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Ochratoxin A-Induced Teratogenesis in Rats: Partial Protection by Phenylalanine

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Ochratoxin A (OA), an important foodborne mycotoxin, is a potent teratogenic and nephrotoxic agent produced by several species of *Aspergillus* and *Penicillium*. OA is a known inhibitor of protein synthesis via competition with phenylalanine (Phe) in the phenylalanyl-tRNA synthetase-catalyzed reaction. It also has been reported that a variety of toxic effects of OA can be prevented by Phe. This study was designed to determine whether Phe could prevent or diminish the teratogenic effects of OA in rats. Pregnant Sprague-Dawley rats were injected with a single individual dose of OA (1.75 mg/kg) alone or in combination with a single dose of Phe (20 mg/kg) or in combination with either a single or daily dose of Phe (25 mg/kg). OA dissolved in 5% sodium bicarbonate and Phe dissolved in normal saline were administered subcutaneously on gestation day 7 to rats. The incidences of OA-induced fetal malformations (gross and skeletal) were significantly diminished in the presence of added Phe. These results indicate that coadministered Phe provides partial prenatal protection from the teratogenic effects of OA.

Ochratoxin A (OA), a chlorinated dihydroisocoumarin derivative connected through an amide bond to L-beta-phenylalanine at the 7-carboxy group is an important foodborne secondary metabolite produced by various species of the fungal genera *Aspergillus* and *Penicillium* (16). It is a potent nephrotoxin in a variety of animal species (12) and a reported teratogen in rats (1, 14), mice (9), hamsters (10), and chicken embryos (S. H. Gilani, J. Bancroft, and M. O'Rahily, Teratology **11**:18A, 1975). OA also has been implicated as a causative factor in the fatal renal disease endemic balkan nephropathy in humans (13).

OA inhibits protein synthesis by competition with the amino acid phenylalanine (Phe) in the phenylalanyl-tRNA synthetase-catalyzed reaction (2, 4). It has been reported that Phe reverses OA-elicited cytotoxicity in tissue culture (3), prevents the lethal effects of OA in mice (5), attenuates OA inhibition of guinea pig macrophage migration (11), and protects against the immunosuppressive effects of OA in BALB/c mice (8).

The present study was undertaken to determine the efficacy of Phe in preventing or diminishing the teratogenic effects of OA in rats.

MATERIALS AND METHODS

Sexually mature (175- to 200-g) virgin, Sprague-Dawley female rats (Timco, Inc., Houston, Tex.) were maintained on feed and water ad libitum at the Texas A&M University Laboratory Animal Resources and Research Facility. After an acclimation period of 1 week, 51 females were mated with mature males of the same strain in filter-top, polycarbonate cages housed in a temperature-controlled and artificially illuminated room (12 h of light and 12 h of dark) free from known sources of chemical contamination. The day on which a vaginal plug was found was designated day 0 of pregnancy.

Pregnant females were randomly distributed into seven groups and treated as follows: group I, untreated; group II, treated with solvent vehicles (5% sodium bicarbonate and saline); group III, Phe (25 mg/kg); group IV, OA (1.75 mg/kg); group V, OA (1.75 mg/kg) plus Phe (20 mg/kg); group VI, OA (1.75 mg/kg) plus Phe (25 mg/kg); and group VII, OA (1.75 mg/kg) plus Phe (25 mg/kg) given once daily for the first 10 days of gestation. OA (Makor Chemicals, Ltd., Jerusalem, Israel) dissolved in 5% sodium bicarbonate and L-beta-phenylalanine (Sigma Chemical Co., St. Louis, Mo.) dissolved in normal saline (0.9% sodium chloride solution adjusted to pH 7.2 with 0.1 M sodium hydroxide) were administered subcutaneously on gestation day 7. All injection volumes were 0.1 ml/100 g of body weight. Six to nine animals were used in each treatment group. Body weights of pregnant rats were monitored daily.

Dams were euthanized by CO_2 asphyxiation on day 20 of pregnancy. The uterine horns were exposed, and the numbers of implants, resorptions, and live fetuses were counted. Live fetuses were removed from the uterus, blotted dry, weighed, and examined for gross abnormalities. Every third fetus was fixed in 95% ethanol, cleared, and stained with alizarin red S as described by Schnell and Newberne (15) before examination for skeletal defects. The remaining fetuses were preserved in Bouin fluid for subsequent detection of internal soft-tissue abnormalities by the method of freehand razor sectioning as described by Wilson (17).

