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Unexpected phenotypes of malformations induced in *Xenopus tropicalis* embryos by combined exposure to triphenyltin and *9-cis*-retinoic acid

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ABSTRACT

Xenopus tropicalis embryos were exposed for 48 hr to the mixtures of 5 μg Sn/L triphenyltin (TPT), which is a well-known endocrine disruptor, and 0.25–5 μg/L *9-cis* retinoic acid (*9c*-RA), which is the natural ligand of retinoid X receptor. The phenotypes induced by combined exposure were more variable than those resulting from single exposure to either TPT or *9c*-RA. The prominent phenotypes included underdeveloped head structures, abnormal eyes, narrow fins, enlarged proctodaeum, etc. Especially, combined exposure induced unexpected notochord malformations, which ranged from small swellings of the surface of the tails to the extension and extrusion of notochord out of the posterior tails. Compared with the 5 μg Sn/L TPT-treated group, the index of fin deficiency was not affected, and the index of axis deficiency was significantly increased with increasing RA concentrations in the mixtures. Our results suggest that combined exposure to TPT and *9c*-RA induced not only more variable phenotypes of malformations than exposure to single compound but also some new and unexpected phenotypes.

Introduction

Global declining amphibian populations have drawn special attention since the early 1990s (Stuart et al., 2004). Increased use of pesticides and other toxic chemicals is considered to be one of the underlying reasons for the decline of amphibian populations (Melvin and Trudeau, 2012). It has been proposed that hind-limb malformation of frogs may be attributed to xenobiotic disruption of retinoid signaling pathways (Degitz et al., 2000). A new finding suggests that cyanobacteria blooms can produce teratogenic retinoic acids (RAs) (e.g. *9c*-RA, *13c*-RA, etc), which are also supposed to be the factor leading to deformed amphibians in eutrophic environments (Wu et al., 2012).

Retinoic acids (RAs) play important roles in the development of vertebrate embryos. They regulate RA signal through the heterodimers named retinoic acid receptor and retinoid X receptor (RXR). *9c*-RA is regarded as the natural ligand of RXR and also used as a drug (alitretinoin) in patients with Kaposi's sarcoma (Cheng et al., 2008). However, excessive RAs will result in teratogenicity in vertebrate embryos (Vieux-Rochas et al., 2010). For example, *9c*-RA has been documented to induce featured malformations including reduced brain, abnormal eyes, bent notochords, and bent tails in embryos of *Xenopus laevis* and *X. tropicalis* (Kraft et al., 1994; Yu et al., 2011).

A lot of chemicals have been proved to disturb retinoid homeostasis at the level of RXR (Li et al., 2008). Triphenyltin (TPT) is a widely used biocide and shows high binding affinity to retinoid X receptor (RXR) (Nishikawa et al., 2004). TPT induces multiple malformations including enlarged proctodaeum and narrow fins in *Xenopus*

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tropicalis embryos (Yu et al., 2011). However, these malformations are much different from those induced by *9c*-RA (Yu et al., 2011). The developmental stages of *Xenopus* that are most sensitive to TPT are different from those sensitive to *9c*-RA, either (Yuan et al., 2011). It seems that TPT and RAs have different modes of action to induce teratogenicity in amphibian embryos.

In the environment, organisms are usually exposed not just to a single pollutant but rather to a mixture of these chemicals. Hence, ecotoxicological evaluations of a single contaminant may not reflect the multitude of antagonistic or synergistic stimuli that wildlife species surely faces (Relyea, 2009). Contamination of coastal waters by TPT and its products of transformation has become a worldwide problem (Yi et al., 2012), and retinoic acids have been detected for the first time in the aquatic environment (Wu et al., 2010).

Anorectal malformations (ARMs) are congenital anomalies involving anus, rectum, urinary and genital tract which can affect both males and females. Rat is often used for the study of ARMs by exposure to all-*trans*-retinoic acid or ethylenthiourea (Qi et al., 2002). The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) is a high-throughput toxicological test used to assess ecological and human health hazards (Buryskova et al., 2006; Yoon et al., 2008). *Xenopus tropicalis* is an emerging test model in developmental toxicology (Berg et al., 2009; Song et al., 2003). In this study, *X. tropicalis* embryos were exposed to the mixtures of *9c*-RA and TPT. Our aim was to determine the combined teratogenicity of two chemically different agonists of RXR in vertebrate embryos.

