

Teratogenic Effects of Ochratoxin A and Aflatoxin B₁ Alone and in Combination on Post-Implantation Rat Embryos in Culture

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Abstract

Objective: The present study was performed to determine effects of simultaneous exposure of ochratoxin A (OTA) and aflatoxin B₁ (AFB₁) on post-implantation rat embryos, in presence and absence of hepatic S9 mix.

Materials and Methods: The embryos were explanted from pregnant Wistar rats on day 10 of gestation and cultured for 24 hours, in medium M-199 containing rat serum, OTA (0.004, 0.008 µg/ml culture), AFB₁ (0.5, 1.0 µg/ml culture) and combination of OTA+AFB₁ (0.004+1.0 µg/ml culture).

Results: Both OTA and AFB₁ interfered with the neural tube development. However, when combined, the effects on the neural tube were reduced but heart defects were observed. The histopathological observations of the embryos confirmed these observations and revealed degenerative changes and vacuolation in heart muscle.

Discussion: The results of the present study suggested that when used simultaneously, the effects caused by OTA were antagonized by AFB₁. Histopathological examination of the embryos revealed that the defects in the neural development were less severe in embryos exposed to both agents together. The defects in heart muscle were seen when the two mycotoxins were used together but not when used alone.

Keywords: aflatoxin B₁, combination, ochratoxin A, rat embryos, teratogenicity

Özet

Okratoksin A ve Aflatoksin B₁'in İmplantasyon Sonrası Sıçan Embriyo Kültüründe Tek veya Kombine Teratojenik Etkileri

Amaç: Bu çalışma, hepatic S9 miks varlığı ve yokluğunda, okratoksin A (OTA) ve aflatoksin B₁'e (AFB₁) implantasyon sonrası sıçan embriyo kültürünün eşzamanlı maruziyeti durumundaki etkilerini belirlemek amacıyla planlanmıştır.

Materyal ve Metot: Embriyolar, gebe Wistar sıçanlarından gebeliklerinin 10. gününde elde edilmişlerdir. Embriyolar 24 saat süreyle sıçan serumu, OTA (0.004, 0.008 µg/ml kültür), AFB₁ (0.5, 1.0 µg/ml kültür) ve kombine OTA+AFB₁ (0.004+1.0 µg/ml kültür) içeren M-199 medyumunda kültüre edilmişlerdir.

Sonuçlar: Hem OTA, hem de AFB₁ nöral tübün gelişimini bozmuştur; bununla beraber kombine kullanımlarında nöral tüp üzerindeki etkiler azalmış ancak kalp defektleri gözlenmiştir. Embriyoların histopatolojik incelemelerinde bu gözlemler doğrulanmış, kalp kasında dejeneratif değişiklikler yanında vakuolizasyon izlenmiştir.

Tartışma: Bu çalışmanın bulguları, kombine maruziyet durumunda OTA'nın etkilerinin AFB₁ tarafından antagonize edildiğini düşündürmektedir. Embriyoların histopatolojik incelemeleri nöral tüpteki defektlerin kombine maruziyet grubunda daha az olduğunu göstermiştir. Kalp gelişiminde olumsuz etkiler, bu miyotoksinlerin kombine kullanıldığı kültürlerde ortaya çıkmış, ancak tek başlarına kullanımları durumunda olumsuz bir etkiye sebep olmamışlardır.

Anahtar sözcükler: aflatoksin B₁, kombinasyon, okratoksin A, sıçan embriyosu, teratojenisite

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Introduction

Ochratoxin A (OTA) and aflatoxin B₁ (AFB₁) are environmentally important food borne mycotoxins produced by various species of *Aspergillus* and *Penicillium fungi* (1). OTA is nephrotoxic and more importantly, it is implicated as a causal factor of Balkan Endemic Nephropathy (BEN) in humans (2). AFB₁ ingestion is associated with hepatotoxicity and is correlated with high incidence of liver cancers in humans (3). Furthermore, strong evidence indicates that transplacental transfer of AFB₁ in humans may increase the risk of childhood cancer (4,5). WHO-IARC (1993) designated AFB₁ as Group-1 and OTA as Group-2B carcinogen (6,7).

The alarming feature of mycotoxins is their occurrence in combination in foods and feeds to exert a greater degree of damage to health and productivity of human and animals (8,9). There is considerable evidence of ochratoxin and aflatoxin exposure in human indicating that exposure is frequent (often >90% of some populations) and at high levels (>200 pg/mg level). The analysis of cord blood samples from pregnant women, human blood, colostrums and milk also revealed the fetal exposure of AFB₁ (10,11), OTA (12,13) and combination of OTA and AFB₁ (14).

OTA has also been reported to be teratogenic in rats (15,16), mice (17), hamsters (18), chick embryos (19) and quail (20) and rabbit (21). Similarly, AFB₁ has also been reported to be teratogenic in rats (22,23), and rabbits (24).

Mammalian whole embryo culture system is an *in vitro* test system used for screening and detection of potentially teratogenic compounds. The use of this method has a major advantage as it can measure the direct effects of a chemical (minus maternal influence) on inhibition of growth and interference with cell differentiation. Furthermore, effects of adding exogenous biotransforming system (hepatic S9) on a compound's developmental toxicity could be closely monitored under *in vitro* conditions. Thus, extra-corporeally maintained post-implantation rat embryos can be used to find out whether the compound requires biotransformation or have direct effects on cell differentiation and growth inhibition.

