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A Post-Implantational Study on the Effects of Zineb on Reproduction Using the Decidualized Pseudopregnant Rat as a Model

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Zineb is an important fungicide widely used in agriculture. After an application, zineb residues may persist up to one month in the field (VILLA et al. 1976). It was calculated that 15 ppm of zineb was left in the whole cabbage 7 days after an initial treatment of 110 ppm (RIPLEY 1979). Zineb has a low order of acute toxicity. The oral LD_{50} to white rats is 5200 mg/kg (FREAR 1961). Ethylene-thiourea has been reported as an in vivo metabolite of zineb in mice (THRUHAUT et al. 1973). In a reproductive study, the size of the uteri of virgin female rats treated with a high dose (1/5 of LD_{502}) of zineb was reduced. These rats, however, were fertile (GHIZELEA and OZERAN 1973). Zineb in single oral doses, 2-8 g/kg, given to pregnant rats on day 11 or 13 of organogenesis, induced congenital anomalies in 12-100% of the fetuses (PETROVA-VERGIEVA 1973). In male rats, doses of 30 mg/kg for five months caused a reduction in dehydrogenase and amino-transferase activities, and a concomitant decrease in fructose contents of accessory sex glands (ORLOVA et al. 1971). DINERMAN et al. (1970) concluded that the maternal rat was more affected than the fetus. The mechanism on which zineb interacts with the reproduction system during pregnancy has not been studied. This study was conducted with this idea in mind. Because pseudopregnant and pregnant rats have an identical mechanism for controlling the onset and loss of uterine sensitivity (CARLSON and DE FEO 1965), the deciduomal response in the decidualized pseudopregnant rats could be used to study possible reproductive disruption as caused by zineb in pregnant uterus.

METHODS AND MATERIALS

Animals and maintenance Albino rats (Sprague-Dawley strain) from Holtzman Co., Wisconsin were housed individually in wire mesh cages in a windowless room (temperature: 20 $\pm$ 2°C; photoperiod: 14 h light and 10 h darkness). The animals were maintained on Purina Lab Chow and tap water ad

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libitum. The rats were allowed to become acclimated to the environment for 1-2 weeks before use. Stages of the estrous cycle of the rat were determined microscopically by vaginal smearing. Rats (200g-300g) which exhibited two consecutive 4-5 day estrous cycles were selected for the experiment.

Pregnancy studies Pregnancy was attained by housing a female rat in the estrus stage with a fertile male. The day on which sperm appeared in the vaginal smear was designated as Day 1 of gestation. The number of implantation sites was counted on Day 6 of pregnancy, following a tail vein injection of Chicago blue dye (LABHSETWAR 1971). The number of developing embryos was counted on Day 12 of pregnancy during laparotomy. The percentage of resorption in implantation sites (I.S.) was calculated by the formula:

$$\frac{\text{Number of I.S. at Day 6} - \text{Number of I.S. at Day 12}}{\text{Number of I.S. at Day 6}}$$

On the day of parturition, the number of dead and alive fetuses was recorded. The fetal survival index of the fetuses was calculated by the formula given by PEPE and ROTHCHILD (1973). The litters were weighed at 0-36 h upon delivery.

Biochemical studies Pregnant rats were sacrificed on Day 16 of pregnancy by cervical dislocation under light ether anesthesia. The ovaries from both uterine horns were extracted over ice for protein assay. The placenta from a living fetus was used for protein and glycogen determinations.

Decidualized pseudopregnant studies Pseudopregnancy was induced by stimulating the uterine cervix of the rat with the introduction of a vibrating fiber glass rod during the proestrus and estrus stages of the rat (DE FEO 1966). The electrical vibrator device was designed by the authors following the guidelines as set by DE FEO (1966). Day 1 of pseudopregnancy was designated as the first day when leukocytes appeared predominantly in the vaginal smear. The decidual cell reaction (DCR) which involved the proliferation of the uterine deciduoma of the maternal placental component, was induced by bilateral uterine traumatization (ZARROW et al. 1964) at Day 4 of pseudopregnancy. On Day 11 of pseudopregnancy, animals were sacrificed. Ovaries were removed quickly for protein determination. Glycogen and protein determinations were conducted on the uterine horns. For water content determination, which is the difference between wet and dry weights, one uterine horn was dried in an oven at 80-100°C for 48 h.

