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Potential risk of acute hepatotoxicity of kodo poisoning due to exposure to cyclopiazonic acid

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Abstract

Kodo millet (*Paspalum scrobiculatum* L.) is a staple food of some sections of people of North India. Consumption of Kodo millet is often found to cause intoxication and poisoning. The grains are frequently infested with *Aspergillus tamarii* Kita, which produced substantial amount of a mycotoxin, cyclopiazonic acid (CPA). Investigations were carried out to evaluate the hepatotoxic/preneoplastic changes in rat liver following single and multiple dose administration of CPA. Results showed a marked increase in the activity of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) following CPA exposures, suggesting acute hepatotoxicity. Significant increase was also observed in gamma glutamyl transpeptidase (GGT) activity following CPA exposures, indicating preneoplastic changes in the liver. The results reveal that Kodo poisoning might cause acute hepatotoxicity in men and animals. The findings thus suggest that the consumption of contaminated Kodo millet is a serious health hazard due to exposure to CPA produced by *Aspergillus tamarii* associated with the millet.

Keywords: Kodo millet; *Aspergillus tamarii*; Cyclopiazonic acid; Hepatotoxic; Hepatocarcinogenic

1. Introduction

Kodo millet (*Paspalum scrobiculatum* L.) is a staple food of some sections of people in parts of North India. However, Kodo poisoning is a toxic syndrome encountered in men and animals in areas where this millet is grown and consumed. The consumption of Kodo millet is often reported to cause intoxication and poisoning. The millet is reported to be toxic to animals when consumed (Bazlur, 1960). The symptoms of Kodo poisoning in the affected people are characterized by nausea, vomiting, delirium, depression, intoxication, and unconsciousness. Kodo millet is often found heavily infested with *Aspergillus tamarii* Kita. Janardhanan et al. (1984) isolated fumigaclavin A from *Aspergillus tamarii* associated with Kodo millet and suggested that the compound might be responsible for part of Kodo poisoning symptoms. The fungus was also reported to produce significant amount of a mycotoxin, cyclopiazonic acid (CPA; Fig. 1) (Dorner, 1983; Rao and Husain, 1985). CPA has been reported to be isolated from a number of fungi namely *Penicillium cyclopium*, *Penicillium camembertii*, *Aspergillus versicolor*, *Aspergillus oryzae*, *Aspergillus flavus* (Holzapfel, 1968; Ohmomo et al., 1973; Orth, 1977; Luk et al., 1977; Still et al., 1978). Morrissey et al. (1985) reported that CPA was acutely toxic to rats and produced focal necrosis in many organs. In chickens, CPA exposure caused reduced weight gain and produced proventricular lesions (Dorner et al., 1983). Neuhring et al. (1985) found that main target organs of CPA-induced toxicity in dogs were gastrointestinal tract and kidneys. In this communication, we report the acute hepatotoxicity and preneoplastic changes in rat liver following oral administration of CPA, isolated from *Aspergillus tamarii* associated with Kodo millet collected from Uttar Pradesh, India, in the month of September. The voucher specimen of *Aspergillus tamarii* was deposited in CAB International Mycological Institute, UK (IMI-260311).

2. Materials and methods

Gamma glutamyl-*p*-nitroanilide hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). Glycylglycine and 2,4-dinitrophenyl hydrazine were obtained from BDH, UK. The rest of the chemicals used were of analytical grade.

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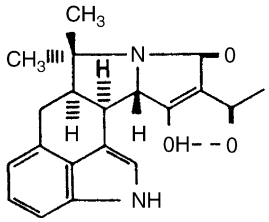

Fig. 1. Structure of CPA.