Data for fetal body weights, implantations, resorptions, live fetuses, and percentage of malformed fetuses per litter in each treatment group were analyzed statistically by Kruskal-Wallis nonparametric analysis of variance and distribution-free multiple-comparison tests (6, 7). In all statistical tests, $P < 0.05$ was accepted as significant.

RESULTS

A single subcutaneous dose of OA (1.75 mg/kg) injected on gestation day 7 produced significant prenatal dysmorphogenesis in comparison with control animals. There was a significant increase in the percentage of embryonic implants resorbed. Approximately 72% of the implants were resorbed. Fetal body weights also were decreased significantly (Table 1).

Phe did not exert any protective effects with regard to OA-induced teratogenicity when coadministered with OA as a single subcutaneous dose on gestation day 7 at levels less

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TABLE 1. Effect of Phe in rats treated with OA (1.75 mg/kg) on gestation day 7

Treatment	No. of litters	No. of implants		Resorptions		Live fetuses		Avg fetal body weights (g)
		Total/ group	Average/ litter	Total/ group	% of implants	Total/ group	% of implants	
Group I (untreated)	7	84	12.0 ± 1.3[a]	5	5.4 ± 2.9[a]	79	94.6 ± 2.9[a]	3.73 ± 0.10[a]
Group II (solvent treated)	7	85	12.4 ± 1.9[a]	2	2.1 ± 1.3[a]	83	97.9 ± 1.3[a]	3.92 ± 0.08[a]
Group III (Phe [25 mg/kg])	6	78	13.0 ± 0.8[a]	6	7.2 ± 4.6[a]	72	92.6 ± 4.6[a]	4.00 ± 0.13[a]
Group IV (OA [1.75 mg/kg])	8	110	13.8 ± 0.4[a]	79	71.8 ± 12.2[b]	31	28.2 ± 12.2[b]	2.69 ± 0.15[b]
Group V (OA [1.75 mg/kg] + Phe [20 mg/kg])	9	113	12.6 ± 0.6[a]	59	52.2 ± 9.5[b]	54	47.8 ± 9.5[b]	2.97 ± 0.12[b]
Group VI (OA [1.75 mg/kg] + Phe [25 mg/kg])	8	107	13.4 ± 1.1[a]	49	46.0 ± 10.0[b]	58	54.0 ± 10.0[b]	2.66 ± 0.17[b]
Group VII (OA [1.75 mg/kg] + Phe [25 mg/kg] given daily for the first 10 days of gestation	6	53	8.8 ± 1.9[a]	26	58.3 ± 13.2[b]	27	41.7 ± 13.2[b]	3.16 ± 0.28[b]

[a,b] Percent resorptions and live fetuses are based on the number of implants. Average and percent values are given as mean ± standard error of the mean. Group means within a category which do not share the same superscript differ significantly from one another ($P < 0.05$); all such means which share the same superscript do not differ significantly ($P > 0.05$).

than 20 mg/kg (data not shown). However, when Phe was administered as a single dose of 20 mg/kg or as a single or daily dose of as much as 25 mg/kg, the effect of OA on embryos was diminished, but the decrease was not significant when compared with rats treated with OA alone (Table 1). Approximately 52.0, 46.0 and 58.0% of implants were resorbed in groups V, VI, and VII, respectively, versus 72% in the group treated with OA alone. There was no significant difference in fetal body weights between groups treated with OA and Phe and the group treated with OA alone.

OA (1.75 mg/kg) induced significant gross, visceral, and skeletal malformations (Table 2). The incidences of OA-induced fetal malformations (gross and skeletal) were decreased significantly in the presence of Phe when compared with rats treated with OA alone (Table 2). Rats treated with OA in combination with Phe (20 mg/kg) had 26% grossly malformed fetuses versus 61.3% in the group treated with OA alone. Likewise, rats treated with OA in combination with a daily dose of Phe (25 mg/kg) had only 42% of fetuses with skeletal defects versus 100% in the group treated with OA alone. The incidence of grossly malformed fetuses in groups VI and VII and skeletal defects in groups V and VI were decreased in the presence of Phe but were not statistically significant when compared with the group treated with OA alone. OA-induced soft tissue defects were not decreased in the presence of Phe.

A comparison of percentages of specific fetal abnormalities associated with prenatal exposure to OA with and without Phe is presented in Table 3. Although the types of OA-induced specific abnormalities remained similar in the presence of Phe, the incidences of most of these abnormalities, especially skeletal defects in group VII, were greatly reduced.

Fetal development in animals treated with a single dose of Phe (25 mg/kg) on gestation day 7, in the absence of OA, appeared like untreated and solvent-treated controls (Tables 2 and 3).