1 Materials and methods

1.1 Chemicals

TPTCl (purity > 95%; CAS#, 639-58-7), *9c*-RA (purity ⩾ 97%; CAS#, 5300-03-8), 3-amino-benzoic acid ethyl ester (MS-222), and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

1.2 Exposure experiments

The husbandry of *X. tropicalis* adults and breeding were performed as described previously (Yu et al., 2011). The exposure experiments were conducted following the FETAX protocol with some modifications (ASTM, 1998). In brief, twenty embryos with jelly coats at stage 10 were put into each acid-washed glass Petri dish (10 cm diameter) with FETAX medium, DMSO solution and test chemical solution. The nominal concentration of DMSO was 0.05% in the solvent control and test substance solutions. The embryos were exposed to 5 μg Sn/L TPT, 0.25–5.0 μg/L *9c*-RA and their mixtures. We chose 5 μg Sn/L TPT because TPT induces low mortality and obvious teratogenicity at this concentration based on our previous study (Yu et al., 2011). Eight replicate dishes were used for each group. The dishes were incubated at (26 ± 0.5)°C with 24 hr dark to avoid the photodecomposition. At 24 hr intervals, the dead embryos were removed and the media were renewed.

1.3 Observations and measurements of embryos

After 48 hr exposure, the surviving embryos were collected and anaesthetized with 100 mg/L MS-222. The embryos from four replicates were fixed with 4% formalin for 24 hr, washed with tap water, and preserved with 70% ethanol. The embryos were observed with an Olympus SZX16 dissecting microscope (Olympus Corporation, Tokyo, Japan), and images were taken with an Olympus DP 25 camera. The whole body length was measured in five tadpoles of each replicate using computer-assisted image analysis (iSee V3.873). The main phenotypes of malformation were distinguished in all surviving embryos, and the incidence of each phenotype was evaluated. The index of axis deficiency (IAD) was evaluated following the method of Kao and Elinson (1988) with some modifications; the index of fin deficiency (IFD) was also evaluated following our classifications (**Table 1**).

Table 1 Classification methods to score the degree of malformations based on the specific phenotypes induced in *Xenopus tropicalis* embryos by teratogens

Score	Index of axis deficiency (IAD)	Index of fin deficiency (IFD)
0	Normal forehead, eyes, and cement gland	Normal fin, proctodaeum and skin pigmentation
1	Reduced forehead, eyes smaller than normal and sometimes joined	Slight narrow fins, enlarged proctodaeum, and skin hypopigmentation
2	Eyes fused or cyclopic, but at least some eye pigment visible, cement gland present	Moderate narrow fins, enlarged and transparent proctodaeum, and skin hypopigmentation
3	No visible eye pigment, otic vesicles or single vesicle still visible	Severe narrow fins, enlarged and transparent proctodaeum, and skin hypopigmentation
4	No otic vesicle(s) present, somites present in trunk or portion thereof	Absence of fin, enlarged and translucent proctodaeum, reduced forehead
5	No somites present, trace of tail mesenchyme occasionally seen	No fins, enlarged and opaque proctodaeum, loss of eye pigmentation, enlarged trunks; bent tails

1.4 Scanning electron microscopy (SEM)

Embryos from another four replicates were fixed with 2.5% glutaraldehyde. The embryos were transferred to 0.1 mol/L phosphate buffer (pH 7.2–7.4), rinsed several times in the same buffer, and incubated for 1 hr in 1% osmium tetraoxide (dissolved in 0.1 mol/L phosphate buffer). The embryos were dehydrated in a graded series of 30%–100% ethanol, critical-point dried using CO_2, and sputter-coated with platinum. Digital micrographs were obtained with a JSM-5610LV scanning electron microscope (JEOL, Tokyo, Japan).

1.5 Statistical analysis

Data were analyzed using SPSS 16.0 software. Each dish of 20 embryos was considered one replicate, and four replicate dishes were evaluated per treatment group (n = 4). Dish to dish variation was handled using one-way ANOVA, followed by Dunnett's test.

