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Toxic Effects of Ochratoxin A and Citrinin, Alone and in Combination, on Chicken Embryos

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The embryotoxic potential of ochratoxin A and citrinin was studied after administering, either subgerminally or intraamniotically, single mounting doses of the mycotoxins to chicken embryos on days 2, 3, and 4. The beginning of the embryotoxicity dose range was found to be between 0.01 to 0.05 μg for ochratoxin A and 1 to 10 μg for citrinin. The maximum response to both mycotoxins occurred after administration on day 3. In addition to significant growth retardation of fetuses, exencephaly, microphthalmia, cleft beak, reduction deformities of the limbs, and abdominal wall and ventricular septal defects were encountered on day 8 of incubation. When 4 μg of citrinin was constantly added to ochratoxin A administered in the dose range 0.03 to 0.5 μg, a strictly additive effect was seen. It may be supposed that citrinin produced together with ochratoxin A in some strains of *Penicillium viridicatum* Westling does not potentiate the clear-cut embryotoxic action of the latter mycotoxin.

Ochratoxin A and citrinin are mycotoxins produced by some species of *Aspergillus* and *Penicillium*. Several strains of *Penicillium viridicatum* Westling that customarily parasitize grains cause nephropathy in pigs and fowls (3, 9). Ochratoxin A was shown to be a teratogen in rats by Moré and Galtier (10, 11) and Brown et al. (1), in hamsters by Hood et al. (7), and in the embryonic chick by Gilani et al. (5). Biological effects of patulin and citrinin in the chicken embryo were studied by Ciegler et al. (2). This paper aims to evaluate the embryotoxicity of ochratoxin A and citrinin, administered both separately and in combination, in the chicken embryo.

MATERIALS AND METHODS

Ochratoxin A was isolated from *P. verrucosum* var. *verrucosum* grown on wheat (13). Citrinin was prepared from *P. janthinellum* F-391 of the Brno collection CCM and cultivated on fluid medium containing 10% saccharose and 1% yeast autolysate. The purity of each mycotoxin was confirmed by melting point, UV spectrometry, thin-layer chromatography, and nuclear magnetic resonance. The basic solutions were prepared by dissolving 1 mg of ochratoxin A or 100 mg of citrinin in 10 ml of sterile 1% $NaHCO_3$ solution. Further dilutions were made by adding water only. The effects of mycotoxins were evaluated with the Chick Embryotoxicity Screening Test (CHEST II; 8).

Fertilized eggs of white Leghorns were incubated at 39°C and 50 to 60% relative humidity in a forced-draft thermostatic oven. The eggs were candled and opened, using the standard window technique, on days 2, 3, and 4. Ten selected normal embryos for each dose and day of administration were injected subgerminally on day 2 and intraamniotically on days 3 and 4 with 10-μl volumes, using a glass micropipette. For more detailed analysis, the dosage was considerably extended on day 3. Then, the windows were covered by glass slides and sealed with paraffin, and the eggs were further incubated until day 8. After careful removal, the living embryos were weighed and inspected under preparation microscope for the presence of abnormalities of the selected morphogenetic systems. The proportions of dead, malformed, and significantly retarded (i.e., weighing <650 mg) fetuses served as an index of embryotoxicity. The beginning of the embryotoxicity range was estimated at the point of intersection of the dose-response curve with the level of naturally occurring embryotoxicity phenomena that equals 0.3. Statistical analysis of dose-response relationships was performed with a log-logistic model applied to quantal response data (4).

RESULTS

The most frequent malformations observed after ochratoxin A administration in doses of >0.02 μg were exencephaly, microphthalmia, bilateral cleft beak, and interventricular septal defects of the heart. Also, reduction defects of the limbs (amelia and micromelia of the upper extremities) as well as abdominal wall closure defects and growth-retarded fetuses were encountered after ochratoxin administration of day 2. The gross dose-response relationships of ochratoxin A and citrinin (Table 1) are depicted in Fig. 1. The beginning of the embryotoxicity dose range for ochratoxin was between 0.01- and 0.5-μg doses; for the apparently less toxic citrinin, it

TABLE 1. Proportion of embryotoxicity manifestations and organ affliction in surviving fetuses within embryotoxicity dose ranges

Mycotoxin	Day	No. of embryos	Proportion of embryotoxicity/affliction								
			Death	Growth retardation	Brain	Eye	Face	Body wall	Extremities	Rump	Heart
Ochratoxin A	2	40	0.53	0.10	0.13	0.20	0.27	0.00	0.07	0.80	0.13
	3	40	0.63	0.27	0.77	0.54	0.08	0.54	0.54	0.00	0.46
	4	40	0.63	0.00	0.67	0.40	0.67	0.27	0.00	0.00	0.13
Mean			0.60	0.12	0.52	0.38	0.34	0.27	0.20	0.27	0.24
Citrinin	2	30	0.40	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.06
	3	30	0.60	0.00	0.08	0.16	0.16	0.16	0.00	0.00	0.16
	4	30	0.45	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.06
Mean			0.48	0.00	0.03	0.07	0.05	0.05	0.00	0.08	0.09
Ochratoxin A	3	110	0.25	0.20	0.41	0.14	0.07	0.28	0.21	0.12	0.26
Ochratoxin A + citrinin	3	90	0.46	0.28	0.62	0.26	0.01	0.09	0.27	0.00	0.25

