HEPATIC HYPERPLASIA AND DAMAGES INDUCES BY ZEARALENONE *Fusarium* Mycotoxins in BALB/c Mice

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Abstract – ***Context*** - Zearalenone is a mycoestrogen and considered a mycotoxin. ***Objective*** - To establish whether zearalenone produced hepatotoxicity via oral administration. ***Methods*** - Zearalenone was orally administered at a dose of 50 μg, 100 μg and 200 μg ZEN/ body weight/daily, respectively, for 14 days to three groups of BALB/c mice. Diagnostic modalities used to evaluate hepatic damage and impaired hepatic function pre- and post zearalenone administration included hepatic marker enzyme activity, pentobarbital sleeping time, cytochrome $P\text{-}_{450}$ activities and histopathologic evaluation of liver. ***Results*** - Significant histopathologic changes viz. sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis and neutrophil infiltration were observed after evaluating of liver section from each group after accumulated zearalenone exposure. Further, zearalenone exposure increased activities of alanine transaminase, aspartate transaminase and lipid peroxides whereas activities of tissue glutathione and cytochrome P_{450} were decreased as compared to control mice. Zearalenone also increased the sleeping time and decreased sleeping latency after pentobarbital through intraperitoneal route as compared to control mice which indicates that the impairment of hepatic metabolizing enzymes by zearalenone. ***Conclusion*** - Zearalenone is a potential hepatotoxin by oral route.

Headings – Zearalenone. Hyperplasia. Liver, pathology. Mice.

INTRODUCTION

Zearalenone (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-Bresorcyclic acid lactone) (ZEN), is a non-steroidal mycoestrogen that activates the estrogen receptors (ERs), ESR1 and ESR2, where it acts as an agonist and partial antagonist to estradiol[5, 8, 10]. ZEN also reported that facilitates carcinogenesis, reproductive toxicity[10] and suppress immunity[9]. ZEN contaminated of cereals and grains and related products causes food and feed-borne intoxications (myco-toxicoses) in man and livestock. This mycotoxins produces by various members of the genus *Fusarium*, including deoxynivalenol (mostly produced by *F. graminearum* and *F. sporotrichoides*) and zearalenone (produced by *F. graminearum* and *F. culmorum* among others) which are major concern in health.

Most of its biological activities of ZEN are attributed to the agonist effect on the ER and certain biological reactions of zearalenone that can not be explained by its estrogenic activity[5]. Estrogens taken by women for birth control purposes are implicated as a cause for development of both benign and malignant hepatic neoplasms[11, 12]. In addition to endogenous steroidal estrogens, a variety of compounds with estrogenic activity also are found in the environment because a number of drugs, insecticides, and natural food products representing many different structures can act like estrogens by products by first phase metabolic transformation after ingestion. Therefore estrogenic activities of ZEN have possibilities to extend the other biological effects - hepatotoxicity.

The aim of the research was to examine the toxic influence of ZEN on liver through estimating mycotoxin influence on markers evaluating hepatic damage.

METHODS

Animals and housing conditions

All the born in the laboratory breeding colony of Central Facility of Animal House, Defence Research Laboratory Tezpur, Assam, India, and maintained under temperature controlled rooms at animal house with 12h alternating light and dark cycles. Food was obtained from Pranav Agrotech, Delhi, India. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal care" (NIH publication 85-23, revised 1985) and approved by Institutional Use and Care Committee.

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Study design

Twenty-four Balb/c mice were divided into control group (I) (n = 6) were given 0.9% saline (5 mL/kg, p.o), ZEN treated groups viz. Groups II, III and IV were given 50 µg,100 µg, 200 µg ZEN/ body weight /daily, respectively, by oral route for 14 days (n = 6). After 14 days mice were euthanized by ether inhalation and sacrificed. Blood samples were obtained from the right ventricle via a left anterior thoracotomy and separated serum. The serum samples were stored at -20°C until used for ALT and AST assays. A portion of liver lobes were fixed in buffered 10% formalin, embedded in paraffin, and used for histopathology by using hematoxylin and eosin (H-E) staining. Another portion of lobe was stored at -20°C for enzymatic assay.

Preparation of mice of liver microsome

Liver microsome was measured as described by previously[18]. Briefly, 100 mg liver was excised and homogenized with a loose-fitting Teflon pestle in 900 mL of 50 mM L^{-1} Tris-HC1buffer, pH 7.4, containing 0.3 M L^{-1} sucrose, 10 mM L^{-1} DTT, and 10 mM L^{-1} EDTA. The homogenate was centrifuged at 20,000 x g for 15 min. The supernatant solution was centrifuged at 10,000 x g for 60 min. The microsomal fraction obtained was suspended in a homogenizing medium without DTT and re-centrifuged at 10,000 x g for 60 min. The resulting microsomal fraction was suspended in 0.1 ML^{-1} phosphate buffer pH 7.4, containing 1 mM L^{-1} EDTA and volume was adjusted to 10 mL with phosphate buffer.

