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The effects of chlormequat chloride on the development of pubertal male rats

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ABSTRACT

Chlormequat Chloride (CCC) is a plant growth regulator that is widely applied in agriculture. Previous studies have shown that long-term exposure of CCC could decrease body weight in animals. However, the underlying mechanisms have not been studied. In this study, CCC was administered to rats daily by gavage on postnatal days 23–60 at doses of 0, 75, 150 and 300 mg/kg bw/d. The results showed that body weight and the length of the right femur were significantly decreased in the 300 mg/kg bw/d group. Histological analysis of proximal growth plates of the right femurs showed narrowed proliferative zones and hypertrophic zones in CCC-treated groups. The mRNA expression of growth hormone, growth hormone receptor and insulin like growth factor 1 were decreased in the CCC-treated group. The results indicated that CCC may affect the expression of growth hormone and insulin-like growth factor 1 and subsequently cause a decrease in body weight and bone length.

1. Introduction

Chlormequat chloride (2-chloroethyltrimethylammonium chloride, CCC) is a widely used plant growth regulator that acts by inhibiting the biosynthesis of gibberellins in order to reduce unwanted shoot elongation without causing a loss of plant productivity (Henriksen et al., 2009; Li et al., 2012). It was reported that in 2002 nearly 2900 t of CCC was used for cereal crops in UK (Reynolds et al., 2004). The European Communities (EC) have established maximum residue limits (MRLs) for CCC in different cereals at 2 mg/kg for wheat, barley, and rye, 5 mg/kg for oat and 0.05 mg/kg for other cereals (Granby and Vahl, 2001). The European Food Safety Authority (EFSA) has reported that the agreed values of CCC are 0.04 mg/kg bw/day for the Acceptable Daily Intake (ADI), 0.04 mg/kg bw/day for the Acceptable Operator Exposure Level (AOEL), and 0.09 mg/kg bw/day for the Acute Reference Dose (ARfD) (EFSA Report, 2008). However, some agricultural products contain excessive residual CCC beyond the agreed values. The highest level of CCC in pears from South Africa was reported to be 5.5 mg/kg (Hau et al., 2000), beyond the Codex MRL of CCC for pears (3 mg/kg) (Codex Alimentarius Comission Report, 1999). Besides, residual CCC was also found in other food such as infant food, milk and noodles (Reynolds et al., 2004). In conclusion, the high level of residual CCC in agricultural products should be a concern.

Clinical symptoms of suicide with CCC are cholinergic crisis such as vomiting, increased salivation and cardiac arrest (Nisse et al., 2015). Acute exposure to CCC would also cause animals to exhibit salivation, lachrymation, malaise, and tremor. The oral LD50 of CCC is 522 mg/kg bw in rats and 589 mg/kg bw in mice (FAO Report, 2003). In a four-week study of toxicity in rats by gavage, it was shown that the main sign of CCC toxicity was reduced body weight gain and the No Observed Adverse Effect Level (NOAEL) was 150 mg/kg bw/d (Schilling et al., 1990). No special organ was significantly affected after short-term oral exposure of CCC in rats. In mutagenicity and long-term toxicity tests, CCC did not show any carcinogenic or genotoxic effects (EFSA Report, 2008). According to a report from the Food and Agriculture Organization of the United Nations (FAO) (Food and Agriculture Organization Report, 1997), when hamsters received CCC at 0, 25, 50, 100, 200, 300, or 400 mg/kg bw once on day 8 of gestation, malformations and delayed development were observed in the latter three treatment groups. However, another study using rabbits that received CCC at 0, 1.5, 3, 6, or 12 mg/kg bw on days 6–18 of gestation reported that only maternal toxicity, not developmental toxicity, was observed.

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Based on the results mentioned above, no clear evidence exists regarding the developmental toxicity of CCC.

Our previous study indicated that treating Sprague-Dawley (SD) rats on days 6–20 of gestation with CCC at 0, 75, 150, or 300 mg/kg bw per day by gavage resulted in no malformation in treated groups. However, the body weight of fetal rats from the high-dose group was significantly decreased compared with that of the control group. the protein expression of insulin-like growth factor 1 (IGF-I) was significantly decreased from fetal rats of the high-dose group compared with that of the control group. In addition, when SD rats on days 6–11 of gestation were dosed orally at the same levels, crown-rump length and head length of fetal rats were not apparently affected by treatment, suggesting that the developmental toxicity of CCC that led to retarded embryonic development by decreasing body weight may function at the late stage of pregnancy.