was between 1- and 10-μg doses (Fig. 1, left). The maximum response to both mycotoxins appeared after administration on day 3 (Fig 1, right). Therefore, this day was chosen for more detailed analysis of the dose-response relations after ochratoxin A or citrinin administration as well as for the study of their effects in combination. The dose-response curves for ochratoxin A and citrinin, administered on day 3, fit the log-logistic model well (chi-square test for goodness of fit, $\chi^2_{(5\ df)} = 5.16$ and $\chi^2_{(3\ df)} = 3.43$, respectively; $P > 0.1$), (Fig. 2). The slopes of the curves are similar; ochratoxin A is significantly shifted to the lower doses. This is also reflected in the 50% effective doses of 0.012 μg (0.009 to 0.017 μg) for ochratoxin and 5.16 μg (3.78 to 7.04 μg) for citrinin. The dose-response curve for the joint action of ochratoxin A and the constant dose of citrinin clearly indicates the additive character of the effects (chi-square test for the difference between the values expected for the additive effect and the empirical values obtained, $\chi^2_{(3\ df)} = 0.73$; $P > 0.5$). Thus, the deleterious embryotoxic action of ochratoxin A does not seem to be potentiated by the presence of citrinin. Similarly, there is no substantial change in the malformation spectra occurring under the joint action of the mycotoxins (Table 1).

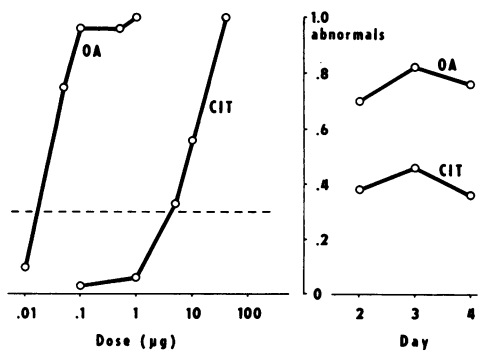

FIG. 1. Empirical dose-response curves as estimated with CHEST II for the population of 2-, 3-, and 4-day chicken embryos (left) and dependence of the response upon day of administration (right). OA, Ochratoxin A; CIT, citrinin. Dashed horizontal line indicates level of nonspecific effects of the experimental interaction.

DISCUSSION

Similar manifestations of embryotoxicity, as observed herein in the chicken embryo after ochratoxin administration, were reported by Hood et al. (7) in the offspring of hamsters treated with 5 to 20 mg of ochratoxin A per kg on days 7 to 9 of pregnancy. Gilani et al. (5) administered 0.5 to 7 μg of the same mycotoxin into the air sacs of hen eggs. In fetuses on day 8, a similar malformation spectrum was observed. The substantially higher dosage of ochratoxin A necessary for inducing the embryotoxic effect of Gilani's experiments could be explained by the different route of administration.

Rumplessness, microphthalmia, bilateral cleft beak, brain herniation, and abdominal wall and ventricular septal defects were observed in our experiments after administration of citrinin in doses higher than 5 μg. The maximum embryotoxic potential of citrinin was expressed in embryos treated on day 3. Ciegler et al. (2) studied the teratogenic action of 10 to 150 μg of citrinin administered to chicken embryos on day 4. Exophthalmia, crossed beak, and crooked neck were encountered. Our results are consistent when one considers the affinity of citrinin to the

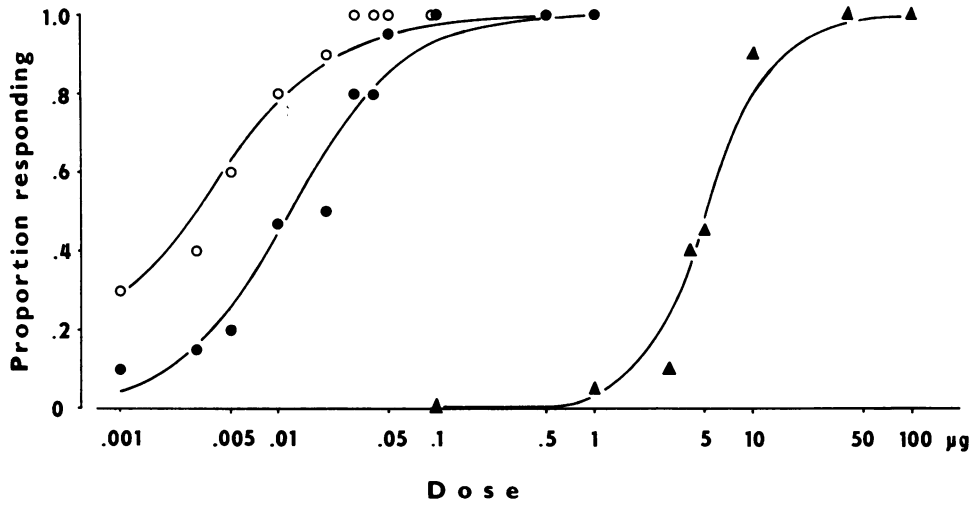

FIG. 2. Theoretical dose-response curves estimated from the empirical values (circles) after administering mounting doses of ochratoxin (●), citrinin (▲), or their combination (○) in the period of maximum sensitivity of chicken embryos on day 3.

target organs; the malformation types are different, however. This fact is by no means surprising because malformation type appears to be determined, besides by the degree of sensitivity of particular morphogenetic systems to the agent or its metabolite or both, by the time of administration, the dose, and the pharmacokinetic parameters of the transport channel (12). According to Thacker (cited in reference 6), ochratoxin A and citrinin exhibited synergistic lethal toxicity in female guinea pigs but additive effects in male guinea pigs.

No data have been found in the accessible literature concerning the effect of the combination of ochratoxin A and citrinin upon embryonic development. The shift of the entire dose-response curve for ochratoxin A to the left when a constant dose of citrinin was added allows us to suppose that no interaction of the two mycotoxins occurred on either a biochemical or a morphogenetic level. This means that even the teratogenicity of ochratoxin A as a specific embryotoxicity manifestation shows no modification in the presence of its connatural companion.

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