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Hepatotoxicity and genotoxicity of patulin in mice, and its modulation by green tea polyphenols administration

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ABSTRACT

Patulin (PAT) is a mycotoxin produced by certain species of Penicillium, Aspergillus, and Byssochlamys. Previous studies demonstrated its cytotoxic, genotoxic, and mutagenic effects in different cell lines. However, there is little information available concerning its toxic behavior *in vivo*. In the present study, we investigated PAT-induced hepatotoxicity and genotoxicity in mice. We also investigated the antioxidant and anti-genotoxicity efficiency of green tea polyphenols (GTP) against PAT-induced toxicity. We found that PAT-treatment induced serum alanine transaminase (ALT) and aspartate transaminase (AST) activities significantly. PAT-induced lipid peroxidation was confirmed with the elevation of thiobarbituric acid-reactive substances (TBARS). Moreover, the increasing of reactive oxygen species (ROS) and decreasing of GSH level implied its oxidative damage mechanism. In bone marrow cell, PAT was found to induce micronucleus and chromosomal aberration formation. In addition, our result suggested that GTP administration has dose-dependent antioxidative and antigenotoxic effect in against PAT-induced hepatotoxicity and genotoxicity.

1. Introduction

Patulin (PAT) is a mycotoxin produced by genera Penicillium, Aspergillus and Byssochlamys (Mahfoud et al., 2002; Sanzani et al., 2012). PAT has been isolated from a number of fruits and vegetables, mainly apples and apple products (Moake et al., 2005). Previous studies demonstrated PAT contamination poses a serious health risk to consumers, various forms of acute and chronic effects of PAT have been characterized (Moake et al., 2005; Sant'Ana et al., 2008). European Union has regulated the maximum permitted levels of PAT in apple juices (50 ppb), purees (25 ppb) and, above all, baby foods (10 ppb) (Sanzani et al., 2012). World Health Organization (WHO) and US Food and Drug Administration (FDA) also limit patulin to 50 ppb as the recommended limit in apple juice (Puel et al., 2010; Van Egmond, 1989).

PAT has obvious electrophilic reactivity, leading to the formation of covalent adducts when it interacts with electrophilic chemicals. The main cellular target of PAT is cysteine-containing tripeptide glutathione (GSH). Indeed, PAT-intoxication induced a quick depletion of GSH in cultured cell (Yang et al., 2011) and in mice (de Melo et al., 2012; Pfeiffer et al., 2005). In addition, PAT also reacts with free cysteine, or cysteine-, lysine-, histidine-containing proteins, and most of the toxic effects of PAT-treatment are considered as the consequences of this adduct-forming activity (Papp et al., 2012). On the other hand, several studies implicated PAT-mediate toxicity *via* oxidative damage pathway. PAT was proved to increase reactive oxygen species (ROS), 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid-reactive substances (TBARS) contents (Ayed-Boussema et al., 2013; Ferrer et al., 2009).

Numerous studies showed that phytochemicals protect against the toxic effects induced by environmental pollutants (Celik et al., 2013). Epidemiological studies indicated the consumption of these phytochemicals is associated with health benefits of reducing oxidative related damages (Borek, 2004). Green tea polyphenols (GTP) are the most important biological active components of green tea. GTP have significant antioxidant properties and exert protective role in the development of cancer, cardiovascular disease and other pathologies (Dreosti, 1996; Dufresne and Farnworth, 2001;

Abbreviations: ALT, alanine transaminase; AST, aspartate transaminase; CAT, catalase; GSH, glutathione; GTP, green tea polyphenols; PAT, patulin; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

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Fujiki et al., 2002; Riegsecker et al., 2013). However, there is no information regarding to the effect of GTP modifying PAT-induced toxic response.

