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Teratogenic Effects in Rat Fetuses Subjected to the Concurrent *in utero* Exposure to Emamectin Benzoate Insecticide

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ABSTRACT

Exposure to insecticides is most considerable due to their untoward effects on the production and reproduction in human and animals. The aim of our study was to assess the teratogenic potential of Emamectin benzoate (EMB) insecticide in rats. Pregnant rats were separated into four equal groups; the first one kept as a control group. The 2nd, 3rd and 4th groups were orally administered EMB on Gestation Day (GD) 6 through GD 15 at doses of 4.4, 8.8 and 17.6 mg kg⁻¹ day⁻¹, respectively. All pregnant rats were exposed to caesarean section on GD 21 and their fetuses were examined for morphological, visceral and skeletal abnormalities. Decreased maternal weight gain, fetal and placental weight and number of viable fetuses and elevated rate of fetal resorptions and post-implantation deaths were recorded in groups exposed to EMB at 8.8 and 17.6 mg kg⁻¹. The percentage of morphological, visceral and skeletal abnormalities were significantly increased in the fetuses of dams of EMB-exposed rats at 8.8 and 17.6 mg kg⁻¹. The retardation in growth of viable fetuses, hydrocephaly, an ophthalmia, lung hypoplasia, incomplete ossification of cranial bones, aplasia of metacarpals, metatarsals, phalanges and caudal vertebrae were the important fetal anomalies. The present study concluded that EMB is teratogenic when given orally to pregnant rats.

Key words: Avermectins, pregnancy, placenta, organogenesis, congenital malformation

INTRODUCTION

Pesticides are used in large amounts in agriculture and the evaluation of their adverse effects is of great interest to public and environmental health (Goldoni *et al.*, 2014). Insecticides

are one of the pesticides that act to kill or repel insects. They are used in agricultural and public health, as well as household purposes. Insecticides can be applied in diverse forms including sprays, dusts and baits. About 40,000 t of insecticides are annually utilized in field crops (Hamid *et al.*,

2012). Current application of insecticides may influence environment including humans and animal's health through soil, water and vegetable contamination (Adjrah *et al.*, 2013). Insecticides are characterized by varying degrees of adverse effects. The genotoxic potential is the major risk factor for long-term effects, such as carcinogenic and degenerative diseases (Bolognesi *et al.*, 2011). At the same time, insecticides are considered as potent pollutants of the environment that have been involved in birth defects and reproductive failure. The adverse health effects correlated with exposure to insecticides during pregnancy have become a considerable public health interest due to the widespread use of insecticides and the rising instability of the fetus and the pregnant mother to toxic exposures (Stillerman *et al.*, 2008).

Emamectin benzoate (EMB) is the 4'-deoxy-4'-epi-methyl-amino benzoate salt of the naturally occurring avermectins (Yen and Lin, 2004). Avermectins are used against external and internal parasites of humans and livestock, including parasitic nematodes causing the human diseases onchocerciasis and lymphatic filariasis (Geary, 2005). The EMB acts against various lepidopteran pests and is being developed for use on major field crops and vegetables (Parsaeyan *et al.*, 2013). The selective toxicity of avermectins against ecdysozoan invertebrates is believed to be based on the binding and blockage of glutamate-gated and γ -aminobutyric acid-gated chloride channels in the invertebrate nervous system (McCavera *et al.*, 2007). The EMB is incompletely absorbed in the gastrointestinal tract of mammals. Oral absorption was estimated at 55% (EFSA., 2012). Contamination of human food by insecticides mostly occurs to farmers and agriculture workers (Litchfield, 2005). Studies suggest an association between environmental exposure of pregnant women to certain agricultural pesticides and malformation in their fetuses (Peiris-John and Wickremasinghe, 2008). The most susceptible period for fetal anomalies to take place is the period of organogenesis (from 6-15 days of gestation). Drugs and chemicals given during this period are more likely to cause fetal defects (Somers, 1962). The objective of this study is to explore the teratogenic potential of EMB in rats.

MATERIALS AND METHODS

Chemicals: The tested insecticide was emamectin benzoate (HEPICLAIM® 5.7% WDG, Zhejiang haben Pesticide and Chemicals Co. Ltd, China). The solutions were prepared by dissolving the insecticide in distilled water in such a way that 1.0 mL of solution contain desired concentration.

Animals: Healthy adult female and male Wistar rats (210-225 g) bred in the laboratory animal unit of our own department were used. All animals were housed in polyethylene (65×25×15 cm) cages, with sawdust-covered floors. Rats were maintained at 22±1°C under conditions of

controlled humidity and on a 12 h light/dark cycle (lights on from 09:00-21:00 h) with *ad libitum* access to food and water.

Estimation of LD₅₀: Five doses were given orally to five groups of rats (n = 5) for the determination of LD₅₀ of EMB starting from 0% mortality to 100% mortality. The animals were observed for 24 h for any toxic signs. After 24 h, the numbers of deceased animals in each group were counted and LD₅₀ was determined according to the method of Miller and Tainter (1944).

Teratogenic effect

Mating: Male and female rats were housed for mating in the ratio of 2:1 during the dark period (between 21:00 and 09:00 h). On the next morning (09:00 h), vaginal smears were obtained from female rats and inspected. The existence of spermatozoa in the vaginal smear was considered as GD 0 (Paumgarten *et al.*, 1998). Delivery in normal rat takes place on days 21-23 of gestation (Rough, 1968).

