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Lambda-cyhalothrin delays pubertal Leydig cell development in rats☆

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ABSTRACT

Lambda-cyhalothrin (LCT) is a widely used broad-spectrum pyrethroid insecticide and is expected to cause deleterious effects on the male reproductive system. However, the effects of LCT on Leydig cell development during puberty are unclear. The current study addressed these effects. Twenty-eight-day-old male Sprague Dawley rats orally received LCT (0, 0.25, 0.5 or 1 mg/kg body weight/day) for 30 days. The levels of serum testosterone, luteinizing hormone, and follicle-stimulating hormone, Leydig cell number, and its specific gene and protein expression were determined. LCT exposure lowered serum testosterone levels at doses of 0.5 and 1 mg/kg and luteinizing hormone levels at a dose of 1 mg/kg, but increased follicle-stimulating hormone levels at doses of 0.5 and 1 mg/kg. LCT lowered *Star* and *Hsd3b1* mRNA or their protein levels at a dose of 1 mg/kg. Immature Leydig cells were purified from pubertal rats and treated with different concentrations of LCT for 24 h and medium androgen levels, Leydig cell mRNA and protein levels, the mitochondrial membrane potential (△Ψm), and the apoptotic rate of immature Leydig cells were investigated. LCT inhibited androgen production at 5 μM and downregulated *Scarb1* at 0.05 μM, *Hsd3b1* and *Hsd11b1* at 0.5 μM, and *Cyp11a1* at 5 μM. LCT also decreased △Ψm at 0.5 and 50 μM. In conclusion, LCT can influence the function of Leydig cells.

1. Introduction

In the last sixty years, human sperm concentration has decreased dramatically (Levine et al., 2017). This decrease could be associated with the increased application of many man-made chemicals, including pesticides. It has been found that pesticide exposure is associated with the increasing rate of the reproductive dysfunction (Abell et al., 2000). One class of pesticides is pyrethroid-related chemicals, of which lambda-cyhalothrin (LCT) is a mixture of isomers of cyhalothrin. LCT is widely used in agriculture, public health facilities, forestry, horticulture, and homes to control a broad spectrum of insects. Due to its extensive use, LCT has been found to be ubiquitous in water sources and lands (Delgado-Moreno et al., 2011; Kuivila et al., 2012; Markle et al., 2014; Weston et al., 2011). Human beings have an increased risk of exposure to this compound (Lu et al., 2013; Morgan, 2012; Trunnelle et al., 2014). A U.S. population investigation of over 1000 children from 6 to 11 years old showed the higher detection rate (~71% population) of LCT metabolite 3-phenoxybenzoic acid with its children's median urinary levels of 1.2–2.2 ng/mL (Morgan, 2012). Usually, LCT is regarded to have less toxicity for mammalians. However, increasing studies have shown that LCT exposure in mammals might cause numerous toxic effects, including genotoxicity (Campana et al., 1999; Cavas and Ergene-Gozukara, 2003), neurotoxicity (Hornychova et al., 1995), and mutagenicity (Cavas and Ergene-Gozukara, 2003; Celik et al., 2003).

Several studies also found that LCT can cause reproductive toxicity as an endocrine disruptor. Al-Sarar et al. reported that adult male mice orally received 0.2, 0.4, and 0.8 mg/kg body weight/day for 6 weeks had a significant decrease in the weight of the seminal vesicle, an androgen-dependent organ, increase in the rate of Leydig cell degeneration, and reduction of sperm motility at the higher dose (Al-Sarar et al., 2014). Study by Yousef also demonstrated that adult male rabbits received 20 mg/kg LCT every other day via gavage for 16 weeks had significantly lower relative testis weight, relative epididymal weight, and lower serum testosterone levels and that these effects were possibly contributed by LCT-induced generation of reactive oxygen species (ROS) since supplement of vitamin E can restore some reproductive parameters to

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normal levels (Yousef, 2010). Apparently, these studies indicated that LCT can adversely affect Leydig cell function per se. However, whether effects of LCT on the development of Leydig cells are still unclear.

The amount of androgens secreted by Leydig cells not only depends on the steroidogenic capacity of the Leydig cell per se, but also on the number of Leydig cells in the whole testis. To achieve the mature level of Leydig cell number, this cell type experiences the proliferation and subsequent differentiation during puberty. Leydig cell development in rats can be conceptually divided into three stages: progenitor stage (at postnatal day 21), immature stage (at postnatal day 28), and adult stage (after postnatal day 56)(Ye et al., 2017). During the late-stage pubertal development from the immature stage, the Leydig cells not only double the cell number but also switch the immature androgen type (mainly androstanediol in the immature stage) to the mature androgen type (testosterone in adult Leydig cells) (Ye et al., 2017). During this stage of development, Leydig cell signaling protein, such as luteinizing hormone receptor (LHCGR), cholesterol-transporting proteins [scavenger receptor class B member 1 (SCARB1) and steroidogenic acute regulatory protein (StAR)], as well as steroidogenic enzymes [P450 cholesterol side chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1), cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), and 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3)] are also significantly upregulated (Li et al., 2016; Payne et al., 1997; Ye et al., 2011), while the androgen-metabolizing enzymes [steroid 5α-reductase 1 (SRD5A1) and 3α-hydroxysteroid dehydrogenase (3α-HSD)] are significantly downregulated (Ge and Hardy, 1998a). Interestingly, a glucocorticoid-metabolizing enzyme, 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1), begins to be expressed in the immature Leydig cells and onward. Therefore, it is a very specific biomarker of Leydig cells at the advanced stages.