In the present study, we aimed to (1) evaluate the hepatotoxicity of PAT, (2) evaluate the genotoxicity of PAT in bone marrow and (3) investigate the protective effect of GTP at three different doses against damages occurred by PAT. Silymarin has been widely used in clinical practice for the treatment of toxic liver diseases (Lee et al., 2007). Its antioxidant capacity and hepatoprotection effect have been extensively reviewed (Polyak et al., 2013; Vargas-Mendoza et al., 2014). In the current study, silymarin was served as a well-characterized antioxidant control for PAT-induced hepatotoxicity and genotoxicity.

2. Materials and methods

2.1. Chemicals

Patulin (PAT, 4-hydroxy-4*H*-furo(3,2-C)pyran-2(6*H*)-one, purity >98.0%, Fig. 1), GTPs (Polyphenon 60 from green tea) and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) were obtained from Sigma–Aldrich (Shanghai, China). The components of GTPs include epicatechin (8.5%), epigallocatechin (17.7%), epigallocatechin gallate (32.1%), epicatechin gallate (10.7%), gallocatechin gallate (3.3%), and catechin gallate (1.4%) (Lee et al., 2008). Diagnostic kits of superoxide dismutase (SOD) and catalase (CAT) activities, serum alanine transaminase (ALT) and aspartate transaminase (AST) activities, GSH and thiobarbituric acid reactive substances (TBARS) content were obtained from the Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). All other chemicals used were of the highest grade commercially available.

2.2. Animals and treatment

All animal experiments were performed in accordance with the guideline of the Animal Care Committee of Southwest University. All efforts were made to minimize suffering. Male Kunming mice (22 ± 2 g) were purchased from Chongqing Tengxin Biotechnology Co. Animals were maintained under standard conditions of humidity (50%), temperature (25 ± 2 °C) in a 12 h light/12 h dark cycle. They were fed standard rodent chow and had free access to water. After one week's acclimation, thirty-six mice were randomly divided into 6 groups with 6 mice in each group. Mice received daily intraperitoneal (i.p.) injection of high dose (100 mg/kg), medium dose (50 mg/kg) or low dose (25 mg/kg) of GTP for 7 days continuously. PAT (1 mg/kg/i.p.) was injected 1 h after the last GTP administration. GTP was dissolved in physiological saline and PAT was dissolved in physiological saline containing 0.1% DMSO. Solvent control group received a physiological saline containing 0.1% DMSO. The last group was given silymarin (100 mg/kg, i.p.) for 7 days continuously prior to PAT treatment. The mice were grouped as follows:

Group 1: vehicle control, received 0.1% DMSO in saline (2 mL/kg BW/d).
Group 2: received PAT (1 mg/kg).
Group 3: received PAT (1 mg/kg) and GTP (25 mg/kg BW/d).
Group 4: received PAT (1 mg/kg) and GTP (50 mg/kg BW/d).
Group 5: received PAT (1 mg/kg) and GTP (100 mg/kg BW/d).
Group 6: received PAT (1 mg/kg) and silymarin (100 mg/kg BW/d).

After the PAT injection for 24 h, mice were anesthetized with CO_2. Blood samples were collected and serum was separated to determine serum ALT and AST activities. Liver were rinsed in ice-cold physiological saline and homogenized in Tris–HCl buffer (0.01 M, pH = 7.4) to give a 10% homogenate. Homogenates were centrifuged at 3000 rpm, 4 °C for 10 min and supernatant was collected for ROS, TBARS, GSH level and antioxidant enzyme activities. Bone marrow was flushed out for micronucleus and chromosomal aberration tests.

2.3. Assay for serum transaminase activity

Serum from individual mice were separated by centrifugation of blood at 600 g for 15 min and stored at −20 °C until use. AST and ALT activities in the serum were determined spectrophotometrically using test kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China), according to the manufacturer's instructions.

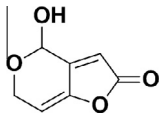

Fig. 1. Chemical structure of PAT.

