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Effects of tris(1,3-dichloro-2-propyl)phosphate on pathomorphology and gene/protein expression related to thyroid disruption in rats

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ABSTRACT

Previous studies demonstrated that tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) caused adverse effects on thyroid hormone (TH) imbalance in aquatic and avian organisms. This study focused on the effects of TDCIPP on thyroid function and hormone homeostasis in mammals. Pubertal female Sprague-Dawley rats were orally administered 50, 100, or 250 mg/kg/d of TDCIPP from postnatal day (PND) 22 to PND42 for 21 days. The serum triiodothyronine (T3) levels increased significantly at 250 mg/kg/d of TDCIPP. There were no significant differences of the body weight, serum thyroxine (T4) and free thyroxine (FT4) levels between control and TDCIPP treated groups. There were significant dose-dependent increases in the mRNA and protein expression level of genes related to drug metabolism (cytochrome-p450-3A1, CYP3A1) and TH clearance (udp-glucuronosyl transferase-1A6, UGT1A6) in the liver. Treatment with TDCIPP increased hepatic type 1 deiodinase (DIO1) mRNA at 250mg/kg/d but down-regulated hepatic TH receptor beta (TRβ) mRNA expression. In addition, TDCIPP exposure induced slight thyroid follicular hyperplasia, and several genes involved in TH biosynthesis *(NIS, TPO, Tg*) were altered at 100 and 250 mg/kg/d of TDCIPP. Nevertheless, serum thyroid stimulating hormone (TSH) levels and the receptor (TSHr) mRNA significantly decreased at only low dose group. Based on these results, we certified that TDCIPP disturbs the normal bioprocess on TH synthesis, biotransformation or clearance, and hepatic detoxication of pubertal female SD rats, causing thyroid function disorder.

Keywords: *TDCIPP, thyroid hyperplasia, hepatic metabolism, pubertal rats*

1. INTRODUCTION

Due to mounting concerns over the exposure and potential health effects of the polybrominateddiphenyl ethers (PBDEs), these compounds were officially banned from use in the European Union (2000) and the USA (2005).[1] Organphosphorus flame retardants (OPFRs) became the primary PBDEs replacement and have been used in various household and industrial products. One OPFR with particular safety advantages related to flammability, tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), is being actively investigated following the phase-out of PBDEs.

With a wide application, TDCIPP has been growing in production volume and is persistently found in the environment and biota. For example, the concentration of TDCIPP was measured at 70-300ng/L in the North Sea and 0.62-5.54μg/kg in the sediment from Taihu Lake in China.[2, 3] TDCIPP was also detected in both dust and air samples in a variety of indoor environments, such as homes, day care centres, hospital wards and offices.[4] In Boston, concentrations of TDCIPP in dust were comparable to PBDEs with a maximum of 56.08 μg/g.[5, 6] In addition, TDCIPP was the most frequently detected flame retardant in upholstered furniture in the United States, with concentrations reaching 110.2mg/g in foam.[7] The same study also analysed polyurethane foam from products intended for use in homes or offices, and TDCIPP was detected at relatively high levels of up to 5% by weight. Moreover, biomonitoring studies detected TDCIPP in human adipose tissue[8], seminal plasma[9], breast milk[10] and muscle samples of freshwater fish from the Pearl River in South China.[11]

In recent years, in vitro and in vivo studies have suggested that TDCIPP is neurotoxic, potentially carcinogenic, a reproductive toxicant, and an endocrine disruptor. In vitro studies showed that TDCIPP exerts a similar developmental neurotoxicity as organophosphorus (OP) pesticides (e.g., decreased cell growth, inhibited DNA synthesis and altered neurodifferentiation) and exhibits cytotoxic and neurotoxic effects on PC12 cells (e.g., increased apoptosis, altered cell morphology, and changes in gene expression of synaptogenesis and neuriteoutgrowth).[12, 13] Two Japanese papers mentioned in secondary sources (WHO, 2004) showed that TDCIPP could cause severe maternal toxicity (e.g., decreased body weight and food consumption) in pregnant rats and produce signs of neurological involvement (e.g., ataxia, convulsions) at lethal doses in mice.[14] Moreover, TDCIPP has been listed as carcinogenic by the California *EPA (2012)* [15], based on the increased occurrences of liver, kidney, testicular and brain tumours in rats.[16]

The variability in the adverse effects of TDCIPP related to endocrine disorders has also been reported for aquatic organisms and avian species. TDCIPP elevated serum estradiol and testosterone levels, impaired reproduction and disrupted genes in the hypothalamic-pituitary-gonad (HPG) axis in zebrafish.[17, 18] Kojima *et al. (2013)* reported that TDCIPP had potential endocrine-disrupting effects and could act as an androgen receptor (AR) antagonist.[19] Moreover, zebrafish embryos that were exposed to different concentrations of TDCIPP showed a dose-dependent developmental

toxicity and altered thyroid hormone (TH) levels (T3 increased and T4 decreased), including thyroid endocrine-disruption activity.[20-23] In cultured chicken embryos, TDCIPP altered the expression of TH-responsive genes.[24, 25] Alterations in TH levels were found in a cohort of men who were exposed to high concentrations of TDCIPP, and even the semen quality was decreased for TDCIPP-exposed men.[26]

Although the adverse effects of TDCIPP on the endocrine system of many organisms including Pisces and Aves have been demonstrated, currently, there is little research on the toxic effects of TDCIPP in mammals. The potential effects of TDCIPP on the thyroid of mammals and related mechanisms are unknown. Therefore, the objective of the present study was to investigate the effects of TDCIPP on thyroid systems in mammals. In this study, adolescent female Sprague-Dawley rats were sacrificed for serum collection and organ (thyroid, liver, and kidney) weights. In addition, the serum concentration of several hormones (*TSH, T3, T4* and *FT4*) and thyroid histopathology were evaluated. Gene and protein expressions in the thyroid and liver were also analysed using real-time (RT)-PCR and western blots to evaluate thyroid hormone disruption. We hypothesized that TDCIPP disturbs normal physiological functions in mammals.

2. MATERIALS AND METHODS

2.1. Chemicals and solutions

TDCIPP (CAS no. 13674-87-8) was purchased from Sigma (USA, purity >95%) and was dissolved in corn oil (Giant Foods, Inc., Tianjin, China) to yield the working concentrations. It was well-mixed and well-distributed in corn oil prior to and throughout dosing.

