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Study of Possible Teratogenic Effects of the Fungicide Maneb on Chick Embryos

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The teratogenicity of the fungicide maneb (80% active ingredient and 20% inert ingredients) in chick embryos was evaluated. Eggs, before incubation, were dipped in 0.2% or 1.2% aqueous dispersions of maneb for 30 seconds. An untreated group, a water group and a formulation ingredient group, at the concentration present in the 1.2% maneb group, ensured control in the study. Eggs were incubated for 18 days then transfered to hatching racks and allowed to hatch normally. Viable chicks and chicks "dead in shell" were assessed for external deformaties. There was no evidence that maneb or its formulation ingredients were teratogenic or embryotoxic to developing chick embryos. © 1989 Academic Press, Inc.

Maneb, an ethylenebisdithiocarbamate (EBDC) fungicide, is widely used on a large number of major food crops. A number of authors have indicated the species variation in the teratogenic potential of maneb. Single oral doses of 1, 2, and 4 g/kg body wt were teratogenic to rats (Petrova-Vergieva and Ivanova-Tchemishanska, 1973). Larsson *et al.* (1976) confirmed this result but did not observe a teratogenic effect in mice. The dose levels used in these studies to induce a teratogenic effect are unlikely to be encountered in the environment.

Using concentrations of maneb (SIPCAM, Milan) ranging from 0.25 to 6.75 times the highest amount recommended, e.g., by BASF for use in the field, Maci and Arias (1987) immersed unincubated White Leghorn hen eggs into concentrations of 0.5 to 13.5 g/liter formulated maneb for 30 sec, identifying lower limb deformities on opening the eggs after 19 days of incubation. The questionable experimental design and procedures led to the conduction of the present study in an attempt to clarify the possible teratogenic effects of maneb on chick embryos.

MATERIALS AND METHODS

A total of 2650 White Leghorn hen eggs[1] were obtained from the same supplier. All eggs had been laid on the same day. The eggs were transported to the laboratory on the day they were laid in an upright position on cardboard trays within a ventilated container. The eggs were stored at a temperature of 20°C for 3 days until the beginning of the study.

A total of 2500 eggs was selected for the study with extremely large and small eggs and those with cracks or shell abnormalities discarded. Eggs were weighed before

[1] Supplied by Lohmann Selected Leghorn (= LSL), Rhein-Main, Geflügelvermehrungsbetriebe GmbH, D-6110 Dieburg/Hessen, FRG.

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treatment in batches of 100. Group mean egg weights were appropriate for hatching eggs, being between 55.1 and 55.3 g.

Maneb (about 80% active ingredient, 20% formulation ingredients) was dispersed in tap water immediately before the eggs were dipped, to yield concentrations of 0.2 and 1.2% maneb (highest concentration as recommended by BASF for use in the field and six times higher). A group of eggs was treated with tap water and a further group was treated with formulation ingredients at a concentration corresponding to the highest maneb concentration. In addition, a group of eggs remained untreated. The temperature of the tap water and the mixtures was adjusted to 20°C.

Eggs were immersed in test liquids for 30 sec on Day 0 of incubation. During the dipping procedure the baskets containing the eggs were moved slowly up and down about six times, ensuring that the eggs were always covered with the appropriate liquid. After dipping, the liquid was allowed to run off for 1 min. The eggs were then placed on filter paper at room temperature (20°C). After 60 to 90 min when the surface of the eggs was dry, they were rolled over the egg equator on dry filter paper and then randomly distributed between groups into three commercial incubators (Type KMB6/V, Ehret, D-7830 Emmendingen 14, FRG). Temperature and relative humidity (%) were maintained within the range 37.3 to 40.1°C (mean 37.8°C) and 60 to 70%. On Day 18 the relative humidity was increased as much as possible and reached 100%. Within the incubators, all eggs were turned every 90 min through an angle of about 180°. Eggs were incubated for 18 days and then transferred to hatching racks equipped with wire mesh floors. Candling with a commercial lamp was performed on Day 8 to check for fertility and early embryonic death and on Day 16 for late embryonic death. One day after hatching the chicks were removed from the incubators and the numbers of healthy chicks, abnormalities, and "dead-in-shell" were recorded. Living chicks were checked for external abnormalities and their gait was observed on corrugated board. In addition, their ability to maintain equilibrium was checked by their being dropped 10 to 20 cm onto corrugated board. The significance of differences between groups was assessed using Fisher's exact test.

RESULTS

The results of candling on Days 8 and 16 are summarized in Table 1. In all groups the number of fertile eggs was high and ranged from 96.2 to 98%. The incidence of early embryonic death appeared to be slightly higher as a result of dipping. Among the untreated controls 2.2% early embryonic death occurred compared with a range 3.5 to 6.3% of fertile eggs in the dipped groups. The incidence was slightly higher in the two maneb-treated groups although the increase was not concentration dependent. The difference was significant ($P < 0.01$) only when compared with the untreated control group and not with the dipped control group. There were no treatment- or compound-related effects on late embryonic deaths where the incidences ranged from 6.3 to 13.5% of fertile eggs with the lowest incidence in the two maneb-treated groups. No compound- or concentration-dependent effect on total embryonic deaths was demonstrated. The highest incidence occurred in the water control group which attained a level of significance ($P < 0.05$) when compared with the untreated control group.

