr of exposure. The nominal concentrations of tributyltin chloride were 0 (a–c) and 200 ng/L (d–i). The transverse sections were chosen from the middle (b, e, h) and posterior trunks (c, f, i) as indicated with the dotted arrows in the corresponding morphological graphs (a, d, g). df: dorsal fin; li: large intestine; lu: lung; lv: liver; in: intestine; n: notochord; oe: oesophagus; pa: pancreas; sc: spinal cord; so: somite; vf: ventral fin.

mass (Fig. 2h, i).

2.3 Enlarged proctodaeums

In the control embryos, the proctodaeums consisted of a simple epithelium of small and rounded cells. The epithelium was surrounded by loose packed mesenchyme (Fig. 3a, b). In TBT treatment groups, the proctodaeums externally expanded, forming a bump-like or columnar structure (Fig. 3c, e, g, i, k). The proctodaeums consisted of multiple layers of yolk-rich cells. The epidermal part of the proctodaeum region extended, arched and formed a lumen inside. Generally, the epidermis developed along and was

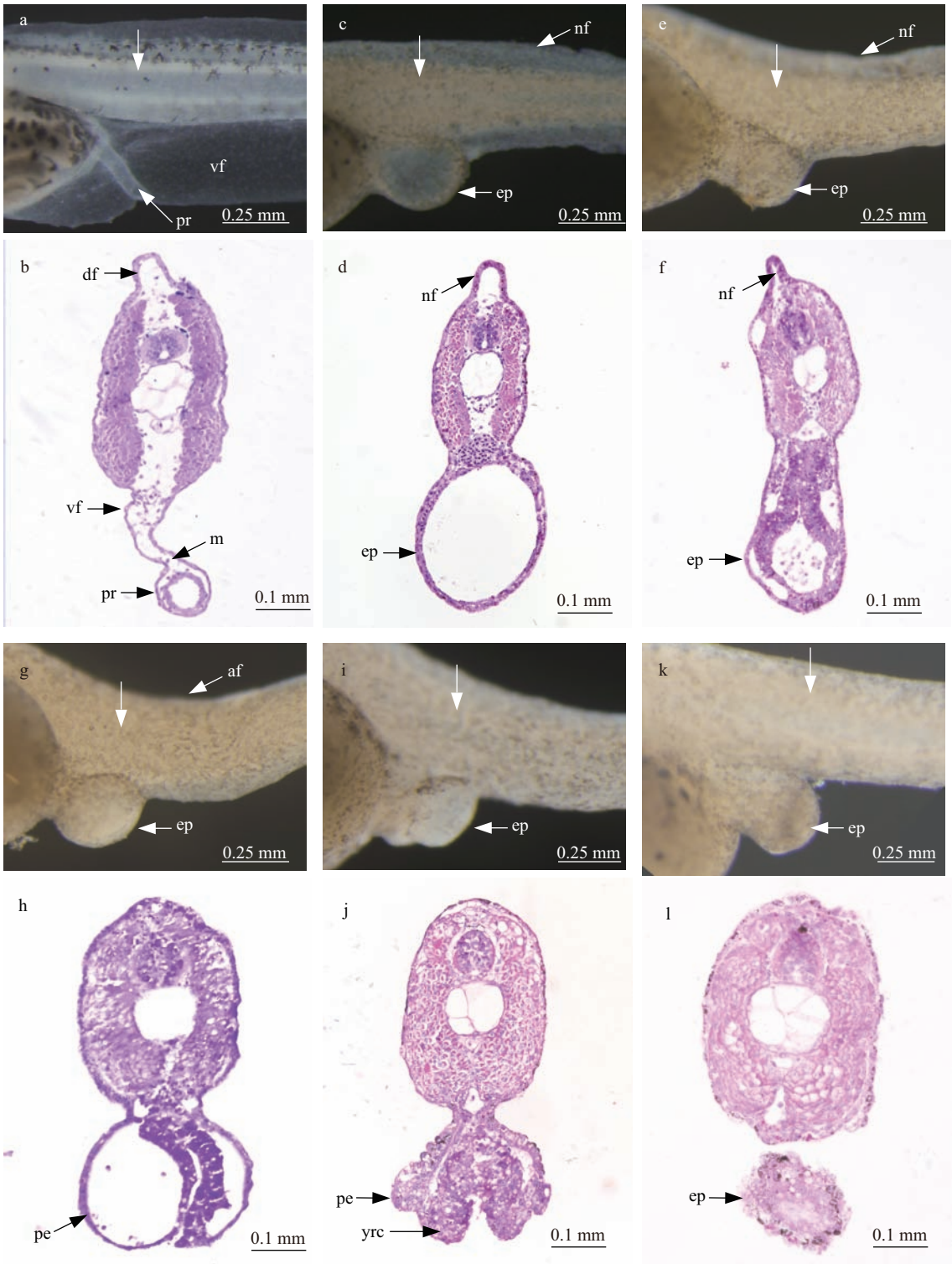

Fig. 3 Phenotypes and histological abnormalities (stained with HE) of proctodaeums induced in *Xenopus tropicalis* tadpoles by tributyltin after 72 hr of exposure. The nominal concentrations of tributyltin chloride were 0 (a, b), 100 (c, d) and 200 ng/L (e–l). The dotted arrows in morphological photographs indicated the positions of transverse sections in the corresponding histological ones. af: absence of fin; df: dorsal fin; ep: enlarged proctodaeum; m: mesenchyme; nf: narrow fin; pe: proctodaeum epidermis; pr: proctodaeum; vf: ventral fin; yrc: yolk-rich cells.