2.1. Isolation of CPA from Aspergillus tamarii

CPA was isolated by the method described by Rao and Husain (1985) with some modifications. The fungus was grown in several 1 l Erlenmeyer flasks containing 200 ml modified Richards medium (g/l: KNO_3 10.0, KH_2PO_4 5.0, $MgSO_4{\cdot}7H_2O$ 2.5, $FeCl_3$ 0.01, sucrose 50.0, and yeast extract 1.0) for 10 days at 25–27 °C. After the incubation period, the cultures were harvested, centrifuged at 4000 rpm for 15 min, and mycelial biomass and culture filtrate were separated. The mycelium was washed, dried at 45–50 °C for 48 h, and powdered. The culture filtrate (10 l) and dried mycelial powder were extracted separately with chloroform. The chloroform extract of culture filtrate and mycelium were pooled and dried over anhydrous Na_2SO_4. The solvent evaporated to dryness under vacuum. The residue, crude toxin (3.5 g), was obtained. The crude toxin was analyzed for the presence of CPA by TLC on silica gel G plates impregnated with 0.4N oxalic acid using chloroform:methylethylketone (80:20) as solvent system. The plates were sprayed with Ehrlich reagent (1 g *p*-dimethyl benzaldehyde in 30 ml of 50% HCl). CPA was detected as violet-colored spot (Rao and Husain, 1985). The crude toxin was further purified by column chromatography using cellulose powder impregnated with formamide:oxalic acid and then eluted with hexane followed by hexane:benzene mixture (Holzapfel, 1968). The hexane:benzene mixture fractions were combined and the solvent evaporated completely at low temperature. Further purification of the toxin was done by preparatory TLC on silica gel G impregnated with 0.4N oxalic acid using chloroform:methylethylketone (80:20) solvent system (Rao and Husain, 1985). CPA band was removed carefully eluted with chloroform–acetone. The fraction was washed several times with water to remove oxalic acid. The solvent was completely evaporated and the residue contained chromatographically pure (99%) CPA (280 mg).

2.2. Treatment schedule

Male Wistar rats (100 ± 5 g) maintained under environmentally controlled conditions and standard solid pellet diet were used for the experiments. The CPA (99% pure) was dissolved in vegetable oil and administered at a dose of 50 mg/kg body weight (5 mg in 200 μl vegetable oil/animal). The animals were divided in four groups. Group I: untreated control (vegetable oil 200 μl/animal) once; Group II: CPA (5 mg in 200 μl vegetable oil) once; Group III: vegetable oil (200 μl/animal) three times alternate days; Group IV: CPA (5 mg in 200 μl vegetable oil/animal) three times alternate days.

2.3. Assay for biochemical parameters

All animals were sacrificed 48 h after the last dose by stunning and their blood was collected by cardiac puncture into clean dry centrifuge tubes. The livers were immediately taken out, cleaned, and weighed. The blood was allowed to clot at 8–10 °C and clear serum was collected after centrifugation. One-fourth of the liver was homogenized (10% w/v) in 0.05 M Tris buffer (pH 7.0) for the estimation of enzyme, gamma glutamyl transpeptidase (GGT), and another part was homogenized in 0.25 M sucrose for the assay of glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and lipid peroxidation. Third portion of the liver was homogenized in 0.15% KCl containing 1 nmol nicotinamide and used for the assay for aryl hydrocarbon hydroxylase (AHH) and aminopyrene-*N*-demethylase. The fraction used for non-protein thiols (NPSH) was homogenized in mannitol–EDTA.

GGT activity was assayed according to the method of Roomi and Goldenburg (1981) in liver and in sera according to the method of Naftalin et al. (1969). GOT and GPT were assayed by the method of Wootton (1964) in liver and serum. AHH was estimated by the method of Dehnean et al. (1973) and aminopyrene-*N*-demethylase was estimated according to Schenkman et al. (1967). Non-protein thiols were estimated according to the method of Sedlak and Lindsay (1968). Lipid peroxidation was measured by the method of Utley et al. (1967) and protein content of the samples was estimated by the method of Lowry et al. (1951).

2.4. Statistical analysis

Experimental data were expressed as mean ± S.D. The Student's *t*-test was applied for detecting the significance. $P < 0.05$ was regarded as significant.

3. Results and discussion

Both single and multiple dose administration of CPA had shown some behavioral changes like breathlessness and lethargy, reluctance to move even on provocation. But a few hours after treatment, they became apparently normal. An oral dose of 50 mg/kg body weight CPA has been reported to cause severe liver lesions and cell necrosis in rats (Purchase, 1974). Hence, this dose was selected for the studies. Activity of aminopyrene-*N*-demethylase was found to be decreased in animals exposed to CPA (Table 1). The induction of aminopyrene-*N*-demethylase is considered to be related with synthesis of cytochrome P-450 (Pelissier and Albrecht, 1976). A significant increase in the AHH

Table 1
Effect of CPA on hepatic aryl hydrocarbon hydroxylase (AHH) and aminopyrene-*N*-demethylase

Parameter	Group			
	I	II	III	IV
AHH(nmol 3-OH B (a) P formed/h/mg protein)	2.90 ± 0.11	4.80 ± 0.31**	2.50 ± 0.26	5.60 ± 0.22***
N-demethylase (nmol formaldehyde formed/min/mg protein)	3.90 ± 0.57	3.45 ± 0.48	4.40 ± 0.45	2.00 ± 0.25**

