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INTERFERENCE OF MYCOTOXINS WITH PRENATAL DEVELOPMENT OF THE MOUSE

I. INFLUENCE OF AFLATOXIN B_1, OCHRATOXIN A AND ZEARALENONE

By

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ARORA, R. G., H. FRÖLÉN and A. NILSSON: *Interference of mycotoxins with prenatal development of the mouse. I. Influence of aflatoxin B_1, ochratoxin A and zearalenone.* Acta vet. scand. 1981, *22*, 524—534. — The prenatal effects of mycotoxins were investigated in CBA mice given by stomach tube a single dose of either aflatoxin B_1 (4 mg/kg), ochratoxin A (8 mg/kg) or zearalenone (20 mg/kg) on pregnancy day 8 or 9.

Aflatoxin caused foetal anomalies (exencephaly, open eyes, and protrusion of intestines) after exposure on gestation day 8 but not on day 9. The effects (increased prenatal mortality, reduced foetal growth, and a wide variety of malformations) caused by ochratoxin were much more severe and occurred after treatment on either of the 2 days of gestation. Among the spectrum of malformations, predominantly involving the craniofacial complex and the axial skeleton, the most striking was the total aplasia/dysplasia of the upper facial structures. These defects were always accompanied by exencephaly and anophthalmia. Zearalenone caused no effects. It is concluded that of the 3 mycotoxins screened with the technique used, ochratoxin is the most potent teratogen in mice.

aflatoxin B_1; ochratoxin A; zearalenone; prenatal development; interference; mouse.

Mycotoxins, a group of biologically active toxic metabolites with considerable structural diversity and complexity, are produced as contaminants in human and animal food by a variety of spoilage molds (*Wilson & Hayes* 1973). Among the different toxicity syndromes reported in man and animals some are well documented and have been directly attributed to the intake of mycotoxin contaminated food (*Krogh et al.* 1973, 1977, *Newberne*

1974, *Krishnamachari et al.* 1975). The toxic effects induced vary according to the specific nature of the toxin present in the food. For instance aflatoxin B_1 has its greatest potential as hepatotoxin and hepatocarcinogen whereas ochratoxin A is primarily nephrotoxic and zearalenone is known to have oestrogenic effects.

It is also now becoming increasingly evident that a number of food contaminating metabolites can cause interference with the foetal developmental process. The foetotoxic and/or teratogenic responses to mycotoxin such as aflatoxin B_1 (*DiPaolo et al.* 1967, *Elis & DiPaolo* 1967), rubratoxin B (*Hood et al.* 1973, *Koshakji et al.* 1973) and ochratoxin A (*Hayes et al.* 1974 a, *Brown et al.* 1976, *Hood et al.* 1976) have been reported in different laboratory animals, such as the rat, mouse or hamster.

Although exposure to mycotoxins chiefly occurs through the ingestion of contaminated food, the route chosen for the teratological testing by the above mentioned authors has often been i.p. As the route of administration can materially influence the teratogenicity of a compound (*Hayes et al.* 1974 a) dosing by stomach tube appears to be a more natural and realistic way for making a reliable assessment of the developmental effects induced by the food borne toxicants. This method not only simulates the natural exposure pattern but also permits the accurate amount of the material to be administered. The current study was, therefore, undertaken to provide further information on the foetocidal and teratogenic effects of maternally ingested mycotoxins such as aflatoxin B_1, ochratoxin A and zearalenone for which the knowledge is sparse.

MATERIALS AND METHODS

Inbred mice of the CBA strain maintained under controlled conditions of housing and breeding at the National Defence Research Institute, Stockholm were used. Standard rat and mouse feed (Astra Ewos) and water were available all the time ad libitum. Virgin females of 60—70 days of age and weighing 20—24 g were caged individually and mated over night with a male of the same strain. Presence of a vaginal plug on the next morning indicated a successful mating and the day was called post conception (p.c.) day 1. Pregnant females were isolated and randomly assigned to one of the treatment or control groups. The

test dosage of each mycotoxin* was freshly dissolved in corn oil and administered in a volume of 10 ml/kg body weight by stomach tube during the most sensitive period of embryonic development, i.e. on p.c. day 8 or 9. Control females were given equivalent volumes of vehicle (corn oil) at the same time. To obtain the near term foetuses for examination all the animals in the experimental and control series were killed by cervical dislocation on p.c. day 19.