morphologically continuous with the wall of the ventral tail rather than that of the posterior abdomen (Fig. 3c–j). The epidermis of the distal proctodaeum seldom separated from the wall of the ventral tail totally (Fig. 3k, l). The mass of yolk-rich cells was usually full of the lumen, blocked the opening of the proctodaeum or even turned inside out (Fig. 3f, h, j, l).

2.4 Absence of fins

Both the dorsal and ventral fins became narrow and even disappeared totally in the trunks and tails of the TBT treatment tadpoles. The tail length (TAL) decreased in 100 and 200 ng/L TBTCl treatment groups, but the proportion of TAL and SVL (TAL/SVL) only decreased in 200 ng/L TBTCl treatment group (Table 1). Both the tail fin

width (TFW) and TFW/TAL decreased in a concentration-dependent manner (Table 1).

Histological observations showed that the fins consisted of flattened epidermal cells elevate into a keel-like structure by a supporting core of mesenchyme in the tails of control tadpoles (Fig. 4b, c). The somatic muscles that bundled on the sides of the notochords were compact and closely contacted each other. In the treatment groups, the histological sections of tails got thicker, and those of the fins got narrower (Fig. 4e, f, h, i). In the tails without fins, the epidermal cells were not smooth, the spinal cords were under differentiated, and the muscular bundles were sparse and loosely arranged (Fig. 4k, l).

3 Discussion

The main phenotypes observed in previous study are further confirmed in this article (Guo et al., 2010). Some phenotypes such as enlarged proctodaeums and absence of fins were more pronounced due to the prolonged exposure.

