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Benchmark dose analysis of multiple thyroid toxicity endpoints in ovariectomized rats exposed to propylthiouracil

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ABSTRACT

Benchmark dose (BMD) analysis is generally recognized superior to generate a point of departure (PoD) to conduct risk assessment on environmental toxicants, comparing with the traditionally employed no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) methods. However, only a few studies compared the two on producing PoD of thyroid toxicity caused by environmental chemicals. Here, we presented BMD analyses on several thyroid toxicity endpoints caused by a model chemical – propylthiouracil (PTU). Adult female rats underwent ovariectomy were randomly assigned into groups receiving different doses of PTU (0, 0.1, 0.5, 1.0, and 5.0 mg/kg bw) through gavage for 8 days. Results show that PTU induces significant dose-dependent changes of serum total thyroxine (tT_4), total triiodothyronine (tT_3), thyroid stimulating hormones, liver type I 5′-deiodinonase (5′-DI) and malic enzyme (ME) activity with profound histopathological exacerbation. BMD and BMDL results (0.03 and 0.01 mg/kg bw respectively) from Hill model of liver 5′-DI activity were accepted based on selection criteria in the benchmark dose analysis. In summary, BMD analysis results in much lower PoD (0.01 mg/kg bw) than LOAEL (0.1 mg/kg bw) in PTU induced thyroid toxicity.

1. Introduction

Thyroid is an endocrine organ that is essential in both normal development and metabolic regulation of living organisms (Mullur et al., 2014). Thyroid disorder and diseases have become a pandemic that induce tremendous social and economic burden. Particularly, the increase of thyroid cancer incidence was much higher than any other malignancy in the last two decades (Vigneri et al., 2015). Etiological studies indicated that many environmental toxicants have been associated as causes of thyroid diseases (Zoeller, 2010; Liu et al., 2017). Thus, risk assessment of environmental toxicant induced thyroid toxicity would better guide future preventative strategies in environmental health.

In risk assessment, a point of departure (PoD) is of great importance as it is the dose level to infer acceptable risk to a certain hazard. PoD marks the level where an estimated lowest observed adverse effect level or no observed adverse effect level, usually known as LOAEL or NOAEL, was determined in a dose-response curve (EPA, 1993). However, the LOAEL and NOAEL methods fail to adequately reflect all information in a dose-response relationship and sample size to extract accurate PoD; they usually fall in the gap between 0 and the actual dose of causing significant effects (Crump, 1995). An alternative method, benchmark dose (BMD) analysis, provids insights to overcome these limitations by utilizing advanced statistical tools to generate PoD (EFSA, 2009). BMD is defined as a dose or an exposure that can cause a prescribed effect in response, known as benchmark dose response (BMR) (Crump, 1995; EFSA, 2009). The statistical lower limit of BMD (BMDL) is usually considered as the PoD in risk assessment.

Propylthiouracil (PTU), a known thyroid function inhibitor, can suppress thyroid peroxidase activity, leading to reduced production of thyroglobulin and thyroxine (T_4) (Taurog, 1976). Thus, PTU should serve as a perfect model chemical to study thyroid toxicity. In the present study, the ovariectomized (OVX) rats were exposed to PTU and samples were collected to measure total T_4 (tT_4), total Triiodothyronine (tT_3), thyroid stimulating hormones (LeVine et al., 2001), type I 5′-deiodinonase (5′-DI), malic enzyme (ME) and histopathology. BMD analysis was employed to screen the most sensitive endpoints and to produce the lowest PoD.

2. Materials and methods

2.1. Chemicals and reagents

PTU (≥ 99% purity), sucrose, and Dowex WX200-400 ion exchange

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resin was purchased from Sigma-Aldrich (St Louis, MO, USA). Corn oil was obtained from COFCO Ltd. (Beijing, China). [I^{125}] labeled reverse T_3 (rT_3) was purchased from Beijing North Biotechnology Institute (Beijing, China). Reverse T_3 (≥ 96% purity) was purchased from Toronto Research Chemicals (Toronto, Canada). Pierce BCA protein assay kit was obtained from Thermo-Fisher (Rockford, IL, USA).

2.2. Animals

Female Sprague Dawley albino rats free of specific pathogens were purchased from Huafukang Bioscience Co. Ltd. (Beijing, China). The animals were housed singly in cages under controlled temperature (20–24 °C) and humidity (40–70%) with a 12-h light/dark cycle and air change of 10 times per hour. Animals were fed with soy and alfalfa free diet (Huafukang Bioscience, Beijing, China) and provided with unlimited purified water throughout the study. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of China National Center for Food Safety Risk Assessment. All experimental procedures were carried out according to *the Guidance for the Care and Use of the Animals Management Rules* of the Ministry of Health of the People's Republic of China.

2.3. Study design

Before the experiment, animals were acclimated to the environment for 7 days. All 50 rats then received bilateral ovariectomy with a twelve-day recovery phase. Sham control, where animals received the surgery without removing ovaries, was not included in this experiment as the same animal model was successfully established in our previously published paper (Chen et al., 2015). Animals were randomly allocated into 5 groups (Control, 0.1, 1.0, 5.0 and 10.0 mg/kg•bw of PTU) based on their body weight. All animals were given either corn oil for control or PTU suspended in corn oil through daily gavage for 8 consecutive days. The animals were observed for any clinical signs of impairment and their body weight was recorded every two days. All animals were fasted for 12 h before the euthanasia with sodium pentobarbital (50 mg/kg•bw). Blood was collected from abdomen aorta and centrifuged at 3000 rpm for 10 min to obtain serum. Slices from right liver lobe weighting about 1 g were immediately rinsed with PBS and frozen with liquid nitrogen. Thyroids were dissected and preserved in 4% paraformaldehyde.