The number of implants, resorption sites, live or dead foetuses were recorded. Living foetuses were weighed individually and examined under stereomicroscope for the gross anomalies. About one half of the foetuses from each litter was cleared and stained with alizarin red-S (*Crary* 1962) for skeletal abnormalities. The remaining half was fixed in Bouin's fluid and processed for paraffin embedding and sectioned serially at 5—6 μm for future histological examination. The sections were routinely stained with haematoxylin and eosin.

RESULTS

Signs of maternal toxicity were not observed after treatment with either of the mycotoxins.

Intrauterine growth, foetal mortality and incidence of anomalies

Data on prenatal mortality, foetal weights and gross anomalies induced by the individual mycotoxin are recorded in Table 1.

In Group I which was given aflatoxin B_1 on p.c. day 8 or 9 the values for embryonic resorptions, late foetal deaths and mean pup weights were comparable to those of vehicle treated controls (Group IV). Grossly observable malformations, though occurring in about 11 % of the foetuses exposed on day 8, were not apparent when the toxin was given on day 9.

With ochratoxin A (Group II) the foetotoxic as well as the teratogenic effects were noticed on both days of treatment. The frequency of late foetal deaths and the occurrence of gross malformations varied according to the day on which the toxin was given. While the foetal mortality rate in the litters treated on

* Mycotoxins-aflatoxin B_1, (AFB_1) and ochratoxin A (OCTA) were obtained from Makor Chemicals Ltd. Jerusalem, Israel, and the zearalenone (Z) was a gift from Mr Bachman, Terre Haute, Indiana.

Table 1. Effects of aflatoxin B_1 (AFB_1), ochratoxin A (OCTA) and zearalenone (Z) on mouse foetal development.*

Group	Mycotoxin dose (mg)/kg	Gestation day	Total implants/ Number of dams	Number of resorptions	Late deaths		Foetal weight (g) $\bar{X}\pm$s.e.m.	Survivors, grossly malformed	
					Number	%		Number	%
I	AFB_1	8	72/8	5	6	9.0	1.06±0.02	7	11.5
	(4)	9	57/7	4	2	3.8	0.92±0.03	—	—
II	OCTA	8	66/8	14	36	69.2	0.60±0.05	8	50
	(8)	9	75/9	16	14	23.7	0.62±0.02	45	100
III	Z	8	79/9	5	1	1.4	1.08±0.03	—	—
	(20)	9	81/9	7	19[a]	25.7	0.97±0.01	—	—
IV	(v) control	8	82/9	5	2	2.6	1.04±0.01	—	—
		9	71/8	5	1	1.5	1.00±0.02	—	—

* toxins were dissolved in corn oil and administered in a volume of 10 ml/kg body weight on p.c. day 8 or 9 by stomach tube.

a all foetuses (9+7) from two mothers, though fully developed were dead.

(v) vehicle controls given only corn oil, 10 ml/kg body weight.

day 8 was about 69 %, it was reduced nearly to 24 % with the same treatment given on day 9 of gestation. Conversely, the malformation rates in foetuses surviving treatment increased from 50 % on day 8 to 100 % on day 9. The increased number of embryonic resorptions and reduced foetal weights noted after exposure to ochratoxin, however, were not influenced by the treatment day.

After administration of zearalenone (Group III), the effects on foetal development were not well marked. Except for a relative increase in the late foetal death rate (about 26 %) with treatment on day 9, other parameters examined were within normal limits. The foetuses totalling 16 in 2 of 9 dams treated on this day were all dead, although, they were fully developed and free from any morphological abnormality.