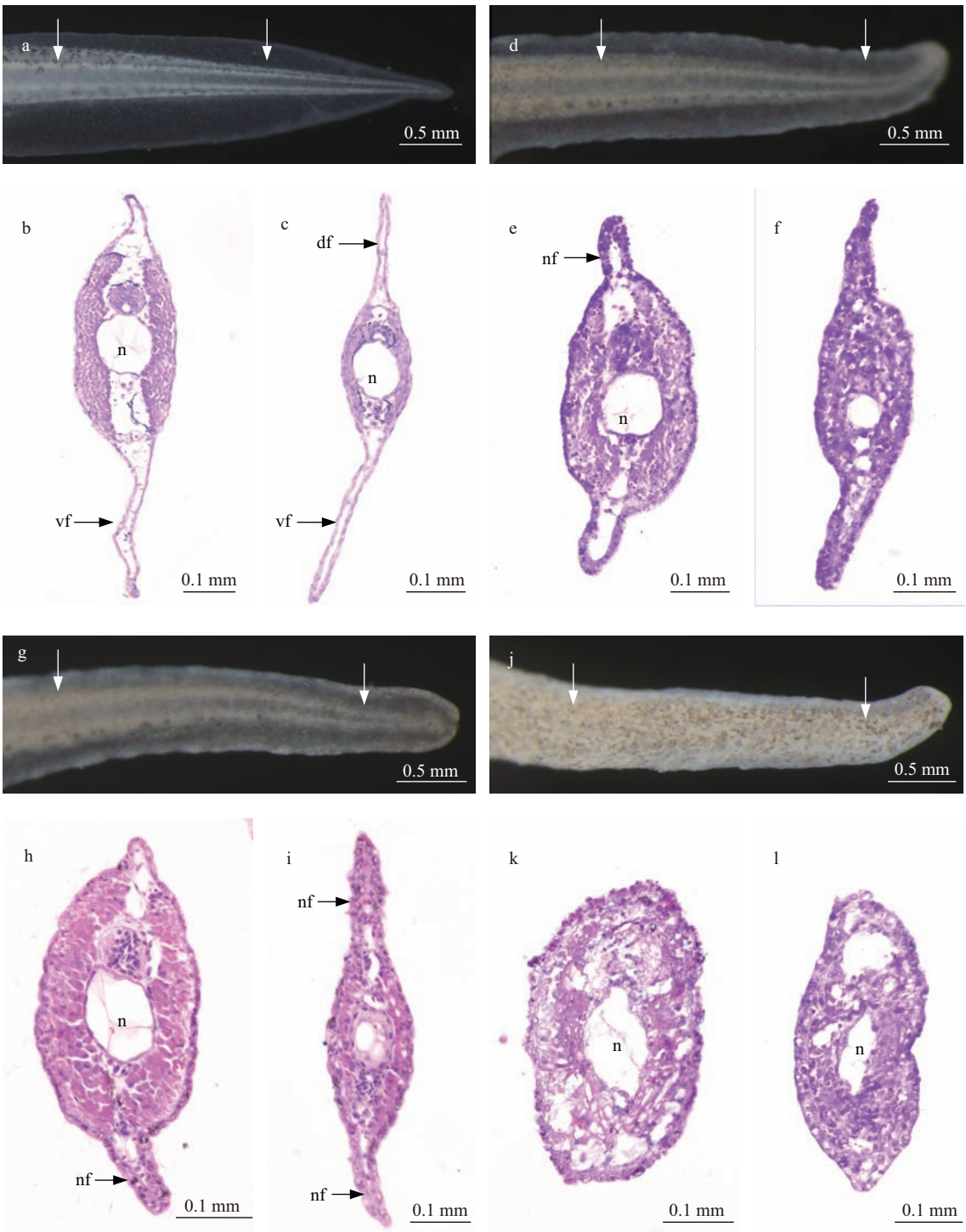

Fig. 4 Histological abnormalities (stained with HE) of fins induced in *Xenopus tropicalis* tadpoles by tributyltin after 72 hr of exposure. The nominal concentrations of tributyltin chloride were 0 (a–c), 100 (d–f), and 200 ng/L (g–l). The transverse sections were chosen from the anterior and posterior tails as indicated with the dotted arrows in the morphological graphs. df: dorsal fin; n: notochord; nf: narrow fin; vf: ventral fin.

TBT induced-malformations nearly included every part of the embryos from the eyes to the fins. The phenotype in each part has its own specific feature. For example, TBT made the proctodaeums extremely large but made the fins totally absent. The phenotypes induced by TBT are much different from those induced by other chemicals (Sone et al., 2004; Bacchetta et al., 2008; Lenkowski et al., 2008). The high sensitivity and specificity of *Xenopus* tadpoles to TBT indicates that TBT-induced malformations might be related to special teratogenic mechanisms. The specific phenotypes might be served as endpoints which facilitate mechanistic study beyond acute toxicity testing in FETAX (ASTM, 1998). Therefore, we should pay more attention to the unique phenotypes induced by TBT.

3.1 Abnormal eyes

In our previous study, we found the loss of pigment in treatment groups after 24, 36 and 48 hr of exposure (Guo et al., 2010). In this study, however, slight loss of pigment was observed. Histological observations showed that the loss of pigment was primarily due to the reduction of the pigment epithelium cells. Slight loss of pigment indicates that the pigment epithelium might develop at later stages though the tadpoles were still exposed to TBT, but the changes of size and location of eyes were not due to the delayed development.