2 Results

2.1 Effects on survival and growth

No significant differences were observed in the percent of survival, the whole-body length or teratogenic effects between FETAX medium and DMSO controls. The DMSO control group was used for the analysis of statistical significance of treatment groups. Compared to the 5 μg Sn/L TPT-treated group, the percentage of survival decreased by 50.8% in embryos following exposure to the mixtures of TPT + 2.5 μg/L *9c*-RA and by 92.6% in embryos to TPT + 5.0 μg/L *9c*-RA (**Fig. 1a**). Compared to the *9c*-RA-treated groups, the whole body length decreased by 9.8%–24.1% in embryos following exposure to the mixtures TPT + *9c*-RA (0.25–2.5 μg/L) (**Fig. 1b**).

2.2 Phenotypes of malformations

The normal embryos had well-developed proctodaeums and fins (**Fig. 2a**). Five μg Sn/L TPT induced typical malformations including enlarged proctodaeums and narrow fins (**Fig. 2b; Table 2**). *9c*-RA induced malformations along anterior-posterior axis including reduced brains, abnormal eyes and bent tails (**Fig. 2c–g**). In embryos following exposure to mixtures of TPT and *9c*-RA, the RA-induced characteristics of malformations were gradually enhanced with increasing RA concentrations (**Fig. 2h–l**). The phenotypes induced by combined exposure were more variable than those resulting from single exposure to either TPT or *9c*-RA (**Table 2**).

In addition to the multiple phenotypes above, combined exposure to TPT and *9c*-RA led to unexpected notochord malformations in the posterior end of the notochords. Scanning electron microscopy allows the documentation of 3-dimensional phenotypes of notochord malformations with excellent detail. The lateral surface of tails was flat in control groups (**Fig. 3a**). In the slightly malformed embryos, small swellings were observed on the surface of posterior tails (**Fig. 3b**), and the swellings expanded and extended backward along the notochord axes (**Fig. 3c, d**). In the moderately malformed embryos, the extruded parts out of the tail skin were more noticeable and can be regarded as notochord. The extruded notochord extended continuously and even went beyond the edge of the tail fin (**Fig. 3e, f**). In the severely malformed embryos, the extruded notochord bent toward different directions, and the tail looked like a fork (**Fig. 3g–i**).

2.3 Combined effects on degree of malformations

Single exposure to 5 μg Sn/L TPT induced a high value of IFD (**Fig. 4a**), and single exposure to *9c*-RA induced an increase in IAD with the increasing concentrations of *9c*-RA (**Fig. 4b**). When embryos were exposed to the mixture of 5 μg Sn/L TPT and *9c*-RA, IFD showed no significant

