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Sub chronic toxicity study of mixed fungal isolates culture filtrates from ground nut hay in rats

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Abstract

Sub chronic toxicity study of mixed culture filtrate of *Rhizopus oryzae*, *Fusarium verticilloides* and *Fusarium xylarioides* isolated from the fungal contaminated ground nut hay was conducted in rats. The fungi were isolated from the contaminated groundnut hay which caused mycotoxicosis in cross bred cattle, exhibited the clinical signs of colic, tenesmus, ruminal atony, anorexia, bleeding from nostrils, rectum and fly bite site. The rats were gavaged with culture filtrate at the dose level of 0.5, 1 and 2 ml daily for 90 days. Clinical signs observed were diarrhea, weakness, severe arching of back, swollen forehead and conjunctival hemorrhage. Cutaneous hemorrhagic patches on back, scrotum, abdomen, ears and legs region seen. There was a significant increase ($P < 0.05$) in serum concentrations of creatinine, urea nitrogen, ALT and AST indicated the renal and hepatic damage which was confirmed by histopathology. There were lesions in brain and GI tract of the treated rats. The present study indicated the toxic feature of the mixed culture filtrates of fungi *Rhizopus oryzae, Fusarium verticilloides* and *Fusarium xylarioides* isolated from ground nut hay.

Key words : Culture filtrate, sub chronic toxicity, groundnut hay, rat

Introduction

Fungal infected hay can infect dairy cattle, especially during stressful periods when they are immune suppressed, causing a disease referred to as mycosis. Molds also produce secondary metabolites or poisons called mycotoxins that affect animals when they consume mycotoxin contaminated feeds. This disorder is called mycotoxicosis (Pier, 1992). The total number of mycotoxins is not known, but the number of potential toxic metabolites of fungi has been estimated to be in thousands, although to date only about 300 different mycotoxins have been identified (CAST, 2003). Mycotoxicosis is well documented in poultry. However there is scanty information on cattle mycotoxicosis.

Cross bred cattle fed with contaminated groundnut hay for duration of 2-3 months exhibited the clinical signs of loss of body condition, colic, tenesmus, ruminal atony and anorexia which was noticed in Pattanayakanahalli village of Tumkur District in Karnataka, India. There was bleeding from nostrils, rectum and fly bite site of the affected animals. Liver function tests of these animals revealed liver damage. The detailed clinical investigation and history revealed that the groundnut hay was fed to these animals and revealed blackish specks or spots indicative of fungal growth. The clinical signs were observed especially during winter season.

In the present study, the sub chronic toxicity of the mixed culture filtrates of fungi *Rhizopus oryzae, Fusarium verticilloides* and *Fusarium xylarioides* isolated from fungal affected groundnut hay was evaluated in rats.

Materials and Methods

Collection of material:

Fungal contaminated groundnut hay which was fed to the ailing animals was obtained from Pattanayakanahalli village of Sira taluk, Tumkur District. Groundnut hay was cultured on potato dextrose agar. The pure cultures were sent to Fungal Identification Service, Mycology and Plant Pathology Group, Agharkar Research Institute, Pune, for identification. Among the grown fungal isolates, *Rhizopus oryzae* (*R. oryzae*), *Fusarium verticilloides* (*F. verticilloides*) and *Fusarium xylarioides* (*F. xylarioides*) were mass cultured on potato dextrose broth. After confirmation of complete growth of the fungus, the supernatant was discarded and the filtrate was used for gavaging the rats. The fungal culture filtrate was analyzed for the presence of aflatoxins (B1, B2, G1 and G2), ochratoxin, T2, citrinin, sterigmatocystin and zeralenone by TLC method.

Experimental design:

Apparently healthy young Wistar albino rats, aged 5 weeks having body weight of 100 ± 10 g were used. Seven groups of rats (n=12) were made and housed in standard polypropylene cages during the experiment. Group I served as control which was gavaged with 2 ml of potato dextrose broth where as group II, III, IV gavaged with all three culture filtrates in equal proportion, where as Group V, VI and VII were gavaged with *R.oryzae* culture filtrate 50%, *F. verticillioides* and *F. xylarioides* 25% culture filtrates at the dose level of 0.5, 1 and 2 ml daily respectively for 90 days. The rats were weighed individually at the beginning of the study and at fortnight interval till day 90. All the rats were observed daily for the clinical signs of toxicity, morbidity and mortality.