In the current study, we systemically examined the effects of LCT on pubertal development of rat Leydig cells by investigating these Leydig cell parameters *in vivo* and *in vitro*.

2. Materials and methods

2.1. Materials and animals

Lambda-cyhalothrin (LCT) was purchased from Sigma-Aldrich (St Louis, MO). Immunohistochemical staining kit (Vectastain Elite, ABC kit, PK-6101) was purchased from Vector Laboratories (Burlingame, CA). SYBR Green qPCR Kit and BCA Protein Assay Kit were purchased from Takara (Otsu, Japan). Trizol Kit was purchased from Invitrogen (Carlsbad, CA). Immulite2000 Total Testosterone Kit and Estradiol Kit were purchased from Sinopharm Group Medical Supply Chain Services Co (Hangzhou, Zhejiang, China). Radio immunoprecipitation Assay buffer was obtained from Bocai Biotechnology (Shanghai, China). The manufacturers of antibodies were listed in Supplementary Table S1. Male Sprague-Dawley rats (21 days of age) were purchased from Shanghai Animal Center (Shanghai, China). The animal study was approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University and was performed in accordance with the Guide for the Care and Use of Laboratory Animals. The animal ethical approval number is wydw2016-0311.

2.2. Animal experiment

Twenty-four male Sprague-Dawley rats (21 days of age) were raised in a 12 h dark/light cycle at temperature of 23 ± 2 °C and relative humidity of 45%–55%. Water and food were accessed ad libitum. Rats were acclimated for a week before they were randomly assigned into 4 groups (6 animals per group): 0 (deionized water, group 1), 0.25 (group 2), 0.5 (group 3), and 1.0 (group 4) mg/kg body weight/day. LCT was dissolved in deionized water. Rats received either water or LCT by gavage. The doses were selected based on the previous studies (Abdallah et al., 2012; Ben Abdallah et al., 2013). Thirty days after administration, the rats were euthanized on postnatal day 58 by asphyxiation with CO_2. Trunk blood was collected, placed in a gel glass tube, and centrifuged at 2000 × *g* for 10 min to collect serum samples. Sera were labeled and stored at −80 °C until being analyzed for testosterone, LH and follicle-stimulating hormone (FSH) levels. Besides, each pair of testes were separated and weighted. One testis per animal was frozen in the liquid nitrogen for subsequent analysis of gene and protein levels. The contralateral testis and epididymis were fixed in Bouin's solution for the histochemical analysis.

2.3. Immunohistochemistry

One testis each rat was used for immunohistochemical staining according to the manufacturer's instructions. Six testes per group were embedded in paraffin as a tissue array as described before (Wu et al., 2017a). Avidin-biotin immunohistochemical staining for CYP11A1 (a general biomarker for all Leydig cells) or 11β-HSD1 (a specific biomarker for Leydig cells at immature and late stages) was conducted following manufacturer's instructions. Antigen retrieval was conducted by boiling the sections in 10 mM (pH 6.0) citrate buffer for 10 min. Then, endogenous peroxidase was blocked with 0.5% of H_2O_2 in methanol for 30 min. Sections were incubated with either CYP11A1 or 11β-HSD1 polyclonal antibody (diluted 1:200) for 1 h at room temperature. Diaminobenzidine was used for visualizing the antibody-antigen complexes, positively labeling Leydig cells by a brown mitochondrial staining (CYP11A1) or endoplasmic reticulum staining (11β-HSD1). Mayer hematoxylin was applied as the counterstaining. Non-immune rabbit IgG was used in the incubation of the negative control sections. Leydig cell number was calculated by a stereological method as below.

2.4. Counting Leydig cell number by a stereological method

To count CYP11A1 or 11β-HSD1-positive Leydig cell number, sampling of the testis was performed according to a fractionator method as previously described (Akingbemi et al., 2004). About ten sections were sampled from each testis per rat. The total number of Leydig cells was calculated by multiplying the number of Leydig cells counted in a known fraction of the testis by the inverse of the sampling probability.

2.5. Computer-assisted image analysis

The images of 8 randomly selected fields of three nonadjacent sections per testis were taken using a Leica DM5500B positive position automatic biological microscope (Germany) equipped with digital camera interfaced to a computer. The images that were displayed on the monitor represented a partial area of a testis. Cell size and nuclear size were estimated using an image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) as previously described (Wu et al., 2017b). Cytosolic size of a cell was calculated by the cell size minus the nuclear size. More than 50 Leydig cells were evaluated in each testis and averaged for each rat for the statistics of the cell size, nuclear size, and cytosolic size. The main area of cell and nucleus were generated by the software and sizes of cell and nucleus were calculated.