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Repeated administration of a *Fusarium* mycotoxin butenolide to rats induces hepatic lipid peroxidation and antioxidant defense impairment

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ABSTRACT

Butenolide, a mycotoxin elaborated by several toxigenic *Fusarium* species, frequently contaminates important agricultural products, and has been considered a potential health risk to humans and animals. However, many toxicology issues including toxicity targets and mechanisms of butenolide remain unclear. Previous study indicated that acute butenolide exposure produced hepatic oxidative toxicity, but its chronic toxicity is still unknown. The present study therefore attempted to reveal the adverse effects of repeated butenolide exposure from a viewpoint of oxidative damage focusing on the liver. Intragastic administration of rats with butenolide for seven consecutive weeks resulted in hepatic injury as shown by obvious changes of serum biochemistry parameters indicating liver function. Repeated butenolide exposure also induced oxidative stress as manifested by impairment of antioxidant defenses including depletion of sulfhydryl groups and reduction of glutathione peroxidase activity, and enhancement of lipid peroxidation both in serum and liver. In conclusion, the present study indicated that repeated butenolide exposure induced a significant liver injury, and oxidative damage may serve as a mediator in the toxicity of butenolide. The current findings contribute to the understanding of the toxic profile of butenolide.

1. Introduction

Fusarium mycotoxins are secondary metabolites of toxigenic *Fusarium* species which distribute ubiquitously in the world. Trichothecenes, zearalenone and fumonisins are most frequently found in a variety of foodstuffs and feedstuffs for human and animal consumption worldwide (Gutleb et al., 2002). There is increasing evidence that *Fusarium* mycotoxins can provoke a broad spectrum of toxicities (Kubosaki et al., 2008; Sudakin, 2003; Voss et al., 2002), and several specific animal mycotoxicoses such as equine leukoencephalomalacia (Wilson et al., 1991) and porcine pulmonary edema (Haschek et al., 2001) have been positively correlated with the exposure of certain *Fusarium* mycotoxins. Dietary intake of *Fusarium* mycotoxins has been implicated in human alimentary toxic aleukia, and is associated with the high incidence of human esophageal cancer in some regions of China and South Africa (Rheeder et al., 1992; Sun et al., 2007). Furthermore, consumption of *Fusarium* mycotoxins-contaminated foodstuffs has long been considered as a suspected etiological factor for Kashin–Beck disease, an endemic osteoarthropathy which is prevalent in China (Haubruge et al., 2001).

Butenolide, 4-acetamido-4-hydroxy-2-butenoic acid γ-lactone (CAS No. 16275-44-8) is a water-soluble *Fusarium* mycotoxin, and frequently invades economically important agricultural products mainly maize and wheat. It was firstly isolated from *Fusarium tricinctum* strain NRRL 3249, subsequently identified from other *Fusarium* species including *F. equiseti*, *F. graminearum*, *F. sporotrichioides, F. moniliforme, F. avenaceum*, *F. nivale*, etc. As a commonly detected mycotoxin, butenolide is capable of inducing diverse toxicities. It had been reported that butenolide caused dermal toxicity in guinea pigs characterized by erythema and induration on skin (Bhavanishankar et al., 1988). Butenolide was considered a vesicant, and steers that received orally butenolide lost weight and developed esophageal and gastric ulcers (Tookey et al., 1972). Repeated exposure of hamsters to butenolide vapor resulted in a subacute inhalation toxicity as shown by salivation, increased liver weight and growth retardation (Feron et al., 1979). It had been implied that butenolide was the causative agent of a livestock mycotoxicosis called "fescue foot" which occurs in cattle grazing on tall fescue grass; the pathogenesis was possibly attributable to significant peripheral vascular disorder (Grove et al., 1970; Tookey et al., 1972). Butenolide is also considered one of the

Abbreviations: DTNB, 5,5-dithio-*bis*-nitrobenzoic acid; GSH, glutathione; GPx, glutathione peroxidase; NPSH, non-protein sulfhydryl group; PSH, protein sulfhydryl group; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TSH, total sulfhydryl group.