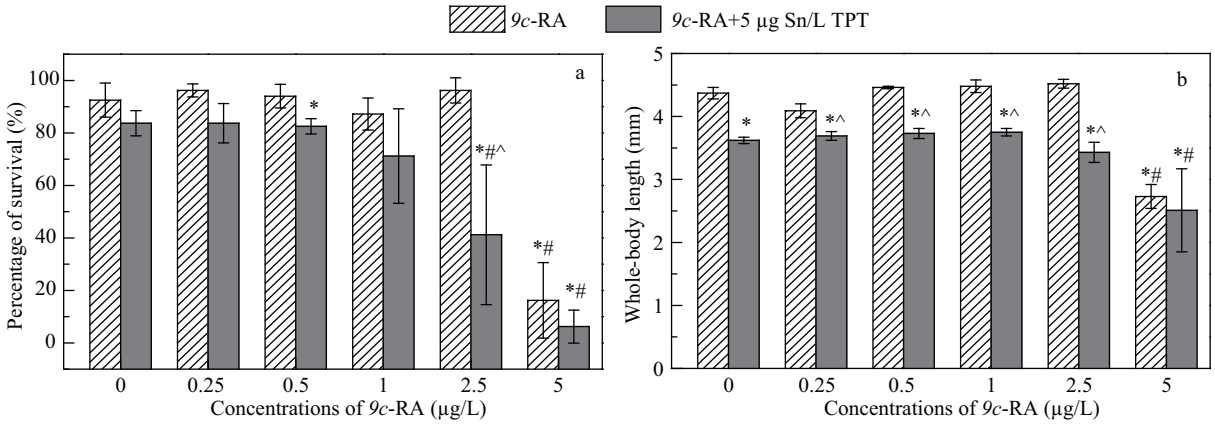

Fig. 1 Changes of the percentage of survival (a) and the body length (b) in *Xenopus tropicalis* embryos following exposure to triphenyltin (TPT) and *9c*-retinoic acid (*9c*-RA). Each vertical bar represents a mean of four replicates, and error bar represents standard deviation. One-way analysis of variance (ANOVA) was used with $^{*}p < 0.05$ compared to the control groups, with $^{\#}p < 0.05$ compared to the 5 μg Sn/L TPT treatment group and with $^{\wedge}p < 0.5$ compared to *9c*-RA treatment groups.

Table 2 Phenotypes of malformations induced in *Xenopus tropicalis* embryos by combined exposure to triphenyltin (TPT) and *9-cis*-retinoic acid

9c-RA (μg/L)	TPT (5 μg Sn/L)	Reduced Brain	Abnormal eye	Narrow fin	Enlarged proctodaeum	Extruded yolk	Wide anus	Bent tail	Extruded notochord	Total malformation
0	–	2.9±5.6	2.8±5.6	0	0	0	2.6±3.1	2.6±3.6	0	4.03±5.3
	+	64.3±10.1^{*a}	12.0±0.7*	100*	83.5±5.8*	0	9.0±3.6	13.2±8.5	0	100±0*
0.25	–	2.6±3.0	1.3±2.6	1.3±2.6	0	0	1.3±2.6	0	0	5.2±4.3
	+	95.8±5.3$^{*\#\wedge}$	22.7±10.0$^{*\wedge}$	100$^{*\wedge}$	94.2±4.5$^{*\wedge}$	0	19.6±3.9$^{*\wedge}$	14.9±7.5$^{*\wedge}$	0	100$^{*\wedge}$
0.5	–	9.2±2.8	6.6±3.0	1.4±2.7	0	0	6.4±4.9	0	0	9.2±2.8
	+	92.4±2.8$^{*\#\wedge}$	19.7±2.6$^{*\#\wedge}$	100$^{*\wedge}$	89.4±5.6$^{*\wedge}$	0	19.5±7.1*	22.5±9.6$^{*\wedge}$	0	100$^{*\wedge}$
1	–	22.2±13.8	16.2±12.1	1.5±2.9	0	0	11.8±6.8	1.6±3.1	0	22.2±13.8
	+	96.8±3.7$^{*\#\wedge}$	26.9±14.2*	100$^{*\wedge}$	73.2±19.9$^{*\wedge}$	1.5±2.9	44.0±18.1$^{*\#\wedge}$	36.5±23.1	5.9±4.6	100$^{*\wedge}$
2.5	–	84.0±11.6*	36.1±14.9*	3.9±2.6	0	0	17.8±11.6	13.0±2.8	0	88.0±10.8
	+	100$^{*\wedge}$	76.0±16.8$^{*\#}$	100$^{*\wedge}$	23.7±20.6	31.2±21.6	56.0±38.6	47.7±16.0$^{*\wedge}$	28.3±15.5$^{*\#\wedge}$	100*
5	–	100$^{*\#}$	100$^{**\#}$	0	4.8±8.2	15.9±16.7	25.4±22.5	88.9±19.2$^{*\#}$	0	100*
	+	100$^{*\#}$	100$^{*\#}$	100$^{*\wedge}$	0	33.3±57.7	11.1±19.2	88.9±19.2$^{*\#}$	50±50	100*

–: TPT was not used; +: TPT was added.

[a] Each value represents the mean ± SD of four replicates. One-way analysis of variance (ANOVA) was used with $^{*}p < 0.05$ compared to the control groups, with $^{\#}p < 0.05$ compared to the 5 μg Sn/L TPT treatment group and with $^{\wedge}p < 0.05$ compared to *9c*-RA treatment groups.