2.2. Animals and experimental protocol

In our study, juvenile female Sprague-Dawley (SD) rats were derived from individually housed pregnant females that were purchased from Weitong Lihua Experimental Animal Central (Laboratory Animal Ltd., Beijing, China) on gestation day12 (GD12) dams. The animals were acclimated for one week under specific-pathogen-free (SPF) conditions in a well-ventilated room at a temperature of 22±2°C, a relative humidity of 55±10%, and a 12-h light/12-h dark cycle. Food and tap water were provided ad libitum. All animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The laboratory is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) certified.

10 litters were available to assure that a sufficient number of juvenile females were available for 8 female pups per treatment group and to avoid the need for placing littermates in the same experimental group. When the pups were weaned on postnatal day (PND) 21, female pups were marked by litter. Then all of the marked female pups were randomly assigned to four treatment groups consisting of 8 animals each

according to the rank of body weight, and littermates were not used in the same experimental group. In the present study, the dose level was chosen from secondary reference literatures of *WHO (2004)* (the NOEL and LOEL for maternal toxicity were 100 and 200 mg/kg/day in Female Wistar rats) and *Moser et al. (2015)*(0, 15, 50 and 150 mg/kg/day in Pregnant Long-Evans rats).[14, 27] Rats in the three experimental groups were administered TDCIPP at 50, 100, or 250 mg/kg/day by oral gavage from PND22 to PND42 for 21 days, whereas a similar vehicle control group was administered treatments of corn oil (1ml/100g). All the animals were dosed between seven o'clock and nine o'clock in the morning of local time.

2.3. Clinical signs of toxicity, body weight and thyroid hormonal measurements

During the study period, each animal was observed at least once daily for clinical signs of toxicity related to chemical treatment. Body weight of each rat was recorded daily to the nearest 0.1 g. At the end of treatment, the blood collection was taken from abdominal aortic after being anaesthetized by injecting 7% chloral hydrate with 0.5ml/100g dose. Serum was prepared immediately and stored at -20°C for later analysis for serum hormone levels. Serum total triiodothyronine (T3), thyroxine (T4), free thyroxine (FT4) and thyroid-stimulating hormone (TSH) concentrations were measured using a commercial ELISA kit specific for rat (Beijing Biotopped Science & Technology CO., Ltd., China) according to the protocol provided by the kit. The detection limits and intra-assay coefficient of variation（CV）for kits are TSH: 0.05-15

mIU/L, <5%; T3:40-10000 pg/ml, <5%; T4:0.5-200 ng/ml, <5% and FT4: 0.5 -150 ng/L, <5%; respectively.

2.4. Measurement of organ weights and histopathological examination

24 hours after the final dose, the animals were sacrificed. And tissue collections were completed before 13 o'clock of the day. The thyroid gland, liver, kidney, adrenal, uterus and ovary were dissected and weighed immediately. The thyroid gland, kidney, liver and brain were then fixed in 10% buffered formalin for at least 5 days. Each tissue was processed in an automatic tissue processor and embedded in paraffin. Thin sections were cut at a thickness of 4-5 mm and stained with hematoxylin and eosin for pathological evaluation under a microscope, and the tissue slices were certified by two individual pathologists.

2.5. RNA isolation and real-time RT-PCR analysis

To determine the effects of TDCIPP on mRNA expression, RT-PCR was used to detect alterations in relative gene expression. Primer sequences of target genes are shown in Table 1, and GAPDH was chosen as an endogenous gene because it was stably expressed in thyroid and liver between control and TDCIPP treatments in our pre-experiment. Isolation of total RNA, synthesis of first-strand cDNA and RT-PCR were all performed as previously described.[28] Briefly, total RNA was isolated from thyroid and liver tissue using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations. To remove genomic DNA contamination,

177 RNase-freeDNaseI (Sigma, USA) was used. The purity and quality of the RNA were
178 determined by measuring 260/280nm ratios and by 1% agarose-formaldehyde gel
179 electrophoresis with ethidium bromide staining. Synthesis of first-strand cDNA was
180 performed using a Prime Script RT Reagent Kit (TaKaRa, Shanghai, China). RT-PCR
181 was performed using a SYBR Real-time PCRMaster-Mix-Plus Kit (Thermo, USA)
182 and was analysed on an ABI 7300 System (PerkinElmer Applied Bio-systems, Foster
183 City, CA, USA) following the manufacturer's instructions. All RT-PCR data were
184 quantified using the $2^{-\Delta\Delta CT}$ method.[29]
185
186 **2.6. Protein extraction and western blotting**
187 Homogenized liver tissue (~200mg) in RIPA protein extraction buffer (Thermo,
188 USA) with freshly added protease inhibitor PMSF (Thermo, USA) was centrifuged at
189 14,000g at 4°C for 10 min. After quantifying the protein concentration with a BCA
190 protein assay (Biyuntian, Beijing, China), the lysates were separated by 10%
191 SDS-PAGE (30 μg of protein/lane) and were then transferred to a polyvinylidene
192 difluoride (PVDF) membrane (Pierce, USA). The membranes were blocked with 5%
193 non-fat dry milk for 1 h at 37ºC and then incubated with primary antibody (purchased
194 from Abcam Company in UK) against GAPDH (Rabbit monoclonal to GAPDH
195 antibody), thyroid hormone receptor beta (TRβ, rabbit polyclonal to TRβ antibdody),
196 transthyretin (TTR, rabbit polyclonal to prealbumin antibody), udp-glucuronosyl
197 transferase-1A6 (UGT1A6, rabbit monoclonal to UGT1A6 antibody) and
198 cytochrome-p450-3A1 (CYP3A1, rabbit polyclonal to CYP3A1 antibody) overnight

at 4ºC. After washing in tris-buffered saline with Tween 20 (TBST) three times, the membranes were incubated with secondary antibody in TBST solution for 30 min at 37ºC and washed as above. The blots were visualised with ECL-plus reagent (Millipore Corporation, Billerica, USA), and the results were analysed with a Gel-Pro analyzer 4.0 (Media Cybernetics, USA).

