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Optimization of the T3-induced *Xenopus* metamorphosis assay for detecting thyroid hormone signaling disruption of chemicals

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ABSTRACT

T3-induced *Xenopus* metamorphosis is an ideal model for detecting thyroid hormone (TH) signaling disruption of chemicals. To optimize the T3-induced *Xenopus* assay and improve its sensitivity and reproducibility, we intend to develop quantitatively morphological endpoints and choose appropriate concentrations and exposure durations for T3 induction. *Xenopus laevis* at stage 52 were exposed to series of concentrations of T3 (0.31–2.5 nmol/L) for 6 days. By comparing morphological changes induced by T3, we propose head area, mouth width, unilateral brain width/brain length, and hindlimb length/snout-vent length as quantitative parameters for characterizing T3-induced morphological changes, with body weight as a parameter for indicating integrated changes. By analyzing time-response curves, we found that following 4-day exposure, T3-induced grossly morphological changes displayed linear concentration–response curves, with moderate morphological changes resulting from 1.25 nmol/L T3 exposure. When using grossly morphological endpoints to detect TH signaling disruption, we propose 4 days as exposure duration of T3, with concentrations close to 1.25 nmol/L as induction concentrations. However, it is appropriate to examine morphological and molecular changes of the intestine on day 2 due to their early response to T3. The quantitative endpoints and T3 induction concentrations and durations we determined would improve the sensitivity and the reproducibility of the T3-induced *Xenopus* metamorphosis assay.

Introduction

Thyroid hormones (THs, T3 and T4) are essential in vertebrate development, and are also required for the normal functions of almost all tissues (Rovet, 2014; McAninch and Bianco, 2014). TH actions are mainly mediated by TH receptors (TRs, TRα, and TRβ) as transcription factors, which stimulate or inhibit transcription of target genes by binding to TH response

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elements (TREs) (Grimaldi et al., 2013). Enough evidence shows that the abnormality of TH levels or TH signaling could result in various developmental disorders, in particular neural defects (Auso et al., 2004). Some environmental chemicals could disrupt TH signaling by interacting with TR due to their similar chemical structures with TH (Darras, 2008). For example, bisphenol A (BPA), polychlorinated biphenyls (PCBs), and tetrabromobisphenol A (TBBPA) have been reported to have potential to disrupt TH signaling as TR agonists or antagonists (Zoeller, 2007; Zhang et al., 2014).

To screen chemicals with TH signaling disrupting activity, a series of *in vitro* assays have been developed, including competitive binding to TR assay, TR-mediated reporter gene transcription assay using mammal cells or yeast, coactivator/corepressor recruitment assay, *etc.* (Freitas et al., 2011; Shiraishi et al., 2003; Terasaki et al., 2011; Ren et al., 2015). Despite *in vitro* assays are rapid and low cost, they cannot completely evaluate TH signaling disruption of chemicals *in vivo* and consequential effects on vertebrate development. It is necessary to use *in vivo* assays to study TH signaling disruption of chemicals and developmental effects.

TR-mediated TH actions are conserved across all vertebrate species during development. Most amphibian species undergo TH-dependent metamorphosis with dramatic morphological and molecular changes. In particular, premetamorphic tadpoles can be induced to metamorphose by adding TH to the rearing water. Moreover, free-living tadpoles are favorable over mammalian models because the interferences from maternal TH and the difficulty in manipulating uterus-enclosed embryo can be avoided. Thus, amphibian metamorphosis serves as an ideal model to study TH signaling and TH-dependent development invertebrates by analyzing morphological and molecular changes induced by TH (Morvan et al., 2008; Tata, 2006). TH actions and molecular mechanisms during development have been revealed from studies based on this model system (Grimaldi et al., 2013; Ishizuya-Oka, 2011). Correspondingly, amphibian metamorphosis offers a unique opportunity to evaluate the possible effects of chemicals on TH signaling during development (Heimeier et al., 2009; Heimeier and Shi, 2010). In particular, T3-induced amphibian metamorphosis has been used to determine whether a chemical can agonize or antagonize TH signaling. In brief, pre-metamorphic tadpoles are treated with test chemicals in the presence or absence of T3 for several days. At the end of treatment, if a chemical inhibits T3-inducedmetamorphosis at morphological and transcriptional levels, this chemical will be proposed to be a TH signaling antagonist. In contrast, if a chemical promotes T3-induced metamorphosis, this chemical will be proposed as a TH signaling agonist (Heimeier et al., 2009; Zhang et al., 2015).