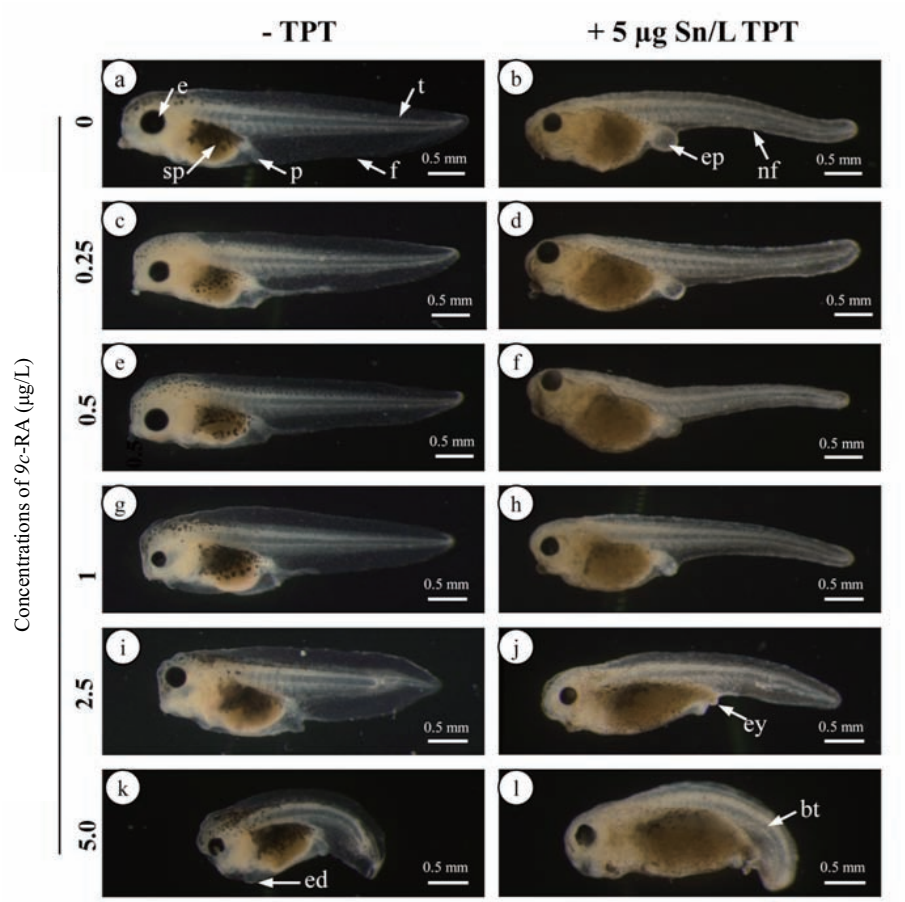

Fig. 2 Combined teratogenic effects of triphenyltin (TPT) and *9c*-retinoic acid (*9c*-RA) on *Xenopus tropicalis* embryos after 48 hr exposure. The embryos were normal in the control (a) and showed multiple malformations in TPT (b), *9c*-RA (c–g) and their mixture (h–l) treatment groups. ae: abnormal eye; bt: bent tail; e: eye; ed: edema; ep: enlarged proctodaeum; ey: extruded yolk; f: fin; hp: hypopigmentation; nf: narrow fin; p: proctodaeum; sp: skin pigmentation; t: tail.

changes with the increase of RA compared to 5 μg Sn/L TPT (**Fig. 4a**), and IAD increased by 40.3% (TPT + 1 μg/L *9c*-RA) and by 6.6% (TPT + 5 μg/L 9c-RA) compared to *9c*-RA (**Fig. 4b**).