2.7. Statistical analysis

All data analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA) and were evaluated for homogeneity of variance using Levene's tests. All data are expressed as the means ± standard deviation (Mean ± SD). The body weights were analyzed by two-way ANOVAs with Bonferroni post-test using both time and dose as factors. One-way analysis of variance (ANOVA) was used to evaluate differences between the control and each exposure group in other dates followed by Tukey's test, and $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Clinical observations and body weights

TDCIPP exposure did not induce clinical signs in juvenile female SD rats at any dose throughout this study. Animals' body weight means are presented in Figure 1. The statistical results by two-way ANOVAs showed that the interaction between time and dose was considered not significant ($F_{(63,558)}=0.92$, $P=0.6432$). No obvious changes of body weight were observed at three TDCIPP doses compared with control.

221 **3.2. Organ weights**

222 TDCIPP significantly and dose-dependently increased the absolute and relative
223 thyroid weight of rats in the two high-dose groups (100 and 250 mg/kg/d). In addition,
224 the statistically significant increases were observed in liver absolute and relative
225 weights at 100 mg/kg/d, and in liver relative weights, kidney absolute and relative
226 weights at 250 mg/kg/d compared to control rats (Table 2). There were no significant
227 changes ($p>0.05$) in average uterine, adrenal or ovarian weights for all TDCIPP
228 groups compared to the control group.

229

230 **3.3. Serum thyroid hormone levels**

231 Serum TSH, T3, T4 and FT4 levels were detected using a commercial ELISA kit
232 following the product manual. As shown in Figure 2, the average TSH level was
233 below the control level at the 50 mg/kg/d ($p=0.025$) and 100 mg/kg/d ($p>0.05$) doses
234 but higher at the 250mg/kg/d ($p>0.05$) dose (Fig. 2.A). Only in the low dose, TSH
235 level was significantly different from control. There were no significant differences
236 between the TDCIPP treated and control groups of serum T3 level, except for a
237 statistically significant rise observed in the 250 mg/kg/d treated group relative to the
238 control ($p=0.008$) (Fig. 2.C). However, serum T4 and FT4 levels were not statistically
239 different between all groups (Fig. 2.B and 2.D).

240

241 **3.4. Histopathology examination**

242 Histopathological examination to visualize an internal lesion was used to

243 determine the effects of TDCIPP treated on the rat organs. Three rats per group were
244 randomly chosen for pathological evaluation. There were no compound-related gross
245 lesions and microscopic changes observed in any of the examined organs (brain, liver
246 and kidney) of the treated rats, except for the thyroid gland. Histological assessments
247 performed on thyroid sections from the different groups are presented in Figure 3. In
248 control rats (Fig.3.A), thyroid follicles were lined by low cuboidal epithelial cells and
249 filled with colloid, and all of the follicles were almost equal and regular in size. The
250 thyroid sections of the low-dose group (Fig.3.B) were similar to control, while in the
251 thyroid of the 100 and 250 mg/kg/d TDCIPP-exposed rats (Fig.3.C and 3.D), there
252 was an increase in the number of irregularly shaped small follicles filled with
253 relatively less colloid, including small hyperplasia of follicular cells. Especially in the
254 highest dose groups, follicular epithelial cells were obvious hyperplasia and some
255 were lack of colloid. The nucleus did not show abnormalities in both 100 and 250
256 dose groups.
257
258 **3.5. TDCIPP induced gene expression in thyroid and liver tissues**
259 To explore possible mechanisms of TDCIPP-induced thyroid hormone disorder
260 and thyroid lesions in rats, mRNA expression was measured by RT-PCR for several
261 key genes involved in drug metabolism and TH synthesis, transport, metabolism and
262 TH receptors in thyroid and liver tissues. There was no amplification in the no-reverse
263 transcriptase or no template controls, and none of the TDCIPP treatments affected
264 GAPDH expression.

Four key genes related to TH synthesis in the thyroid *(NIS, TPO, Tg and TSHr)* were measured to investigate whether TDCIPP exposure affects thyroid hormone biosynthesis. As shown in Figure 4, expression of thyroid peroxidise (TPO) (Fig. 4.B) presented a significant dose-dependent up-regulation in all groups and was induced to a maximum of 1.5-, 1.8- and 2.4-fold in the groups exposed to 50 ($p>0.05$), 100 ($p=0.002$) and 250 ($p=0.017$) mg/kg/d TDCIPP. No statistically significant effects of TDCIPP on either sodium iodide symporter (NIS) (Fig. 4.C) or thyroglobulin (Tg) (Fig. 4.D) mRNA were observed in the 50 or 100 mg/kg/d groups compared with controls, but at the 250 mg/kg/d treatment level, NIS and Tg were significantly increased 1.6-fold ($p=0.037$) and 1.65-fold ($p=0.044$), respectively. Notably, TDCIPP significantly down-regulated TSH receptor (TSHr) mRNA (Fig. 4.A) by approximately 2-fold ($p=0.008$) at a 50mg/kg/d concentration; however, the TSHr mRNA level showed a trend to recover to the normal level with higher doses.

Furthermore, as a vital organ for biological transformation, metabolism and excretion of many xenobiotic compounds, the liver plays an important role in the study of thyroid interference. The expression levels of several common genes known to transport thyroid hormones (transthyretin, TTR), biotransformation of TH (type 1 deiodinase, DIO1), excretion metabolites of TH and compounds (udp-glucuronosyl transferase-1A1, 1A6; UGT1A1, UGT1A6), TH receptors (TRα and TRβ) and a drug metabolism gene cytochrome-p450-3A1 (CYP3A1) were assessed in the liver tissue

of TDCIPP-exposed rat. Of the seven mRNA transcripts assessed, only four were significantly affected by TDCIPP treated (Fig. 5). There were significant dose-dependent increases both CYP3A1 and UGT1A6 mRNA expression following TDCIPP exposure, with a significant maximum induction of 3.7-fold and 2.3-fold ($p<0.05$) at the highest dose treatment groups, respectively (Fig. 5.A). DIO1 (Fig. 5.B) mRNA expression was also significantly up-regulated 1.6-fold ($p<0.05$) with 250mg/kg/d treatment of TDCIPP. TDCIPP-exposed rats had lower TRβ (Fig. 5.B) mRNA expression levels relative to controls at all concentration tests; however, only the decrease at the highest dose was statistically significant (approximately 51%, $p<0.05$). No significant changes in hepatic mRNA expression were observed for the other transcripts (UGT1A1, TTR, and TRα) in animals exposed to TDCIPP.