Estimation of hepatic marker enzymes

Serum was used for the assay of alanine transaminase (ALT) and aspartate transaminase (AST) using assay kits (Transasia, Mumbai, India) according to the manufacturer's instructions.

Determination of cytochome P_{450} (CYP_{450})

Hepatic cytochome P_{450} was measured as described by previously[16]. Briefly 1 mL supernatant of liver microsomes was taken and 10 mg of sodium dithionate was added. Air was bubbled for 10 seconds and then centrifuged for 5 minutes at 3000 x g. Five hundred µl (500 µl) supernatant was mixed with 5 mL Tris HCl buffer (pH 7.4) and absorbance was measured at 450 nm wavelengths using excitation coefficient of 91 mol $L^{-1}cm^{-1}$ for A_{450}.

Light microscopy analysis

Six µm stains of formalin –fixed and paraffin-embedded were taken and stained with H-E. Slide was examined by a pathologist who had no prior knowledge of the treatment groups. Histopathology of liver was evaluated by using a scoring system (Table 1) as described previously[19].

Two types of eosinophilic hepatocellular changes are presumed to be apoptotic in origin viz. round and detached from surrounding hepatocytes (classical Councilman bodies) and shrunken compared to adjacent hepatocytes, but still firmly attached were counted in 5 to 20 fields from each liver to count at least 100 stellate –Abs. The changes in stellate cells were calculated by using a scoring system.

TABLE 1. Scale of histopathology scoring

Histopathology	
Assessment	**Score**
Steatosis (the percentage of liver cells (containing)	
5% to 25% of cells containing fat	1+
26% to 50% of cells containing fat	2+
51% to 75% of cells containing fat	3+
>75% of cells containing fat	4+
Inflammation and necrosis	
One focus/ lobule	1+
Two or more foci/ lobules	2+
Apoptosis	3+
Stellate cells	
If nucleus fragmented present or absent	1+
If nucleus Pyknotic (or those in intact nuclei)	2+
If nucleus Pyknotic, fragmented or absent	3+
If adjacent inflammation present	4+
If located in an acidophilic domain	5+
If nodular hyperplasia	6+
If atrophy present	7+
If necrosis present	8+
If apoptosis present	9+

Determination of lipid peroxides (LPO)

Hepatic LPO was measured as method described[7]. To 100 µl separated microsomes in 0.1(M) phosphate buffer saline, 1 mL of 28% trichloroacetic acid was added and centrifuged at x 2000 g at 4ºC for 20 minutes. One milliliter of supernatant was separated and 900 µl of 1% thiobarbituric acid was added and volume was adjusted to 3 mL by using phosphate buffer (pH 7.0), heated in water bath for 60 min and cooled in ice bath. The absorbance was measured at 532 nm. The lipid peroxidation was calculated on the basis of the molar extinction coefficient (1.56×10^5) of malondialdehyde.

Tissue glutathione (GSH) assays

The hepatic reduced GSH level was determined by the method of Ellman[2]. Briefly, after 0.2 g liver tissues were homogenized in 4 mL of 0.02 M EDTA Na2 (using an all glass homogenizer in an ice bath). In 2.5 mL tissue homogenates (aliquots) were mixed with 2.0 mL of distilled water and 0.2 mL of 50% TCA. All tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 × g. Two milliliter of 0.4 M Tris buffer (pH 8.9) and 0.1 mL of 0.01 M 5,5'-dithiobis- 2-nitrobenzoic acid (DTNB) were added to 2.0 mL of tissue supernatant, and the sample was shaken. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank with no homogenate. GSH levels were calculated using standard curve prepared by known amounts of GSH (Aldrich Chemical Co. Ltd, Germany). The concentration of GSH was expressed as mg/g tissue.

Pentobarbital sleeping time

The pentobarbital sleeping time test was performed using pentobarbital. After 14 days treatment with ZEN animals received pentobarbital (concentration of 3 g/100 mL of solution) through intraperitoneal injection at a dosage of 50 mg/kg of mice body weight (or 0.0017 mL/g of animal weight) and time was measured in minutes, from the loss of position reflex to its gaining reflexes.