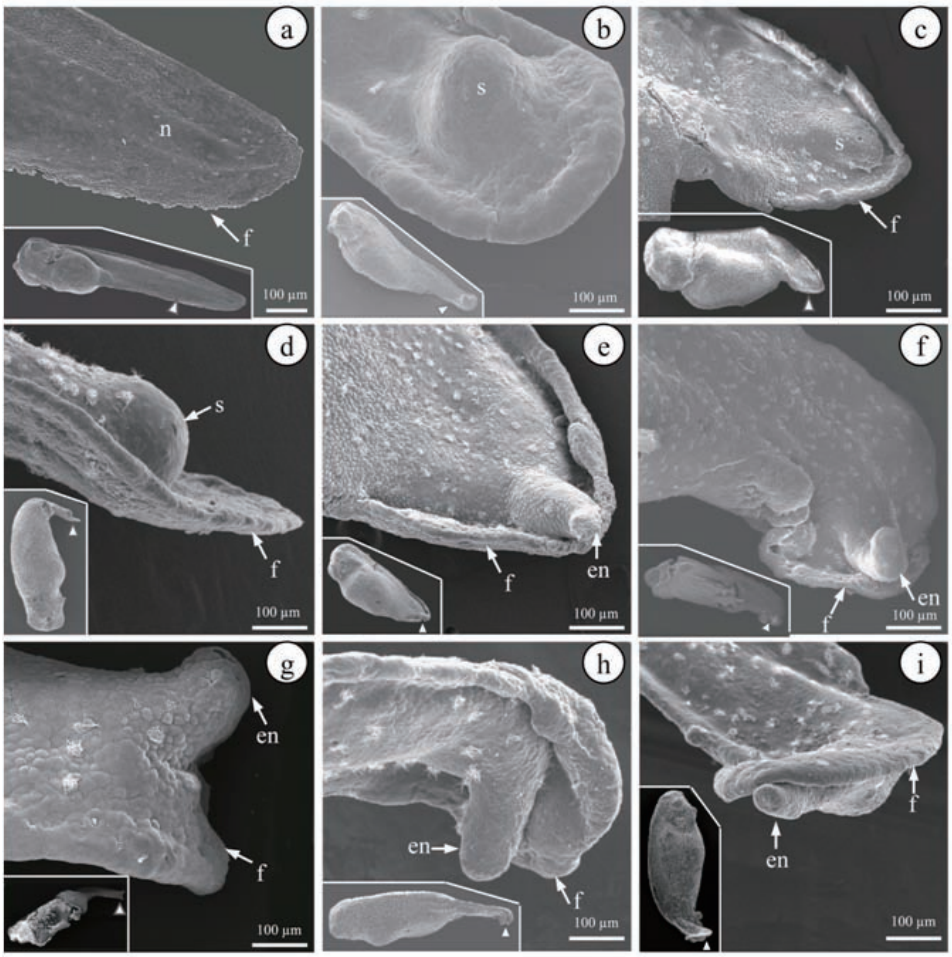

Fig. 3 Scanning electron microscopic photographs of tail notochord malformations induced in *Xenopus tropicalis* embryos by the mixtures of triphenyltin (TPT) and *9c*-retinoic acids (*9c*-RA). The embryos were normal in the control (a) and showed multiple phenotypes in combined treatment groups (b–i). The arrowheads in whole embryos at bottom left of each box indicated the areas of magnification. en: extruded notochord; f: fin; n: notochord; s: swelling.