There are very few reports on the use of whole embryo culture method to detect developmental toxicity of OTA (25,26) and AFB₁ (27). Furthermore, literature scanned showed no report on combined effects of OTA and AFB₁ on rat embryos *in vitro*. Mechanism of teratogenesis caused by OTA and AFB₁ have not been clearly understood and may involve direct and/or indirect effects (18,16).

In our previous *in vivo* studies with rats (23) and rabbits (28), it has been observed that, when used in combination, OTA and AFB₁ have antagonistic interaction. It was of interest to see whether post-implantation rat embryos cultured *in vitro*, when exposed to OTA and AFB₁ in combination would demonstrate the same effects observed *in vivo*, and whether the metabolic biotransformation plays any role in dysmorphic action of these two mycotoxins when used together.

Materials and Methods

All the experimental procedures were conducted with prior permission of Institutional Ethical Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Due to CPCSEA rule of utilizing bare minimum number of experimental animals, the number of embryos used per group was restricted to 10-12 only. The characterization and concentrations of major metabolites produced from OTA and AFB₁ in culture medium in presence of S9 mix was not studied, due to unavailability of sufficient facilities.

Chemicals

OTA and AFB₁ were procured by Sigma Chemical Ltd., USA. Penicillin G, streptomycin sulphate and medium M-199 were obtained from Gibco-BRL, USA. The reagents, as diphenylamine and orcinol were obtained from Central Drug House and Thomas Baker (Chemicals) Limited India, respectively.

Collection of rat serum

Blood was collected from male rats and immediately centrifuged at 1500 rpm for 3 minutes, then allowed to clot at room temperature followed by centrifugation at 2500 rpm for 10 minutes. Serum was heat inactivated at 56°C and stored at -20°C until used.

Experimental animals

Sexually mature (175-200 g), virgin, female Wistar rats were obtained from the Laboratory Animal Resource (LAR) Division of the Institute. Following an acclimatization period of one week, females were mated with mature males of the same strain. The day on which vaginal plug was found or sperms observed in vaginal smear was designated as day zero of pregnancy. Experimental animal room temperature and relative humidity were set at 21±2°C and 50±10%, respectively and light cycle contained 12 hours lights and 12 hours darkness.

Preparation of S9 fraction (liver homogenate)

The liver homogenate (S9 mix) was prepared by the procedure described by Garner *et al.* (29). A young adult rat (150 g) was administered with 0.1% phenobarbital in the drinking water for 7 days. On the seventh day, the rat was sacrificed, liver (6.819 g) was excised and washed with an equal volume of 0.15M KCl (93 ml/g of wet liver) and then homogenized. The homogenate was centrifuged till the clear transparent supernatant was obtained. The supernatant so collected, was labelled as S9 mix. All the steps were performed under aseptic conditions at 0-4°C with cold, sterilized solutions and glassware. The freshly prepared S9 mix was distributed in 2.0 ml aliquots in small sterile plastic tubes, quickly frozen and stored at -80°C. The activity in the S9 mix was verified using Cyclophosphamide and Mytomycin C as positive controls (results not presented).

Embryo culture procedures

In the whole embryo culture experiment, total of 13 pregnant rats were sacrificed on day 10th of gestation by CO₂

anaesthesia. The gravid uterus was removed and placed in sterile petri-dish containing Hank's Balance Salt Solution (HBSS) at 37°C. The embryos were explanted and cultured as described by New (30), with little modification. The implantation sites were taken out and under dissecting microscope the Reichert's membranes were removed except for the portion around the periphery of the developing placenta. The embryos with intact yolk sac and amnion were placed in pre-warmed (37°C) M-199 medium (1 ml/embryo) containing rat serum (2 ml/embryo). The embryos from each pregnant female were apportioned at random among the six groups, control and different test groups of ochratoxin A and Aflatoxin B₁ alone and in combination (Table 1). Antibiotics, penicillin G and streptomycin (100 IU/ml and 10 µg/ml, respectively) were added. The stock solutions of OTA and AFB₁ were prepared in 0.1% dimethyl sulfoxide (DMSO) so that each 0.002 ml of the working solutions will contain 0.004 and 0.008 µg OTA/ml culture and 0.5 and 1.0 µg AFB₁/ml culture and were added to the culture medium before placing the embryos. Control cultures contained 0.002 ml of 0.1% DMSO and were manipulated in an identical manner as the treated embryos. Only the embryos having good heartbeat, active yolk sac and blood circulation and approximately similar somite numbers were selected for culture. Half of the embryos in each treatment group were cultured in the pre-

sence of S9 mix, while the other halves were cultured without the S9 mix.

Whole embryo culture assay

At the end of the culture period of 24 hours, the embryos were examined using stereo zoom microscope in the same sequence as they were placed in culture. The viability was confirmed by beating heart and active yolk sac circulation. The embryonic growth measurements such as yolk sac diameter, crown to rump length and somite numbers in each embryo and embryo weight were determined. Subsequently, the embryos were inspected for external structural defects. Following these observations, the embryos were washed 2-3 times in normal saline and 6 embryos from each group were homogenized in a test tube under ice-cold conditions. The total embryonic proteins were estimated by methods of Lowry et al. (31) and DNA and RNA contents by method of Schmid et al. (32) in the same samples.

