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The toxicity of 3-chloropropane-1,2-dipalmitate in Wistar rats and a metabonomics analysis of rat urine by ultra-performance liquid chromatography–mass spectrometry

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ABSTRACT

3-Monochloropropane-1,2-diol(3-MCPD) fatty acid esters can release free 3-MCPD in a certain condition. Free 3-MCPD is a well-known food contaminant and is toxicological well characterized, however, in contrast to free 3-MCPD, the toxicological characterization of 3-MCPD fatty acid esters is puzzling. In this study, toxicological and metabonomics studies of 3-chloropropane-1,2-dipalmitate(3-MCPD dipalmitate) were carried out based on an acute oral toxicity test, a 90-day feeding test and ultra-performance liquid chromatography–mass spectrometry (UPLC–MS) analysis. The LD_{50} value of 3-MCPD dipalmitate was determined to be 1780 mg/kg body weight (bw) for Wistar rats. The results of the 90-day feeding test in male Wistar rats showed that 3-MCPD dipalmitate caused a significant increase in blood urea nitrogen and creatinine in the high-dose group (267 mg/kg bw/day) compared to control rats. Renal tubular epithelium cell degeneration and renal tubular hyaline cast accumulation were the major histopathological changes in rats administered 3-MCPD dipalmitate. Urine samples obtained after the 90-day feeding test and analyzed by UPLC–MS showed that the differences in metabolic profiles between control and treated rats were clearly distinguished by partial least squares-discriminant analysis (PLS-DA) of the chromatographic data. Five metabolite biomarkers which had earlier and significant variations had been identified, they were first considered to be the early, sensitive biomarkers in evaluating the effect of 3-MCPD dipalmitate exposure, and the possible mechanism of these biomarkers variation was elucidated. The combination of histopathological examination, clinical chemistry and metabolomics analyses in rats resulted in a systematic and comprehensive assessment of the long-term toxicity of 3-MCPD dipalmitate.

1. Introduction

3-Monochloropropane-1,2-diol(3-MCPD) is a well-known food contaminant, and has been detected in a wide range of foods and ingredients. It was first identified in foods prepared using acid-hydrolyzed vegetable proteins (acid-HVP) [1]. Toxicological studies have shown that 3-MCPD has carcinogenicity in rats and genotoxic activity *in vitro* [2–3]. Based on these studies, a provisional maximum tolerable daily intake (TDI) of 2 μg/kg body weight has been established by the Scientific Committee on Food of the European Union [4]. Subsequent studies have demonstrated that 3-MCPD is not only present in typical acid-HVP-containing foods, such as soy sauce and related products, but is also found in a wide range of retail outlet and home-made foods as well as in various food ingredients formulated without the addition of acid-HVP [5–11]. Therefore, the mechanism of 3-MCPD formation is not fully understood. Several studies showed that in heat-processed fat-containing foodstuffs with low water, 3-MCPD was formed from glycerol or/and acylglycerols and chloride anions [12,13]. These studies have also led to the assumption that 3-MCPD is formed from triacylglycerides in the presence of chloride ions under acidic conditions, where a chloride anion may replace an acyl group by a nucleophilic attack on a carbon atom which is

Abbreviations: 3-MCPD, 3-monochloropropane-1,2-diol; acid-HVP, acid-hydrolyzed vegetable proteins; TDI, tolerable daily intake; 3-MPCD dipalmitate, 3-chloropropane-1,2-dipalmitate; UPLC, ultra performance liquid chromatography; MS, mass spectrometry; LD_{50}, median lethal dose; Cr, creatinine; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HE, hematoxylin–eosin; ESI-TOF-MS, electrospray ionization time-of-flight mass spectrometry; RT, retention time; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; BPI, base peak intensity; VIP, Variable Importance in the Project; IDO, indolamine-2,3-dioxygenase.

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activated by neighboring ester groups [7,14]. Further acid-mediated ester hydrolysis may then lead to the release of free 3-MCPD. Seefelder et al. [15] demonstrated using intestinal lipase as a substrate *in vitro*, that the yield of 3-MCPD from 3-MCPD monoesters was greater than 95% in approximately 1 min, and that the release from diesters was slower, reaching about 45%, 65% and 95% of 3-MCPD after 1, 5 and 90 min of incubation, respectively.

Recent findings have shown that only a small fraction of food-borne 3-MCPD is present as free 3-MCPD, and in much larger amounts is mostly esterified with long-chain (C_{14}–C_{18}) fatty acids [12]. These 3-MCPD-esters are also known as bound 3-MCPD. The occurrence of high levels of 3-MCPD-esters (monoesters and diesters) in some foods, such as vegetable oils [15,16], baby foods [17] and coffee [11], has been reported. However, only a few recent studies have investigated the potential toxicity of 3-MCPD fatty acid esters using *in vivo* and *vitro* approaches [18], and in contrast to free 3-MCPD, the toxicological characterization of 3-MCPD fatty acid esters is puzzling.