Malformations

The various types of malformations induced by aflatoxin B_1 and ochratoxin A are listed in Table 2. The anomalies caused by aflatoxin B_1 varied from case to case and included exencephaly, open eye lids and protrusion of intestines. The mean number of

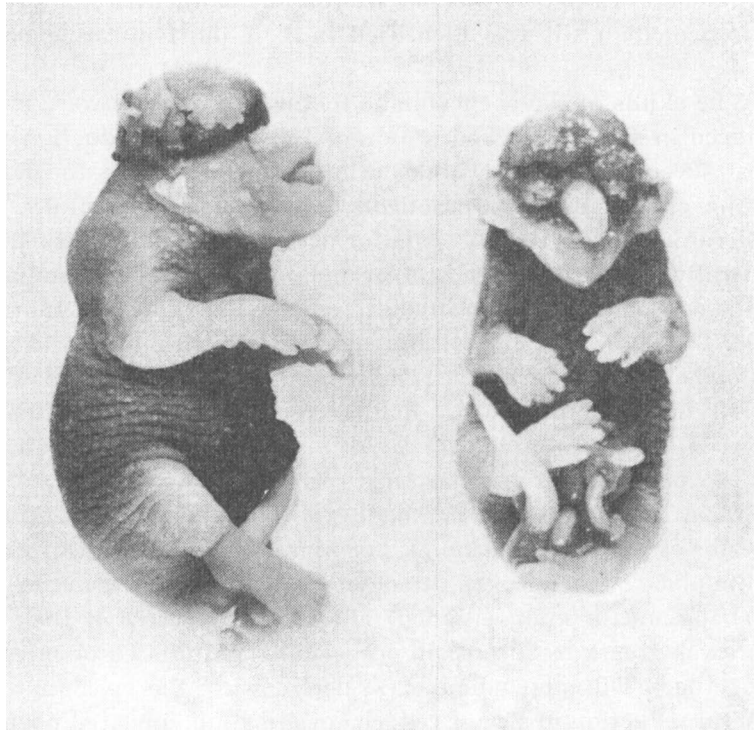
Table 2. Frequency of various types of malformations associated with prenatal exposure to AFB_1 (4 mg/kg) and OCTA (8 mg/kg) on gestation day 8—9.

Type of malformation	AFB_1		OCTA	
Gestation day	8	9	8	9
External				
Number malformed/ total number examined	7/61	0/51	8/16	45/45
Exencephaly	4 (6.6)		2 (12.5)	41 (91.1)
Anophthalmia			2 (12.5)	28 (62.2)
Microphthalmia				7 (15.6)
Open eye lids	3 (4.9)		3 (18.8)	
Cleft pinna/open ear			1 (6.3)	10 (22.2)
Anotia				4 (8.8)
Agenesis of external nares			6 (37.5)	9 (20.0)
Cleft lip				4 (8.8)
Median cleft face				7 (15.6)
Malformed jaws/short jaws with protruding tongue			5 (31.3)	12 (26.7)
Protrusion of intestine/ gastroschisis	2 (3.3)			13 (28.9)
Club foot				5 (11.1)
Short and hooked tail				9 (20.0)
Skeletal				
Number malformed/ total number examined	0/31	0/26	2/4	13/24
Fused ribs			1 (25.0)	9 (37.5)
Bifurcated ribs				4 (16.7)
Fused vertebrae				11 (45.8)
Malformed sternebrae			1 (25.0)	6 (25.0)
Mean number of gross anomalies per foetus	0.15		1.69	3.98

Figures in parentheses represent percentage.

malformations per foetus was 0.15. No obvious anatomic or structural alterations in the skeleton were detectable.

With ochratoxin A a wide variety of defects involving the craniofacial complex, abdomen and axial skeleton were produced. The average number of malformations per foetus increased from 1.69 on exposure day 8 to 3.98 on day 9. The predominant and most consistantly observed craniofacial anomalies included exencephaly, anophthalmia, exo- or microphthal-



Figures 1—2. Various foetal anomalies in mice treated with ochratoxin A on post conception day 9. Examination was made on day 19.

Figure 1 (left). Mouse foetus with exencephalic brain, anophthalmia, bifid pinna, subcutaneous oedema and blebs, shorter jaws and club feet.

