TERATOGENIC EFFECTS OF OCHRATOXIN A IN RABBITS

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ABSTRACT: Ochratoxin A, is a food-borne mycotoxin produced by several fungal species of the genera *Aspergillus* and *Penicillium.* Ochratoxin A was dissolved in corn oil and given by gastric intubation to rabbits on days 6-18 of gestation with the dose levels of 0.025, 0.050 and 0.100 mg/kg body weight. When compared with controls (4.16 %), in case of 0.100 mg/kg dose group, there was a significant increase in the incidence of gross ($P<0.05$) and skeletal ($P<0.10$) anomalies. The number of live fetuses in the case of 0.100 mg/kg dose group was significantly less than those of the 0.025mg/kg dose group. When compared to controls and 0.025mg/kg dose group, the mean fetal weights and mean fetal crown to rump lengths of dose group 0.100 mg/kg were significantly lower. Major gross anomalies caused by ochratoxin A included wrist drop, rudimentary tail, knuckling of fetlock and agenesis of tail. Skeletal anomalies were agenesis of caudal vertebrae, incomplete ossification of skull bones and wavy ribs. The soft tissue anomalies included internal hydrocephalus, microphthalmia and kidney agenesis.

Key words: ochratoxin A, teratogenicity, rabbit.

INTRODUCTION

Ochratoxin A (OA) has gained great importance due to its biological effects and widespread toxicity. OA is nephrotoxic and more importantly, it is implicated as a causal factor of Balkan Endemic Nephropathy (BEN) in humans (STOEV, 1998).

WHO-IARC (1993) designated OA as Group-2B carcinogen (HUSSEIN and BRASEL, 2001). A widespread awareness of the teratogenic impact of xenobiotics

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dates back to the discovery of human thalidomide teratogenicity in 1960s (LENZ, 1961). It is now becoming increasingly evident that a number of food-contaminating metabolites can cause interference with fetal developmental processes. Mycotoxins have emerged as a potential threat to animal and human health in view of their teratogenicity, but a perusal of the reports and the literature scanned shows that the studies in the field of mycotoxin teratogenesis are very limited. Ochratoxin A (OA) has been shown to be teratogenic in rats (STILL *et al.,* 1971; MORE and GALTIER, 1974; BROWN *et al.,* 1976; MAYURA *et al.,* 1982, 1984a, 1984b; ABDEL-WAHHAB *et al.,* 1999), mice (HAYES *et al.,* 1974; ARORA; 1982; FUKUI *et al.,* 1987); hamsters (HOOD *et al.,* 1976), chick embryos (GILANI *et al.,* 1975; LALITHA KUNJAMMA and NAIR, 1997) and in quail (DWIVEDI, 1984). Earlier reports of OA teratogenicity in rats, showed that OA caused a marked increase in the number of foetal deaths and resorptions (STILL *et al.,* 1971; MORE and GALTIER, 1974). The systematic study of BROWN *et al.* (1976) using various doses of OA (0.25 to 4 mg/kg body weight) during gestation days 6-15 by oral route induced various gross, visceral and skeletal abnormalities. In a series of studies using the subcutaneous route MAYURA *et al.* (1982, 1984a, 1984b) studied embryocidal, fetotoxic and teratogenic effects of OA, effects of protein deficiency, impaired renal function and protective effects of phenylalanine in OA induced teratogenicity in pregnant rats.

The rabbit is among the most sensitive species to OA toxicity (MARQUARDT and FROHLICH, 1982), however, no systematic teratogenic studies of mycotoxins, especially OA, appear to have been conducted in rabbits, which are considered as the most suitable laboratory animal models for domestic animals as well as for studying the teratogenic potential of chemicals (WHO, 1967).

Although exposure to mycotoxin chiefly occurs through the ingestion of contaminated food, the route chosen by earlier workers had often been subcutaneous or intra-peritoneal. As the route of administration can materially influence the teratogenicity of a compound, dosing by oral route appears to be a natural and realistic way for reliable assessment of developmental disorders induced by food borne mycotoxins (ARORA, 1982).

Therefore a detailed, systematic study was planned to elucidate the teratogenic effects of ochratoxin A in rabbits by administering orally in different doses during 6-18 days of gestation.

MATERIALS AND METHODS

Sexually mature (1.5±0.5 Kg), virgin, New Zealand White female rabbits obtained from the Laboratory Animal Resource Division of Institute were housed in still cages and maintained on food tested free of any mycotoxins. The representative feed samples from each lot (around 250gm) were analysed, spotted on chromatoplate along with the standards mycotoxins (aflatoxin B_1 and ochratoxin A). The chromatoplates (TLC) were developed and observed under ultraviolet light in a dark chamber for presence of any mycotoxins. All the procedures, conducted on the experimental animals were duly approved by the Institute's Ethics Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The number of animals used per group was restricted to five because of CPCSEA rules and due to the non-availability of a sufficient quantity of mycotoxins.