Clinical biochemistry:

The blood samples were obtained by retro-orbital plexus puncture method on day 0, 7, 14, 21, 35, 50, 75 and 90 and the serum was used to estimate concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRT) and blood urea nitrogen (BUN) using Semi-Automatic Biochemical Analyzer (ARTOS, Bangalore) and commercially available diagnostic kits (Swemed Diagnostics, Bangalore).

Gross and histopathological studies:

Necropsy of rats was conducted which succumbed during the experiment and the survived rats were sacrificed at the end of the study. Organs were weighed and representative tissue samples of liver, kidney, brain, intestines and stomach were collected in 10 % normal buffer formalin solution (NBF) and were subjected to histopathology (Luna, 1968).

Statistical analysis:

The data was analyzed by one-way ANOVA with Tukey's post test using GraphPad Prism Software (Trial version 5.00 for Windows) GraphPad Software, San Diego, California, USA.

Results

In the present study, the predominant fungal species isolated from the fungal contaminated ground nut hay were *R. oryzae, F. verticilloides* and *F. xylarioides*. Perusal of the literature revealed no reports of the same fungal species identified on groundnut hay. The fungal culture filtrate was negative for all the nine mycotoxins analyzed.

Clinical signs in rats observed were diarrhea, weakness, reduced feed /water intake, loss of body weight, recumbency, slight arching of back, swollen forehead and torticollis. The rats lost balance of hind limbs and rarely the forelimbs. The rats lost balance of hind limbs and rarely the forelimbs. Hemorrhages were seen on conjunctiva, sub cutis on back, scrotum, abdomen, ears and legs region.

There was a significant increase ($P<0.001$) in serum ALT and AST concentrations in the samples of day 21, 35, 50, 75 and 90 (Table 1 and 2). The gross changes in the liver comprised of hemorrhage, congestion and the typical histopathological lesions like severe congestion, centrilobular necrosis, vacuolar degeneration, mild biliary hyperplasia, fibrotic change at periportal areas and karyomegaly in some of the hepatocytes (Fig 1 and Fig 2).

The serum creatinine concentration in rats increased significantly (P<0.001) from day 35 to 90 in groups treated with 1 and 2 ml of culture filtrate and no significant change in the serum urea nitrogen concentration observed (Table 3 and 4). The histopathological changes in kidney comprised of congestion along with vacuolar degeneration, necrosis of the tubules and fibrosis in the interstitium (Fig 3). Mild congestion of intestinal mucosa and hemorrhage was observed. Congestion of blood vessels of stomach was observed in all group of the rats.

In the present study, lesions in the brain comprised of congestion of blood vessels, perivascular cuffing with mononuclear cells, multiple focal areas of necrosis with infiltration of few inflammatory cells and occasional glial cell aggregation (Fig 4).

Discussion

Hemorrhages were seen on conjunctiva, sub cutis on back, scrotum, abdomen, ears and legs region. These results were similar to the earlier findings of Yiannikouris and Jouany (2002), who reported that mycotoxins caused weight loss, vomiting, severe skin problems and bleeding and death of animals.

The gross changes in liver comprised of hemorrhage, congestion and the typical histopathological lesions confirmed the liver damage due to culture filtrate treated groups in rats. The results of the present study is in accordance with the findings of Sharma *et al*. (1983).

The elevated serum AST and ALT concentration compared to control group is suggestive of the possible role of mycotoxins present in gavaged culture filtrate in liver damage. This was further supported by the gross and histopathological lesions in the treated groups, by the presence of lesions of severe congestion, centrilobular necrosis, vacuolar degeneration, mild biliary hyperplasia, fibrotic change at periportal areas and karyomegaly in some of the hepatocytes. Such hepatic damage in rats and mice due to mycotoxicosis was also reported by other workers with elevation of serum AST concentration (Voss *et al*. 1995; Kellerman *et al*. 1990; Fodor *et al*. 2006).

