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# Toxic Effects of Aflatoxin $B_1$ and Ochratoxin A, Alone and in Combination, on Chicken Embryos

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Aflatoxin $B_1$ (AFB), a member of a group of closely related, biologically active mycotoxins, is produced by strains of *Aspergillus flavus* and *A. parasiticus* and occurs naturally in several important animal feeds including corn, cottonseed, and peanuts. Aflatoxins damage the liver, kidney, and thymus producing a variety of effects including disruption of carbohydrate, protein, and lipid metabolism, immunosuppression, decreased growth rate and lowered productivity (Cheeke and Shull, 1985). Additionally, aflatoxins have been shown to be potent hepatocarcinogens, strong mutagens, and potential teratogens (Patterson, 1976). Ochratoxins, of which ochratoxin A (OA) is the most prevalent, are produced by species of *Aspergillus* and *Pencillium* and occur as natural contaminants of many feedstuffs including corn, barley, wheat, oats, and peanuts (Krogh, 1977). Toxic effects of OA include liver and kidney damage, growth retardation and reduced food consumption (Cheeke and Shull, 1985). Ochratoxin A has been shown to be teratogenic in mice (Hayes et al., 1974), rats (Brown et al., 1976), hamsters (Hood et al., 1976), and chick embryos (Gilani et al., 1978).

The Chick Embryotoxicity Screening Test (CHEST) bioassay is a rapid, inexpensive technique for testing the embryotoxic potential of numerous compounds including fungal metabolities. The CHEST bioassay has been used to examine the effects of mycotoxins including aflatoxin (Cilievici et al., 1980; Vesely et al., 1983; Dietert et al., 1985) and ochratoxin (Gilani et al., 1978; Harvey et al., 1987). Because AFB and OA can occur simultaneously in feedstuffs and the demonstrated impact of mycotoxin interactions (Huff et al., 1988; Kubena et al., 1988), the present study was designed to determine the effects of AFB and OA administered in combination, using the CHEST bioassay.

## MATERIALS AND METHODS

In two experiments, embryonating chicken eggs (Hy-Line W-36) were injected with AFB, OA, or a combination of the two mycotoxins on d 3 of incubation. Mycotoxins were dissolved in 25% ethanol and water and .1 mL of mycotoxin solution injected into a false air-cell created on the chorio-allantoic membrane. The holes were sealed with melted paraffin and eggs candled daily throughout the experimental period. Crystalline AFB was purchased from Sigma Chemical Company, St. Louis, MO. The OA was produced,

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extracted, and purified by methods previously described by Huff et al. (1974).

In the first experiment, 3-day embryonating eggs were randomly divided into eight groups and administered the following treatments (25 eggs/treatment): 1) Non-injected control; 2) 0 μg AF, 0 μg OA (sham-injected control; 3) .05 μg AFB (HIAF); 4) .025 μg AFB (LOAF); 5) 2.3 μg OA (HIOA); 6) 1.15 μg OA (LOOA); 7) .05 μg AFB + 2.3 μg OA (HIC); or 8) .025 μg AFB + 1.15 μg OA (LOC). Mortality was recorded through d 13 of incubation at which time eggs were opened and embryos examined. Visible abnormalities were noted and measurements of weight and length recorded.

In the second experiment, 320 eggs were randomly allotted to eight groups (40 eggs/group) and injected as described above. Mycotoxin treatments were identical to those used in the first experiment, with the following exceptions: HIOA = 2.0 μg OA; LOOA = 1.0 μg OA; HIC = .05 μg AFB + 2.0 μg OA; and LOC = .025 μg AFB + 1.0 μg OA. Eggs were candled daily and mortality and abnormalities recorded through d 18 of incubation, at which time eggs were transferred to a commercial hatcher. Unhatched eggs were examined and live chicks individually weighed and wing-banded. Chicks were placed into electrically heated batteries under continuous fluorescent lighting with a commercial chick starter ration and water available ad libitum. Pen feed consumption and individual body weights were recorded weekly for two weeks at which time the study was terminated.

Data were subjected to analysis of variance appropriate for a completely randomized design using the GLM procedure of SAS (SAS Institute Inc., 1988). Means were separated by the LSD test (Steel and Torrie, 1980).

