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Change in kidney damage biomarkers after 13 weeks of exposing rats to the complex of *Paecilomyces sinclairii* and its host *Bombyx mori* larvae

Mihye Jeong [a], Young-Won Kim [b], Jeong-Ran Min [b], Min Kwon [b], Beom-Suk Han [b], Jeong-Gyu Kim [c], Sang-Hee Jeong [b,*]

[a] Agro-Material Safety Evaluating Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea
[b] Department of Applied BioToxicology, Hoseo Toxicology Research Center, Hoseo University, Asan 336-795, Republic of Korea
[c] Environmental Science and Ecological Engineering, Korea University , Seoul 136-701, Republic of Korea

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ABSTRACT

Complex of *Paecilomyces sinclairii* and host larvae, *Bombyx mori,* is a well known health food; however, concerns about nephrotoxicity have been raised. Kidney toxicity was investigated after 13 weeks of administering the complex orally to rats with parameters including blood urea nitrogen (BUN), creatinine, and kidney damage biomarkers, beta-2-microglobulin (β2m), glutathione *S*-transferase alpha (GST-α), kidney injury molecule 1 (KIM-1), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), vascular endothelial growth factor (VEGF), calbindin, clusterin, cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), and osteopontin. Dose-dependent kidney cell karyomegaly and tubular hypertrophy were observed, with higher severity in males. There was a dose-dependent increase in KIM-1 and TIMP-1 levels in kidney and urinary KIM-1, cystatin C, β2m, and osteopontin levels. KIM-1 and TIMP-1 increased in male kidneys had not recovered by 2 weeks after stopping exposure. Cystatin C in kidney was significantly lowered in all treatment groups at 13 weeks of administration. All the changes were more noticeable in males. These data indicate that the complex damage renal tubule cells with histopathological lesions and changes in biomarker levels. Kidney and urinary KIM-1 and cystatin C were the most markedly affected and early increased indicators among biomarkers tested, whereas BUN and creatinine were not affected.

1. Introduction

Cordyceps is an entomogenous fungi that is formed from the larvae and pupae of insects, and is an established human health supplement or potential drug causing beneficial effects, such as anti-tumorigenicity, immunostimulation, hypoglycemic effects, and decreased production of peroxisomes (Kuo et al., 1994; Kiho et al., 1996; Shim et al., 2000). In particular, *Cordyceps* fungi formed from silk worm larvae have been used as a traditional folk remedy, or food ingredient, to strengthen the immune function and regain vitality in several Asian countries, including China, Japan, and Korea (Ji et al., 2011). In addition, extracts of silk worms that are the host of *Cordyceps* have been reported to alleviate pain and decrease blood glucose levels (Jeon et al., 2000). Recently, Kang et al. (2010) indicated that silk worm extracts attenuate neurotoxicity induced by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) through the inhibition of monoamine oxidase-B (MAO-B) in the mouse brain.

Paecilomyces sinclairii is a new *Cordyceps* spp., which is isolated from cicada larvae, and has been successfully mass-produced through the artificial cultivation from silk worm larvae infected with *P. sinclairii* (Kim et al., 2003; Shin et al., 2003). It may be assumed that the complex formed from *P. sinclairii* and larvae of its host, *Bombyx mori* (silk worms), may have enhanced effects compared to the separate application of the *P. sinclairii* fruit body or silk worm larvae, because of the similar effect promoted by each component.

Toxicological effects were not observed when adult Sprague Dawley (SD) rats were administered orally with the complex powder suspension of *P. sinclairii* and larvae of *B. Mori* at doses ranging from 0.008 to 5 g/kg body weight (bw) for 2 weeks, except for a decline in the weight of the thymus in males (Kwack and Lee, 2009). A single oral toxicity study of the complex showed that doses of 50% lethality (LD50) exceeded 5–10 g/kg bw in rats and dogs, indicating that the material was negligibly toxic (Kim et al., 1996). The complex did not induce any genotoxic effects in a battery of tests, including bacterial reverse mutation assays, mammalian cell chromosome aberration assays, and rodent micronucleus assays (Ahn et al., 2004b).