Histopathological evaluation

For the histological examination, the embryos showing the gross abnormalities from treatment group or otherwise control embryos were fixed in Bouin's fluid, processed and embedded in paraffin. There were three embryos per treatment and control groups both in absence and presence of S9 mix. The horizontal serial sections (3-4 µm) of embryos were cut and stained with hematoxylin and eosin and observed under light microscope.

Table 1. Effects of OTA, AFB₁ and OTA+AFB₁ on growth parameters, macromolecular content and percent malformations of post-implanted embryos cultured in absence and presence of the S9 mix

Groups/Parameters Doses (µg/ml culture)	Control (0)	Ochratoxin A (OTA) (0.004)	Ochratoxin A (OTA) (0.008)	Aflatoxin B ₁ (AFB ₁) (0.5)	Aflatoxin B ₁ (AFB ₁) (1.0)	OTA+AFB ₁ (0.004+1.0)
In presence of S9 Mix						
No. of embryo exposed	10	10	10	10	10	12
Yolk Sac diameter (mm)	5.90±0.09	5.68±0.41	5.64±0.41	5.70±0.20	5.69±0.25	5.70±0.38
Crown rump length (mm)	3.87±0.04	3.78±0.14	3.73±0.18*	3.77±0.13	3.76±0.15	3.79±0.14
Embryo weight (g)	0.032±0.0014	0.031±0.0020	0.029±0.0017*	0.030±0.0014	0.030±0.0008*	0.031±0.0011
Somite No.	25.8±0.63	24.9±0.74	24.6±1.07*	25.2±0.98	24.9±1.20	24.7±0.98*
Protein content	344.38±2.00	291.25±2.67*	267.37±2.70*	301.87±4.06*	293.96±3.23*	294.13±3.17*
DNA content	34.63±0.95	28.12±1.26*	26.90±0.98*	28.5±0.98*	28.04±1.27*	26.38±1.06*
RNA content	25.94±1.15	19.87±0.35*	19.50±0.55*	20.88±2.14*	20.28±1.65*	20.83±1.57*
Morphological defects (%)	0	30.00	40.00 ^a	20.00	30.00	25.00
In absence of S9 Mix						
No. of embryo exposed	11	10	10	11	11	10
Yolk Sac diameter (mm)	5.80±0.34	5.70±0.31	5.67±0.31	5.76±0.34	5.72±0.34	5.73±0.40
Crown rump length (mm)	3.84±0.14	3.80±0.12	3.78±0.15	3.83±0.10	3.79±0.13	3.80±0.10
Embryo weight (g)	0.032±0.0012	0.032±0.0011	0.031±0.0016*	0.031±0.0011	0.030±0.0010*	0.031±0.0013
Somite No.	25.9±0.30	25.3±0.82	24.7±0.82*	25.2±0.75	25.5±0.82	25.6±0.70
Protein content	348.75±3.10	299.26±1.91*	279.50±11.25*	339.37±1.97	316.32±5.43*	299.75±3.76*
DNA content	36.25±0.71	29.87±0.78*	28.79±1.21*	32.38±0.67*	32.00±0.21*	30.88±1.05*
RNA content	27.05±0.47	19.50±2.42*	19.08±1.51*	30.88±1.05	23.54±1.84	21.50±3.63
Morphological defects (%)	9.09	20.00	30.00	18.18	27.27	20.00

* Significantly different from controls ($p<0.05$). ^a Significantly different from controls ($p<0.1$).
All the values mentioned are mean ±SD.

Statistical analyses

Statistical analysis of growth parameters such as yolk sac diameter, crown to rump length, somite numbers and embryo weights and macromolecular contents (i.e. protein, DNA and RNA) contents were performed by analysis of variance (ANOVA) followed by Duncun's test as *post hoc* test. To determine the significance of malformation incidence, 2x2 contingency table test was used (33). The statements of significance were based on a probability level of 0.05.

Results

The effects of OTA and AFB₁ alone and in combination on growth parameters, macromolecular content and percent malformations of post-implanted embryos cultured in presence and absence of S9 mix have been shown in Table 1.

There was 100% viability of the post-implantation embryos in all the treatment groups. The embryos from the control groups (Figure 1) did not show any malformations except one embryo (9.1%), which revealed stunted growth in the plain control culture without S9 mix.

Ochratoxin A

In presence of the S9 mix, yolk sac diameters of embryos exposed to OTA in two different concentrations were comparable to that of controls at both OTA concentrations, although a slight decrease was noticed. Crown to rump length, embryo weights and somite numbers were significantly decreased only at the higher OTA concentration. The protein, DNA and RNA contents were decreased significantly at both concentrations. As compared to controls, the incidence of malformations was significantly increased at the higher OTA concentration.

In absence of S9 mix, the yolk sac diameter and crown to rump length were comparable to those of controls in both OTA concentrations. Significant decrease in embryo weights and somite numbers was observed only at the higher OTA concentration but, significant decrease in the protein, DNA and RNA contents was observed at both the OTA levels. The



Figure 1. Rat embryo (control); showing normal development.

incidence of malformations was non-significantly higher in embryos cultured at 0.008 µg OTA/ml culture (30%) as compared with that of controls (9.09%).