## RESULTS AND DISCUSSION

Data from Experiment 1 are presented in Table 1. Injection of AFB and OA, either singly or in combination, resulted in greater ($P < .01$) embryo mortality when compared to sham- and non-injected eggs. Mortality, through d 13, was highest (80%; $P < .01$) in eggs injected with 2.3 μg OA (HIOA) compared to all other treatments. Eggs treated with HIAF and HIC had 52 and 56% mortality, respectively, while LOAF and LOC treatments both produced 32% mortality. Mortality for sham- and non-injected controls averaged 4%. Embryo weight decreased ($P < .01$) in eggs injected with HIAF (6.1 g) and HIC (6.5 g) compared with sham- (7.4 g) and non-injected (7.7 g) controls. Embryo length was similarly reduced ($P < .01$) in HIAF, HIC, and LOC treated embryos. Abnormalities observed included everted viscera, exencephaly, crossed beak, small head and eyes, and crooked limbs. The number of embryo abnormalities observed was greatest ($P < .01$) for the HIC treatment, with 36.4% of the embryos observed at d 13 expressing one or more abnormalities. The HIAF, LOOA, and LOC treatments likewise produced visible abnormalities (16.7, 16.7, and 10.5%, respectively), however, this was not significantly different from the controls. No abnormalities were observed in any control, LOAF, or HIOA treated embryos.

Embryonic mortality and abnormality data from Experiment 2 are presented in Table 2. Embryonic mortality (d 14-18) increased ($P = .02$) in the HIC treatment compared to control, AFB, and HIOA treated embryos. No differences in mortality were seen from d 3-13 ($P = .50$) or during hatching (d 19-23; $P = .12$). Total mortality (d 3 - hatch) was higher ($P < .01$) in HIOA (27.5%), LOC (27.5%), and HIC (37.5%) treatments compared

Table 1. Effect of aflatoxin $B_1$ and ochratoxin A, administered singly and in combination on d 3 of incubation, on embryonic mortality, abnormalities and embryo size at d 13 of incubation (Exp. 1)

| | Treatment[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | NI | CON | HIAF | LOAF | HIOA | LOOA | HIC | LOC | SEM[b] |
| Mortality, % | 4[g] | 4[g] | 52[de] | 32[ef] | 80[c] | 28[f] | 56[de] | 32[ef] | 3.40 |
| Abnormalities, % | 0[d] | 0[d] | 16.7[cd] | 0[d] | 0[d] | 16.7[cd] | 36.4[c] | 10.5[d] | 2.40 |
| Egg wt., g | 46.5 | 46.9 | 47.8 | 46.9 | 47.2 | 47.9 | 47.1 | 47.3 | .24 |
| Embryo wt., g | 7.7[de] | 7.4[de] | 6.1[f] | 7.3[de] | 8.1[c] | 7.6[cd] | 6.5[f] | 7.0[e] | .08 |
| Embryo length, mm | 55.6[c] | 54.8[cd] | 49.7[f] | 54.1[cd] | 55.4[c] | 53.2[d] | 49.6[f] | 52.2[e] | .30 |

[a]NI = not injected; CON = sham injected; HIAF = .05 μg $AFB_1$; LOAF = .025 μg $AFB_1$; HIOA = 2.3 μg ochratoxin A; LOOA = 1.15 μg ochratoxin A; HIC = .05 μg $AFB_1$ + 2.3 μg ochratoxin A; LOC = .025 μg $AFB_1$ + 1.15 μg ochratoxin A.
[b]Standard error of the mean.
[c-g] Means within a row with no common superscripts differ significantly ($P<.05$).

Table 2. Effect of aflatoxin $B_1$ and ochratoxin A, administered singly and in combination on d 3 of incubation, on embryonic mortality and abnormalities (Exp. 2)

