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Acute oral toxicity of 3-MCPD mono- and di-palmitic esters in Swiss mice and their cytotoxicity in NRK-52E rat kidney cells

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ABSTRACT

The acute oral toxicity of 1-palmitoyl-3-chloropropanediol (3-MCPD 1-monopalmitate) and 1,2-bis-palmitoyl-3-chloropropanediol (3-MCPD dipalmitate) in Swiss mice were examined, along with their cytotoxicity in NRK-52E rat kidney cells. LD_{50} (median lethal dose) value of 3-MCPD 1-monopalmitate was determined 2676.81 mg/kg body weight (BW). The results showed that 3-MCPD 1-monopalmitate dose-dependently decreased the mean body weight, and caused significant increase of serum urea nitrogen and creatinine in dead mice compared to the control and survived mice. Major histopathological changes in mice fed 3-MCPD 1-monopalmitate were renal tubular necrosis, protein casts and spermatids decrease in the seminiferous tubules. According to the limit test for 3-MCPD dipalmitate, LD_{50} value of 3-MCPD dipalmitate was presumed to be greater than 5000 mg/kg BW. Obvious changes were not observed on mean body weight, absolute and relative organ weight or serum urea nitrogen and creatinine levels in mice fed 3-MCPD dipalmitate. However, renal tubular necrosis, protein casts and spermatids decrease were also observed in the dead mice. In addition, MTT and LDH assay results only showed the cytotoxicity of 3-MCPD 1-monopalmitate in NRK-52E rat kidney cells in a dose-dependent manner. Together, the results indicated a greater toxicity of 3-MCPD 1-monopalmitate compared to 3-MCPD dipalmitate.

1. Introduction

Fatty acid esters of 3-chloro-1,2-propanediol (3-MCPD), a known carcinogen, have been detected in many foods, including bread, coffee, refined vegetable oils, infant formula, salty crackers, dark malt, French fries, doughnuts, pickled olives and herrings (Hamlet and Sadd, 2004; Pudel et al., 2011; Svejkovská et al., 2004; Weißhaar, 2011; Zelinková et al., 2009; Zelinková et al., 2006). In 2008, esters of 3-MCPD were also detected in human breast milk at a concentration from below 300 to 2195 μg/kg fat or 6–76 μg/kg milk, indicating that 3-MCPD esters could be absorbed and distributed to human tissues and organs (Zelinková et al., 2008). This has raised a food safety concern assuming that 3-MCPD esters might be converted to free 3-MCPD which is a common food contaminant (Weißhaar, 2011) and may cause toxic effects such as nephrotoxicity and carcinogenesis.

A few recent studies have investigated the potential toxicity of 3-MCPD esters using *in vitro* and *in vivo* approaches (EFSA, 2011; Tee et al., 2011). For instance, Tee et al. (2011) evaluated cytotoxicity of 1-palmitoyl, 1-steroyl, 2-oleoyl and 1-palmitoyl-2-oleoyl-3-chloropropanediol using agarose overlay and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) methods. The results suggested that 1-palmitoyl and 1-steroyl-3-chloropropanediol had cellular toxicity at 200,000 ppm level, with 50% inhibitory concentrations (IC_{50}) of 70,000 and 31,000 ppm, respectively, while 2-oleoyl-3-chloropropanediol showed a toxic effect at 781 ppm treatment concentration with a IC_{50} value of

Abbreviations: 3-MCPD, 3-chloro-1,2-propanediol; BW, body weight; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EU, European Union; GHS, Globally Harmonized Hazard Classification and Labelling Scheme; H&E, hematoxylin and eosin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, 50% inhibitory concentration; LD_{50}, median lethal dose; LDH, lactate dehydrogenase activity; OECD, Organization for Economic Cooperation and Development; SEM, standard error of mean.