2.4. Liver 5′-DI activity assay

As previously described (Chen et al., 2015), liver tissue was homogenized in ice-cold buffer (320 mM sucrose, 1.0 mM dithiothreitol (DTT), 10 mM HEPES buffer, pH 7.0) at a 1:39 wt/volume ratio. The suspensions were centrifuged (3000 × g) at 4 °C for 10 min to collect supernatant. 1 mL buffer was added to the centrifuged pellets and suspended thoroughly and recentrifuged (20,000 × g) at 4 °C for 10 min. The supernatant was collected and combined with that from the first centrifugation. 20 μL supernatant was incubated with a buffer (0.005 μM [^{125}I] rT_3, 0.495 μM rT_3, 2 mM DTT, 1 mM EDTA, 10 mM PBS, pH 7.0) at 37 °C. The reaction was stopped by adding 200 μL cold T_4/PTU solution. The stopped reaction solution was centrifuged through a filter loaded with Dowex acidic ion exchange resin at 1500 × g for 5 min. The filters were eluted twice with 1.0 mL 10% acetic acid and all supernatants were collected and used to measure the released $^{125}I^-$ ion in a gamma counter (Xi'an Nuclear Instrument Factory, Shanxi, China). Counts per minutes (CPM) were recorded and normalized with total protein concentrations for all supernatant using Pierce BCA protein kit.

2.5. Liver malic enzyme activity assay

The method of measuring ME activity was described previously with some minor modifications (Schmutzler et al., 2004). Liver was homogenized on ice at a w:v = 1:10 ratio in a buffer containing 0.25 mM sucrose, 50 mM Tris-HCl, 0.1 mM EDTA, pH = 7.4. Homogenate was centrifuged at 15,000 g for 10 min at 4 °C. Supernatant was collected for total protein assay and malic enzyme activity assay. 200 μL of reaction buffer (67 mM triethanolamine, 1.5 mM L-malic acid, 4 mM $MnCl_2$, and 0.5 mM $NADP^+$, pH = 7.4) was added in 96 well plate. 20 μL of liver sample supernatant or homogenate buffer as control was added in the reaction buffer. Absorbance was measured at 340 nm every 31 s for 7 times in each sample well. NADPH concentration was determined by applying NADPH (10–500 μM) standards. The speed of NADPH formation ($^{\Delta e}/_{\Delta t}$) was calculated based on 7-time results. The ME activity was labeled as μmol NADPH per mg protein per min under equation:

$$\text{ME activity} = \frac{\textit{Total reaction volume} \times {}^{\Delta e}/_{\Delta t}}{\textit{Sample volume} \times \textit{protein concentration}}$$

2.6. Serum thyroid hormones

Serum levels of tT_3 and tT_4 were measured using a radioimmunoassay kit (North Biotechnology Institute, Beijing, China). Serum TSH level was determined by an ELISA assay kit (Endocrine Technology, Newark, CA, USA). All experiments were carried out according to the manufacturers' protocols.

2.7. Histopathology and morphometry

Thyroid weight and thyroid/body weight ratio was recorded and calculated. Fixed thyroid tissues were embedded in paraffin wax and sectioned at approximately 46 μm. Sections were then deparaffinized and stained with hematoxylin and eosin (H&E) for histopathologic and morphologic analysis. An area ratio of thyroid follicular epithelium/colloid was measured and calculated as described previously (Chen et al., 2015; Schmutzler et al., 2007).

2.8. BMD analysis

Benchmark dose software (BMDS) version 2.4 (EPA, Washington D.C., USA) was used for dose-response relationship analysis. Mean, standard deviation (SD), and sample size of each endpoint were imputed to calculate BMD and BMDL. Benchmark dose (BMD) and benchmark dose lower limit (BMDL) was generated based on following modeling criteria from EPA (EPA, 2012):

- ➢ Because all quantitative endpoints are continuous in the study, we apply each dataset to all five available continuous model options in BMDS (polynomial, exponential, hill, linear, and power model).
- ➢ Dataset will be excluded from modeling if: 1) the dose-response relationship curves is drastically "wavy or bumpy", because such variances are very unlikely to occur from a biological point of view; 2) the tested endpoint shows no significant change ($P > 0.05$) at any treatment doses comparing with control.
- ➢ Benchmark dose response (BMR) will be defined as 1 SD of control group for continuous data. Evidence has shown BMR of 1 SD of control group for continuous data is quantitatively similar 10% extra risk as BMR for categorical data (Crump, 1995).
- ➢ Models were statistically accepted when P value is greater than 0.10. Among these accepted models for each endpoint, the one with the smallest akaike information criterion (AIC) value was considered most fit (EFSA, 2009). Finally, the most appropriate BMD and BMDL were the smallest set among all endpoints.

2.9. Statistics

One-way analysis of variance (ANOVA) was used to determine the