3.6. Relative protein expression level in rat liver exposed to TDCIPP

To verify the reliability of gene expression changes in liver tissue of rats exposed to TDCIPP, western blot experiments were carried out for four target proteins (TRβ, TTR, UGT1A6 and CYP3A1), as well as an internal protein GAPDH (as shown in Fig. 6.A). The experimental results showed that the TRβ protein expression level decreased in a dose-dependent manner compared to control. The 100 and 250 mg/kg/d concentrations of TDCIPP reduced TRβ protein expression 0.83 and 0.65 times ($p<0.05$), respectively (Fig. 6.B). Unlike the expression of TRβ, the UGT1A6 and CYP3A1 protein expression levels demonstrated a significant up-regulation in a dose-dependent manner. Compared with the control, liver UGT1A6 protein

309 expression increased 1.4-, 1.65- and 1.8-fold ($p<0.05$) and CYP3A1 increased 1.4-,
310 2.4- and 3.3-fold ($p<0.05$) in the 50, 100 and 250 mg/kg/d treatment groups,
311 respectively. No significant change in TTR protein expression was observed between
312 the TDCIPP treated groups (Fig. 6.B). Together, there was a strong correlation
313 between western blot and RT-PCR results.
314

315 **4. DISCUSSION**

316 Several studies have suggested that TDCIPP has adverse effects on endocrine
317 function in aquatic animals, showing that the T4 level decreases and that mRNA
318 expression associated with thyroid function increases.[20,25,26] However, until our
319 investigation, little study explored the potential of repeated oral exposure to TDCIPP
320 in mammals to disrupt thyroid function or the related molecular mechanisms of such a
321 disruption. Therefore, our research is the first to find that TDCIPP exposure from
322 PND22-PND42 has adverse effects on thyroid function in pubertal female rats. The
323 effects of TDCIPP were observed at all endpoints examined, including the following:
324 1) thyroid gland weight; (2) thyroid gland morphology; (3) serum hormone levels; (4)
325 mRNA expression in thyroid and liver tissues; and (5) protein expression levels
326 associated with drug metabolism, TH synthesis, transport, excretion and TH receptor
327 expression.
328

329 In the present study, during exposure to TDCIPP, pubertal female rats that were
330 given 250mg/kg/d displayed a significant increase in absolute and relative kidney

weights. Moreover, a significant increase in absolute and relative thyroid weights and relative liver weight were observed in the two high-dose groups after PND42. Similar, Moser *et al. (2015)* reported TDCIPP exposure (150mg/kg/d) increased relative liver weight in dams and were lower both absolute liver weights at weaning and weight gain in offspring.[27] These results suggest that TDCIPP exposure could have an adverse effect on normal growth and development in pubertal female rats, especially a high dose of TDCIPP.

Thyroid hormone is essential for a number of normal physiological processes related to brain development, metabolism, reproduction, and cardiovascular health. Therefore, changes in the function of the thyroid gland or interference with the ability of thyroid hormone could produce serious adverse effects on normal physiological functions.[30] Our results revealed a positive relationship between both thyroid dysfunction and lesions with TDCIPP exposure in pubertal female rats. With regard to thyroid hormone levels, serum TSH levels were significantly decreased in low dose TDCIPP treated group, and T3 levels were increased in highest dose. Compared to recent studies, Wang et al *(2013)* reported that TDCIPP exposure significantly decreased whole-body T4 concentrations and increased whole-body T3 concentrations in larval zebrafish.[20] Chick embryos exposed to TDCIPP (7.64ug/g) showed there was a decrease in plasma T4 level.[25] In a human epidemiological study, high concentrations of TDCIPP in house dust were associated with decreased T4 levels in a cohort of men. [26] However, in our present study, serum thyroxine (T4) and free

thyroxine (FT4) levels had no significant differences between control and TDCIPP treated groups. Interestingly, Moser et al *(2015)* administered TDCIPP (15, 50,150 mg/kg/d) to pregnant Long-Evants rats from gestational day 10 to weaning for evaluating the potential developmental neurotoxicity of TDCIPP, and thyroid hormones (T3 and T4) also were measured in dams and offspring. They pointed out that TDCIPP would not alter levels of T3 and T4 in dams and offspring, which did not support the potential for thyrotoxicity.[27] As for mammals, there is a higher bioactivity level of thyroid during pregnancy and lactation, and dams produced a large amount of thyroid hormone to maintain normal physiology of themselves and their offspring. Physiological functions such as placental barrier, metabolism and detoxification of dams protect offspring from poisonous effects of compounds. In addition, adolescent development is closely related to hormone homeostasis, and is more sensitive to the stimulations of compounds. Relative to other species, mammals have excellent immune system and powerful self-regulating mechanism against outside interference. The negative feedback regulation of pituitary gland and hypothalamus or the compensatory mechanism of thyroid gland hyperplasia could still maintain TH homeostasis when the TH levels of the body were perturbed. Besides, ovipara (like fish, check) are different with mammals in absorption, distribution, metabolism, and excretion of compounds, and also in the abilities of immunization, or resisting external disturbances. These differences may partly explain that ovipara are more sensitive to external stimuli. And monitoring the levels of thyroid hormone via a multiple-time-point method is more meaningful than a single-time-point way during

chemical exposure. What's more, it is incomplete to evaluate the thyroid disruption only by the changes of thyroid hormone levels and it is necessary to implement a comprehensive evaluation including pathological morphology or molecular level changes of thyroid. Polychlorinated biphenyls (PCBs) or PBDE exposure in rats resulted in mild to severe hypertrophy of the thyroid and irregularly shaped follicle cells with hyperplasia.[31,32] Similarly, in present study, histopathological examination revealed that there were thyroid follicular hyperplasias in high-dose groups, and hyperplastic thyroid might be a compensatory mechanism of body, which prompts the thyroid epithelium cells to produce thyroid hormone to keep homeostasis in blood.