Xenopus laevis is the most used species for the T3-induced amphibian metamorphosis assay, while other species are also used, such as *Rana rugosa* (Kitamura et al., 2005), *Pseudacris regilla* (Veldhoen et al., 2006), *etc.* Some chemicals, including acetochlor, BPA, and TBBPA, have been demonstrated to have a potential to disrupt TH signaling pathway using the T3-induced *X. laevis* metamorphosis assay (Crump et al., 2002; Kudo et al., 2005; Heimeier et al., 2009; Zhang et al., 2014). Generally, however, morphological endpoints used are descriptive but not quantitative in these studies. Thus, our study aimed to develop quantitatively morphological endpoints to improve the sensitivity of this assay. Moreover, the T3 concentrations used in previous studies varied from 1to 100 nmol/L, whereas exposure duration ranged from 2 to 7 days. T3 induction concentration and duration decide the sensitivity of the T3-induced metamorphosis assay to detect TH signaling disruption of chemicals. Excessively both dramatic and weak metamorphosis induced by T3 at inappropriate concentrations and exposure durations are unfavorable to detect TH signaling disruption of chemicals. Thus, another aim of our study is to choose appropriate T3 induction concentrations and exposure durations that result in moderate metamorphosis, which ensures high sensitivity of this assay for detecting TH signaling disruption.

1. Materials and methods

1.1. Chemicals

T3 from Geel belgium (New Jersey, USA) was dissolved in distilled water with NaOH to prepare a stock solution of 7.31 mmol/L. 3-Aminobenzoic acid ethyl ester (MS-222) was from Sigma-Aldrich (St. Louis, MO, USA). Human chorionic gonadotropin (HCG, Yantai North Pharmaceutical Co. Ltd., Shandong, China) was dissolved in 0.6% NaCl. All the stock solutions were subpackaged and stored at −20°C. PCR primers were synthesized by Sangon Biotech (Beijing, China). Picric acid, formaldehyde, acetic acid, chloroform and ethanol were taken from Beijing Chemical Reagent Co., Ltd. (Pure analysis, Beijing, China). G-Red (Nucleic acid dye) was purchased from Bio Teke (Beijing, China).

1.2. Animals

X. laevis frogs (Nasco, Fort Atkins, WI, USA) and tadpoles are routinely maintained in glass tanks containing charcoal-filtered tap water. The water quality was as follows: chlorine concentration < 5 μg/L, pH 6.5–7.0, the dissolved oxygen concentration > 5 mg/L, and water hardness ($CaCO_3$) approximately 150 mg/L. Housing and breeding conditions were reported in our previous studies (Lou et al., 2013). Adult female and male *X. laevis* were raised separately in glass tanks with a 12 hr light/12 hr dark cycle and fed with chopped pork liver three times a week. Water temperature was maintained at (22 ± 1)°C. Adult frogs were injected by HCG (400 IU for male, female 600 IU) to induce breeding. Fertilized eggs were incubated in the dechlorinated tap water at (22 ± 1)°C. On the fifth day post-fertilization, tadpoles were fed with live Artemia.

1.3. Exposure of *X. laevis* to T3

In the T3-induced *Xenopus* metamorphosis assay, tadpoles at stages 52–54 (Nieuwkoop and Faber, 1994) were used in previous studies (Crump et al., 2002; Kudo et al., 2005; Heimeier et al., 2009; Zhang et al., 2014). In the present study, we chose stage 52 *X. laevis* because the responsiveness of tadpoles to THs has been demonstrated to decrease with development in previous studies (Opitz et al., 2006; Zhang et al., 2015). Tadpoles were exposed to series of concentrations of T3 for 6 days. In a pre-experiment, we found that 5 nmol/L T3 resulted in death of some tadpoles following 3 day-exposure. Thus, we chose T3

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