The malformations consisted almost exclusively of defects in neural tube development, which included open neural tubes, hypoplasia in the area dorsal to the mandibular arches (Figure 2). Other malformations observed were retarded embryonic growth, poor flexion, curling of tail and stunted growth in the limb bud development (Table 2). The malformations observed in absence and presence of S9 mix were similar except for hypoplasia in the area dorsal to the mandibular arches and stunted growth in the limb bud development which were not seen in embryos in absence of S9 mix.

The histopathological examination of embryos revealed congestion and degenerative changes in the neural tube and at places focal areas of hemorrhages. The organization and differentiation of various parts of neural folds were imperfect. As compared with that in the controls, the cephalic flexure was too much widened and the ventricles appeared dilated, pituitary gland was hypoplastic, underdeveloped and rarefied. The degeneration and haemorrhages in vitreous humour were seen in optic vesicle of the OTA treated embryos. The histopathological changes were seen at both OTA levels, although the changes were milder at the lower OTA concentration.

AFB₁

In presence of S9 mix, the yolk sac diameter, crown to rump length and somite numbers of the embryos exposed to AFB₁ were decreased non-significantly as compared with those of the controls. The decrease in the embryo weights was significant only at the higher AFB₁ concentration. The protein, DNA and RNA contents were significantly decreased but the incidence of malformations was non-significantly increased at the both AFB₁ levels.

In the absence of S9 mix, the yolk sac diameter, crown to rump length, somite numbers and RNA contents remained

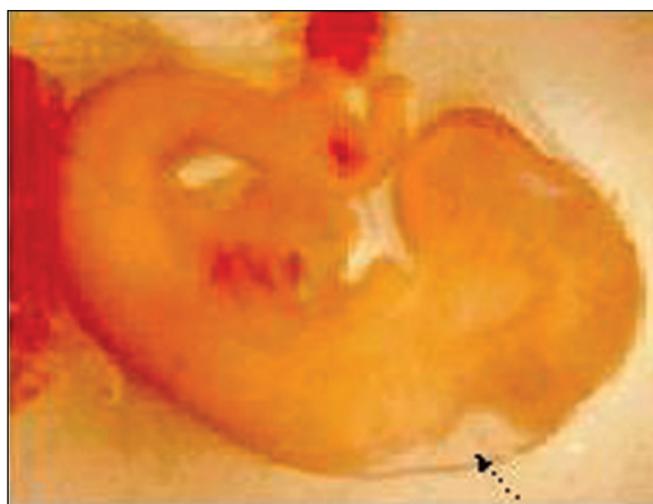


Figure 2. Rat embryo (OTA 0.008 µg/ml culture without S9); showing neural tube defect and curling of tail.

Table 2. Individual type of morphological defects observed in embryos exposed to ochratoxin A and aflatoxin B₁ alone and in combination

Groups	Number of embryos							
	Obs	Affected n	%	With S9 mix Description	Obs	Affected n	%	Without S9 mix Description
Control	10	0		-	11	1	9.09	Retarded embryonic growth (1)
OA (0.004)	10	3	30.00	Neural tube defect (1) Retarded embryonic growth (1) Curling of tail	10	2	20.00	Neural tube defect (1) Retarded embryonic growth (1) Curling of tail (1)
OA (0.008)	10	4	40.00 ^a	Neural tube defect (4) Hypoplasia of mandibular arches (3) Poor flexion (4) Stunted limb bud development (2) Curling of tail (3)	10	3	30.00	Neural tube defect (3) Poor flexion (2) Curling of tail (2)
AFB ₁ (0.5)	10	2	20.00	Neural tube defect (1) Poor flexion Curling of tail Retarded embryonic growth (1)	11	2	18.18	Poor flexion (1) Curling of tail (1) Retarded embryonic growth (1)
AFB ₁ (1.0)	10	3	30.00	Neural tube defect (3) Poor flexion Retarded embryonic growth (3) Curling of tail (2)	11	3	27.27	Neural tube defect (2) Poor flexion (1) Retarded embryonic growth (3) Curling of tail (2)
OTA+AFB ₁ (0.004+1.0)	12	3	25.00	Neural tube defect (1) Heart defects (3) Poor flexion Curling of tail Retarded embryonic growth (2)	10	2	20.00	Neural tube defect (1) Heart defects (2) Poor flexion (1) Curling of tail (1) Retarded embryonic growth (1)

^a- Significantly different from controls ($p < 0.1$).

unaffected by AFB₁ at both levels. The embryo weights and protein contents were significantly decreased only in embryos cultured with 1.0 µg AFB₁/ml culture as compared with those of controls. However, the DNA contents were decreased significantly at both the doses. The increase in the incidence of malformations was non-significant at both AFB₁ levels.

The types of malformations observed both in presence and absence of S9 mix were similar and included neural tube defects, retarded embryonic growth, poor flexion and curling of tail. However, the incidence was higher in presence of S9 mix than in its absence.