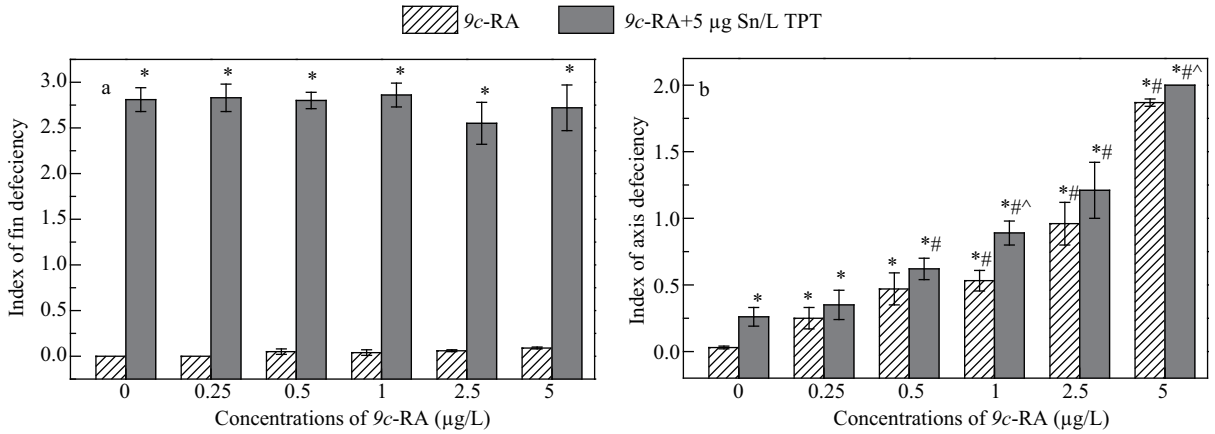

Fig. 4 Changes of the index of axis deficiency (IAD) and the index of fin deficiency (IFD) in *Xenopus tropicalis* embryos following exposure to triphenyltin (TPT) and *9c*-retinoic acids (*9c*-RA). All the surviving embryos (**Table 2**) from each of four replicates were used for the analysis of malformation degree. The values of IAD and IFD were calculated to evaluate the degree of specific phenotypes of malformation based on the classification method in **Table 1**. One-way analysis of variance (ANOVA) was used with * $p < 0.05$ compared to the control groups, with $^{\#}p < 0.05$ compared to the 5 μg Sn/L TPT treatment group and with $^{\wedge}p < 0.05$ compared to *9c*-RA treatment groups.

3 Discussion

Specific phenotypes of malformations are usually induced in animal models by some known teratogens. These phenotypes are very useful in the study of congenital anomalies of human being (Kluth, 2010). We also found unexpected notochord malformations at the posterior end of notochord in *X. tropicalis* embryos. A search of the literature has revealed a few examples of notochord malformations following early life stage exposure to various toxicants. In most cases, however, the main malformations were wavy notochords.

Haendel et al. (2004) documented one special phenotype of notochord malformation. They found the notochord cells of zebrafish (*Danio rerio*) embryos extend beyond the normal boundaries in small region(s) of the posterior trunk after the embryos were exposed to dithiocarbamate. This phenotype has some similarities to the slight notochord malformation found in this study. Nevertheless, no further extension of notochord is observed in zebrafish exposed to contaminants. In addition, the swelling and extension of notochord only occurred in the posterior end of notochord in this study, but the occurrence of swelling was not limited to such a specific position in zebrafish. Fish and amphibian sometimes show species differences in the response to the same chemical. For example, though TPT induces eye defects and morphological malformation in fish, neither narrow fin nor enlarged proctodaeum is found (Zhang et al., 2008; Hu et al., 2009). Therefore, the unique and noticeable notochord malformations in this study have not been documented in previous studies.

The results of this study confirmed previous reports about the sensitivity of index of fin deficiency and index of axis deficiency (Yu et al., 2011). In this study, IFD can be used to indicate the effects of TPT, and IAD can be used to indicate the effects of LG69. Our results suggested *9c*-RA did not show any effect on the TPT-induced teratogenicity in *Xenopus* embryos. The IAD in embryos following exposure to the mixture of TPT and *9c*-RA was equal to or even less than the addition of IAD induced by single compound. Therefore, TPT did not show any effect on the *9c*-RA-induced teratogenicity, either. These results confirmed our hypothesis that TPT and *9c*-RA have different modes of action to induce teratogenicity.

Nevertheless, new and unexpected phenotypes in embryos following combined exposure indicated that new mode of action might occur during the interaction of TPT and *9c*-RA in *Xenopus* embryos. The findings further demonstrate the importance of addressing the issue of chemical mixtures of pollutants acting through dissimilar mode of action (Sária et al., 2011). We have found that TPT and tributyltin are strong teratogens in amphibian embryos (Guo et al., 2010; Yu et al., 2011). Besides organotins, inorganic tin compounds (e.g. $SnCl_2$) has been proved to induce teratogenicity in aquatic organisms (Sisman, 2011). RAs, which have been found in aquatic environments (Wu et al., 2012), represent another classification of teragoens (Kraft et al., 1994). It is possible for them to coexist in the real environments. Therefore, more work is required to reveal the interaction of pollutants with different modes of actions in future.

4 Conclusions

In brief, we found that combined exposure to TPT and RA induced multiple malformations in *X. tropicalis* embryos. The phenotypes in combined exposure group were more variable than those in single compound exposure. Especially, some unexpected phenotypes (e.g. extruded notochord) occurred in combined exposure groups.

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