The total incidence of "dead-in-shell" was higher in all dipped groups and ranged from 4.3 to 5.8% of fertile eggs compared with 2.3% in the untreated control group

TABLE 1

RESULTS OF CANDLING ON DAYS 8 AND 16

Group:	0 Untreated control	1 Water control	2 Formulation ingredients	3 0.2% Maneb	4 1.2% Maneb
Eggs set	500	500	500	500	500
Fertile eggs	490	481	485	490	484
% Of eggs set	98	96.2	97	98	96.8
Early embryonic deaths	11	19	17	31	26
% Of fertile eggs	2.2	4.0	3.5	6.3**	5.4**
Late embryonic deaths	50	65	61	32	44
% Of fertile eggs	10.2	13.5	12.6	6.3	9.1
Total embryonic deaths	61	84	78	63	70
% Of fertile eggs	12.5	17.5*	15.9	12.9	14.5
Living embryos	429	397	407	427	414

Note. Group 1 versus groups 2, 3, and 4, not significant.
* $P < 0.05$ compared with value for untreated control group.
** $P < 0.01$ compared with value for untreated control group.

(Table 2). Among dipped groups the highest incidence was in the water control group. The number of deaths in the hatcher was minimal with zero to two deaths recorded in any group.

The slightly higher number of total embryonic deaths and "dead-in-shell" in the dipped groups resulted in a slightly higher rate of hatched chicks of fertile eggs amounting to 85.3% in the untreated control compared with the dipped groups with rates from 76.7 to 82.3% (Table 2). The number of living hatched chicks in fertile eggs was significantly lower ($P < 0.01$) in the water control group and in the formulation ingredient and 1.2% maneb groups ($P < 0.05$) than in the untreated control group.

The total number of "dead-in-shell" with externally visible abnormalities was higher in all dipped groups compared with the untreated control group (Table 3). The total incidence observed in the group treated with 1.2% Maneb attained a level of significance ($P < 0.05$) when compared with either the untreated or the water controls. However, the different types of abnormalities observed were similarly distributed among all groups including the controls.

The incidence of chicks with externally visible abnormalities among hatched living chicks was very low with a range 1.44 to 2.33% between the groups (Table 4). There was no indication of a compound-related effect. The maneb-treated groups had an incidence of abnormalities similar to those of the untreated and water control groups. The abnormalities observed in the study were mostly deformations of the toes. Two cases of a closed cloaca and one case of an external cyst were detected and in one case an open skull was seen. The incidences recorded in this study accord with the reports of the breeder of the strain, whereby deformation of the toes, closed cloaca, and unilateral anophthalmia account for a total of less than 1% of the population.

TABLE 2

INCIDENCE OF "DEAD IN SHELL" AND HATCH RATE

Group:	0 Untreated control	1 Water control	2 Formulation ingredients	3 0.2% Maneb	4 1.2% Maneb
Dead in pipped shell (TIS)[a]	6	10	4	12	6
Dead in undamaged shell (TIIS)[b]	5	18	17	12	19
Total "dead in shell" as % of fertile eggs	2.3	5.8**	4.3*	4.9*	5.2*
Dead in hatcher	2	2	1	1	0
No. of hatched	418	369**	386*	403	389*
% Hatched living chicks of No. of fertile eggs	85.3	76.7	79.6	82.3	80.4

Note. Group 1 versus groups 2, 3, and 4, not significant.

[a] TIS, chicks viable and had pipped the shell but failed to liberate themselves.

[b] TIIS, chicks considered to be fully formed; no evidence of pipped shell.

* $P < 0.05$ compared with value for untreated control group.

** $P < 0.01$ compared with value for untreated control group.

Table 5 summarizes the total number of abnormalities found in the study and demonstrates the low incidence of abnormalities resulting from the different treatments. Comparison between the dipped groups and the untreated control group confirms that dipping alone increased the number of chicks with abnormalities. No significant difference was found in a comparison of the maneb-treated groups with the water control group. The small increase in abnormalities in the maneb-treated groups is not considered to be of sufficient magnitude to be of biological relevance; the differences were not statistically significant.

DISCUSSION

A single 30-sec dipping treatment of unincubated eggs into aqueous solutions of maneb or its formulation ingredients was not teratogenic nor did the treatment have any effect on the incidences of embryonic deaths, on chicks "dead-in-shell," or on the hatch rate. These results conflict with those of Maci and Arias (1987) who reported unilateral lower limb deformities in White Leghorn hen embryos following dipping of unincubated eggs in 0.5 to 13.5 g/liter maneb in aqueous solution for 30 s.

Maci and Arias treated eggs in group sizes varying between 157 and 207 and achieved viable embryos in 73.6 to 82.9% of the groups. This compares with a group range in the present study of 82.5 to 87.6%. No information was provided on the origin, storage, or handling of the eggs before incubation. It is generally recognized that unfavorable incubation conditions, caused by a variety of different factors, e.g., unfavorable humidity conditions, may increase the incidence of malformed embryos.