Following an acclimatization period of one week, females were mated with mature males of the same strain. For mating, the female rabbits (in oestrus) were placed in the cages of males at evening and mating was observed. A grunting sound made by the male, who turned to its side after mating was considered as successful mating. Females were separated the next morning, which was considered as day zero of pregnancy.

Ochratoxin A was produced on sterile maize, using *Aspergillus ochraceus* NRRL- 3174 culture, procured from National Center for Agricultural Utilization Research (NCAUR) Peoria, Illinois, USA as per the method of TRENK *et al*. (1971). For purification of OA, procedures of AOAC (1995) were followed and OA was estimated by using thin layer chromatography along with standard OA procured from Sigma Chemicals Ltd. The quantitative estimation was done by spectrophotometric

analysis. The areas covering the spots were marked under the UV light and the silica gel covering each spot was scraped off and collected in separate tubes and extracted with benzene: acetic acid (99:1) and the optical density was recorded at 333nm in UV spectrophotometer and the concentration of OA was calculated. OA was dissolved in corn oil and the rabbits were dosed by gastric intubation at the rate of 0.025, 0.050 and 0.100 mg/kg body weight on days 6-18 of gestation.

Physical condition of each rabbit was assessed daily and on day 30 of gestation the dams were sacrificed and the fetuses were taken out by uterine incision. Each fetus was carefully examined for gross anomalies. Two third of the fetuses were randomly selected and fixed in Bouin's solution and examined for visceral anomalies by Wilson's free hand razor slicing method (WILSON, 1965). The remaining fetuses were fixed in 70% alcohol, stained with alizarin red S and processed for evaluations of skeletal defects.

Data for maternal observations, such as number of corpora lutea, number of implantations, number of live and dead fetuses, pre- and post-implantation losses and fetal crown to rump lengths and fetal weights were statistically evaluated by analysis of variance (ANOVA) followed by Duncan's test. For the evaluation of gross, skeletal and visceral anomalies a 2 X 2 contingency table test was used (SNEDECOR and COCHRAN, 1967). In case of ANOVA, a probability of $P < 0.05$ and in case of 2 x 2 contingency table test $P<0.05$ and $P<0.10$ was accepted as significant.

RESULTS

Litter data

The effects of ochratoxin A on total number of corpora lutea, number of implantations, per cent resorptions, number of live and dead fetuses, per cent pre- and post-implantation losses and fetal crown to rump lengths and fetal weights are summarized in Tables 1 and 2. There were no maternal mortalities in any group. There was non-significant decrease in the number of corpora lutea and implantations;

Table 1: Litter characteristics (mean±SE) of rabbits treated with ochratoxin A.

Groups	Rt	Lt	Total
No. of corpora lutea			
Control	1.80±0.58	3.60±0.68	5.40±0.98
0.025	2.80±0.37	2.80±0.58	5.60±0.51
0.050	2.40±0.40	2.60±0.60	5.00±0.71
0.100	2.60±0.40	1.20±0.37	3.80±0.20
No. of implantation sites			
Control	1.60±0.51	3.40±0.68	5.00±0.89
0.025	2.80±0.37	2.80±0.58	5.60±0.51
0.050	2.40±0.40	2.60±0.60	5.00±0.71
0.100	2.40±0.51	1.20±0.37	3.60±0.24
Live foetuses			
Control	1.60±0.51	3.20±0.49	4.80±0.86[ab]
0.025	2.80±0.37	2.60±0.40	5.40±0.40[b]
0.050	2.20±0.37	2.60±0.60	4.80±0.73[ab]
0.100	2.00±0.32	1.20±0.37	3.20±0.20[a]

Means within a row with different superscripts differ ($P<0.05$).

however, the number of live fetuses in the 0.100 mg/kg dose group was significantly lower than those of the 0.025 mg/kg dose group. The 0.100 mg/kg dose caused a non-significant increase in the resorptions, pre- and post-implantation losses as compared with those in the control group. There were no dead fetuses in any group. As compared with the control and 0.025 mg/kg dose group, the mean fetal weights and mean crown to rump lengths were significantly reduced in the case of the 0.100 mg/kg dose group.

Table 2: Means and standard error of the fetal losses.

