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Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin

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ABSTRACT

Moniliformin is a *Fusarium* mycotoxin highly prevalent in grains and grain-based products worldwide. In this study, the acute oral toxicity of moniliformin was assessed in Sprague–Dawley male rats according to OECD Guideline 423 with a single-dose exposure. Clinical observations and histopathological changes were recorded together with the excretion of moniliformin via urine and feces, utilizing a novel liquid chromatography–mass spectrometry method.

According to our study, moniliformin is acutely toxic to rats with a rather narrow range of toxicity. Moniliformin can be classified into category 2 (LD_{50} cut-off value 25 mg/kg b.w.), according to the Globally Harmonized System for the classification of chemicals. The clinical observations included muscular weakness, respiratory distress and heart muscle damage. Pathological findings confirmed that heart is the main target tissue of acute moniliformin toxicity. A significant proportion (about 38%) of the administered moniliformin was rapidly excreted in urine in less than 6 h. However, the toxicokinetics of the majority of the administered dose still requires clarification, as the total excretion was only close to 42%. Considering the worldwide occurrence of moniliformin together with its high acute toxicity, research into the subchronic toxicity is of vital importance to identify the possible risk in human/animal health.

1. Introduction

Mycotoxins, secondary metabolites produced by filamentous fungi, are a worldwide problem, especially in grains and grain-based products. Mycotoxins evoke a broad range of toxic effects, including carcinogenicity, neurotoxicity, immunotoxicity, as well as reproductive and developmental toxicity, leading to possible adverse effects on both humans and animals. As a result of modern risk management systems in the developed countries, disease outbreaks caused by mycotoxins have occurred very infrequently.

Fusarium is the most prevalent fungal genus infecting small-grain cereals in temperate regions of the world. Most *Fusarium* species are capable of producing a variety of mycotoxins. The most commonly studied *Fusarium* mycotoxins include trichothecenes, zearalenone and fumonisins. However, nearly all of the most prevalent *Fusarium* species universally infecting grains are also apparently capable of producing other toxic metabolites, such as moniliformin (MON) produced by *Fusarium avenaceum*, *Fusarium subglutinans* and *Fusarium proliferatum* (Jestoi et al., 2009). The worldwide natural occurrence of MON has been reviewed in a number of studies (Jestoi, 2008; Jestoi et al., 2009; Peltonen et al., 2010). The highest concentrations have been observed in maize, whereas in small-grain cereals the levels detected have usually been markedly lower. Due to the worldwide distribution of natural MON contamination, humans and livestock might be daily exposed to this mycotoxin. However, the significance of this exposure is currently unclear, as little information is available on the toxicity, absorption, distribution, metabolism or excretion of

Abbreviations: b.w., body weight; ESI, Electrospray ionization; GHS, Globally Harmonized System; HILIC, hydrophilic interaction chromatography; HT-2, HT-2 Toxin; LD_{50}, dose lethal to 50% of animals; LOD, limit of detection; LOQ, limit of quantification; MON, moniliformin; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; OECD, Organisation for Economic Cooperation and Development; T-2, T-2 Toxin; TCA cycle, tricarboxylic acid cycle; UPLC, ultra performance liquid chromatography; Q-Tof, quadrupole-time-of-flight.

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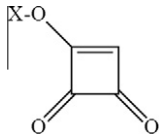

Fig. 1. The chemical structure of moniliformin. X = H (free acid), Na (sodium salt) or K (potassium salt).

MON after oral administration. There is consequently a need for further assessment of MON toxicity.

MON has not been associated with fatal animal disease outbreaks, but has been highly toxic in experimental settings involving birds and rodents (Nagaraj et al., 1996; Abbas et al., 1990; Kriek et al., 1977). The acute toxicity of MON is reported to be comparable to the most toxic trichothecenes, T-2 and HT-2 (Burmeister et al., 1979,1980; Ueno, Y., 1983). It has also been suggested that MON may be one of the factors associated with two endemic diseases in China: Keshan disease, an endemic cardiomyopathy (Zhu et al., 1982) and Kashin-Beck disease, a chronic deformative osteoarthropathy (Cao et al., 2007). However, subsequent studies have demonstrated that the association between MON contamination and Keshan disease is not very strong (Yu et al., 1995). On the other hand, MON has been found to inhibit the synthesis of aggrecan and type II collagen in human chondrocytes, stimulating a pro-catabolic effect on the metabolism of articular cartilage (Zhang et al., 2010). The findings do not provide any cause-effect relation, but do indicate the potential association between MON and Kashin-Beck disease.

MON exists as a sodium or potassium salt of the semisquaric acid (Fig. 1) and it is structurally related to pyruvate. The exact mechanism of toxicity and the toxicokinetics of MON are still unclear. It has been proposed that the mode of action in acute toxicoses is inhibition of the oxidation of tricarboxylic acid (TCA) cycle intermediates, resulting in respiratory stress and myocardial degeneration, and even in the mortality of experimental animals (Kriek et al., 1977; Thiel, P.G., 1978; Burka et al., 1982; Gathercole et al., 1986; Nagaraj et al., 1996; Morgan et al., 1999; Engelhardt et al., 1989).