The histological examination of the embryos treated with AFB₁ showed degenerative changes in the neural tube epithelium. As compared with that in the controls, the ventricles were dilated and all the parts of the neural tube were hypoplastic and underdeveloped. Exposure to the lower AFB₁ concentration caused only mild degenerative changes of the neural tube.

Combination of OTA and AFB₁

When the embryos were exposed to both the mycotoxins simultaneously, OTA+AFB₁ (0.004+1.0 µg/ml culture), the

yolk sac diameter, crown to rump length and embryo weights were comparable with those of controls, both in presence and absence of S9 mix.

In the presence of S9 mix, there was significant decrease in the somite numbers, protein, DNA and RNA contents. The incidence of malformations was non-significantly increased compared to the controls.

In absence of S9 mix, there was significant decrease in the protein and DNA contents only. The incidence of malformation was non-significantly higher compared to the controls.

The malformations observed in presence and absence of S9 mix were developmental defects of the neural tube and the heart, retarded embryonic growth, poor flexion and curling of tail in embryos cultured in presence of the S9 mix.

Histopathological examination of the embryos revealed degenerative changes in the neural tube and the heart muscle. As compared with that of control embryos (Figure 3), the heart of the embryos treated with combination of OTA and AFB₁ were enlarged and auricles were dilated (Figure 4), which resulted in stretching, breaking and loss of muscle fibers and absence of compartments in auricles. Ventricles we-

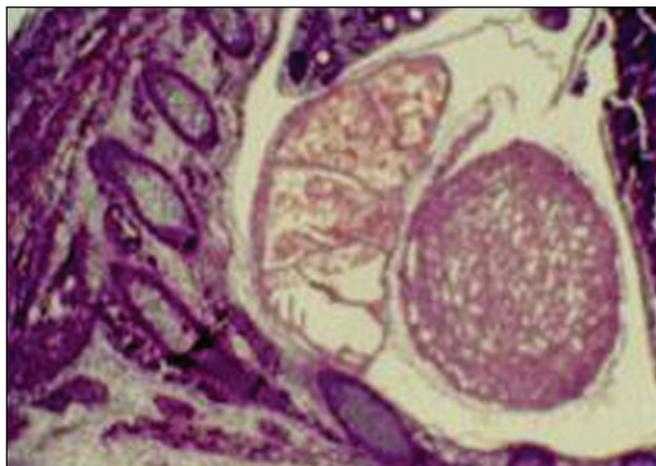


Figure 3. Histological section of heart of rat embryo (control); note the normal development of heart compartments and muscle fibres.

re reduced in size; degenerative changes, vacuolation and loosening were seen in muscle bundles making it less compact, as compared with those in the controls.

Comparative evaluation of toxicity of OTA and AFB₁ alone and in combination

The effects of the combination treatment were different from those when these mycotoxins were used individually. When these two mycotoxins were present in combination, the embryo weights both in absence and presence of the S9 mix and somite numbers in absence of the S9 mix were less severely affected than when either of the mycotoxins were used

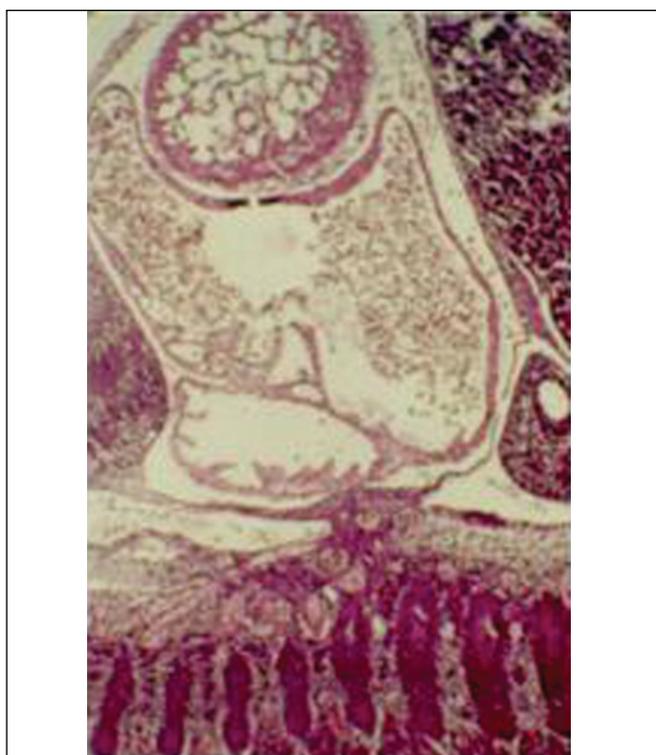


Figure 4. Histological section of heart of rat embryo (OA 0.004+AFB₁ 1 µg/ml culture with S9); degeneration and vacuolations of the muscle bundles and dilatation of heart compartments.

alone. The incidence of the abnormalities was lower than those seen at the higher level of AFB₁. The defects in heart development were seen only in embryos cultured with both mycotoxins, in presence and absence of the S9 mix.

Discussion

The whole embryo culture method was utilized in the present study as it permits monitoring the early organogenesis *in vitro* and especially allows the measurement of the direct effect of chemicals on growth and cell differentiation. Further, effects of adding exogenous enzymatic biotransformation system (the S9 mix) on a compound's developmental toxicity can also be closely monitored.