Feeding Zineb (purity 99.9%, obtained from the EPA) was mixed in a pulverized lab chow to form a desired dietary concentration. Rats were given free access to the diet during the test. Food consumption rates, and body weights were monitored for each individual. Feeding of zineb was conducted from days 6-15 of pregnancy and days 6-9 of pseudopregnancy. These feeding periods coincided with the period of elevated plasma titers of progesterone during pregnancy (PEPE and ROTHCHILD 1974) and the period of deciduomal growth during pseudopregnancy (ZARROW et al. 1964).

Protein determination Protein analysis was based on the method of LOWRY et al. (1951) using bovine serum albumin as the standard. Fresh tissue sample (0.20-0.25 g) was grinded in a chilled Potter Elvehjem homogenizer with glass distilled water to form a 2.0% homogenate (g/ml). Following an incubation period at 37°C for 15 minutes, the homogenate was centrifuged at 15,000xg at 2°C for 20 minutes. Absorbance values were read on a Beckman (Model 24) spectrophotometer at 660 mμ.

Glycogen determination Glycogen analysis was based on the method of SEIFTER et al. (1950), modified by ZARROW et al. (1964), using Type III rabbit liver glycogen as the standard. Absorbance values were read on a Bausch and Lomb Spectronic 20 colorimeter set at 620 mμ.

Statistic analysis Analysis was accomplished by one-way analysis of variance and the Student's t-test. Differences among the experimental groups were tested for statistical significance using Duncan multiple range test (DUNCAN 1955). A probability value of less than .05 is considered to be significant.

RESULTS AND DISCUSSION

In Table 1, at the levels of zineb tested, the body weight of decidualized pseudopregnant rats was maintained. The intrauterine environment, in terms of uterine weight, uterine water, protein and glycogen contents, was not affected. In comparison with the control, pseudopregnant ovarian weight was increased. This increase was not dosage dependent (Table 2). Ovarian protein content was not affected. In biochemical studies of pregnant rats exposed to zineb, placental enzymatic activities as reflected on placental protein content (Table 3) were not affected. In comparison with the control, placental glycogen content was increased. This increase was not dosage dependent. The differences may be attributed to mere random changes (WEIL and CARPENTER 1969). Pregnant ovarian weight was not affected (Table 2). In comparison with the control, ovarian protein

TABLE 1

UTERINE WEIGHT, WATER, PROTEIN AND GLYCOGEN CONTENTS OF DAY 10 DECIDUALIZED PSEUDOPREGNANT RATS GIVEN ZINEB

Diet[a] PPM	Daily intake[b] of zineb (mg/day)	Body weight[b] of rats (g)	Uterine[b] weight (g)	Uterine[b,c] water content (%)	Uterine[b] protein (100μg/100mg uterus)	Uterine[b] glycogen (mg/100g uterus)
control	---	241±11	3.8±0.3	81.0±0.7	45.6±3.6	111±21
500	10.1±0.4	258± 9	3.2±0.2	80.3±0.6	41.4±4.4	89±17
1000	21.6±0.8	271± 7	3.4±0.4	80.7±0.8	37.7±5.1	109±39
1500	31.1±0.6	249±11	3.6±0.3	79.8±0.3	39.6±4.5	100±28
2000	42.9±1.1	270± 4	3.2±0.4	79.6±0.5	34.0±2.4	99±10
2500	50.4±1.4	258± 3	3.3±0.1	79.9±0.2	44.3±2.1	89± 4
		NS[d]	NS[d]	NS[d]	NS[d]	NS[d]

[a] Rats were given a diet of respective concentrations from Days 6 through 9 of decidualized pseudopregnancy.

[b] Mean±SE, for results of 5 rats.

[c] Percentage (%)= $\frac{\text{wet weight (g) - dry weight (g)}}{\text{wet weight (g)}} \times 100$

[d] Probability of treatment (control vs. exposed groups), using analysis of variance.

TABLE 2

OVARIAN WEIGHT AND OVARIAN PROTEIN CONTENT ON DAY 10 OF DECIDUALIZED PSEUDOPREGNANT RATS AND ON DAY 16 of PREGNANT RATS GIVEN ZINEB

Diet[a] PPM	Pseudopregnant rats		Pregnant rats	
	Ovarian[b] weight (g)	Ovarian[b] protein (100µg/100mg uterus)	Ovarian[b] weight (g)	Ovarian[b] protein (100µg/100mg uterus)
control	0.07±.01	50.3±8.0	.16±.02	39.1±5.0
500	0.11±.00	48.4±2.6	.16±.01	40.5±3.7
1000	0.10±.00	51.1±5.3	.11±.02	34.6±4.0
1500	0.09±.01	52.6±8.8	.15±.01	39.3±5.6
2000	0.10±.00	50.6±5.7	.15±.02	36.4±4.5
2500	0.10±.00	47.1±7.8	.13±.00	55.3±1.9
	$p < 0.05$[d]	NS[d]	NS[d]	$p < 0.05$[d]

[c] Values joined by the line represent a cohort of values which do not differ significantly from each other, using Duncan's test.