DISCUSSION

OA is a very potent foodborne teratogen. It recently has been demonstrated (14) that this mycotoxin results in significant prenatal dysmorphogenesis after subcutaneous administration on gestation day 7 with a dose of only 1.75 mg/kg in rats. Hence, this same treatment regimen was chosen to initially characterize the antagonistic potential (regarding OA-induced teratogenesis) of coadministered Phe.

OA has been reported to inhibit protein synthesis by competition with Phe in the phenylalanyl-tRNA synthetase-catalyzed reaction (2, 4). It also has been reported that a variety of toxic effects of OA can be prevented by Phe (3, 8, 11). Most notably, Creppy et al. (5) have reported that the simultaneous intraperitoneal administration of Phe in a single dose slightly higher than the lethal dose of OA counteracted the acute lethal effects of OA in mice. Our results

TABLE 2. Rat litters with malformations from maternal exposure to OA (1.75 mg/kg) in combination with Phe on day 7

Treatment	Malformations					
	External		Visceral		Skeletal	
	No. examined	% Malformed	No. examined	% Malformed	No. examined	% Malformed
Group I (untreated)	79	0.0[a]	52	0.0[a]	27	0.0[a]
Group II (solvent treated)	83	0.0[a]	53	0.0[a]	30	3.3[a]
Group III (Phe [25 mg/kg])	72	0.0[a]	43	0.0[a]	29	13.8[a]
Group IV (OA [1.75 mg/kg])	31	61.3[c]	20	65.0[b]	11	100.0[c]
Group V (OA [1.75 mg/kg] + Phe [20 mg/kg])	54	25.9[b]	34	70.6[b]	20	90.0[c]
Group VI (OA [1.75 mg/kg] + Phe [25 mg/kg])	58	48.3[c]	36	66.7[b]	22	90.9[c]
Group VII (OA [1.75 mg/kg] + Phe [25 mg/kg] given daily for the first 10 days of gestation)	27	37.0[b,c]	15	60.0[b]	12	41.7[b]

[a,b,c] Group means within a category which do not share the same superscript differ significantly from one another ($P < 0.05$); all such means which share the same superscript do not differ significantly ($P > 0.05$).

TABLE 3. Fetal malformations associated with prenatal exposure to OA (1.75 mg/kg) in combination with Phe on day 7

Malformation	% of examined fetuses in:			
	Group IV	Group V	Group VI	Group VII
External				
Anophthalmia	32.3	20.4	32.8	37.0
Hydrocephaly	54.8	16.7	32.8	25.9
Omphalocele	3.2	16.7	6.9	3.7
Ectopia cordis		9.3	3.4	3.7
Short snout	6.5	3.7	6.9	
Hematoma	3.2		1.7	
Micrognathia	3.2			
Visceral				
Hydrocephaly	65.0	70.6	55.6	46.7
Shift in position of esophagus	10.0	14.7	2.8	13.3
Skeletal				
Bipartite sternebrae	36.3	40.0	40.9	16.7
Sternebrae agenesis	72.7	50.0	45.5	8.3
Bipartite vertebral centra	36.6	45.0	22.7	25.3
Vertebral centra agenesis	18.2	35.0	27.2	
Incomplete ossification of skull bones	18.2	5.0	9.1	
Fused ribs	36.3	40.0	45.5	8.3
Missing ribs		15.0	31.8	
Extra ribs	63.6	30.0	45.5	
Wavy ribs	27.3			
Broken ribs	9.1	5.0	9.1	
Irregular arrangement of ribs	18.2	30.0	36.4	

indicated that Phe, when administered at a dose in excess (i.e., 11 to 14 times) of the minimum teratogenic dose of OA, provides partial prenatal protection from this potent mycotoxin. Moreover, multiple treatment with Phe appears to be more efficacious with regard to antagonism of teratogenesis than single-dose treatment and may indicate either a delay in the delivery of OA or of active metabolite to a fetal site of action or the necessity of threshold levels of Phe required to prevent the teratogenicity of OA, or both.

The teratogenic mechanism of OA appears to be more complex than the mechanism postulated for acute toxicity. Although previous studies have attempted to correlate OA toxicity with inhibition of Phe tRNA synthetase (8), our data indicate that a Phe-directed site of action may be only partially involved in the modulation of prenatal toxicity of OA. Perhaps the antidotal activity of Phe may be further enhanced via combination with other amino acids or congeners. Clearly, dietary manipulation may hold the key to the preventive toxicology of mycotoxins such as OA. Further studies are now warranted to ascertain the ability of Phe to prevent teratogenicity of OA via the diet.

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