TABLE 3

ABNORMALITIES IN CHICKS "DEAD-IN-SHELL"[a]

No. of chicks with	0 Untreated control	1 Water control	2 Formulation ingredients	3 0.2% Maneb	4 1.2% Maneb
Unilateral lower limb defects	0	0	0	1	0
Bilateral lower limb defects	0	1	0	0	0
Unilateral defects on toes	3	3	0	1	2
Bilateral defects on toes	0	1	0	0	0
Beak malformations	1	3	2	7	5
Tongue missing	0	0	0	0	1
Exencephaly (open skull with open brain)	1	3	0	3	3
Anophthalmia or microphthalmia	0	1	2	3	4
Open coeloma or hernia abdominalis	1	0	1	2	2
Distended abdomen	0	3	5	9	4
External edemas	1	3	0	3	2
Extremities (wings and legs) double	0	0	0	0	1
Double headed	1	0	0	1	0
Total number of chicks "dead-in-shell" with abnormalities	4	13	7	16	19*
Total number of "dead-in-shell"	11	28	21	24	25
% "Dead-in-shell" with abnormalities of total "dead-in-shell"	36%	46%	33%	67%	76%

[a] More than one abnormality can occur in one chick.
* $P < 0.05$ compared with value for untreated control group or water control.

The chief external abnormalities, as an index of incubation conditions, "consist in a marked shortening of the extremities and frequent deformities of the skull" (=chondrodystrophy) (Landauer, 1951). Furthermore several authors found that there is a definite seasonal change in incidence of chondrodystrophic embryos, the frequency decreasing with advancing spring, and "available evidence points to nutritional deficiency as the cause of sporadic chondrodystrophy of chicken embryos" (Landauer, 1951). In addition, the absence of a nondipped control group was a failing in the study design as it is known that humidity on the eggshell promotes the penetration of bacteria into the egg (Scriba, 1985).

For the present study it was decided to allow the chicks to emerge from their shells naturally, in order to avoid misinterpretation in the assessment of lower limb deformities in embryos which are pressed in a compact form within the egg. This may have been a major source of error in the study of Maci and Arias who encountered "lower limbs that were contracted and often shortened."

TABLE 4

NUMBER OF HATCHED LIVING CHICKS WITH ABNORMALITIES

Kind of abnormalities	0 Untreated control	1 Water control	2 Formulation ingredients	3 0.2% Maneb	4 1.2% Maneb
Outer toe (left) bent inward	1				1
Outer toe (right) bent inward			1	1	
Outer toes (left and right) bent inward	1	3	2		1
Outer and middle toe (right) bent inward	2	1	1	1	2
Outer and middle toe (left and right) bent inward			2		
Inner toe (right) bent inward	1				
Middle toe (right) bent inward			1		
Three toes (left and right) bent inward		1	2	1	
Three toes (left) bent downward				1	
Three toes (left and right) bent downward					2
Open skull				1	
Cyst filled with reddish fluid		1			
Closed cloaca	1			1	
Total No. of living chicks with abnormalities	6	6	9	6	6
% of living hatched chicks	1.44%	1.63%	2.33%	1.49%	1.54%

The highest incidence of bilateral limb deformities reported by Maci and Arias was in their water control group and inert ingredient group, 6.3 and 4.7%, respectively, of viable embryos. Similarly, in the present study the highest comparable incidences (of "dead-in-shell" and hatched chicks) occurred in the water control and formulation ingredients groups with five and six chicks, respectively, affected in each group, an incidence of about 1% of the viable embryos and similar to the incidence reported by the breeder of the strain.

In the present study the incidence of living chicks with abnormalities was low in all test groups and ranged from 1.4 to 2.3% with no indication of a treatment or concentration effect. These findings conflict with those of Maci and Arias who found a significant concentration-dependent effect with up to 70% of viable embryos with abnormalities, of which 65.8% of viable embryos showed unilateral limb defects at

TABLE 5

TOTAL NUMBER OF ABNORMALITIES

	0 Untreated control	1 Water control	2 Formulation ingredients	3 0.2% Maneb	4 1.2% Maneb
Total living chicks with abnormalities plus total "dead-in-shell" with abnormalities	10	19*	16	22*	25**
Living chicks and embryos with abnormalities as % of fertile eggs	2.0	4.0	3.3	4.5*	5.2**
Living chicks and embryos with abnormalities as % of living embryos	2.3	4.8*	3.9	5.2*	6.0**

Note. Group 1 versus groups 2, 3, and 4, not significant.
* $P < 0.05$ compared with value for untreated control group.
** $P < 0.01$ compared with value for untreated control group.

the highest concentration of maneb (13.5 g/liter). This apparent asymmetric response to maneb is difficult to understand as few xenobiotics preferentially affect only one limb; among those recently reviewed by Scott (1985) neither maneb nor any dithiocarbamate derivative was included.

CONCLUSION

Under the conditions of this study there was no evidence that maneb or its formulation ingredients were teratogenic or embryotoxic to developing chick embryos.

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