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Chlormequat chloride retards rat embryo growth *in vitro*

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ABSTRACT

Chlormequat chloride is the most widely used plant growth regulator in agriculture to promote sturdier growth of grain crops by avoidance of lodging. Therefore, human exposure to chlormequat chloride is very common, but its developmental toxicity has not been studied. Thus, we investigated the developmental toxicity of chlormequat chloride by applying rat whole embryo culture (WEC) model, limb bud micromass culture and 3T3 fibroblast cytotoxicity test. Chlormequat chloride at 150 μg/ml (0.93 mM) retarded the rat embryo growth without causing significant morphological malformations and at 500 μg/ml (3.1 mM) caused both retardation and morphological malformation of the embryos. However, the proliferation and differentiation of limb bud cells were not affected by chlormequat chloride at as high as up to 1000 μg/ml (6.2 mM) applied. This concentration of chlormequat chloride did not affect the cell viability as examined by 3T3 fibroblast cytotoxicity test either, suggesting that cellular toxicity may not play a role in chlormequat induced inhibition of rat embryo growth. Collectively, our results demonstrated that chlormequat chloride may affect embryo growth and development without inhibiting cell viability.

1. Introduction

Plant growth regulators are a family of chemicals widely used in agriculture to reduce the length but enlarge the straw of grain crops to prevent lodging and hence better and easy harvest. These chemicals also increase green color of the leaves and indirectly affect flowering without causing any noticeable malformations. In 1960s, a new type of plant growth regulator, chlormequat chloride, also known as chlorocholine chloride (CCC), was first noticed because of its capacity in reducing the height of wheat while increasing the diameter of its stem (Tolbert, 1960) and initiating the flowering of azalea (Stuart, 1961). Chlormequat chloride was also shown to affect the metabolism of gibberellins system in plants (Lockhart, 1962). Recently, more studies showed that the application of chlormequat chloride causes alterations in global DNA methylation levels during floral transition of azalea (Meijón et al., 2011). Increased global DNA methylation is one of the major potential mechanisms of epigenetic control of flowering in azalea (Meijón et al., 2009). However, it remains unclear whether chlormequat chloride alters global DNA methylation by disturbing the metabolism of phytohormoneor by influencing gibberellins system *via* epigenetic control. Nevertheless, the lack of information regarding the activity of chlormequat chloride in plants has not prevented it becoming one of the most widely used plant growth regulators around the world today. Chlormequat chloride absorbed by wheat foliage undergoes no significant metabolism. This is also true for animal kingdom, chlormequat chloride ingested by rats remains unchanged and 98% of it is excreted in its intact form within 46.5 h after ingestion (Blinn, 1967). In human body, chlormequat chloride has a similar biological fate. In a study, chlormequat chloride was administered orally at a dose of 1/2 Accepted Daily Intake (ADI) which was determined as 0.05 mg/kg.bw by WHO (Lu, 1995). After 46 h, all of the chlormequat chloride was eliminated from the body in its intact form (Lindh et al., 2011). Collectively, these studies suggest that chlormequat chloride is metabolized neither by plants nor by mammalians, hence chlormequat chloride exerts its action in its intact form. Chlormequat chloride exists in its non-target organisms. Chlormequat chloride was detected in the milk of lactating cows (Lampeter and Bier, 1970) and also in the pig serum by LC-MS/MS after the pigs were fed with chlormequat chloride (Poulsen et al., 2007). Because of the wide range of distributions of chlormequat chloride in non-target organisms, it is necessary to investigate its potential effects on human developmental process.

Nowadays, chlormequat chloride is mainly used in agricultural production of cereals to shorten and strengthen the stem to prevent the risk of lodging. Its application has also been extended to vegetables, fruits, and ornamental plants. Therefore, human beings are widely exposed to chlormequat chloride. Elevated concentrations of chormequat chloride in rural farmer's body was noticed when pesticides were applied in nearby field (Littorin et al., 2012). The levels of chlormequat chloride in the horticulture workers were increased substantially after work (Littorin et al., 2012). Besides occupational exposure, existence of chlormequat chloride in the ordinary population was also noticed. The mean chlormequat chloride levels in the 24-h urine samples collected

Abbreviations: IC50, 50% inhibition concentration; SRB, sulforhodamine B; TMS, total morphological score; VYS, visceral yolk sac; WEC, whole embryo culture.

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from hundred non-occupationally exposed individuals were determined to be about 4 ng/ml (Lindh et al., 2011). This is probably due to the widespread chlormequat chloride contamination in our food supply. For example, the maximum residue level (MRL) of chlormequat chloride in tomatoes has been set in European Union as 0.05 mg/kg, but reports indicated that, sometimes the residual chlormequat chloride could reach as high as 9 mg/kg (Xi et al., 2011). The researchers from UK reported that the concentration of chlormequat chloride in pears could reach as high as 11 mg/kg (Reynolds et al., 2004) despite EU MRL for pears was set at 3 mg/kg.

There was little knowledge about the biological effects that chlormequat chloride exert on physiological processes of animals until Danish scientists reported that cows displayed impaired reproduction, mainly impaired estrus, while being fed by grain from crop treated with chlormequat chloride (Sorensen and Danielsen, 2006).This is supported by a study that the epididymal spermatozoa of mice that were fed with chlormequat chloride-treated crops had compromised fertilizing competence *in vitro* (Torner et al., 1999). Importantly, the estimated intake of chlormequat chloride in pigs and mice mentioned above was 0.0023 mg/kg bw/day and 0.024 mg/kg bw/day, respectively, which are below the acceptable daily intake value of 0.05 mg/kg bw/day set by WHO (Lu, 1995). It was reported recently that the metabolisms of the offspring of the rats were affected if the rats were exposed to low dose pesticides including chlormaquat chloride during pregnancy (Nathalie et al., 2014). These together suggest that chlormequat chloride might exert its effect on non-target organisms at a low concentration.