TH-responsive genes are critical developmental signals in various animal species. Therefore, these genes need to be identified to improve our understanding of the molecular basis of the TH disorder and thyroid dysfunction induced by TDCIPP during pubertal development in mammals. Several key genes and proteins associated with drug metabolism and thyroid hormone synthesis, transport, metabolism, clearance and receptors were assayed in our study. The current study provides evidence that TDCIPP acts as an inducer of selected hepatic genes (CYP3A1, UGT1A6, DIO1 and TRβ) in pubertal female rats that exposed TDCPP for 21 day from PND22-PND42 when dosed daily at 50 mg/kg/d or greater. CYP3A genes encode monooxygenases, which catalyse many reactions involved in drug metabolism and the synthesis of cholesterol, steroids and other lipids. In addition, the hepatic UGT1A system is actively involved in hepatic metabolism and biliary clearance of

thyroid hormone, which suggests that T4 depletion by exposure to xenobiotics mainly occurs through T4-glucuronide and UGT1A1 or UGT1A6, the major two isozymes in rat liver. In fact, TDCIPP-exposed rats or chicken embryos exhibited an elevation of circulating cholesterol and bile acid levels.[33, 34] Van den Eede *et al. (2013)* confirmed that the metabolic process of TDCIPP involves oxidative enzymes (namely CYPs) rather than hydrolases in vivo in liver and in human liver S9 subcellular fractions and rat liver microsomes.[35-37] Consistent with previous reports, TDCIPP treated affects CYP3A37, CYP2C45, UGT1A1 and UGT1A9 in cultured embryos or hepatocytes of chicken.[24, 25] Interestingly, biological processes of hepatic induction are most often driven by several key nuclear receptors, including the activation of aryl hydrocarbon receptor (AhR), the peroxisome proliferator-activated receptor (PPAR), constitutive androstane receptor (CAR) and the pregnane X receptor (PXR).[38,39] Similar sensitivity of these molecules to a variety of FRs has also been shown, such as tris(1-chloro-2-propyl) phosphate (TCPP), PBDEs, and polychlorinated biphenyls (PCBs), among others. Our study observed a significant dose-dependent up-regulation from 2.2- to 3.7-fold and from 1.6- to 2.3-fold for liver mRNA expression of CYP3A1 and UGT1A6, respectively, following TDCIPP exposure, indicating that enhanced induction of hepatic biotransformation related to CYPs and UGTs can be activated by nuclear receptors. We found that mRNA expression levels of CYP3A1 and UGT1A6 were well-consolidated by western blot results. Most convincingly, hepatic biotransformation induced by TDCIPP was associated with increased liver and thyroid weight, as well as increased hormone catabolism including thyroid hormones and

419 accelerated T4 clearance.

420

421 Deiodination is another critical process for inactivation of serum T4, in addition
422 to glucuronidation. The increase in DIO1 mRNA expression can enhance thyroidal
423 5'-deiodination from T4 to T3 and approximately 80% of serum T3 in mammals is the
424 product of this conversion.[40] These results are consistent with our results showing a
425 rise of T3 levels after 250mg/kg/d TDCIPP exposure. A previous study suggested that
426 a possible mechanism for the reduction in T4 could involve increased tissue-specific
427 deiodinase activity that converts T4 to T3.[41] However, Hood and Klaassen *(2000)*
428 revealed that the PCB mixture Aroclor 1254 leads to a decrease in hepatic DIO1
429 activity, which leads to conclusions that are diametrically opposed to ours.[42] The
430 differences in compound exposure and the inconsistency between mRNA expression
431 and enzyme activity mainly caused this discrepancy. T4 and T3 hormones exert their
432 major effects by binding to thyroid receptors (TRs), such as TRα and TRβ, whose
433 most important functions are the regulation of metabolism and the development of the
434 organism.[43, 44] Treatment with TDCIPP caused transcriptional responses in the
435 TRα-associated pathway in zebrafish.[21] Bogazzi *et al. (2003)* found that a commercial
436 mixture of PCBs (Aroclor 1254) exhibited specific binding to the rat TRβ.[45] Our
437 results show that TDCIPP-exposed rats had lower TRβ mRNA and protein expression
438 levels compared to controls at all concentrations tested, with no change in TRα.
439 Recently, a study showed that in spite of the activation of the pregnane X receptor
440 (PXR), TDCIPP did not have any thyroid hormone receptor (TR) agonistic or

antagonistic activity in cultured simian kidney COS-7 cells.[19] It is unknown whether the decrease in TRβ is directly induced by TDCIPP or is the adverse effect of a hypermetabolism-triggered organism defence response by down-regulated TRβ to inhibit the activity of TH to prevent energy and nutrient consumption. In addition, TTR is important for the transport of the thyroid hormone. A number of chemicals with a structure similar to T4 have been shown to displace T4 from TTR and bind to TTR with higher affinity than T4 itself.[30] However, our study found that the consequences of TDCIPP exposure on TTR mRNA and protein expression in all groups were not different, which could have been caused by chemical structure diversity.

Intracellular adenosine 3',5'-cyclic monophosphate induces TSH to bind to its receptor (TSHr) on the surface of thyroid follicle cells; then, Tg, NIS, and TPO are activated to promote TH synthesis.[44] In larval zebrafish, TDCIPP altered genes related to thyroid synthesis, metabolism, and development at subtoxic concentrations.[20] Chick embryos exposed to TDCIPP impaired embryo growth, and altered metabolizing enzymes.[25] In our study, expression of TPO was up-regulated 1.8- and 2.4-fold in the two high-dose groups, and NIS and Tg were significantly increased 1.6- and 1.65-fold after 250mg/kg/d TDCIPP exposure. These results show that multiple drugs or environmental compounds, including TDCIPP, can induce thyroid gland hyperplasia in rats through enhanced metabolism or clearance of thyroid hormones, which leads to increased TRH from the hypothalamus and TSH secretion by the pituitary gland as a

463 feedback mechanism.[46] Subsequently, thyroid gland hyperplasia induces TPO, NIS
464 and Tg up-regulation for the synthesis of more thyroid hormone to maintain TH
465 homeostasis. It is also worth mentioning that TDCIPP significantly down-regulated
466 TSHr mRNA in the thyroid by approximately 2-fold at only 50mg/kg/d concentration,
467 but a trend of recovery to normal levels was observed in the two high-dose treatment
468 groups without being statistically significant. There was a very visible degree of
469 uniformity between serum TSH level and thyroid TSHr mRNA expression in our
470 study, but the reason for this phenomenon is inexplicable. This result may be related
471 to regulation of hypothalamus and pituitary involved in thyroid system disruption
472 within the upstream of HPT axis.
473