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mycotoxins which are implicated in Kashin–Beck disease, and several studies indicate that butenolide can provoke chondrocyte injuries in recent years (Wang et al., 2007; Zuo et al., 2006).

Although it has been recognized that butenolide poses a health risk to animals and human, only a few studies have concentrated on the toxicology profile of butenolide over the past decades, and many important issues including potential targets and mechanisms of toxicity remain to be thoroughly elucidated. It is shown recently that repeated administration of rats with butenolide induces a significant cardiotoxicity (Liu et al., 2007), but its adverse effects on other organs still need to be revealed. It was also indicated that acute exposure of rats to butenolide resulted in hepatic lipid peroxidation, suggesting the occurrence of oxidative liver injury (Liu et al., 1999). Based on these above observations, the present study attempted to reveal the toxic effect of repeated butenolide exposure from a viewpoint of oxidative damage focusing on the liver. The present study may contribute to the understanding of the toxicity profile of butenolide and to the elucidation of the toxic mechanism.

2. Materials and methods

2.1. Reagents and chemicals

Thiobarbituric acid, glutathione (GSH), 1,1,3,3-tetraethoxypropane and 5,5-dithio-*bis*-nitrobenzoic acid (DTNB) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Butenolide was chemically synthesized by Beijing Institute of Pharmacology and Toxicology (Beijing, China). Briefly, it was prepared by photochemical oxidation of furfural to 4-ethoxy-4-hydroxyl-2-butenoic acid-γ-lactone; this pseudoester was hydrolyzed before reaction with acetamide to give the butenolide in yield of 27.2% (Feng et al., 1988). This synthetic product was shown to be identical to the natural mycotoxin, and the purity was more than 99.0%. All other chemicals were of analytical grades commercially available (Sinopharm Chemical Regent Beijing Co., Ltd., Beijing, China).

2.2. Animals husbandry and maintenance

Male Wistar rats weighing 200–220 g (8–10 weeks of age) were supplied by Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). Rats were housed at 22 ± 2 °C in 50 ± 10% relative humidity with 12 h/12 h light–dark cycle and were allowed free access to standard rodent chow (Rodent Diet JD-2000-015, Certified, Beijing Vital Keao Feed Co., Ltd., Beijing, China) and drinking water. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Academy of Military Medical Sciences.

2.3. Experimental protocol

After an acclimatization period of 1-week, 21 rats were randomly divided into three groups of 7 animals each as follows:

Group 1: Control group, rats were intragastrically administered vehicle physiologic saline.

Groups 2 and 3: Rats were treated with intragastric administration of butenolide at doses of 10 and 20 mg/kg body weight daily for 7 successive weeks, respectively.

The doses used in the present study were primarily based on the following factors: firstly, the medial lethal dose (LD50) of butenolide was determined to be about 200 mg/kg in male Wistar rats in a preliminary experiment and 1/20 and 1/10 of LD50, i.e. 10 and 20 mg/kg can avoid serious toxicity or unexpected deaths during a relative long-term administration; secondly, our recent study has shown that repeated administration of male Wistar rats with 10 and 20 mg/kg of butenolide for 2 months induces moderate cardiotoxicity. Therefore, the doses in the present study were set at 10 and 20 mg/kg, which maintain the consistency and comparability of the similar studies.

At the end of the 7-week treatment period, all animals were subjected to pentobarbital sodium anaesthesia, and blood samples were collected by heart puncturing. The livers were removed immediately by dissection, washed in ice-cold physiologic saline, blotted on filter papers, and stored in liquid nitrogen until further analyses.