Groups	Resorption	Dead fetus	Pre-impl. loss (%)	Post- impl. loss (%)
Control	0.11±0.20	0.00±0.00	0.22±4.15	0.22±3.33
0.025	0.20±0.20	0.00±0.00	0.00±0.00	2.86±2.86
0.050	0.20±0.20	0.00±0.00	0.00±0.00	4.00±4.00
0.100	0.40±0.24	0.00±0.00	5.00±5.00	10.00±6.12

Teratogenic findings

With regard to fetal malformations, OA produced various gross, skeletal and visceral anomalies particularly at the higher dose level (0.100 mg/kg body weight). Table 4 shows various anomalies induced by OA in rabbit fetuses. The incidence of gross anomalies was 3.70%, 8.53% and 31.25% in OA (0.025, 0.050 and 0.100 mg/kg body weight) treated groups, respectively as compared with that in the control group (4.16%). The gross anomalies observed included wrist drop, rudimentary tail, knuckling of fetlock and agenesis of tail, as compared with that in controls (Fig. 1).

Figure 1: Rabbit fetus (control); Showing well developed tail and normal legs. Fetus on upside (OA 0.100 mg/kg bw); Showing agenesis of tail and knuckling of fetlocks.

Figure 2: Rabbit fetal skeleton (control); well-developed ribs and caudal vertebrae. Fetal skeleton on downside (OA 0.100 mg/kg); note wavy ribs and incomplete ossification of skull bones (arrows).

Table 3: Fetal weight and lenght (mean±SE).

Groups	Mean fetal Weight (g)	Total fetal Weight (g)	Mean CR Length (cm)	Total CR Length (cm)
Control	36.32±37.73[bc]	165.41±0.98[ab]	7.07±6.03[bc]	32.66±0.28[b]
0.025	37.77±0.39[c]	204.14±15.54[b]	7.00±0.05[c]	37.84±2.97[b]
0.050	34.70±24.66[c]	166.18±0.35[ab]	6.24±4.41[ab]	29.84±0.11[a]
0.100	33.66±6.22[a]	107.60±0.44[a]	6.09±1.12[a]	19.48±0.06[a]

Means within a row with different superscripts differ ($P<0.05$).

There was a significant increase ($P<0.05$) in percent gross anomalies observed at the 0.100 mg/kg dose level than those of controls. Skeletal anomalies were agenesis of caudal vertebrae, incomplete ossification of skull bones and wavy ribs (Fig. 2). The skeletal anomalies observed at 0.100 mg/kg dose level showed a significant increase ($P<0.10$) as compared with that of controls. The soft tissue anomalies included internal hydrocephalus, microphthalmia and kidney agenesis (Fig. 3 and 4).

Figure 3: Section of the rabbit fetus (control) passing through abdomen; Note well developed right and left kidneys.
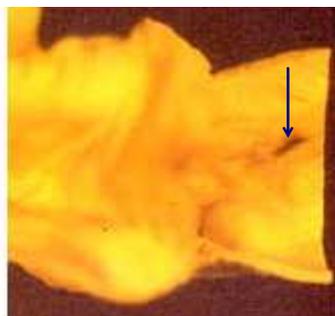
Figuere 4: Section of the rabbit fetus (OA 0.100 mg/kg bw) passing through abdomen, showing agenesis of left kidney and presence of fissure at its place (arrow).

DISCUSSION

The literature scanned showed no report on teratogenic effects of ochratoxin A in rabbits, although these are the preferred laboratory animal species and are exclusively recommended for such toxicological studies by regulatory bodies. Such studies may have widespread applications in different species of domestic animals. Further, OA has been reported to cause outbreaks in rabbits, affecting their production and reproduction (SHALINI, 1996). Thus, there is a clear need to study the effects of prenatal exposure to ochratoxin A on developing rabbit fetuses. The oral route of administration of OA to pregnant rabbits was selected to simulate the natural mode of ingestion of the toxin under field conditions.

BROWN *et al.* (1976) reported that LD_{50} of OA in female rats is 22 mg/kg bw but multiple exposure of doses of OA as small as 0.25 to 0.5 mg/kg bw were teratogenic. The LD_{50} of ochratoxin A in rabbits, determined in this laboratory was 10 mg/kg body weight (MIR *et al.,* 1999). Moreover, these levels of toxins are also present in the range of natural contamination of the feedstuff ingredients with OA under field conditions (PETZINGER and ZIEGLER, 2000). Therefore the doses of 0.025, 0.050 and 0.100 mg/kg body weight were selected for present study.

The occurrence of resorptions, pre- and post implantation losses were increased at the highest doses (OA 0.10 mg/kg body weight). There were no dead fetuses in any of the dose groups. The results obtained were in accordance with the earlier reports in rats (BROWN *et al*., 1976; MAYURA *et al*., 1982) mice (ARORA, 1982) and hamsters (SCHMIDT and PANCIERA, 1980).