474 **5. CONCLUSIONS**

475 Our study was designed to examine potential thyroid disruption and the related
476 molecular mechanism following short-term TDCIPP exposure in adolescent female
477 rats. The present results demonstrated that high-dose TDCIPP exposure from
478 PND22-PND42 induced alterations in mRNA and protein expression that are crucial
479 to the TH pathway and hepatic detoxification. In addition, thyroid follicular
480 hyperplasia disclosed the negative impact to animals after TDCIPP exposure. We
481 certified that TDCIPP disturbed the normal bioprocess on TH synthesis, clearance,
482 and hepatic detoxication of pubertal female SD rats, causing thyroid function disorder.
483 Furthermore, the effects of prolonged or low-dose exposure to TDCIPP and involve in

484 regulation of hypothalamus and pituitary on thyroid dysfunction in mammals are
485 worth further investigation.
486

487 **CONFLICT OF INTEREST**

488 The authors have disclosed all financial sources and confirm that there are no
489 conflicts of interest.
490

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496

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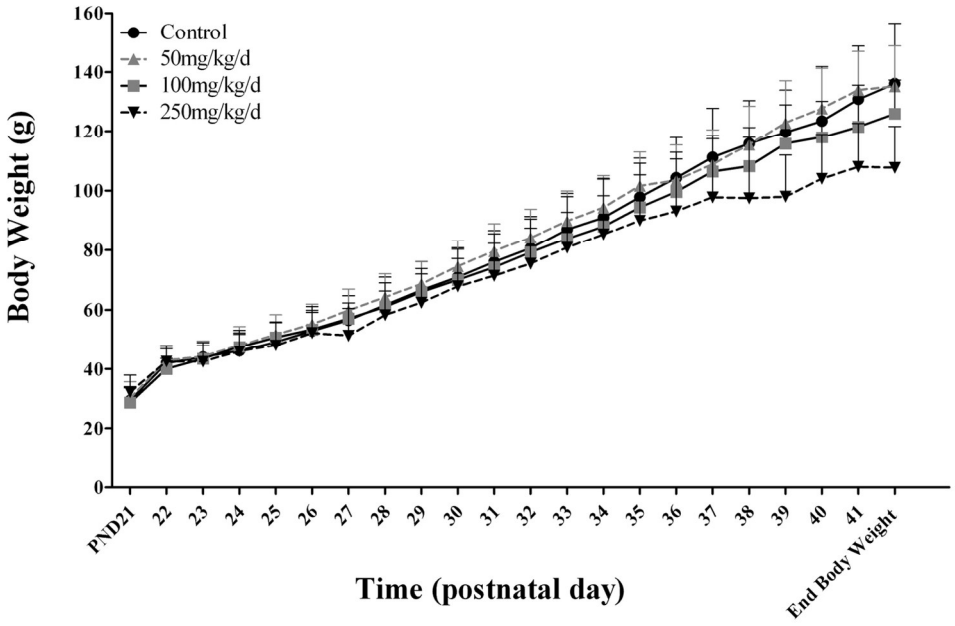

Figure 1. Change in body weight induced by TDCIPP
133x97mm (300 x 300 DPI)

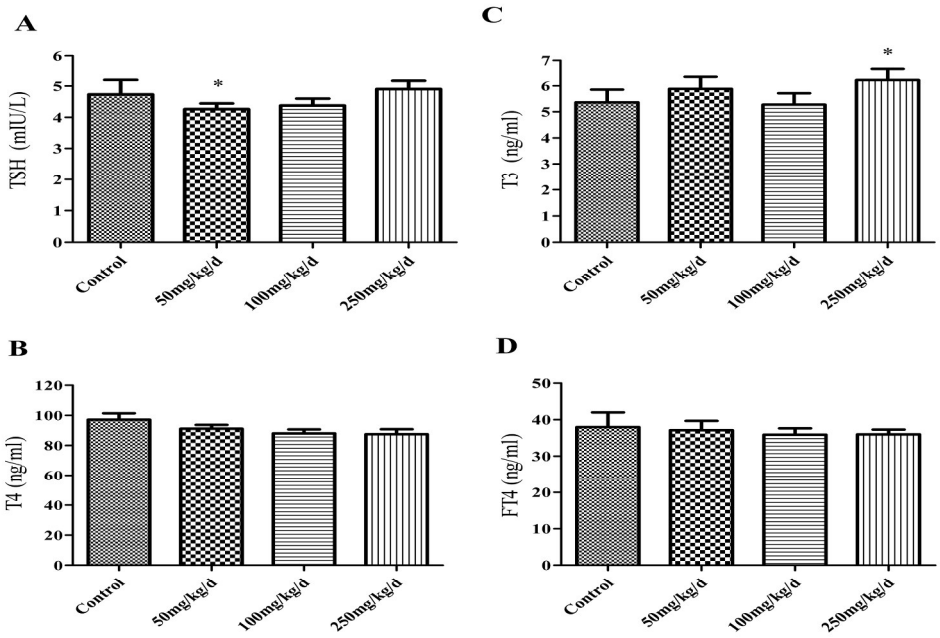

Figure 2. Alteration of serum hormone levels after TDCIPP treatment
112x79mm (600 x 600 DPI)

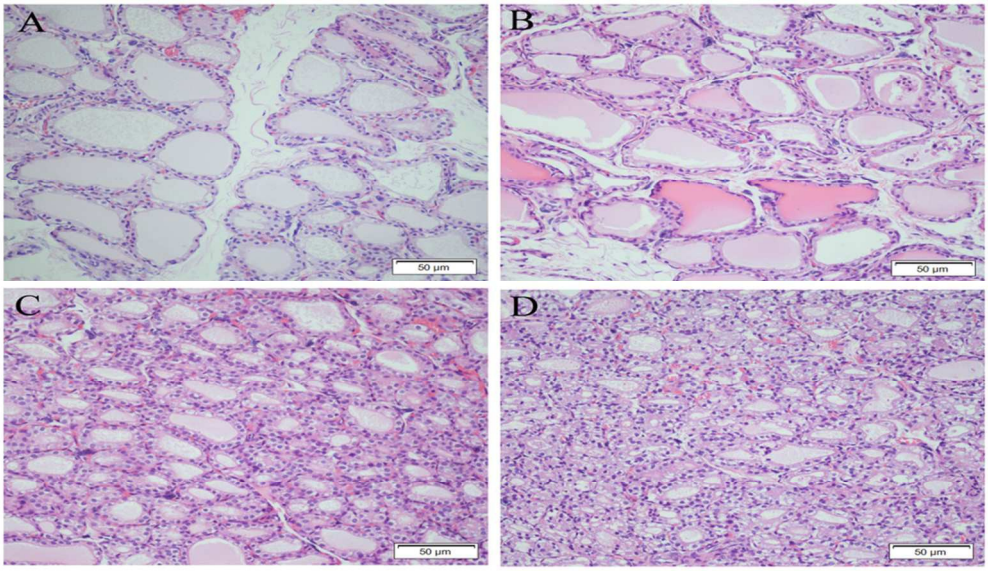

Figure 3. Histopathology of TDCIPP-induced damage in thyroid sections (200×)
49x30mm (600 x 600 DPI)