The endoplasmic liver activity of the liver-donor animals was induced with phenobarbital as it has been reported that pretreatment with phenobarbitone enhances the metabolism of aflatoxin B₁ (34) and OTA (35).

The literature scanned showed that no studies on the combined effects of OTA and AFB₁ with the whole embryo culture method, despite the demonstration of the presence of these mycotoxins together in different foods and feeds (8). Yokoyama and Akita (26) reported that ochratoxin A treatment (0.001 µg/ml) caused no effects on heart rate, systemic blood circulation and crown to rump length of cultured rat embryos. However, Mayura *et al.*, (25) observed that addition of ochratoxin A at concentrations of 75 µg/ml and above caused significant decrease in yolk sac diameter, crown to rump length, somite number and protein and DNA contents in post-implantation rat embryos. Geissler and Faustman (27) reported that 10-day old cultured rat embryos when exposed to AFB₁ (15 µM) alone and in presence of co-factors and hepatic S9 fractions, developed abnormalities in neural tube. Joshi and Joshi (36) investigated effects of AFB₁ on early embryonic stages of the chick embryos cultured *in vitro* and reported that primitive streak stage was found to be the most susceptible stage to sublethal dose of AFB₁ (0.5 µg/ml).

In the pilot study before that reported here, it was observed that OTA and AFB₁ at concentrations as low as 0.004 µg/ml culture (OTA) and 0.5 µg/ml culture (AFB₁) were found effectively toxic (results not shown). Taking into consideration previous reports and pilot study results, the concentrations of 0.004 and 0.008 µg/ml culture of OTA and 0.5 and 1.0 µg/ml culture of AFB₁ were selected for the study presented here. The dose for combination treatment (OA+AFB₁) selected was 0.004+1.0 µg/ml culture, as these two mycotoxins never appear in exactly equal quantities in the samples of different food and feedstuffs screened in this laboratory.

The control cultures did not reveal any adverse effect on the growing embryos. Significant decreases as observed in the present study, in protein, DNA and RNA contents and effects on different developmental parameters in rat embryos after treatment with OTA (25,26,37) or AFB₁ (27), as well as with AFB₁ in chicken embryos (36) and mice embryos (38) have

also been reported earlier by others. These earlier observations of macromolecular changes and developmental defects were observed in embryos exposed to either OTA or AFB₁, both in presence and absence of S9 mix.

The present study, however, has provided evidence of direct as well as indirect actions through metabolites of these toxins resulting in the developmental defects. The simultaneous exposure to OTA and AFB₁ resulted in increased incidence of malformations and decreased growth parameters in presence than in absence of the S9 mix. When compared with exposures to the individual mycotoxins, it was observed that the combination treatment had less severe effects than that caused by OTA or AFB₁ when used alone. The effects of combined mycotoxins were, thus, less than additive, suggesting sparing effects.

The histological examination of embryos supported these observations, indicating that the neural tube lesions were less severe with the mycotoxins combined than with OTA or AFB₁ used alone. However, when used in combination, these mycotoxins severely affected the heart development.

There was no report on the combined teratogenic effect of OTA and AFB₁ using post implantation rat embryos, however, Small et al. (39) have reported synergistic effect of OTA and cyclopiazonic acid (CPA) in cultured rat embryos.

Earlier authors, while studying the *in vivo* teratogenesis in different laboratory animals have reported that OTA caused exencephaly, incomplete closure of skull, hypognathia, micromelia, scoliosis, curled tail, skeletal defects involving fused, bifurcated ribs as well as soft tissue anomalies like the ectopic or polycystic kidney, renal agenesis, hydrocephaly and microphthalmia (15,16,21,23). Similarly, the reported *in vivo* teratogenic anomalies induced by AFB₁ in different laboratory animals involved exencephaly, gastroschisis, abnormal head along with open eye, incomplete ossification of skull bones, hydrocephalus, microphthalmia and cardiac defects such as fusion of auriculo-ventricular valve, enlargement of auricles and increased ventricular cavity (17,21,22,24).

When the findings of *in vivo* studies observed in different laboratory animals were compared with those of the *in vitro* findings, the results were matching. Even the findings of combination usage of mycotoxins in this study are in accordance with our previous results in rat and rabbit fetuses obtained from the dams treated with combination of OTA and AFB₁ (23,28). These changes were also confirmed by histopathological findings. Although, the detailed studies on histopathological changes of embryos in the *in vitro* studies are meager, microscopic lesions such as degenerative changes in the neural tube and the heart muscle observed in the present study were in accordance with earlier studies, where marked degeneration of neural tube epithelium and vacuolization of the tissue layer and significant sloughing of debris into neural tube lumen were recorded in embryos treated with AFB₁ (27) and OTA (25). These results

are matching with those observed in rat fetuses obtained from the dams treated with combination of OTA and AFB₁ (40).