The serum creatinine concentration in rats increased significantly (P<0.001) from day 35 to 90 from day 35 to day 90 in groups treated with 1 and 2 ml of culture filtrate and no significant change in the serum urea nitrogen concentration observed. The elevated serum creatinine concentration in comparison to control concentration, is suggestive of the possible role of the toxins in causing kidney damage. This was further supported by histopathological lesions like, congestion along with vacuolar degeneration, necrosis of the tubules and fibrosis in the interstitium. Similar findings were also reported by Fodor *et al.* (2006).

Mild congestion of intestinal mucosa and hemorrhage was observed. T-2 toxin is a very potent mycotoxin in cattle which was associated with gastroenteritis and intestinal hemorrhages (Petrie *et al*. 1977). However T2 toxin was negative in the screening of the culture filtrate which indicates either concentration of T2 might be too low to be detected by TLC method or rats might be susceptible to such low concentration.

Congestion of blood vessels of stomach was observed in all group of the rats. Similar findings were reported by Junsuk *et al.* (1999) in rats which were fed with fungal contaminated the diets. The changes in the brain described as necrosis, liquefaction and hemorrhages in the present study were similar to those of earlier reports of Uhlinger, (1997).

Further studies are essential to confirm the changes seen under natural disease process in large animals by considering many factors including dose, concentration of the fungal culture extract and the form in which the test material is administered.

Conclusion

In mixed culture filtrate administered group, i.e., all three culture filtrates in equal proportion administered rats were diarrheic, had arching of back and swollen forehead. Loss of balance in the legs region seen. The present study revealed the hepatotoxic, nephrotoxic and cardiotoxic nature the culture filtrate in rats at the dose of 0.5, 1 and 2 ml in the sub chronic toxicity study obtained from mouldy ground nut hay, which had caused toxicity in cattle. The toxin/s present in the culture filtrate has to be identified and isolated.

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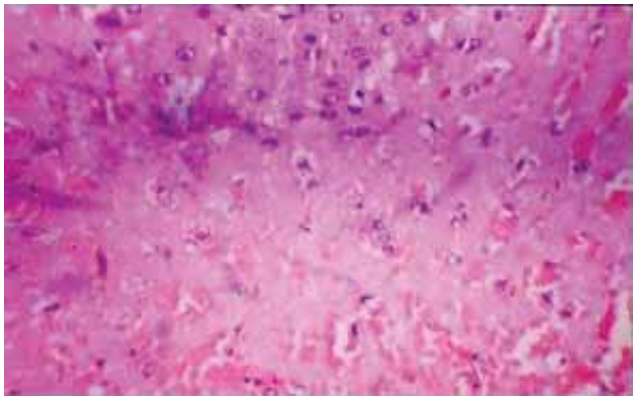

Fig 1:Section of liver from rat treated with *Rhizopus oryzae* culture filtrate showing extensive with loss of normal architecture. Note hepatomegaly in some of hepatocytes adjacent to normal cells .

H&E x 500

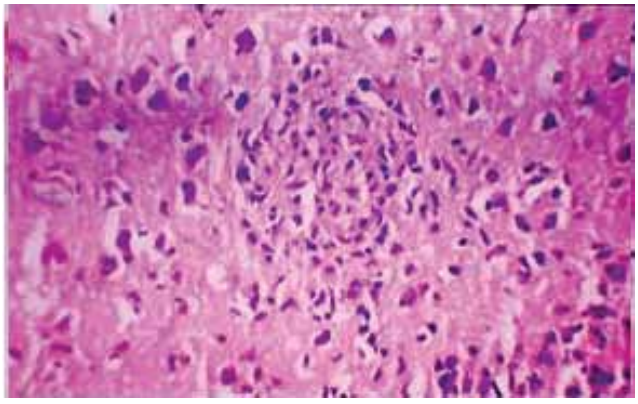

Fig 2: Section of liver from rat treated with *Fusarium xylarioides* culture filtrate showing prominent biliary hyperplasia in periportal region