Statistical analysis

Data are expressed as mean ± S.D. The statistical significance between data means was determined by Student's *t*-test. *P*-values $P<0.05$ were considered as significant

RESULTS

Effect of ZEN on hepatic marker enzymes and cytochrome P_{450}

ZEN increases significantly ($P<0.01$) AST, ALT levels whereas cytochrome P_{450} levels decreases as compared to controlled mice. Activities of ALT and AST were 55.23 ± 7.1 and 52.19 ± 9.40 $I.UL^{-1}$, respectively, in controlled mice which increased to 87.96 ± 4.64 and 70.33 ± 3.59.UL^{-1}, respectively, in ZEN treated (200 µg/kg body weight) mice.

Microsomal, cytochrome P_{450} levels in the control mice were 0.66 ± 0.032 nML^{-1} mg^{-1} proteins which decreased to 0.29 ± 0.017 nML^{-1} mg^{-1} proteins after treatment for 14 days with ZEN 200 µg/kg body weight (Table 2).

Effect of ZEN on LPO, GST, sleeping time and sleep latency

After treatment with ZEN significantly ($P<0.01$) increased LPO levels and GST levels were significantly decreased ($P<0.01$) as compared to control group. Activities of LPO and GST were 0.7 ± 0.05 nML^{-1} MDA mg^{-1} of protein and 52.40 ± 4.90 mM L^{-1} of 1-chloro 2,4-dinitrobenzene (CDNB) conjugated min^{-1} 100 mg^{-1} protein in control groups and after treatment with ZEN (200 µg/kg body weight) increased to 5.22 ± 0.80 nML^{-1} MDA mg^{-1} of protein whereas GST decreased to 36.20 ± 3.77 mM L^{-1} of 1-chloro 2,4-dinitrobenzene (CDNB) conjugated min^{-1} 100 mg^{-1} protein.

Microsomal, cytochrome P_{450} levels in the control mice were 0.66 ± 0.032 nML^{-1} mg^{-1} proteins which decreased to 0.29 ± 0.017 nML^{-1} mg^{-1} proteins after treatment for 14 days with ZEN 200 µg/kg body weight (Table 3). Pentobarbital along with ZEN increased significantly ($P<0.01$) sleeping time as compared to control mice (Table 4).

Effect of ZEN on liver histopathology

Liver histopathology was evaluated based on sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis, and neutrophil infiltration (Table 5). Histopathology examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoids spaces and central vein (Figure 1a). ZEN treated group showed that disarrangement and degeneration of normal hepatic cells with intense centri-lobular necrosis extending to mid– zone and sinusoidal hemorrhages and dilation.

TABLE 2. The effect of ZEN on ALT, AST, cytochrome P_{450} levels after 14 days treatment

Groups	[a]ALT	[a]AST	[b]Cytochrome P_{450}
Control mice (Group I)	55.23 ± 7.10	52.19 ± 9.40	0.66 ± 0.032
ZEN treated (Group II) (50 µg/kg body weight)	62.11 ± 5.32*	60.73 ± 4.47*	0.50 ± 0.047
ZEN treated (Group III) (100 µg/kg body weight)	70.33 ± 6.91*	64.77 ± 5.06*	0.33 ± 0.053*
ZEN treated (Group IV) (200 µg/kg body weight)	87.36 ± 4.64*	70.33 ± 3.59*	0.29 ± 0.017*

Results are expressed as mean ± SD (n = 6). *Statically different ($P<0.01$) from control. [a]Expressed in I.U. L^{-1}, [b]Expressed as nML^{-1} protein

TABLE 3. The effect of ZEN on GST and LPO after 14 days oral administration

Groups	[a]GST	[a]LPO
Control mice (Group I)	52.40 ± 4.90	0.7 ± 0.05
ZEN treated (Group II) (50 µg/kg body weight)	43.27 ± 6.66	2.36 ± 0.77
ZEN treated (Group III) (100 µg/kg body weight)	40.16 ± 2.75	3.56 ± 0.82
ZEN treated (Group IV) (200 µg/kg body weight)	36.20 ± 3.77	5.22 ± 0.80

Results are expressed as mean ± SD (n = 6). *Statically different ($P<0.01$) from control. [a]Expressed as nM L^{-1} of 1-cloro 2,4-dinitrobenzene (CDNB) conjugated min^{-1} 100 mg^{-1} protein for GST; [b]Expressed as nML^{-1} MDA mg^{-1} of protein

TABLE 4. The effect of ZEN on sleeping time and sleep latency after 14 days oral administration of pentobarbital