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520 ppm, suggesting that both substitution location and the type of fatty acid might alter the cellular toxicity of 3-MCPD monoesters. Also reported from this research was that 1-palmitoyl-2-oleoyl-3-chloropropanediol showed no cytotoxic effect at the concentration of 24.41–781 ppm under the same experimental condition, suggesting that introducing the 1-palmitoyl reduced the toxicity of 2-oleoyl-3-chloropropanediol. In another words, diesters might have lower toxicity than its counterpart 2-fatty acid monoester. Tee et al. (2011) also fed Sprague–Dawley rats (n = 5/sex/group) with a single dosage of 1-palmitoyl, 1-steroyl, 2-oleoyl or 1-palmitoyl-2-oleoyl-3-chloropropanediol at 50, 200 and 400 mg/kg for 14 days, and found out that the four 3-MCPD esters had no toxic effects measured as mortality, changes in body and organ weight, or clinical observation under the experimental conditions. Furthermore, the median lethal dose (LD_{50}) of 3-MCPD esters was determined to be greater than 400 mg/kg.

Another 90-day toxicological study using 10 male and female Wistar rats compared free 3-MCPD and its di-palmitic esters on an equimolar dosage basis for their effects on urinary excretion of metabolites (EFSA, 2011). Urinary metabolites in the dipalmitate fed rats were about 30% lower than that of the 3-MCPD fed groups, reflecting a significant bioavailability of 3-MCPD from orally given 3-MCPD dipalmitate and the significant metabolism of the diester. The study also found out that the kidney and testes in male rats might be the critical organs for the dose-dependent 3-MCPD dipalmitate toxicity (EFSA, 2011). Taking together, these previous studies confirmed the potential toxic effects of 3-MCPD mono- and di-fatty acid esters, their primary toxic organs, and the possible influence of fatty acids on their toxic activities, warranting future research to further determine the LD_{50} values of individual mono- and di-fatty acid esters of 3-MCPD, and estimate their toxic degrees according to the Globally Harmonized Hazard Classification and Labelling scheme (GHS) or the European Union (EU) classification system based on their LD_{50} values.

In the present study, 1-palmitoyl-3-chloropropanediol (3-MCPD 1-monopalmitate) and 1,2-bis-palmitoyl-3-chloropropanediol (3-MCPD dipalmitate) were evaluated for their acute oral toxicity in Swiss mice and cytotoxicity in rat kidney NRK-52E cells. LD_{50} values were measured for the 3-MCPD 1-monopalmitate and dipalmitate to determine their GHS and EU hazard degrees. The histopathological examinations were also performed for kidney, testes, liver, thymus and spleen tissues, along with serum urea nitrogen and creatinine measurements. In addition, cytotoxicities of 3-MCPD 1-mono- and di-palmitates were examined in NRK-52E rat kidney cells using the MTT and lactate dehydrogenase (LDH) activity assays. Free 3-MCPD was included as a reference in the cell-based study. This would be the first report of the LD_{50} values for 3-MCPD 1-monopalmitate and dipalmitate.

2. Materials and methods

2.1. Chemicals and reagents

3-MCPD 1-monopalmitate (CAS 30557-04-1,1-palmitoyl-3-chloropropanediol, purity > 98%) was provided by Prof. Tao Lu (China Pharmaceutical University, Nanjing, China). 3-MCPD dipalmitate (CAS 51930-97-3, 1,2-bis-palmitoyl-3-chloropropanediol, purity > 98%) was provided by Prof. Tiankui Yang (Dalian University of Technology, Dalian, China). The 3-MCPD 1-mono- and di-palmitates were confirmed for their chemical structures and purity using ^{1}H, ^{13}C NMR and GC–FID methods. 3-MCPD (CAS 96-24-2, purity > 98%) was purchased from Shanghai Darui finechemical Co. Ltd. (Shanghai, China).

2.2. Acute oral toxicity study in Swiss mice

2.2.1. Animals and care

Male and female Swiss mice were received at approximately 4 weeks of age (14–16 g) from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). Animals were housed in stainless steel cages in a temperature controlled room maintained on a 12 h light/dark cycle, and were allowed free access to drinking water and standard diet. They were acclimatized for 5 days prior to the initiation of the test. Male and female Swiss mice were fasted for 4 h before the experiment to eliminate feed from gastro-intestinal tract. A single-dose study was conducted employing 4-week old (18–22 g) mice of Swiss strain. Mice were fasted for another 1 h after giving 3-MCPD fatty acids esters.