H&E x 500

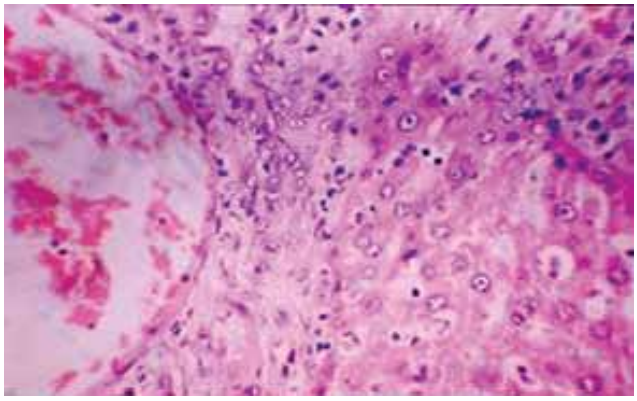

Fig 3: Section of kidney from rat treated with *Fusarium verticilloides* culture filtrate showing severe congestion of inter tubular vessels and focal areas of tubular necrosis with loss of architecture

H&E x 500

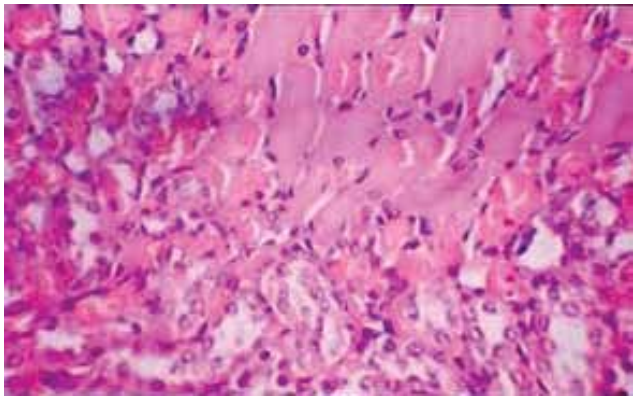

Fig 4:Section of brain from rat treated with *Fusarium xylarioides* culture filtrate showing severe congestion of blood vessels and perivascular mononuclear cuffing

H&E x 500.

Table 1: The effect of mixed fungal culture filtrates on serum ALT concentration (U/L)

Type of culture filtrate	Days							
	0	7	14	21	35	50	75	90
Group I (PD broth, Control)	34.05±1.15	36.22±1.04	38.63±1.06	39.41±0.97	43.06±0.64	45.49±1.18	47.74±1.15	46.55±1.85
Group II	35.28±1.53	41.99±1.63	51.54±2.50	54.28±2.54***	57.85±2.78***	65.58±1.84***	71.47±1.94***	66.36±1.72***
Group III	35.06±1.41	40.38±1.9	45.58±1.75	48.15±2.02*	50.96±2.09*	59.30±2.25***	65.86±2.30***	61.20±2.11***
Group IV	33.90±1.52	38.28±1.77	42.34±2.33	44.50±2.43	46.81±2.45	53.99±1.93*	61.92±2.10***	57.71±2.26**
Group V	35.74±1.50	42.02±1.72	55.77±1.84	58.20±1.95***	60.93±2.04***	69.03±1.66***	74.90±1.97***	69.75±1.39***
Group VI	34.62±1.58	40.36±1.52	50.82±1.77	53.20±1.73**	55.47±1.66***	63.45±2.01***	68.93±1.81***	64.92±1.77**
Group VII	32.79±1.37	38.57±1.61	44.59±2.11	47.18±2.13*	50.01±2.14	62.35±1.68***	66.32±2.17***	62.70±2.18**

Values are mean ± SE, n = 12, *** P < 0.001,* * P < 0.01.