The effects of OTA on the neural tube development were due to the inhibition of protein synthesis, these observations are in agreement with report of Monnet-Tschudi et al. (41), who reported that toxic effects of OTA on brain cells in culture are due to the inhibition of protein synthesis. The reasons for the occurrence of antagonistic effects of AFB₁ over those caused by OTA in embryos might be depend upon their biotransformation and formation of DNA adducts. The antagonistic effects of the combined mycotoxins on the proteins, DNA and RNA contents observed in presence of the S9 mix indicated the biotransformation might have played some role in the results obtained in the present investigation. Juchau et al. (42), correlated the prenatal susceptibility to onset of detectable levels of oxidative enzymes such as CYP3A7. Coupled with this, interference with other cellular and molecular targets (20), as well as induced apoptosis and damage to plasma membrane (43) might be responsible for the antagonistic effects observed in the present study.

The mechanism of action of both these mycotoxins is complex and neither act by a single mechanism. However, inhibition of cellular macromolecule synthesis and oxidative stress are the mechanisms that are shared by both these toxins (44,45). It is possible that production of reactive oxygen species by these toxins might have led to derailment in the genetic control of development and the coordinated cascade of events that occur during normal development. Kimmel, et al. (46) have reported disturbance in the coordinated expression of developmental genes involved in cell-cell interaction, cell migration, differentiation, and segmentation which could possibly be attributed to the effects observed here.

Additional studies to determine the exact mechanism of antagonistic reaction of OTA and AFB₁ are required. However, the findings of the present *in vitro* study further correlated and substantiated the previous observations of *in vivo* studies in rats (23) and rabbits (28) of reduced teratogenic activity of OTA in presence of AFB₁.

References

1. Scott PM, Van Walbeek W, Kennedy B, Anyeti D. Mycotoxins (ochratoxin A, citrinin and sterigmatocystin) and toxigenic fungi in grains and other agriculture products. *J Agric Food Chem* 1972;20:1103-9.
2. Stoev SD. The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation. *Vet Human Toxicol* 1998;40(6):352-60.
3. Trail F, Mahanthi N, Linz J. Molecular biology of aflatoxin biosynthesis. *Microbiol Reading* 1995;141(4):755-65.
4. Autrup H. Transplacental transfer of genotoxins and transplacental carcinogenesis. *Environ. Health Perspect* 1993;101:33-8.
5. Doi AM, Patterson PE, Gallagher EP. Variability in Aflatoxin B1-Macromolecular Binding and Relationship to Biotransformation Enzyme Expression in Human Prenatal and Adult Liver. *Toxicol Appl Pharmacol* 2002; 181:48-59.
6. World Health Organization. International Agency for Research on Cancer (IARC), Monograph on Evaluation of Carcinogenic Risks to Humans, 1993.
7. Hussein HS, Brasel JM. Toxicity, metabolism and impact of mycotoxins on