* Corresponding author. Fax: +82 41 540 9675.
E-mail address: Jeongsh@hoseo.edu (S.-H. Jeong).

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However, histopathological lesions of the kidney manifested when the complex of *P. sinclairii* fruiting body and silkworm larvae was provided via feed at 12,500, 25,000, 50,000, and 100,000 ppm for 13 weeks (Ahn et al., 2004a). Tubular cell abnormalities were observed in male and female rats that were treated with all doses, while tubular edemas or tubular destruction was observed at doses of 50,000 and 100,000 ppm, with approximately 30% occurrence. In another study, *Cordyceps* mushrooms have been reported to support kidney functions through an increase in 17-hydroxycorticosteroid and 17-ketosteroid levels (Zhu et al., 1998). Hence, further studies are required to elucidate whether the complex is beneficial or toxic to kidney function, with the delineation of dose-dependent effects, if it is a nephrotoxic material.

Kidney histopathology and biochemistry markers, such as serum creatinine and blood urea nitrogen (BUN), have been commonly used to evaluate the nephrotoxicity of chemicals and biomaterials. However, these indicators only show changes when renal function has been significantly damaged (Perazella, 2009). Hence, these indicators should be treated with caution when used as markers to predict and diagnose kidney damage.

Recently, the USFDA and the European Medicine Agency validated seven urinary renal biomarkers including kidney injury molecule 1 (KIM-1), clusterin, cystatin C, and beta-2-microglobulin (β2m) for monitoring nephrotoxicity in preclinical toxicity tests. These biomarkers have been proposed for use by the Nephrotoxicity Working Group of the Predictive Safety Testing Consortium (Dieterle et al., 2010). Zhou et al. (2008) suggested the use of a number of biomarkers including KIM-1, neutrophil gelatinase-associated lipocalin (NGAL), and glutathione *S*-transferase alpha (GST-α) for the detection of acute kidney damage. These biomarkers are more sensitive than traditional biomarkers (such as BUN and serum creatinine). These biomarkers have been proposed as being sensitive, and include specific markers of site-specific renal tubular damage involving necrosis and glomerular filtration dysfunction following ischemia or exposure to renal toxicants (Harpur et al., 2011). Extensive studies have identified that the gene expression of osteopontin, vascular endothelial growth factor (VEGF), calbindin, and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) are specifically altered at the level of the kidney tissue or those proteins, in urine or the kidney following renal damage (Amin et al., 2004; Guha et al., 2011; Kondo et al., 2009; Minowa et al., 2012; Vaidya et al., 2008).

The current study used sensitive biomarkers of kidney damage to determine whether the complex of *P. sinclairii* with larvae of its host, *B. mori*, induces kidney toxicity.

2. Materials and methods

2.1. Animals

Specific pathogen-free 5-week-old male and female SD rats were purchased from a commercial animal breeder (Hanrim Lab animal Institute, Gyeongido, Korea). A total of 50 male and 50 female rats were acclimated for 1 week in an environmentally controlled room, in which the conditions set to 22 ± 2 °C, 50 ± 10% humidity, air ventilation of 10–15 times/h, and a 12 h light/dark cycle. The rats were provided commercial pellets (Mouse/rat feed, Purina Co., Korea) and UV-sterilized and filtered tap water *ad libitum*. The animal experiments were conducted in accordance with the Ethics for the Care and Use of Laboratory Animals prepared by Hoseo Toxicological Research Center of Hoseo University.

2.2. Preparation of test materials

P. sinclairii endophytically parasitizing dead or living *Cicadae* subspecies was isolated from conidiospores, and cultured in potato dextrose agar medium, and subsequently inoculated onto silk worms, Korea (Rural Development Administration, KOREA). The fruiting bodies of *P. sinclairii* and its silk worm larvae hosts were collected together, and then dried and homogenized into powder-form and stored at 4 °C. Each dose of the powdered complex was mixed with feed powder (Rat and Mouse 18% 5L79, PMI Nutrition International, MO, USA) at 5000, 10,000, and 50,000 ppm. The mixture was kneaded together by adding distilled water, and then made into pellet feed by drying at a temperature of 57 °C overnight. The feed was prepared every week.