TBT and another organotin compound, triphentyltin (TPT), can lead to eye malformation in fish embryos (Hano et al., 2007; Hu et al., 2009). The food web magnification usually results high concentrations of TPT in fish (Hu et al., 2006). Ocular abnormal development has been found in the Chinese sturgeon (*Acipenser sinensis*) larvae from the field, and TPT is proved to be the causal agent to induce the abnormal eyes (Hu et al., 2009). Due to the high binding affinity with the retinoid X receptor (RXR), TPT could modulate this receptor in a way that could lead to the observed deformities in fish (Hu et al., 2009). The ocular malformations in amphibian larvae by TBT may be also via similar mechanism, and further studies are necessary.

3.2 Enlarged trunks

The increase trend of TRW/SVL in the treatment groups suggests that the trunks failed to elongate. During embryogenesis, the gut is transformed from a straight tube to a complex coiled structure and elongates approximately 3.5 times (Chalmers and Slack, 2000). In this study, the enlarged trunks were well accompanied by the failure of elongation and coiling of guts. This indicated that the enlarged trunks were partly due to the delayed development of guts.

The morphological differentiation of the guts is closely correlated with the consumption of yolk platelets (Jorgensen et al., 2009). All the embryonic cells inherit yolk platelets from the egg cytoplasm and consumed them intracellularly during embryogenesis. The yolk-rich mass in TBT treatment tadpoles suggest that the gut tissue was poorly differentiated, which led to the failed elongation of the guts and subsequently the enlarged trunks. Therefore, the histological observation provides a good explanation at the cellular and organic levels for the external changes.

3.3 Enlarged proctodaeums

The proctodaeum follows the distal large intestine. In the control tadpoles, there were significant differences between the large intestine and proctodaeum. The change from the columnar epithelium of the large intestine to the epithelium of the proctodaeum occurs roughly at the position that the gut passes through the body wall (Chalmers and Slack, 1998, 2000). In TBT treatment groups, both the large intestine and proctodaeum were rich in yolk granules, sharing the similar cell types. Therefore, the thick proctodaeums still came from the original undifferentiated gut tissues.

The externally enlarged proctodaeum was one of the most notable characteristics induced by TBT. Our results suggest that at least three reasons led to this syndrome. First, the proctodaeum failed to enlongate and made itself thicker; second, the expansion of epidermal part made the outer ring of the proctodaeum large; finally, the narrow fins or absence of fins made the proctodaeum relatively protruding.

3.4 Absence of fins

Our results suggest that TBT disrupted all the fins in the tadpoles. In the tail, the relatively steady value of TAL/SVL and sharp decrease of TFW/TAL indicate that TBT showed asynchronous effects on the development of tails and fins; that is, TBT specifically inhibited the development of fins. In terms of cell composition, the dorsal fin mesenchyme core arises from neural crest cells, while the mesenchyme of the ventral fin has a dual origin. The ventral fin contains neural crest cells that migrate in from the dorsal side of the embryo, but a contribution is also made by cells from the ventral mesoderm (Tucker and Slack, 2004). In this study, both the dorsal and ventral fins were disturbed, which indicates that TBT might affect the neural crest and the ventral mesoderm at the same time.

TBT can induce dorsal curvature and severely twisted tails in *Sebastiscus marmoratus* embryos, and apoptosis is a possible mechanism leading to twisted tails abnormality (Zhang et al., 2011). Apoptosis also plays an important role in the tail resorption during amphibian metamorphosis. The narrow fins or absence of fins in *X. tropicalis* larvae might be due to the apoptosis induced by TBT. The fin defects were always companied by enlarged protodaeums in this study. The development of proctodaeums and ventral fins are closely related to bone morphologenetic protein (BMP) signaling (Reversade et al., 2005). Therefore, BMP signaling might be involved in TBT-induced fin and proctodaeum abnormality. Further work is great in need to identify the role of RXR, apoptosis and BMP signaling in TBT-mediated teratogenicity as well as their interactions.

4 Conclusions

We found great changes of histology corresponding to the unique phenotypes induced in *X. tropicalis* larve by TBT. The gut tissue was poorly differentiated, which led

to the failed elongation of the guts and subsequently the enlarged trunks. The enlarged proctodaeums were due to the undifferentiation of inner layer, the expansion of outer epidermal part and the absence of fins around them. In brief, the histological observations provided insights into the cause of the unique external malformations in some degree. In future, we will choose some specific molecular markers for the RXR, apoptosis and BMP signal pathway to reveal the possible mechanisms of TBT mediated-teratogenicity.

Acknowledgments

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