Figure 2 (right). Total mid facial cleft with tissue remnants of two halves of the upper face displaced on either side. Note the tongue and mandible are intact and the brain exencephalic.

mia, bifid pinna, short jaws with protruding tongue and agenesis of external nares (Fig. 1). Particularly severe and grotesque malformations of the face, which occurred in 7 foetuses treated on day 9 were characterized by a total aplasia/dysplasia of the median and paramedian structures of the upper face. The tongue and mandible in these foetuses with mid facial clefts remained intact but with no exception the brain was exencephalic (Fig. 2). In addition to these effects a generalised oedema and clear fluid filled blebs on the skin of the face were observed in 2 foetuses exposed on day 8 and in 5 on day 9. Cleft palate independent of

a total cleft of the face was not seen in any of the cases, although a deep median slit was identified in 3 of the foetuses exposed on day 9.

The skulls of the exencephalic foetuses in general were much reduced in size and lacked the cranial cavity. The reduction was most severe in the anterio-posterior diameter. While the bones of the calvarium were practically absent or rudimentary, the basicranium was severely malformed. The facial bones were generally reduced in size and/or malpositioned. The anomalies of the rest of the skeleton involved chiefly the spine and thoracic cage. The spine was curved and shorter than normal due to anomalous and fused vertebrae. The fusions of vertebral bodies were usually seen in the thoraco-lumbar region whereas the fusions between the neural arches of adjacent vertebrae were more common in cervical or upper thoracic region. The deformities of the thoracic cage were represented by a fan like configuration of the ribs owing to the fusion of 2 or more adjacent ribs, especially at the place of their costal attachment. Sternebrae were irregular in shape and/or malpositioned. The xiphoid process of the sternum was greatly reduced and occasionally bifurcated or missing altogether. The appendicular skeleton with the exception of general reduction in size or reduced ossification appeared normal.

DISCUSSION

While several investigations have shown the toxicity and carcinogenicity of various food contaminating mycotoxins, the information on the foetocidal and teratogenic effects due to maternal ingestion of these compounds is limited. In addition studies on the teratogenicity of aflatoxin have led to reports of unequivocal results.

The aflatoxin induced foetal anomalies observed in 7 out of 67 foetuses examined have not previously been reported to occur either in mice (*DiPaolo et al.* 1967, *Hayes et al.* 1974 b) or in rats (*LeBreton et al.* 1964, *Butler & Wigglesworth* 1966, *DiPaolo et al.*). On the other hand *Elis & DiPaolo* (1967) working with golden hamster reported the teratogenic effects of aflatoxin similar to those observed by us. However *Schmidt & Panciera* (1980) using methods and test animals similar to those of *Elis & DiPaolo* failed to confirm these results. The growth inhibition (without morphological abnormalities) noted in the foetal hamster (*Schmidt & Panciera*) as well as in the rat (*Butler & Wiggles-*

worth) was believed to be an indirect effect of aflatoxin on the maternal liver. The possibility of such a mechanism of action of the aflatoxin in initiating changes that alter the course of subsequent development of foetal mice seems to be unlikely by the fact that aflatoxin in adult mice does not cause any of the morphological or biochemical alterations that occur in the liver of susceptible animals (*Akao et al.* 1971).

This statement is supported by our own experiments (*Arora* 1981) which failed to reveal any detectable lesions in the liver or other organs of mice treated even with a dose 10 times higher than that used in the present experiments. We have also shown by whole body autoradiography that the toxin is able to cross the maternal barrier to reach the foetus (*Arora et al.* 1978). In view of all these facts, it seems reasonable to assume that a direct effect of the aflatoxin on the developing embryo may well account for its teratogenicity in mice.

As seen from Table 1 the foetocidal and the teratogenic effects of ochratoxin were much more severe and contrary to the aflatoxin occurred after treatment on either of the day (8—9) of pregnancy.