2.2.2. LD_{50} test

Acute oral toxicity of 3-MCPD 1-monopalmitate was determined by estimating the LD_{50} value. Mice were randomly divided into six groups (untreated control, treatment I-1000 mg/kg body weight (BW), treatment II-1428 mg/kg BW, treatment III-2040 mg/kg BW, treatment IV-2914 mg/kg BW, and treatment V-4162 mg/kg BW) consisting of 10 animals (n = 5/sex/group). The treatment levels were selected according to the preliminary experiments. 3-MCPD 1-monopalmitate was dissolved in a vehicle of blended edible oil, and a dosing volume of 0.6 ml/20 g BW was used by oral gavage. Mice in the control group received oral gavage administration of vehicle only (0.6 ml/20 g BW).

2.2.3. Limit test

Acute oral toxicity of 3-MCPD dipalmitate was determined following the Organization for Economic Cooperation and Development (OECD) guideline for testing of chemicals, OECD TG420 (OECD, 2001), since it showed very low acute oral toxicity in the preliminary test. This method is commonly used to efficiently test chemicals with low toxicity. Thirty Swiss mice were divided into two groups: the control group (n = 5/sex/group) and the test group (n = 10/sex/group). 3-MCPD dipalmitate was dissolved in the blended edible oil vehicle and administered to each mouse at 0.6 ml/25 g BW by oral gavage for a dose level of 5000 mg/kg BW. The mice in the control group received oral gavage administration of same volume of vehicle only.

2.2.4. Body weights and objective observation

All the mice including those in the control groups were observed for 14 days. Body weights were recorded on a daily basis. Animals were observed closely for any sign of toxicity, including morbidity and mortality. Signs included changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity.

2.2.5. Serum biochemistry

On day 15, blood samples were collected from the retro orbital sinus. Blood samples were collected in 1.5 ml micro test tube (CNW, Shanghai, China) and serum was obtained by centrifugation at 3500 rpm for 10 min at 4 °C. Serum was immediately subjected to clinical biochemistry determinations for urea nitrogen and creatinine using an Olympus AU640 autoanalyzer (Tokyo, Japan).

2.2.6. Histology

On day 15, mice were sacrificed by cervical dislocation and then a complete gross necropsy was performed. Liver, kidney, thymus, spleen, and testis (or ovary) were collected and weighed. Liver, kidney, thymus and spleen of 50% mice in the control and test groups were fixed in 10% neutral buffered formalin, while testis and ovary were fixed in the Bouin's solution (Cho et al., 2008a). They were trimmed for embedding in paraffin and sectioned (5 μm), and then stained with hematoxylin and eosin (H&E) (Cho et al., 2008a). Histopathological changes were evaluated under an Olympus DP72 microscope (Tokyo, Japan).

2.3. Cytotoxicity study in NRK-52E rat kidney cells

2.3.1. Cells culture

NRK-52E rat kidney cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences), derived from normal rat renal proximal tubular cell line, were cultured in DMEM (Dulbecco's Modified Eagle's Medium) (GIBCO BRL, Carlsbad, California, USA) containing 4 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 5% (v/v) bovine calf serum (GIBCO BRL, Carlsbad, California, USA), 100 U/ml of penicillin and 100 U/ml of streptomycin (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) in a humidified incubator (37 °C, 5% CO_2).

2.3.2. MTT assay for cell viability

MTT assay was performed to determine effects of 3-MCPD 1-monopalmitate, 3-MCPD dipalmitate on NRK-52E cell viability. Free 3-MCPD was included for comparison. Briefly, NRK-52E cells were seeded on 96-well plates at an initial density of 2×10^4 cells/ml for 24 h, followed by incubation with different concentrations of 3-MCPD 1-monopalmitate, 3-MCPD dipalmitate or 3-MCPD for 48 h. At 4 h before the end of incubation, 20 μl of MTT (5 mg/ml in PBS) (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) was added to each well, and incubated for 4 h. After the medium was removed at the end of incubation, 150 μl of DMSO (dimethylsulfoxide) was added to each well, and shaken at ambient temperature for 10 min until the absorbance was measured at 490 nm using an Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). The inhibition rate (%) to reflect the cell viability was calculated as:

$$\text{Inhibition Rate } (\%) = 100 \times (A_{\text{vehicle}} - A_{\text{treatment}})/A_{\text{vehicle}}$$