Groups	Duration of sleep time	Sleep latency
Control mice + Phenobarbitone (Group I)	80.02 ± 2.77	2.84 ± 0.88
ZEN treated + Phenobarbitone (Group II) (50 µg/kg body weight)	91.54 ± 3.56*	2.35 ± 0.54*
ZEN treated + Phenobarbitone (Group III) (100 µg/kg body weight)	107.89 ± 9.23*	2.33 ± 0.47
ZEN treated + Phenobarbitone (Group IV) (200 µg/kg body weight)	125.64 ± 11.54*	1.97 ± 0.75*

Results are expressed as mean ± SD (n = 6). *Statically different ($P<0.01$) from control. Sleep time, sleep latency were expressed in minutes

TABLE 5. The effect of ZEN on histopathology of liver after 14 days oral administration

Histopatological features	Groups			
	Control groups		ZEN treated groups	
	Round AB	Stellate AB	Round AB	Stellate AB
a. Nucleus fragmented or absent	5/105 (5%)	15/400 (4%)	77/189 (41%)	59/510 (12%)
b. Nucleus Pyknotic (intact nucleus)	4/110 (4%)	13/200 (7%)	40/70 (57%)	190/620 (31%)
c. Nucleus Pyknotic fragmented or absent	Not significant	1/125 (2%)	102/140 (72%)	240/550 (44%)
d. Adjacent inflammation present	Not significant	Not significant	87/109 (80%)	222/487 (46%)
e. Located in an acidophilic domain	2/108 (2%)	6/330 (2%)	104/188 (55%)	98/175 (56%)
f. Nodular hyperplasia	4/125 (3%)	17/220 (8%)	103/127 (81%)	104/125 (83%)
g. Atrophy	4/116 (3%)	4/155 (3%)	105/160 (66%)	69/104 (66%)

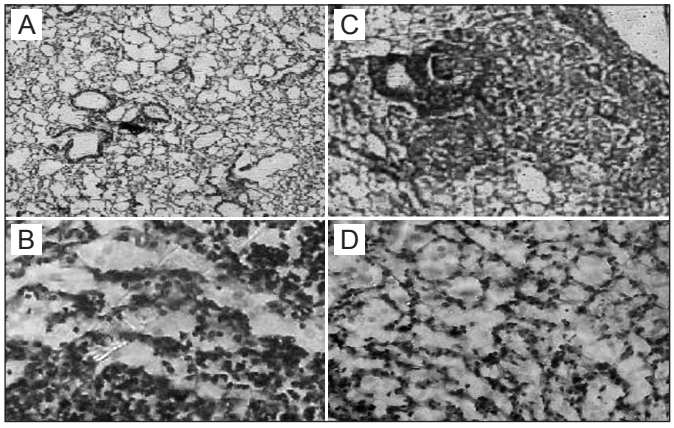

FIGURE 1. Showing histopathology of liver sections after staining with H-Eosin (H-E, x.400). (A) Liver section of control mice showing normal architecture of liver with central vein. (B) Liver section of ZEN (50 μg/kg body weight) administered showing sinusoidal congestion and cytoplasmic vacuolization. (C) Liver section of ZEN (100 μg/kg body weight) administered showing hepatocellular necrosis and neutrophil infiltration. (D) Liver section of ZEN (200 μg/kg body weight) administered showing severe nodular hyperplasia, pyknotic nucleus and necrosis

There was chronic inflammatory cells infiltrate in the portal tracks. There was also extensive hepatocellular necrosis, sinusoidal congestion, and neutrophil infiltration. Shrunken acidophilic hepatocytes were both round and detached from surrounding hepatocytes (round – Abs) and stellate – shaped firmly attached to adjacent hepatocytes (stellate – Abs). In 44% of round – Abs, the nucleus was fragmented or absent and among those with an intact nucleus, 80% had nuclear pyknosis stellate. Stellate – Abs often clustered in acidophilic domain usually without significant lymphoid infiltrate or necrosis. Round- Abs were usually not clustered and seen more often with an adjust lymphocytes infiltrate. Also apoptotic bodies, in the form of round or stellate – Abs, were seen (Figure 1b).

DISCUSSION

In this present investigation ZEN showed hepatotoxicity in BALB/c mice which characterized by increasing of hepatic marker enzymes (ALT, AST), decreasing of microsomal cytochrome P_{450} and histopathology study showed that ZEN decreased in the number of perfused sinusoids and hepatocellular hypoxia.