It has been hypothesized that MON substitutes pyruvate, because of the structural similarity of the two molecules, by inhibition of the physiological function of thiamine pyrophosphate-dependent enzymes (Pirrung and Nauhaus, 1996). This would result in the obstruction of pyruvate incorporation into the TCA cycle. MON may also interfere with carbohydrate metabolism through the inhibition of gluconeogenesis and aldose reductase (Deruiter et al., 1993). Furthermore, oxidative damage in myoblasts has been demonstrated, possibly due to the inhibition of glutathione peroxidase and glutathione reductase by MON (Chen et al., 1990).

In this study we determined the acute oral toxicity of MON according to Guideline 423 of the Organisation for Economic Cooperation and Development (OECD). In addition to estimation of the level of acute toxicity, the approach enables the evaluation of possible target organs and clinical symptoms. The use of metabolic cages allowed us to assess MON excretion in urine and feces. Information on the acute toxicity of MON can also be utilized in a dose-finding trial for subacute toxicity studies.

2. Materials and methods

2.1. Chemicals

Synthetic potassium salt of MON, used in *in vivo* settings, was provided by Prof. Ilkka Kilpeläinen, University of Helsinki. The structure of MON was verified with NMR and MS and the purity of the mycotoxin was accordingly demonstrated to be >99%.

Acetonitrile, methanol, potassium chloride and formic acid of HPLC grade were purchased from J.T. Baker (Deventer, The Netherlands). Ammonium formate of *p.a.* quality and leucine enkephalin were obtained from Sigma–Aldrich (Munich, Germany). Water was purified using a Milli-Q Plus system (Millipore, Espoo, Finland). Analytical MON standard was purchased from Sigma (St. Louis, MO, USA; Cat. No. M5269) and a 100 μg/mL stock solution of MON in methanol was prepared.

2.2. Experimental procedure

The *in vivo* toxicological properties of MON were investigated according to OECD Guideline 423, consisting of a single-dose 14-day acute oral toxicity study. The acute toxicity study described in OECD Guideline 423, is a stepwise procedure with the use of 3 animals of a single sex per step. The range of acute toxicity of the test substance depends on the mortality and/or moribund status of the animals. Two to four steps may be necessary to determine the toxicity category (Fig. 2).

Male Sprague–Dawley rats obtained from Harlan Laboratories Inc. (Horst, The Netherlands) were used. The animals were 9–10 weeks old and weighed 211–302 g at the beginning of the experiments. The rats were acclimatized to the laboratory conditions and metabolic cages for one week prior to administration and were judged healthy before testing. The animals were housed individually in metabolic cages for rats (Techniplast 3701M081, UK). The study rooms were maintained under controlled conditions appropriate for the species (temperature 22 ± 2 °C; relative humidity 45–65%), with a reversed photoperiod of 12 h light. Standard, ionized rodent diet (Tekland 2916, Harlan, Indianapolis, IN, USA) and filtered tap water were available *ad libitum*.

According to the Globally Harmonized System (GHS) for the classification of chemicals that cause acute toxicity, a low dose of 5 mg/kg b.w. and a high dose of 50 mg/kg b.w. were administered to the rats. The high dose was expected to produce mortality in dosed rats. The test procedure is illustrated in Fig. 2. Three additional rats were exposed to 10, 25 or 40 mg/kg b.w. MON, respectively, to help in assessing the appropriate dose levels for future studies on the subacute toxicity of MON. Only one rat/dose was used to reduce the number of experimental animals. The health condition of the animals was monitored at least twice a day for 13 days. However, monitoring was frequent for the first 24 h and continuous for the first 4 h post dosing. Clinical observations comprised changes in clinical appearance, behavior, body position, movements, reflexes, respiratory and circulatory functions and assessment of the color and composition of feces and urine.

The laboratories of the Finnish Food Safety Authority Evira are accredited according to the ISO 17025 standard and all experiments were conducted under a permit from the National Animal Experiment Board of Finland.

2.3. Dose administration and sampling

A single dose (max volume 1 mL) of synthetic potassium salt of MON, dissolved in filtered tapwater (0.45 μM Whatman, Germany), was administered to three rats per group, by gavage using stainless steel feeding needles (Popper & Sons, NY, USA). An additional two rats were assigned as controls (1 animal/dose) and were each exposed to 5 or 50 mg/kg b.w. potassium chloride. The rats were fasted for 12 h (o/n) prior to dosing and 3 h post-dosing.

At pre-defined time points (24 h pre-exposure, 6, 12, 24, 48, 168 and 336 h post-exposure), urine and feces samples were collected and stored at −70 °C until analysis. At the end of the experiments (14 days), all surviving animals were euthanized by carbon dioxide inhalation followed by cervical dislocation to ensure death. The 10 mg/kg b.w. dose rat was euthanized at 48 h post-exposure. All animals were subjected to gross necropsy.

2.4. Necropsy and histopathological methods

A complete necropsy was performed for each rat with thorough evaluation of macroscopic changes. Tissue samples were collected from the liver, spleen, kidneys, lungs, adrenal glands, thymus and heart. In addition, samples of stomach, intestine, testicles, pancreas, skeletal muscle and brain were collected in the 50 mg/kg b.w. MON dose group. Samples were fixed in 10% buffered formalin, routinely processed, embedded in paraffin, sectioned at 4 μm and stained with haematoxylin and eosin.