2.3. Treatments

Forty SD male and 40 female rats were randomly divided into four groups (Control, 5000, 10,000, and 50,000 ppm) of 10 animals for each dose group and sex. A further 5 males and 5 females were allocated into each control and the highest dose group, to observe recovery from any toxic effects at 2 weeks after stopping exposure. The control group was provided with normal feed, without any addition of the powdered complex. Male and female rats were administered with the powdered complex at 0, 5000, 10,000, and 50,000 ppm through the food for 13 weeks. Change of body weight and daily feed intake was measured every week during the treatment of test materials.

After 13 weeks of treatment or a further 2 weeks after stopping exposure, the animals were anesthetized, and blood was collected from the abdominal artery. Serum samples were collected by centrifugation at 13,000*g* for 15 min, and supernatants were stored at −80 °C until analysis. The kidneys were removed and weighed. Half of the vertical and horizontal sections of each kidney were fixed in formalin for histopathological examination. The remaining parts of the kidney were weighed on a milligram scale, and then transferred to 25 μl of lysis buffer (consisting of 500 mM NaH_2PO_4/Na_2HPO_4 buffer and 0.1% tween 20) in H_2O, with a protease inhibitor cocktail (Roche, Germany) per mg of wet weight tissue. The tissue was ground using 7 strokes of a motor glass-Teflon homogenizer (DaiHan Scientific Co., Ltd., Korea) on ice, and centrifuged at 16,000*g* for 15 min at 4 °C. The supernatant was collected and stored at −20 °C until analysis.

On days 13, 55, and 90 of treatment, and day 13 of recovery, the rats were transferred to individual metabolic cages, and 24-h urine samples were collected on ice in 50-mL polypropylene tubes containing 1 mL 1% sodium azide. The volume of urine was measured and stored at −80 °C until analysis.

2.4. Serum and urine biochemistry analysis

BUN and creatinine levels in the blood serum or urine were measured using a clinical chemistry analyzer (HITACHI 7020) with respective assay kits (Chema Diagnostica Com., Italy).

2.5. Determination of kidney damage biomarkers

Serum, kidney tissue homogenates, and urine samples were analyzed for KIM-1, TIMP-1, VEGF, osteopontin, clusterin, NGAL, β2m, GST-α, calbindin, and cystatin C by using commercially available rat multiplex immunoassay kits (Merck KGaA Darmstadt, Germany) on the multiplex flow cytometer (Luminex MAP system). KIM-1, TIMP-1, VEGF, β2m, and GST-α levels were analyzed using the rat kidney toxicity panel 1 assay kit (Widescreen®, Merck KGaA Darmstadt, Germany). The levels of cystatin C, osteopontin, clusterin, NGAL, and calbindin were assayed using rat kidney toxicity panel 2 (Widescreen®, Merck KGaA Darmstadt, Germany). In brief, the samples were diluted with sample dilution buffer to be adequately included into the standard curve ranges, and were then incubated with capture beads for 2 h at room temperature in the dark. The primary antibody for each biomarker and then streptavidin–phycoerythrin was added and incubated at each separate step. Finally, the concentrations of the biomarkers in each sample were measured simultaneously using a calibration curve prepared for each experiment. The quantity of biomarkers in urine was presented after normalization, based on the concentration of urinary creatinine.

2.6. Statistical analysis

Data are expressed as mean ± SD. Statistical significance between the control and treated groups was determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple tests, using the STATISTICA program. A difference in the mean values of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Changes in body weight, daily feed intake and kidney weight

Body weight and the amount of food and water consumption were not different between the control group and the treatment groups for 13 weeks of treatment, or for further 2 weeks after stopping exposure in both sexes (Fig. 1).

The absolute and relative kidney weight did not differ between the control group and the treatment groups after 13 weeks of treatment, or after a further 2 weeks after stopping exposure in male and female rats (Table 1).