Zearalenone is resorcylic acid lactones and functionally is mycoestrogen which found in contaminating grain. Mycoestrogen increased incidence of adenomas (pituitary, liver) was detected in one species after a 2-yr oral carcinogenicity study[11]. In present investigation ZEN shows the potential hepatotoxicant may due to estrogenic property. Thus, the impaired hepatic function caused after administration of ZEN, that might be a reason for activation of hepatocytes led to increase hepatic marker enzymes, as seen in the present work. The pathophysiology of hepatic injury is complex and it is thought that hepatotoxin activates hepatic cells and subsequently causes free radical- mediated tissue injury and by series of chain reactions produces Lipid peroxidation (LPO)[17] (Figure 1).

Ayed et al.[1] reported the genotoxicity of ZEN and concluded that biotransformation of ZEN involved only partial detoxification and remaining metabolites are relatively toxic. Another study of Frizzell et al.[3] reported that ZEN and its metabolites showed potential endocrine disruptors by altering hormone production. Further, ZEN ingestion of animals from contaminated feed decreased the TNF-α synthesis and IL-8 synthesis[14]. Another study of Marin et al.[13] reported similar immunosuppressive effects of ZEN and its derivatives (alpha-ZOL, beta-ZOL, ZAN) in swine. Thus, ZEN showed multiple side effects and our observation reported first time hepatotoxicity of ZEN.

Formation of LPO leads to many pathological changes in tissue including liver necrosis[6]. The consequences of lipid peroxidation may be manifested as alternation in membrane integrity of membrane- associated functions in sub-cellular organelles. Reduced glutathione (GSH) is known to function as an antioxidant and a physiological reservoir for cysteine and is involved in DNA synthesis, protein synthesis regulation, and detoxification, etc. Cellular GSH deficiency affects the mitochondrial GSH pool and the cytosolic GSH pool. Mitochondrial GSH is important for the detoxification of ROS generated by the respiratory chain, conjugation of xenobiotics, maintenance of thiol-containing proteins, and regulation of the mitochondrial membrane potential[15] (Table 5).

In our present study indicates that in parallel increased AST and ALT, increased levels of LPO and decreased GSH activity occurred liver injury by ZEN.

Another point to be considered in this context is that there are increased sleeping times of pentobarbital with combination of ZEN. This result indicates that ZEN impaired metabolism of pentobarbital by hepatic damage with inhibits the activity of CYP_{450}. Previous study shows that ZEN activates the nuclear receptor PXR and induces the expression of the drug-metabolizing enzyme CYP3A4, a hepatic monooxygenase involved in the metabolism of about 60% of clinically used drugs[4]. Further, in our present investigation decreased of CYP_{450} activities correlates the increasing sleeping time of pentobarbital. Thus, it is likely that ZEN inhibits CYP_{450} activities by hepatotoxicity.

In summary, the present study provides the first functional anatomical evidence that phenobarbitone sleep time increase may predispose the liver to significant oxidative injury and subsequently hepatic damage, CYP_{450} deactivation by ZEN. Although the detailed mechanisms concerning the deactivation of CYP_{450} are not fully understood, the current findings present important insights into the potential detrimental effect of oxidative stress and impaired hepatic function of ZEN.

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RESUMO – ***Contexto*** - Zearalenone é um micoestrógeno e considerado como micotoxina. ***Objetivo*** - Avaliar se o Zearalenone produz hepatotoxicidade por administração via oral. ***Métodos*** - Zearalenone foi administrada por via oral em doses de 50 µg, 100 µg e 200 µg/peso corporal/dia/14 dias, respectivamente, para três grupos de camundongos BAB/C. Modalidades diagnósticas usadas para avaliar o dano hepático e comprometimento da função hepática pré- e pós-administração de Zearalenone incluíram atividade enzimática de marcadores hepáticos, tempo de sono por pentobarbital, atividade do citocromo P-450 e avaliação histopatológica hepática. ***Resultados*** - Alterações histopatológicas significantes como congestão sinusoidal, vacuolização citoplasmática, necrose hepatocelular e infiltração neutrofílica foram observadas após avaliação histológica de cada grupo após exposição acumulada de Zearalenone. Além disto, a exposição à Zearalenone incrementou a atividade das enzimas alanina transaminase e aspartato transaminase e peróxidos lipídicos, ao passo que as atividades teciduais de glutationa e citocromo P-450 diminuiram, quando comparadas com camundongos-controle. Zearalenone também aumentou o tempo de sono e diminuiu a latência do sono após a administração de pentobarbital por via intra-abdominal, quando comparados com camundongos-controle, o que indica o comprometimento das enzimas do metabolismo hepático por ela. ***Conclusão*** - Zearalenone é uma potente hepatotoxina quando administrada por via oral.

DESCRITORES – Hiperplasia. Zearalenona. Fígado, patologia. Camundongos.

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