| | Treatment[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | NI | CON | HIAF | LOAF | HIOA | LOOA | HIC | LOC | SEM[b] |
| Mortality, number (%) | | | | | | | | | |
| d 3 - 13 | 0/40 (0) | 2/38 (5.3) | 4/40 (10.0) | 3/40 (7.5) | 3/40 (7.5) | 1/40 (2.5) | 4/40 (10) | 4/40 (10) | 1.4 |
| d 14 - 18 | 0/40 (0)$^{d}$ | 0/36 (0)$^{d}$ | 1/36 (2.8)$^{d}$ | 1/37 (2.7)$^{d}$ | 3/37 (8.1)$^{cd}$ | 0/39 (0)$^{d}$ | 5/36 (13.9)$^{c}$ | 2/36 (5.6)$^{cd}$ | 4.0 |
| d 19 - hatch | 1/40 (2.5) | 2/36 (5.6) | 1/35 (2.9) | 2/36 (5.6) | 5/34 (14.7) | 4/39 (10.3) | 6/31 (19.4) | 5/34 (14.7) | 8.8 |
| Total | 1/40 (2.5)$^{e}$ | 4/38 (10.5)$^{de}$ | 6/40 (15.0)$^{d}$ | 6/40 (15.0)$^{d}$ | 11/40 (27.5)$^{c}$ | 5/40 (12.5)$^{de}$ | 15/40 (37.5)$^{c}$ | 11/40 (27.5)$^{c}$ | 2.2 |
| Abnormalities, % | 0$^{e}$ | 0$^{e}$ | 0$^{e}$ | 0$^{e}$ | 2.9$^{de}$ | 0$^{e}$ | 12.9$^{c}$ | 8.8$^{cd}$ | 1.0 |

[a] NI = not injected; CON = sham injected; HIAF = .05 μg $AFB_1$; LOAF = .025 μg $AFB_1$; HIOA = 2.0 μg ochratoxin A; LOOA = 1.0 μg ochratoxin A; HIC = .05 μg $AFB_1$ + 2.0 μg ochratoxin A; LOC = .025 μg $AFB_1$ + 1.0 μg ochratoxin A.

[b] Standard error of the mean.

[c-e] Means within a row with no common superscripts differ significantly ($P < .05$).

with non-injected (2.5%) and sham-injected controls (10.5%). Abnormal embryos were observed only in HIOA (2.9%), HIC (12.9%), and LOC (8.8%) treatments, with only the combination treatments differing significantly from the controls. No differences were seen in chick BW at hatch ($P$ = .83), at two weeks of age ($P$ = .76), or in BW change over the 14-d feeding period ($P$ = .76). Feed efficiency was not different ($P$ = .56) among treatments (data not shown).

Embryonic mortality increased following exposure to both AFB and OA, however administration of these mycotoxins in combination did not result in greater mortality compared to either mycotoxin administered alone. Embryonic mortality has been reported by others following exposure to AFB (Cilievici et al., 1980; Vesely et al., 1983; Dietert et al., 1985), OA (Choudhury and Carlson, 1973; Gilani et al., 1978; Harvey et al., 1987), and when OA and citrinin were administered in combination (Vesela et al., 1983). Differences in the lethal dose reported here and by others may be explained by the different routes of administration, embryo age at time of injection, and by type and volume of carrier solvent injected. Furthermore, differences in mortality seen in Experiment 1 vs Experiment 2 could also be related to variations in embryo sensitivities among batches of eggs and not dosing protocol as was reported by Prelusky et al. (1987).

The number of abnormalities observed was greater when both mycotoxins were administered in combination, particularly those embryos injected with the HIC treatment. However, the incidence of abnormalities was not consistent among treatments in the experiments. The HIAF and HIOA treatment groups produced abnormalities, but only in one experiment each. The reason no abnormalities were seen in the HIOA group in Experiment 1 may relate to the small number of embryos examined at d 13, due to the high mortality rate (80%). Some differences may also be explained by variations in chick sensitivity as mentioned earlier. Embryo weight and length were reduced by .05 μg AFB administered alone (HIAF) and in combination (HIC). However, if this same reduction in embryo size occurred in Experiment 2, compensatory growth might have occurred between d 14 and hatching, since no differences were observed in chick body weight at hatch. Growth retardation and similar abnormalities including twisted limbs, evisceration, exencephaly, and microphthalmia have been reported in embryos exposed to AFB (Cilievici et al., 1980) and OA (Gilani et al., 1978; Vesela et al., 1983). No differences in body weight change or gain/feed were observed. Dietert et al. (1985) also reported no significant differences in body weight up to 26 weeks of age following embryonic exposure to AFB.

In summary, AFB and OA produced embryonic mortality and abnormalities when administered to the embryonating chicken. However, only the number of abnormal embryos increased when these mycotoxins were administered in combination.

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