[a] Rats were given a diet of respective concentrations from Days 6 through 9 of decidualized pseudopregnancy, or from Days 6 through 15 of pregnancy.

[b] Mean±SE, for results of 5 decidualized pseudopregnant rats and for results of 6 pregnant rats.

[d] Probability of treatment effect (control vs. exposed groups), using analysis of variance.

content was increased at the highest level (2500 ppm) tested. In Table 4, pregnant rats exposed to zineb for 10 days did not show a dosage dependent decrease in body weight. Development of implantation sites in pregnant rats after 7 days of exposure to zineb was not affected. The fetal survival rate of the fetuses from dams exposed to zineb for 10 days was not changed. Fetal weight was reduced (Table 4). This decrease was not considered to be compound-related since it was not dose related. In the present studies on zineb, neither embryotoxicity of the fetus, nor biochemical lesions in the maternal reproductive system was observed. The test levels used were believed to be considerably greater than those which ordinarily might be ingested by the rodents in the field. The results

TABLE 3

PLACENTAL PROTEIN AND GLYCOGEN CONTENTS ON DAY 16 OF PREGNANT RATS GIVEN ZINEB

Diet[a] PPM	Daily[b] intake of zineb (mg/day)	Maternal[b] body weight (g)	Placental[b] protein (100µg/100mg tissue)	Placental[b] glycogen (mg/100g tissue)	
cont	---	297±16	52.2±4.9	479±22	
500	11.8±0.6	294± 9	49.7±3.5	588±27	c
1000	21.1±1.3	269±11	53.8±2.8	554±94	
1500	30.3±2.4	298± 8	59.1±3.0	528±81	
2000	38.6±4.9	285± 5	51.7±2.1	573±73	
2500	54.8±0.3	285± 2	56.3±1.6	654±12	
		NS[d]	NS[d]	$p < 0.05$[d]	

[a]Rats were given a diet of respective concentrations from Days 6 through 15 of pregnancy.

[b]Mean±SE, for results of 6 rats.

[c]Values connected with the same line represent a cohort of values which do not differ from each other, using Duncan's test.

[d]Probability of treatment effect (control vs. exposed groups), using analysis of variance.

of the current studies, together with those of other investigations, indicate that zineb has low toxic effects on the fetus and maternal reproduction system. This may be attributed to the fact that zineb does not accumulate to a large extent in mammalian tissues (PICCO 1962; IVANOVA-CHEMESHANSKA 1971), and could be metabolized readily (THRUHAUT et al. 1973).

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TABLE 4

THE EFFECT OF ZINEB ON THE OUTCOME OF PREGNANCY

Diet[a] PPM	Daily intake of zineb (mg/day)	Number of litter	Implantations at Day 6 per dam $\bar{x} \pm SE$	Number of conceptuses at Day 12 $\bar{x} \pm SE$	Number of fetuses at birth Dead	Alive	Percentage of fetal survival rate per litter $\bar{x} \pm SE$	Fetal weight (g) $\bar{x} \pm SE$	Maternal[b] weight gain $\bar{x} \pm SE$
cont	---	6	13± 0.5	13± 0.5	33	45	57.2± 8.8	7.2±0.3 [c]	70±6
500	10.0±0.5	6	13± 0.4	13± 0.4	33	44	58.7±12.2	7.3±0.2	56±3 [c]
1000	19.8±1.1	6	10± 0.6	10± 0.6	28	34	54.3± 6.8	7.2±0.3	53±3
1500	31.5±0.5	6	11± 0.6	11± 0.6	23	40	63.2±12.3	6.7±0.3	50±2
2000	41.5±0.6	6	10± 0.3	10± 0.3	29	29	49.0± 9.2	7.1±0.1	51±6
2500	51.7±1.2	6	12± 0.3	12± 0.3	38	31	46.4± 2.9	6.9±0.1	45±2
							NS[d]	$p < 0.05$[d]	$p < 0.05$[d]

[a] Rats were given a diet of respective concentrations from Days 6 through 15 of pregnancy.

[b] Maternal weight gain=maternal weight after delivery (g)-maternal weight at Day 6 of pregnancy (g).

[c] Values joined by the same line represent a cohort of values which do not differ significantly from each other, using Duncan's test.

[d] Probability of treatment effect (control vs. exposed groups), using analysis of variance.

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