Table 2: The effect of mixed fungal culture filtrates on serum AST concentration (U/L)

Type of culture filtrate	Days							
	0	7	14	21	35	50	75	90
Group I (PD broth, control)	100.43±1.41	104.16±0.61	105.88±0.70	107.98±0.69	110.40±0.72	115.05±0.78	108.08±0.77	101.46±0.66
Group II	101.90±1.58	115.05±3.51	150.76±3.53**	195.79±4.06***	235.88±4.26***	254.10±10.10***	240.77±8.82***	225.13±6.20***
Group III	100.57±0.92	112.95±2.47	144.00±2.85	188.91±4.31	225.54±4.67	259.23±4.52***	245.03±3.31***	233.83±2.64**
Group IV	101.55±1.53	109.56±2.21	132.46±3.58	179.44±3.19	208.79±5.02	230.71±8.32	217.32±5.77***	205.30±4.19**
Group V	100.48±1.72	115.68±4.09	157.90±3.03***	209.38±4.92***	240.88±2.78***	267.71±5.73***	246.81±4.452***	230.21±3.72***
Group VI	101.36±1.46	114.35±2.63	149.24±3.49**	198.095±3.91***	223.01±4.65***	260.59±3.81***	243.79±3.01***	226.40±2.95**
Group VII	100.85±1.74	112.50±2.20	140.99±3.02	191.76±2.22	221.46±3.58	251.62±4.88***	239.29±3.89***	218.50±4.45**

Values are mean ± SE, n = 12, *** P < 0.001,* * P < 0.01

Table 3: The effect of mixed fungal culture filtrates on serum creatinine concentration (mg/dl)

Type of culture filtrate	Days							
	0	7	14	21	35	50	75	90
Group I (PD broth, Control)	0.49±0.033	0.54±0.030	0.57±0.036	0.61±0.025	0.58±0.044	0.58±0.035	0.56±0.03	0.55±0.027
Group II	0.46±0.031	0.51±0.021	0.67±0.03	0.78±0.01**	0.80±0.022***	0.91±0.034***	0.96±0.04***	0.84±0.026***
Group III	0.46±0.02	0.52±0.021	0.59±0.014	0.69±0.021	0.75±0.027***	0.77±0.022***	0.82±0.03***	0.76±0.031***
Group IV	0.45±0.028	0.49±0.023	0.57±0.021	0.63±0.019	0.72±0.019*	0.75±0.019**	0.80±0.08***	0.74±0.023**
Group V	0.48±0.027	0.53±0.024	0.57±0.021	0.62±0.023	0.67±0.022	0.76±0.018***	0.86±0.03***	0.79±0.023***
Group VI	0.46±0.028	0.50±0.021	0.57±0.020	0.63±0.023	0.70±0.023	0.75±0.018***	0.76±0.02***	0.69±0.013**
Group VII	0.47±0.03	0.50±0.025	0.56±0.020	0.63±0.018	0.69±0.023	0.71±0.014*	0.79±0.01***	0.69±0.022**

Values are mean ± SE, n = 12, *** P < 0.001,* * P < 0.01.

Table 4 : The effect of mixed fungal culture filtrates on serum urea nitrogen concentration (mg/dl)

Type of culture filtrate	Days							
	0	7	14	21	35	50	75	90
Group I (PD broth, Control)	38.38±1.30	39.66±1.33	40.99±1.14	42.43±1.39	44.06±1.75	46.00±1.92	41.75±1.72	38.07±1.34
Group II	40.50±0.96	41.52±0.98	43.14±1.22	46.04±2.24	49.84±1.99	59.54±2.93***	52.57±2.12***	46.03±1.31**
Group III	39.04±1.02	40.13±1.08	40.99±1.28	42.05±1.57	45.11±1.60	55.06±2.11**	47.09±1.68	41.91±1.33
Group IV	37.016±0.81	38.10±0.82	38.25±1.08	38.91±1.02	40.29±1.60	47.94±2.29	43.31±1.99	38.24±1.65
Group V	41.19±0.75	42.86±0.86	44.24±0.90	47.79±1.81	52.10±2.29**	57.73±2.17***	50.20±1.77**	44.22±1.48
Group VI	39.39±1.15	40.24±1.12	41.10±1.20	42.14±1.63	45.27±1.71	52.43±1.55	44.84±1.54	39.03±1.17
Group VII	37.87±0.94	39.54±1.48	40.18±1.58	41.60±2.19	43.78±2.54	50.47±2.23	46.18±1.71	38.89±1.01

Values are mean ± SE, n =12, *** P < 0.001,* * P < 0.01. ■