Each value represents mean ± S.D. of six rats.
** $P < 0.01$.
*** $P < 0.001$.

activity was observed in both single (Group II) and multiple dose (Group IV)-treated animals (Table 1) which indicated that there might be some involvement of polycyclic aromatic hydrocarbon (PAH)-specific modulation of aminopyrene-*N*-demethylase. The two-fold increase in AHH activity indicated the possibility of CPA to induce only CYP1A1- and CYP1A2-dependent induction, which might convert CPA to its toxic metabolite forms that could bind to the macromolecules inside the cell (Ioannides et al., 1984). Therefore, it may be suggested that the AHH thus induced may activate CPA to its intermediate forms for the expression of their mutagenic/preneoplastic/carcinogenic potential.

A significant increase in the activity of GGT, which is a marker enzyme for the assessment of preneoplastic changes (Hanigan and Pitot, 1985; Periano et al., 1983), was observed in both liver and serum in the animals of the Groups II and IV (Table 2). GGT is known to catalyze the transfer of gamma glutamyl group of compounds containing this group to a wide variety of amino acceptors (Meister, 1973). Abnormally high levels of GGT were also observed in tumors of a variety of tissues including hepatocellular carcinomas (Brelsterili, 1979).

The parameters examined to evaluate hepatic damage in this study were tissue and serum glutamate oxaloacetate transaminase (GOT and SGOT) and tissue and serum glutamate pyruvate transaminase (GPT and SGPT). Significant increase in the activity of both the enzymes in tissue and serum of CPA-exposed animals were observed. Induction in the activity of these two enzymes are reported to be associated with generalized hepatotoxicity.

The levels of lipid peroxidation in the liver of CPA-exposed animals did not show any appreciable variation. This indicates that free radicals are not involved in CPA-induced hepatotoxicity (Table 3). However, significant increase in the non-protein thiols was found in animals exposed to CPA (Table 3). The increase in the levels of non-protein thiols may be due to the formation of deoxyribonucleoside diphosphate (dNdp). The formation of dNdp in relatively more efficient manner appears to be of metabolic

Table 2
Effect of CPA on gamma glutamyl transpeptidase (GGT), glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT) in liver and serum of rats

Parameter	Group			
	I	II	III	IV
GGT (nmol *p*-nitroaniline liberated/min/mg protein)	3.89 ± 0.35	6.44 ± 0.45**	4.5 ± 1.1	18.0 ± 2.7***
GOT (μmol/min/g tissue)	7.84 ± 0.75	9.24 ± 0.82*	6.22 ± 0.43	16.33 ± 1.77**
GPT (μmol/min/g tissue)	39.87 ± 5.1	48.4 ± 2.7*	38.91 ± 3.20	98.53 ± 6.30***
SGGT (nmol *p*-nitroaniline liberated/min/mg protein)	29.42 ± 1.42	35.49 ± 1.30*	27.33 ± 1.7	61.24 ± 2.98***
SGOT (μmol/min/g protein)	0.306 ± 0.029	0.459 ±0.017**	0.497 ± 0.013	0.752 ± 0.025***
SGPT (μmol/min/g protein)	3.269 ± 0.250	4.68 ± 0.23**	2.89 ± 0.17	4.949 ± 0.15***

Each value represents mean ± S.D. of six rats.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
Non-protein thiols and lipid peroxidation in liver of rats following exposure to CPA

Parameter	Group			
	I	II	III	IV
Non-protein thiols (nmol/mg protein)	17.80 ± 0.97	21.40 ±1.02*	20.2 ± 1.18	34.3 ± 1.62*
Lipid peroxidation (nmol malonaldehyde formed/h/mg protein)	0.478 ± 0.024	0.56 ± 0.370	0.475 ± 0.04	0.60 ± 0.04

Each value represents mean ± S.D. of six animals.
* $P < 0.05$.

importance in cell proliferation subsequent to cellular damage (Holmgren, 1976).

The present investigations reveal that single or repeated oral administration of CPA may exert hepatotoxic/hepatocarcinogenic risk to the exposed population. Kodo millet is often found contaminated with *Aspergillus tamarii*, which is reported to produce significant amount of CPA. The extract of the infected millet with the fungus has also been found to produce this mycotoxin (Rao and Husain, 1985). The findings thus suggest that consumption of contaminated Kodo millet is a serious health hazard to humans because of the risk of exposure to CPA. Although *Aspergillus tamarii* isolated from kodo millet has been reported to produce an indole alkaloid, fumigalavin A (Janardhanan et al., 1984), no information is available on the involvement of this compound in hepato-toxicity.

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