2.4. Determination of ROS level

ROS level was determined using DCFH-DA as fluorescent probe (Pinto et al., 2012). In brief, the assay buffer contained 20 mM Tris–HCl, 130 mM KCl, 5 mM $MgCl_2$, 20 mM NaH_2PO_4, 30 mM glucose and 5 μM DCFH-DA. The mixture was incubated at 37 °C for 15 min and terminated the reaction by H_2O_2. The fluorescence was recorded with an excitation wavelength of 488 nm and an emission wavelength of 526 nm (F7000, HITACHI). The average fluorescence of the control group was set to 100%.

2.5. Determination of reduced GSH content

Reduced GSH levels were determined using Ellman's reagent (Ellman, 1959). Tissue suspension was double diluted and 5% trichloroacetic acid was added to precipitate protein. The supernatant was centrifuge at 10,000 g for 5 min, Ellman's reagent was added and the absorbance was measured at 412 nm using a UV–Vis spectrophotometer (UV-2450, SHIMADZU). A standard curve was formulated using different concentrations of GSH solution.

2.6. Determination of lipid peroxidation products

Lipid peroxidation was detected by measuring the concentration of TBARS in fluorescence at 532 nm (F7000, HITACHI), using a TBARS detection kit according to the manufacturer's instructions. The absorbance of was measured at 535 nm. TBARS concentrations of the samples were calculated using the extinction co-efficient of 156,000 M^{-1} cm^{-1}.

2.7. Determination of antioxidant enzyme activities in liver

SOD and CAT activities were measured with commercial assay kits, according to the previously described method (Liu et al., 2012). SOD activity was determined based on the reaction of SOD and nitrotetrazolium blue chloride (NBT). Briefly, 2.8 mL of reactive mixture (xanthine 0.3 mM, EDTA 0.67 mM, 150 μM NBT, Na_2CO_3 0.4 M, bovine albumin 30 mg/30 mL) is added to 0.1 mL sample and 50 μL xanthine oxidase (0.2 M), incubated at 25 °C for 20 min and mixed with 0.1 mL 8 M $CuCl_2$. The absorbance was recorded at 550 nm. CAT activity was determined by measuring the rate of H_2O_2 decomposition at 240 nm in a reaction mixture containing 10 mM H_2O_2, 50 mM KH_2PO_4 and 50 μM of sample. The results were expressed as units of enzyme activities calculated per milligram of protein (U/mg protein).

2.8. Bone marrow micronucleus test

Ends of the femurs were cut off and the bone marrow was flushed out using 0.5 mL of fetal calf serum. Bone marrow cells were centrifuged at 1200 rpm/10 min and the pellet was re-suspended in 0.56% KCl solution and incubated at 37 °C for 25 min. Cells were re-centrifuged at 1200 rpm/10 min and fixed in cold Carnoy's fixative (acetic acid/methanol, 1:3, v/v) three times. Cell was spread on a slide by pulling one drop of bone marrow suspension behind a cover glass held at a 45°. Smears were air-dried, fixed in methanol and stained with May-Grünwald/Giemsa and evaluated with a fluorescence microscope (OLYMPUS IX71). For each experimental group, 6 mice were used, and 1000 erythrocytes were observed from each animal to determine micronuclei in polychromatic erythrocyte (MNPCE, ‰), micronuclei in normochromatic erythrocytes (MNNCE, ‰) and polychromatic erythrocyte/polychromatic erythrocyte + normochromatic erythrocyte in 1000 cells [PCE/1000 (PCE + NCE)].

2.9. Bone marrow chromosomal aberration test

Fixed bone marrow cells were re-suspended and dropped onto chilled slides, flame-dried and stained the following day in 5% buffered Giemsa (pH 6.8). Well spread 100 metaphases per animal (600 metaphases per group) were examined to score the aberrations, as described previously (Savage, 1976). Metaphases with chromatid/chromosome gaps and breaks were recorded and expressed as percentage of total metaphases per group.

2.10. Statistical analysis

Results were expressed as the means ± S.D. Statistical significance was determined by one-way analysis of variance (ANOVA) using SPSS 18.0 software. *Post hoc* testing was performed for inter-group comparisons using the least significance difference (LSD) test. $p < 0.05$ was considered to be significant.