In close agreement with earlier work (*Hayes et al.* 1974 a, *Hood et al.* 1976) the mycotoxin caused increased prenatal mortality, reduced foetal growth and a wide variety of anomalies. As regards the latter, it was reported (*Hayes et al.* 1974 a) that the highest effects were produced following i.p. injection of ochratoxin on p.c. day 8, where as in the present study the maximum effect (100 % malformations) was found when the toxin was supplied orally on p.c. day 9. Since the route of administration, the strain of the animals and vehicle used as solvent were not identical, anyone of these or all might have contributed for the discrepancies between the results of the present and the earlier studies. In view of the fact that the exposure to food borne mycotoxins chiefly occurs via ingestion, we preferred oral route over the i.p. injection. The results obtained under these conditions provide additional evidence that ochratoxin is a highly potent teratogen and the developing embryo is much more sensitive when the toxin is ingested by the mother on day 9 of pregnancy as opposed to on day 8 when given i.p. This conclusion is further strengthened by the results of our subsequent experiments with other doses administered on these 2 days of pregnancy (*Arora & Frölén* 1981).

The spectrum of malformations which predominantly involved the soft as well as the skeletal elements of the craniofacial region indicate that ochratoxin primarily interferes with the process of brain closure. The varying degree of facial clefts which in extreme cases were represented by the aplasia of the upper face with remnants of the two facial halves displaced on either side of the head appear to be similar to those caused by hypervitaminosis A (*Giroud et al.* 1969) and are considered most likely to be the result of a damage to the prechordal mesoderm (*DeMyer* 1971, *Hayes et al.* 1974 a). Many of the other skeletal anomalies involving vertebrae and the thoracic cage were similar to those reported by *Hayes et al.* (1974 a) although the defects of the appendicular skeleton (polydactyly, syndactyly, etc.) were not seen in our study. The pathogenetic mechanism of ochratoxin induced prenatal effects is not known. Studies in this regard are in progress in this laboratory and will form the subject of future communications.

The developmental effect attributed to zearalenone administration during the stages of embryogenesis on p.c. day 8 or 9 did not occur. It remains, however, to be investigated whether the embryonic stages other than these are sensitive to this mycotoxin. *Ruddick et al.* (1976) reported decreased weights and increased incidence of some minor skeletal anomalies (delayed ossification, malpositioned sternebrae or extra ribs) in the foetuses of rats given zearalenone daily during the period of major organogenesis (day 6—15). The cause of the death of the foetuses in 2 of the 9 mice treated on day 9 could not be determined. However, it is not likely to be related to zearalenone as no such effect was evident in the subsequent experiments with this mycotoxin (to be published).

In conclusion, the current study has provided further evidence that mycotoxins present in the food chain not only affect the health of mature individual but the unborn is also at risk. Among the 3 mycotoxins investigated ochratoxin is the most potent teratogen. The extremes of severity and the extent of developmental defects induced by ochratoxin indicate the need for further investigations on its teratogenesis.

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SAMMANFATTNING

Mykotoxiners interferens på den prenatala utvecklingen hos mus. I. Påverkan av aflatoxin B_1, ochratoxin A och zearalenone.

Mykotoxinernas prenatala effekter undersöktes hos CBA-möss som via magsond fick en engångsdos av antingen aflatoxin B_1 (4 mg/kg), ochratoxin A (8 mg/kg) eller zearalenone (20 mg/kg) vid 8 eller 9 dagars dräktighet.

Hos möss som exponerades för aflatoxin B_1 under det 8:e dräktighetsdygnet utvecklades foetala missbildningar (exencephalon, öppna ögon, bukspalt med tarmframfall). Dessa missbildningar återfanns inte hos de möss som exponerats för aflatoxin under 9:e dräktighetsdygnet.

De effekter som orsakades av ochratoxin (ökad prenatal dödlighet, reducerad fostertillväxt samt ett stort antal varierande missbildningar) var mycket gravare, och drabbade foster, från honor vid båda behandlingstillfällena. Missbildningarna var främst lokaliserade till det kraniofasciella komplexet och det axiella skelettet, varvid total hypoplasi/aplasi av ansiktets övre delar var de mest iögonfallande defekterna. Dessa åtföljdes alltid av exencephalon och anophthalmi.

Zearalenone hade inga teratogena effekter.

Undersökningen har således visat, att av de tre mykotoxinerna med den undersökningsteknik som använts, har ochratoxin den mest potenta teratogena effekten hos mus.

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