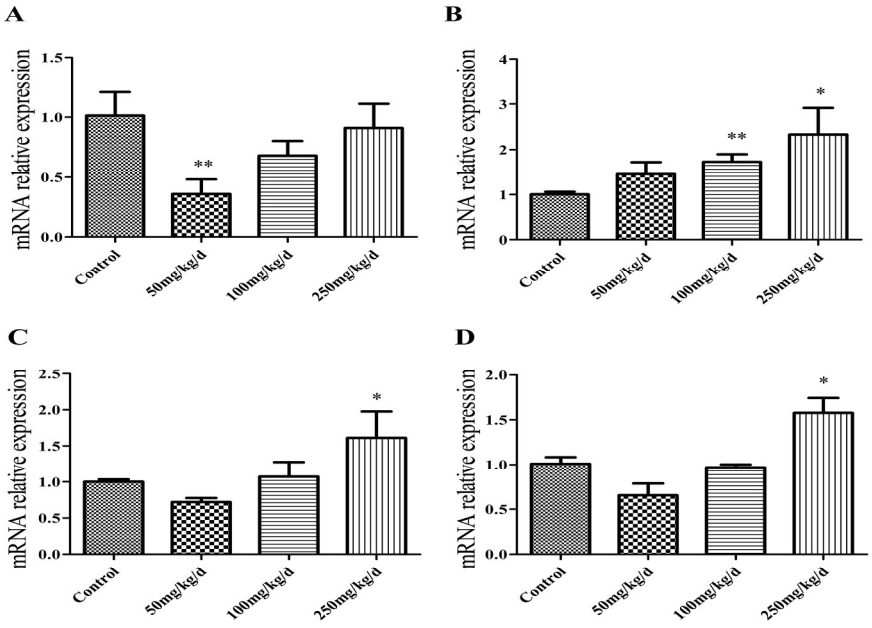

Figure 4. RT-PCR detected expression of thyroid hormone biosynthesis genes
110x77mm (600 x 600 DPI)

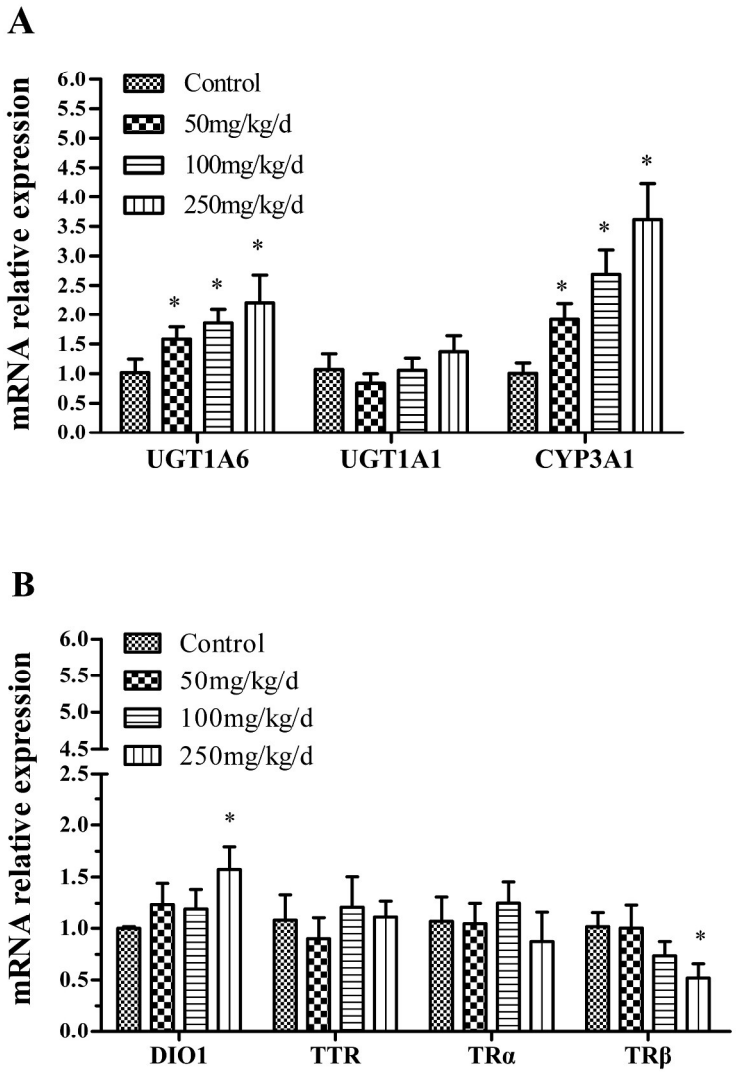

Figure 5. Effect of TDCIPP exposure on liver mRNA expression
115x160mm (600 x 600 DPI)