Although the potential reproductive toxicity of chlormequat chloride was noticed, its developmental toxicity remains unclear. Thus, we studied the developmental toxicities of chlormequat chloride using rat whole embryo culture model, limb bud micromass model and 3T3 fibroblasts cytotoxicity test.

2. Materials and methods

2.1. Chemicals

Chlormequat chloride (≥99% pure, CAS: 999-81-5) and Poly-D-lysine (PDL) were purchased from Sigma Chemical Company (St Louis, Mo, USA). Dimethyl sulfoxide (DMSO), acridine orange (AO), neutral red and alcian blue were purchased from Amresco Company (Solon, Ohio, USA). Hoechst33342 was purchased from Invitrogen Company. TUNEL kit was from Roche Company (Indianapolis, IN, USA). Other analytical reagents were of analytical grade and purchased from Beijing Chemical Company (Beijing, China). Chlormequat chloride was dissolved in Hank's solution or PBS for storage before applied to the culture medium.

2.2. Experimental animals

Virgin female and adult males of Sprague-Dawley (SD) rats were supplied by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All rats were housed under controlled conditions of temperature (20–26 °C), humidity (40–70%), and illumination (12/12 h light/dark cycle). Standard rodent chow (Chinese Academy of Medical Science, China) and autoclaved tap water were freely available. Female and male rats were mated overnight in a ratio of 2:1 and the presence of sperm was confirmed following morning by vaginal smear. The day that sperms were detected was considered to be the gestation day (GD) 0. All the experimental procedures with animals were approved by the Institutional Animal Care and Use Committee of Peking University according to the government guidelines for animal care.

2.3. Determination of exposure concentrations of chlormequat chloride

The value of chlormequat chloride in the serum of pigs fed at a dose equals to European ADI was detected as 5 ng/ml (Poulsen et al., 2007). This value was expanded 1000 times (safety factor), considering the differences between species and individuals. So the dose of 5 μg/ml (0.031 mM) was applied as the lowest dose in pilot experiments to determine the dose range in this study. The final exposure concentrations of chlormequat chloride in WEC test were determined as 0, 50, 150, 500, 1000 μg/ml (equals to 0, 0.31, 0.93, 3.1, and 6.2 mM) according to the regulations of INVITTOX Protocols No. 123.

2.4. Rat whole embryo culture (WEC) test

2.4.1. WEC test

In vitro post-implantation whole embryo culture was performed according to the method previously described (New, 1978; Xing et al., 2010) and INVITTOX Protocols No. 123. Briefly, on day 9.5 of gestation, female rats were sacrificed after anesthetization. The gravid uteri were removed from dams and transferred into sterile Hank's solution (pH 7.2). Maternal decidua tissue was removed, and complete visceral yolk sac (VYS) was harvested. The embryos displaying three to five somites were selected and randomly divided into different groups. Culture medium is 100% male rat serum, which was immediately centrifuged, heat inactivated (56 °C for 30 min), 0.2 μm filter sterilized, and supplemented with 100 μ/ml penicillin G and 100 μg/ml streptomycin. The embryos were then cultured for 48 h at 37 ± 0.5 °C in sealed 50-ml glass bottles (four embryos per bottle, one embryo per milliliter culture medium) and rotated at 30 rpm. The culture medium was initially gassed for 2.5 min with a mixture of 10% O_2: 5% CO_2: 85% N_2. Subsequent gassing for 2.5 min duration occurred at 16 h (20% O_2:5% CO_2:75% N_2) and 26 h (40% O_2:5% CO_2:55% N_2). The different type of gas mixtures was premixed and prepared commercially.

For the teratogenicity evaluation, chlormequat chloride at different concentrations was added into the culture medium at the beginning of the culture. The final concentrations of chlormequat chloride were 0, 50, 150, 500, 1000 μg/ml (equals to 0, 0.31, 0.93, 3.1, and 6.2 mM).

2.4.2. Growth and morphology assessment

The embryos were cultured for 48 h and transferred to a Petri dish with Hank's solution for evaluation under a stereomicroscope. Only viable embryos with yolk sac circulation and heartbeat were examined. Embryonic morphology and growth assessment were conducted according to a standard scoring system established by Brown and Fabro (Brown and Fabro, 1981). The system gave a numerical score (0–4) to 16 morphological parameters which include embryonic flexion, heart, tail neural tube, cerebral vesicles (fore-, mid-, and hindbrain), visual organs, auditory organs, olfactory organs, branchial arch, maxillary and mandibular processes, limb buds (forelimb and hindlimb), yolk sac circulation and allantois. A total morphological score (TMS), the sum of the scores of parameters listed above, was presented as a general morphological parameter and was used as an end point-value in prediction model of embryo toxicity (Table 1). Two Prediction Models (PM1 and PM2) were developed by ECVAM for the WEC test to classify chemicals into 3 categories: if the result of Function I exceeds the results of Function II and III, the chemical is classified non-embryotoxic; if the result of Function II exceeds the results of Function I and III, the chemical is classified weak-embryotoxic; if the result of Function III exceeds the results of Function I and II, the chemical is classified strong-embryotoxic. The total somite number was counted and recorded as another parameter for morphological assessment. Besides, diameter of the VYS, crown-rump length, and head length of each embryo were measured under the stereomicroscopes as growth parameters.