- human and animals. *Toxicol* 2001;167:101-34.
8. Roberts BA, Glancy EM, Patterson DSP. Rapid economical method for determination of aflatoxin and ochratoxin in animal feed stuffs. *J Assoc Off Anal Chem* 1981;64:961.
 9. Adebajo LO. Survey of aflatoxins and ochratoxin A in stored tubers of *Cyperus esculantus* L. *Mycopathol* 1993;124:41-6.
 10. Yousef M, Abdulrazzaq NO, Ahmed I. Fetal exposure to aflatoxins in the United Arab Emirates. *Ann Trop Paediatr* 2002;22:3-9.
 11. Abdulrazzaq YM, Osman N, Yousif ZM, Trad O. Morbidity in neonates of mothers who have ingested aflatoxins. *Ann Trop Paediatr* 2004;24:145-51.
 12. Kovacs F, Sandor G, Vanyi A, et al. Detection of ochratoxin A in human blood and colostrum. *Acta Vet Hung* 1995;43(4):393-400.
 13. Skaug MA, Stormer FC, Saugstad OD. Ochratoxin A: a naturally occurring mycotoxin found in human milk samples from Norway. *Acta Paediatr* 1998;87(12):1275-8.
 14. Jonsyn FE, Maxwell SM, Hendrickse RG. Human fetal exposure to ochratoxin A and aflatoxins. *Ann Trop Paediatr* 1995;15:3-9.
 15. Brown MH, Szezech GM, Purmalis BP. Teratogenic and toxic effects of ochratoxin A in rats. *Toxicol Appl Pharmacol* 1976;37:331-7.
 16. Mayura K, Reddy RV, Hayes AW, Berndt WO. Embryocidal, fetotoxic and teratogenic effects of ochratoxin A in rats. *Toxicol* 1982;25:175.
 17. Arora RG. Mycotoxin induced effects on the prenatal development in mice: a teratopathologic and autoradiographic study of aflatoxin B1, ochratoxin A and zearalenone. Ph.D. Thesis submitted to Swedish University of Agricultural Sciences (1982), Uppsala, Sweden.
 18. Hood RD, Naughton MJ, Hayes WA. Prenatal effects of ochratoxin A in hamsters. *Teratol* 1976;13:11-4.
 19. Lalitha Kunjamma CR, Nair MK. Pathomorphology of the combined effects of ochratoxin A and citrinin in chicken embryos. *Ind Vet J* 1997;74:22-5.
 20. Dwivedi P. The immunological and pathological changes in poultry induced by ochratoxin A. Ph.D. Thesis, University of Edinburgh, UK 1984.
 21. Wangikar PB, Dwivedi P, Sinha N. Teratogenic effects of ochratoxin A in rabbits. *World Rabbit Sci* 2004a;12:159-171.
 22. Mayura K, Abdel-Wahhab MA, McKenzie KS, et al. Prevention of maternal and developmental toxicity in rats via dietary inclusion of common aflatoxin sorbents: potential for hidden risks. *Toxicol Sci* 1998;41:175-82.
 23. Wangikar PB, Dwivedi P, Sinha N. Effect in rat of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. I. Maternal toxicity and fetal malformations. *Birth Defects Res Part B Deve Reprod Toxicol* 2004b;71(6):343-51.
 24. Wangikar PB, Dwivedi P, Sharma AK, Telang AG. Effects of aflatoxin B1 on embryo fetal development in rabbits. *Food Chem Toxicol* 2005a;43(4):607-15.
 25. Mayura K, Edwards JF, Maull EA, Phillips TD. The effects of ochratoxin A on post-implantation rat embryo in culture. *Arch Environ Contam Toxicol* 1989;18:411-5.
 26. Yokoyama A, Akita M. Toxicological study of ochratoxins in cultured rat embryos. (Abs) *Teratol* 1998;57(3):19A,B-24.
 27. Geissler F, Faustman EM. Developmental toxicity of aflatoxin B1 in the rodent embryo *in vitro*: Contribution of exogenous biotransformation system to toxicity. *Teratol* 1988;37:101-11.
 28. Wangikar PB, Dwivedi P, Sharma AK, Telang AG. Teratogenic effects in rabbits of simultaneous exposure to ochratoxin A and aflatoxin B1 with special reference to microscopic effects. *Toxicol* 215 (2005) 37-47.
 29. Garner RC, Miller EC, Miller JA. Liver microsomal metabolism of aflatoxin B1 to a reactive derivative toxic to *Salmonella typhimurium* TA 1530. *Cancer Res* 1972;32(10):2058-66.
 30. New studies on mammalian fetuses *in vitro* during the period of organogenesis. In: *The mammalian fetus in vitro*. C.K. Austin, (Ed), Chapman and Hall, London. p.15.
 31. Lowry OH, Rosebrough NJ, Farr AB, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
 32. Schmid BP, Trippmacher A, Bianchi A. Teratogenicity induced in cultured rat embryos by the serum of procarbazine treated rats. *Toxicol* 1982;25:53-60.
 33. Snedecor GW, Cochran WG. Statistical methods. Sixth edition. Oxford and IBH Publishing Co. 1968;pp 215-20.
 34. Lotlikar PD, Raj HG, Bohm LS, et al. A mechanism of inhibition of aflatoxin B1-DNA binding in the liver by phenobarbital pretreatment of rats. *Cancer Res* 1989;49(4):951-7.
 35. Adlouni CEI, Pinelli E, Azémar B, et al. Phenobarbital increases DNA adduct and metabolites formed by ochratoxin A: Role of CYP 2C9 and microsomal glutathione-S-transferase. *Environ Mol Mutagen* 2000;35(2):123-31.
 36. Joshi MS, Joshi MV. Effects of aflatoxin B1 on early embryonic stages of chick *Gallus domesticus* cultured *in vitro*. *Ind J Exp Biol* 1981;19:528-31.
 37. Han SY, Park KS, Park KL, et al. Ochratoxin A-induced embryotoxicity and preventive effect of catalase in cultured rat embryonic midbrain cells. *Teratol* 1999;59(6):403.
 38. Irvin TR, Poorniyi ALS, Stevens EK, Martin JE. Parental toxicity of aflatoxins: Application of mammalian cell culture systems to characterize the developmental toxicity of aflatoxin mycotoxins. *Cancer Health* 1991;167:82.
 39. Small M, Smith E, Braithwaite C, et al. Evaluation of the developmental toxicity of ochratoxin A and cyclopiazonic acid in post-implantation rat embryos. *Toxicologist* 1991;11(1):297.
 40. Wangikar PB, Dwivedi P, Sharma AK, Sinha N. Effect in rat of simultaneous prenatal exposure to ochratoxin A and aflatoxin B1. II. Histopathological features of teratological anomalies induced in fetuses. *Birth Defects Res Part B Deve Reprod Toxicol* 2004c;71(6):352-8.
 41. Monnet-Tschudi F, Sorg O, Honegger P, et al. Effects of the naturally occurring food mycotoxin ochratoxin A on brain cells in culture. *Neurotoxicol* 1997;18(3):831-9.
 42. Juchau MR, Lee QP, Fantel AG. Xenobiotic biotransformation/ bioactivation in organogenesis-stage conceptual tissues: Implications for embryotoxicity and teratogenesis. *Drug Metab Rev* 1992;24:195-238.
 43. Marquardt RR, Frohlich AA. A review of recent advances in understanding ochratoxicosis. *J Anim Sci* 1992;70:3968-88.
 44. O'Brien E, Dietrich DR. (2005). Ochratoxin A: The continuing enigma. *Critical Reviews Toxicol* 1973;35:33-60.
 45. Shen HM, Shi CY, Shen Y, Ong CN. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. *Free Radic Biol Med* 1996;21(2):139-46.
 46. Kimmel CA, Generoso WM, Thomas RD, Bakshi KS. A new frontier in understanding the mechanisms of developmental abnormalities. *Toxicol Appl Pharmacol* 1993;119(2):159-65.