IGF-I belongs to the growth hormone/insulin-like growth factor axis (GH-IGFs), which is a determinant of the regulation of liner growth. GH-IGFs include the growth hormone system and the insulin-like growth factor system. The growth hormone system is made up of growth hormone (GH), growth hormone receptor (GHR), and growth hormone binding protein (GHBP). The insulin-like growth factor system is made up of IGF-I, insulin-like growth factor 2 (IGF-II), insulin-like growth factor 1 receptor (IGF-IR), insulin-like growth factor 2 receptor (IGF-IIR), and insulin-like growth factor binding proteins 1–6 (IGFBP1-6) (Giustina et al., 2008). GH and IGF-I, as the key components of the GH-IGFs, have pleiotropic effects on many tissues, including regulating cell proliferation, differentiation and promoting postnatal growth. After secreting from the anterior pituitary gland, circulating GH is transported to the liver and binds to its receptor GHR followed by the stimulation of a cascade of signaling events including JAK2-STAT pathway and the PI3K/Akt pathway, resulting in the synthesis of IGF-I, IGFB*P*-3, and the acid-labile submit (ALS) (Vottero et al., 2013; Waters and Brooks, 2011). IGF-I binds to IGFB*P*-3/5 and ALS, circulating as a 150-kDa complex in the blood (Kim et al., 2006). After transporting to target tissues, IGF-1 regulates cell proliferation and differentiation. Different proteins of the GH-IGFs begin to work at different stages of development. GH-deficient mice did not display apparent changes compared with normal mice until two weeks after birth (Ohlsson et al., 2009). The IGF-I-deficient phenotype in mice is first significant at E13.5 (Nakae et al., 2001). Based on the studies mentioned above, GH and IGF-I may have more effects in the late stages of embryonic development and postnatal development.

Connecting our previous observations that the toxic effects of CCC may work at the late stages of pregnancy with the temporal characteristics of GH and IGF-I, Whether CCC decreases body weight and causes growth retardation because of the decreased expression of GH and IGF-I are the main focus of this article.

2. Materials and methods

2.1. Animals

Fifty-two weanling male Sprague-Dawley rats weighting 50.0–60.0 g were supplied by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All rats were housed individually in polycarbonate cages and in a barrier system under controlled conditions of lighting (12/12 h light/dark cycle), temperature (20–26 °C), and relative humidity (40%–70%). Rats were given access to standard rodent chow and purified tap water *ad libitum*. All procedures were approved by the Animal Care Committee of Peking University.

2.2. Chemicals

CCC (99% pure, CAS: 999-81-5) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The melting point and temperature of decomposition was 236 °C with decomposition. The solubility was above 500 g/L at 20 °C in deionized water. In this study CCC was dissolved in purified water to concentrations of 0, 7.5, 15 and 30 mg/ml.

2.3. Animal treatment

SD rats were assigned to four groups (0, 75, 150 or 300 mg/kg bw/d) using an unbiased randomization procedure. CCC was administered to each rat daily by gavage on postnatal days 23–60; the gavage capacity was 1 ml/100 g bw. All rats were observed daily throughout the experimental period for clinical signs. Body weight and food utilization were recorded daily. On postnatal day 61, rats were weighed, anesthetized by isoflurane inhalation, and killed by withdrawing blood from the arteria femoralis after anesthesia. Necropsies were conducted on all rats, blood from the arteria femoralis was collected, and serum was prepared and frozen at −80 °C for further analysis. Organ weights were recorded for the brain, pituitary gland, liver, kidneys, spleen and testes. The right femur was dissected and the length was measured.

2.4. Serum chemistry

Acetylcholine (ACH), GH, and IGF-I in serum were analyzed by ELISA kits (Freemore, Beijing, China) according to the manufacturer's instructions. Cholinesterase (CHE), AST, and ALT were analyzed using a biochemical analyzer (HITACHI Automatic Analyzer 7020)

2.5. Blood analysis

Blood (40 μl) from the femoral artery was dissolved in 4 ml blood sample dilution buffer, followed by analysis using the automatic blood cell counter (Nihon Kohden Celltac 2).

2.6. Histology

Tissues including the brain, liver, spleen, kidneys, testis, and right femur from five randomly selected rats from each group were fixed in 4% formaldehyde solution and embedded in paraffin blocks. Tissue blocks were sectioned and stained with haematoxylin and eosin, followed by microscopic examination.

2.7. mRNA expression of GH, GHR and IGF-I

Total RNA was extracted from the pituitary gland and liver using TRIzol reagent (Invitrogen, San Diego, CA) based on the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg RNA using the reverse-transcriptase kit (Takara, Dalian, Liaoning, China). Real-time PCR (RT-PCR) was performed using SYBR Green PCR Master Mix (Takara, Dalian, Liaoning, China) according to the product manual. The primers used for RT-PCR are shown in Table 1. The relative expression of genes was measured using the $2^{-\triangle\triangle Ct}$ method.

2.8. Statistics

All statistical analyses were carried out using Statistical Package for Social Sciences for Windows (version 13.0; SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Statistical significance was tested by one-way ANOVA followed by homogeneity of variances test and Dunnett's *post hoc* test. If equal variance did