Considering that diesters were identified as the predominant form of bound 3-MCPD (in the case of edible oils, 3-MCPD monoesters do not exceed 15%) [15], in this study, we examined the toxicity of 3-MPCD dipalmitate using acute oral toxicity testing in Wistar rats. As a result, the LD_{50} value of this substance in Wistar rats was proposed for the first time. The long-term toxicity of 3-MPCD dipalmitate which was administered orally in different doses in feedstuff for 90 days was also examined in male Wistar rats. Histopathological examinations were performed on the kidney, testes, liver and brain of rats exposed to 3-MCPD dipalmitate, along with clinical chemistry measurements. In addition, we used an ultra-performance liquid chromatography (UPLC) system combined with electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) and multivariate statistical analysis to determine changes in metabolic profiles in the urine of 3-MCPD dipalmitate-treated Wistar rats and control rats. The structures of 3-MCPD and 3-MCPD dipalmitate were showed in Scheme 1.

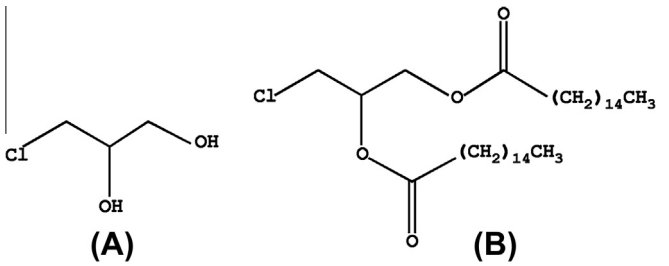
2. Materials and methods

2.1. Chemicals and reagents

3-MCPD dipalmitate (purity >95%) was obtained from Changchun Discovery Sciences Co. Ltd. (Changchun, China). The assay kits for serum creatinine (Cr), blood urea nitrogen (BUN), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) standards were purchased from Roche Technology and Science Inc. (Shanghai, China). HPLC grade methanol, acetonitrile and formic acid were purchased from Dikma Technologies Inc. (Lake Forest, USA). Distilled water was filtered using the Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Animal treatment

The experiments were performed on SPF grade Wistar rats weighing 180–220 g. A total of 50 rats (16 rats in the acute toxicity test and 34 rats in the long-term toxicity test) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were kept at a controlled humidity (50–60%) and temperature (22 ± 2 °C) with a 12 h light–dark cycle. Before treatment, maintenance diets for rodents and distilled water were provided ad libitum, and all rats were allowed to acclimatize in communal plastic cages for 5 days followed by metabolic cages for 3 days. Animal handling was approved by the Institute of Zoology Animal and Medical Ethics Committee and was in accordance with the current Chinese legislation.



Scheme 1. Structures of 3-MCPD (A) and 3-MCPD dipalmitate (B).

2.2.1. Acute toxicity test

After acclimatization, the 16 Wistar rats were randomly divided into four groups (each group included two males and two females). The dose of 3-MCPD dipalmitate in these groups was 3160, 1000, 316 and 100 mg/kg bw/day, respectively. 3-MCPD dipalmitate was diluted with soybean oil, and was given to rats by oral gavage. Symptoms of toxicity in the treated rats were closely observed and mortality as well as time of death was recorded.

2.2.2. Long-term toxicity test

34 male Wistar rats were housed individually in metabolic cages after acclimatization and were randomly divided into three groups: the high-dose group ($n = 12$), which received 3-MCPD dipalmitate at 267 mg/kg bw/day; the low-dose group ($n = 12$), which received 12.3 mg/kg bw/day; and the control group ($n = 10$). 3-MCPD dipalmitate was continuously administered to rats in the treatment groups for 90 days by incorporation into the maintenance diet. The amount of 3-MCPD dipalmitate in the diet in each group was adjusted once a week according to the weight of rats. Control rats were given approximately the same weight of diet only. After 90 days of treatment, all rats were sacrificed by exsanguination from the abdominal aorta under 2% pentobarbital sodium anesthesia via intraperitoneal injection.

The following methods (Section 2.3–2.6) were used in the long-term toxicity test only.

2.3. Sample collection and preparation

Urine samples were collected each week from rats in the metabolic cages over 24 h to avoid the effects of diurnal variation on urine metabolite profiles. All samples were centrifuged at 12,000 rpm for 10 min, and the supernatants were collected and stored at −80 °C for UPLC–MS analysis.

Blood samples were collected on the 20th, 40th, 60th, 80th and 90th day for clinical chemistry testing. The blood samples on the 20th, 40th, 60th and 80th day were collected from an incision in the rats' tail tip, while the blood samples on the 90th day were obtained from the abdominal aorta before sacrifice. All blood samples were drawn and allowed to clot, and serum was then obtained by centrifugation at 3000 rpm for 15 min. The serum supernatants were immediately analyzed using an Autolab-PM7100 automated biochemical analyzer (HITACHI, Tokyo, Japan) to test for AST, ALT, BUN and Cr.

A complete necropsy was performed on all animals on day 90. The testes, liver, brain, spleen and kidneys of the sacrificed rats were excised, dissected free of connective tissue and fat, weighed and fixed in buffered 10% formalin.

2.4. Histopathology

The formalin-fixed organs were processed in wax blocks and serial transverse sections were prepared. For each organ, individual sections were stained with hematoxylin–eosin (HE) and examined by light microscopy.