The occurrence of gross, skeletal and visceral anomalies was increased in the 0.100 mg/kg dose group. The anomalies predominantly occurred in the region of legs involving extremities. Skeletal anomalies involved caudal vertebrae, ribs and skull bones. Various soft tissue anomalies indicated the effect of OA on brain and eye of developing fetuses. No such anomalies were observed in control fetuses. The agenesis of kidney observed in one of the fetuses of the higher dose group indicated

Table 4: Gross, skeletal and visceral anomalies observed in fetuses of ochratoxin A treated rabbit.

NUMBER OF FOETUSES

Groups	GROSS ANOMALIES				SKELETAL ANOMALIES				VISCERAL ANOMALIES			
	Obs	Affected N	%	Description	Obs	Affected N	%	Description	Obs	Affected N	%	Description
Control	24	1	4.16	Wrist drop (1)	8	0	0.00	-	16	0	0.0	-
0.025	27	1	3.70	Wrist drop (1)	9	1	11.11	Caudal vertebrae agenesis (1)	18	1	5.55	Mildly dilated lateral ventricles.
0.050	24	2	8.53	Wrist drop (1) Rudimentary tail (1)	8	1	12.5	Caudal vertebrae agenesis (1)	12	2	16.66	Hydrocephalus (2)
0.100	16	5	31.25*	Wrist drop (2) Knuckling of fetlock (2) Agenesis of tail (1)	6	3	50†	Caudal vertebrae agenesis (1) Skull- Incomplete ossification (2) Wavy ribs (1)	10	4	40	Hydrocephalus (2) Microphthalmia (1) Kidney agenesis (1)

*$P<0.05$. †$P<0.10$.

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sensitivity of rabbit kidney to OA (MARQUARDT and FROHLICH, 1992), which is known to be the most potent nephrotoxic agent. Agenesis of kidney has also been reported by MAYURA *et al*. (1982) in rat fetuses, thus supported the present findings. The absence of kidney observed might be attributed to nephrotoxic effects of OA. The patterns of anomalies observed in rabbit fetuses were similar to those observed in rats (BROWN *et al*., 1976), mice (ARORA, 1982) and hamsters (SCHMIDT and PANCIERA, 1980).

These results indicated that OA is also teratogenic in rabbits, and doses as low as 0.050 mg/kg body weight, when given orally during gestation days 6-18, could cause anomalies in fetuses. The dose of 0.050 mg/kg body weight can also be considered as the minimum oral teratogenic dose for rabbits, however further studies are needed to substantiate this finding.

For the teratogenic mechanism of OA, role of maternal protein deprivation, impaired glycolysis and inactivated phosporylase-b-kinase have been suggested by previous workers (MAYURA, *et al*., 1983). OA is known to inhibit mitochondrial respiration and has a direct effect on fetuses, rather than having its action mediated through an effect on the dam (HOOD *et al*., 1976).

The localization of ^{14}C-labelled OA in various organs of fetal mice (APPELGREN and ARORA, 1983) indicated that this mycotoxin caused interference in the development of these organs and continuous exposure due to added doses of the toxin during the organogenesis period were the factors responsible for the appearance of different anomalies of these organs. There is a delicate balance among cell proliferation, cell differentiation and apoptosis in the developing embryo; impairment in these mechanisms caused by OA during the development stages might have been responsible for the anomalies observed.

Although the exact mechanism of OA induced teratogenesis is not clear, the wide spread toxicity of OA as inhibition of DNA, RNA and protein synthesis (MARQUARDT and FROHLICH, 1992), intracellular transport and subsequent lipid per

oxidation resulting from generation of free radicals (WEI and SULIK, 1993), direct effect on osteoblasts and osteoclasts (DWIVEDI, 1984) and interference in the calcium homeostasis (KHAN *et al*., 1989) involved in excessive embryonic cell death might be responsible for the teratogenic effects caused.

Presence of OA in cord blood samples of pregnant women and long serum half-life in humans (JONSYN *et al*., 1995), might be correlated with the potential threat of teratogenicity in humans as well as domestic animals, as observed in the rabbits of the present study.

From these results, it can be concluded that ochratoxin A is teratogenic in rabbits when given by the oral route. A dose of 0.050 mg/kg can be considered as the minimum oral teratogenic dose. From the perusal of literature, it appears to be the first study on teratogenic effects of OA in rabbits. There are several fields where information is not available. Further studies are required on the combined effects of various mycotoxins and the pathogenesis of OA-induced teratogenesis.

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