2.4. Serum biochemical analyses

Biochemical analyses of serum obtained from the collected blood were conducted. Activities of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), and levels of cholesterol (CHOL), triglycerides (TG) and albumin (ALB) were measured by an automated biochemistry analyzer (Hitachi, Japan) using commercial kits (Biosino Biotechnology and Science Inc., Beijing, China).

2.5. Assay for biochemical parameters

Fresh liver homogenate prepared in 10 mM ice-cold phosphate buffered saline (PBS, pH 7.4) and serum were used to estimate lipid peroxidation, contents of sulfhydryl groups and activity of glutathione peroxidase (GPx).

2.5.1. Assay for lipid peroxidation

Lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) formation was measured as described previously (Ohkawa et al., 1979). In brief, 0.25 ml of sample was mixed thoroughly with 1 ml of 20% trichloroacetic acid and 0.5 ml of 0.67% thiobarbituric acid. The reaction mixture was heated for 30 min at 95 °C. After cooling, the pink substances were extracted by *n*-butanol, and 200 μl aliquots of the organic layer were spectrophotometrically measured at 532 nm.

2.5.2. Measurement of total, protein and non-protein sulfhydryl groups

The contents of total, protein and non-protein sulfhydryl groups (TSH, PSH and NPSH) in serum and liver homogenate were quantified by the method of Sedlak and Lindsay (1968). Briefly, for assay of TSH, 1 ml of reaction mixture containing 50 μl of sample, 150 μl of 0.2 M Tris buffer (pH 8.2), 10 μl of 0.01 M DTNB and 790 μl of methanol was allowed to stand at ambient temperature for 15 min, 200 μl aliquots of the mixture supernatants were then spectrophotometrically measured at 412 nm. For assay of NPSH, the reaction system contains 300 μl of supernatant of protein-free sample, 600 μl of 0.4 M Tris buffer (pH 8.9) and 15 μl of DTNB, and absorbance of the supernatant was read at 412 nm. PSH was calculated by subtracting the NPSH from the TSH.

2.5.3. Determination of GPx activity

GPx activity was determined with the DTNB colorimetric assay (Hafeman et al., 1974). The enzyme assay tubes were incubated at 37 °C and contained: 0.5 ml of 2.0 mM GSH, 0.5 ml of 0.40 M sodium phosphate buffer (pH 7.0) and 20 μl of sample. The reaction was initiated by addition of 0.5 ml of 1.25 mM H_2O_2 after a 5-min preincubation. Two milliliters of metaphosphoric acid solution was then added 5-min later. GSH in the protein-free supernatant was measured by mixing 1.0 ml of supernatant with 1.0 ml of DTNB, and absorbance was recorded at 412 nm. A blank (with buffer substituted for enzyme source) was carried through the incubation simultaneously since non-enzymatic GSH oxidation occurred during incubation. GPx activity was expressed as units/g tissue (for homogenate) or units/L (for serum), and one unit was defined as a decrease in GSH of 1 mM per minute after the decrease in GSH per minute of the non-enzymatic reaction was subtracted.

2.6. Statistical analysis

Results were presented as mean ± SD. Data were analyzed by using one-way ANOVA and Student's *t* test. *P*-values less than 0.05 were considered as statistically significant.

3. Results

3.1. Clinical observations

All animals appeared normal during the first four weeks. One rat treated with 20 mg/kg of butenolide was found dead in Week 5. Abnormal observation was noted in butenolide-treated animals beginning on Week 5, including lassitude, rough hair and haemoid mucus around pinna nasi; a loss of appetite and reduction of food consumption were also seen in butenolide-treated animals from Week 5 (data not shown). No abnormality was observed in vehicle-treated animals throughout the treatment period.

The changes of body weights following intragastric administration of butenolide were shown in Fig. 1. There were no significant differences in mean body weights during the first four weeks. However, in accordance with the observations of food intake reduction, remarkable losses in body weights were seen in 20 mg/kg butenolide-treated animals, and to a lesser extent in 10 mg/kg animals beginning on Week 5, and continued for the remainder of the treatment period.