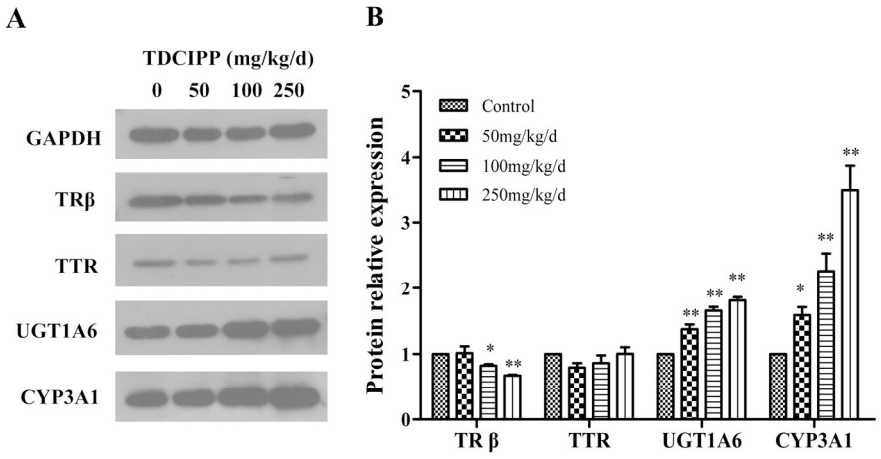

Figure 6. Western blot analysis of TDCIPP-induced protein expression changes in livers of rats
90x47mm (600 x 600 DPI)

FIGURE CAPTIONS

Figure 1. Change in body weight induced by TDCPP

Means ± standard (SD, n=8) of individual body weights of SD rats given TDCPP by oral gavage daily for 21days.

Figure 2. Alteration of serum hormone level after TDCPP treatment

Serum TSH (A), T4 (B), T3 (C) and serum FT4 (D) levels of animals exposed to TDCPP for 21 days. Each pillar denotes the mean ± SD, n=8. Mean differences across groups were determined by ANOVA followed by Turkey's post hoc test. ($^{*}p<0.05$ compared to control).

Figure 3. Histopathology of TDCPP-induced damage in thyroid sections (200×)

Histopathology for vehicle controls (A) and exposure groups treated with 50mg/kg/d of TDCPP (B), 100mg/kg/d of TDCPP (C), and 250mg/kg/d of TDCPP (D).

Figure 4. RT-PCR detected expression of thyroid hormone biosynthesis genes

The effect on mRNA expression of thyroid hormone biosynthesis genes (A) TSHr, (B) TPO, (C) Tg, and (D) NIS in rat thyroid exposed to TDCPP, as measured by quantitative real-time PCR. Fold changes are presented relative to the vehicle control. Values are expressed as the mean ± SD (n=4-5 per group, $^{*}p<0.05$, $^{**}p<0.01$ indicates significant changes in expression compared to control).

Figure 5. Effect of TDCPP exposureon liver mRNA expression

RT-PCR analyses of hepatic mRNA expression for (A) excretion metabolites of TH and compounds (UGT1A1, UGT1A6) and a drug metabolism gene (CYP3A1), (B) biotransformation of TH, (DIO1)transport thyroid hormone (TTR), and TH receptors (TRα and TRβ) in animals administered corn oil and TDCPP at different dose. Relative expression levels of the target genes are presented as the mean ± SD (n=4-5 per group, $^{*}p<0.05$ indicates significant changes in expression compared to control).

Figure 6. Western blot analysis of TDCPP-induced protein expression changes in the livers of rats

A representative western blot of TRβ, TTR, UGT1A6 and CYP3A1 expression in the livers of rats is shown in (A), and the relative quantification of protein expression shown in (B). The data represent the means from three replicate experiments. All data are expressed as the mean ± SD of fold change relative to the control. $^{*}p<0.05$ indicates significant changes in expression compared to control.

Table 1 Primer sequences used for RT-PCR

Gene name	GenBank accession no.	Primer Sequence
GAPDH	NM_017008	F:5'-GACAACTTTGGCATCGTGGA-3'
		R:5'-ATGCAGGGATGATGTTCTGG-3'
Tg	NM_001270784	F:5'-AGAATGGAGCAACCTGGCGTA-3'
		R:5'-AGTGATTGCAGGGCAGCAGA-3'
NIS	NM_052983	F:5'-ATCAGGGCATCGCTCCTGTC-3'
		R:5'-CGAGCATTACCACAACCTGGAAC-3'
Tshr	NM_012888	F:5'-ACCTGAAGACCATTCCCAGTCTTG-3'
		R:5'-AGTCGCTGCAGAGTGGCATCTA-3'
TPO	NM_019353	F:5'-CCTACATGCTAGGTGAGGATGAGAA-3'
		R:5'-TGGCCAAACCACCAATGAGA-3'
UGT1A6	NM_057105	F:5'-CTGTGGTGTGATCCTGGCTGA-3'
		R:5'-GGGCTTTGACCAAGCATGTG-3'
UGT1A1	NM_012683	F:5'-GCCATGCAGCCTGGATTTG-3'
		R:5'-CATGCGATCTGTGTTCGAGGA-3'
CYP3A1	NM_013105	F:5'-CAGCAGCACACTTTCCTTTGTC-3'
		R:5'-CTCCTCCTGCAGTTTCTTCTGTGTA-3'
DIO1	NM_021653	F:5'-GTGGTGGTGGACACAATGCAG-3'
		R:5'-TTGTAGTTCCAAGGGCCAGGTTTA-3'
TTR	NM_012681	F:5'-TGCCTCGCTGGACTGATATTG-3'
		R:5'-TTGAACACTTTCACGCCACA-3'
TRα	NM_001017960	F:5'-GACAAGGCCACCGGTTATCACTAC-3'
		R:5'-GATCTTGACGATGACGCAGCA-3'
TRβ	NM_012672	F:5'-GGGGTACCACTATCGCTGCATCAC-3'
		R:5'-TCCCACTGCCTTGAGGACAAC-3'

F= forward; R= reverse.

Table 2 Absolute and relative (to body weight) organ weights of SD rats

Organ		TDCIPP (mg/kg/d)			
		0	50	100	250
Thyroid	g [a]	0.011±0.002[c]	0.011±0.003	0.013±0.002*	0.016±0.005*
	%[b]	0.008±0.003	0.008±0.002	0.010±0.002*	0.013±0.004*
Liver	g	5.98±0.89	6.73±0.74	7.38±0.99*	7.16±2.05
	%	4.42±0.44	4.97±0.59	5.85±0.47*	5.92±0.40*
Kidney	g	1.29±0.23	1.55±0.22	1.71±0.21	1.82±0.52*
	%	0.95±0.07	1.14±0.15	1.35±0.47	1.51±0.11*
Terminal body weight	g	136.1±10.4	135.2±13.6	125.9±11.4*	119.9±16.3*

*$p<0.05$ Compared to control;

[a] g, Grams.

[b] %=[Organ weight/terminal body weight]×100.

[c] Mean ± SD for n=8.