Treatment: After mating and ensuring successful conception, 40 pregnant rats were distributed into four groups (n = 10). The first group served as control and received the vehicle (distilled water). The 2nd, 3rd and 4th groups were orally received EMB at doses of 4.4, 8.8 or 17.6 mg kg⁻¹, respectively. The EMB doses (4.4, 8.8 and 17.6 mg kg⁻¹) were chosen in the present work according to its calculated LD₅₀ (1/20th, 1/10th and 1/5th of LD₅₀, respectively). The vehicle and the insecticide were administered orally by gavage in a volume of 5 mL kg⁻¹ once daily on GD 6 through GD 15. This period is considered as the critical period for the structural development span of the embryonic stage for rats. Body weights of dams were recorded every 3 days beginning on GD 0 through GD 21.

Morphological examination of the uteri and fetuses: At GD 21, the dams were weighed, sacrificed and the laparotomy process was performed with exposure of the uterine horns. The uteri from each female were removed, opened using a scissor and then the fetuses were separated. Numbers of implantation sites (using a magnifying lens) and viable, dead and resorbed fetuses were calculated. The percentages of post-implantation loss were calculated:

$$\text{Post-implantation loss} = \frac{\text{No. of implantation sites} - \text{No of living fetuses}}{\text{No. of implantation sites}} \times 100$$

The live fetuses were dried on a blotting paper, weighed, measured (crown-rump length) and examined for external morphological abnormalities. Moreover, the placental weights were recorded.

Visceral examination of fetuses: Two third of the fetuses from each dam were kept in Bouin solution for 1-2 weeks.

Then, the fetuses were rinsed with cold water and several cross sections were made throughout the fetal body. Fetal sections were grossly examined under a dissecting microscope for any visceral malformations (Hayes, 1986).

Skeletal examination of fetuses: The remaining fetuses were preserved in 95% ethanol solution for 7 days for dehydration. The abdominal wall of each dehydrated fetus was opened and the internal organs were removed. The eviscerated fetuses were placed in 2% potassium hydroxide solution for 24-35 h according to the size of the fetus till complete digestion and clearance of the muscles. After clearing, the fetuses were immersed in Mallsch's solution with alizarin red stain for 24 h as described by Taylor (1986). The stained fetuses were kept in Mallsch's solution alone for 2 days, then rinsed with water and preserved by successive passage in graded concentrations of glycerin watery solution (70, 80, 90 and 100%). Different parts of the axial and appendicular skeleton of the stained fetuses were inspected under the dissecting binocular microscope for any anomalies.

Statistical analysis: Statistical significance was evaluated by one-way ANOVA using SPSS Version 19 and the individual comparison was obtained by LSD method. Values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Estimation of LD₅₀: Abundant use of insecticides in agricultural products continue to risk the human life. Thus, it is very important to know the LD₅₀ of the pesticide before using it in the fields. The LD₅₀ of EMB in animals following oral administration was calculated to be 88.0 mg kg⁻¹. Consequently, the tested insecticide is considered safe since agents having LD₅₀ higher than 50 mg kg⁻¹ are non toxic (Buck and Osweiler, 1976). The insecticidal activity of EMB involves stimulation of high-affinity GABA receptors and a resultant increase in membrane chloride ion permeability. Some previous studies recorded a wide margin of safety of EMB in animals because mammals are much less sensitive due to lower GABA receptor affinities and relative impermeability of the blood-brain barrier (Yen and Lin, 2004). The experimental rats treated with high oral doses of EMB (25-100 mg kg⁻¹) displayed appreciable changes in physical activity and signs of apparent toxicity symptoms (fatigue, writhing and loss of appetite) and mortality up to 24 h post treatment.

Teratogenic effect: It is known that pesticides cross the placental barrier and enter fetal circulation (Acosta-Maldonado *et al.*, 2009). Therefore, exposure to pesticides during pregnancy has a probability to induce some kind of structural anomalies in the fetuses. The embryonic period of rats (6-15 days) is when organogenesis takes place;

therefore, this duration is one of high susceptibility to toxic materials. During this period, each organ or morphological trait has its own critical period of development.

In the present study, the average maternal body weights for the control and experimental groups recorded on the GD 0, 3, 6, 9, 12, 15, 18 and 21 are represented in Fig. 1. The data indicated that the pregnant females of both the control and all experimental groups showed a steady increase in the body weight during the first 6 days of gestation before administration of EMB. On the 9th day of gestation, the body weight of rats of the control group continued to increase approximately at the same rate, while those of EMB-exposed groups showed a less rate of increase in the body weight gain. On the 21st day, pregnant females of the control group exhibited high percentage of body weight gain (53.89%), while those which were treated with low dose of EMB recorded 42.39%. The rate of increase in body weight reduced as the dose of EMB was increased. The rate of increase in weight of dams exposed to 8.8 mg kg⁻¹ decreased than the control one being 29.28%, while those, which were treated with EMB at 17.6 mg kg⁻¹ recorded 19.04%. The results could be explained by the fact that animals exposed to EMB at 8.8 and 17.6 mg kg⁻¹ had a lower number of viable fetuses as well as increased rates of post-implantation deaths. In accordance with our results, Sangha *et al.* (2011), found a significant decrease in the body weight gain after treatment of pregnant rats with endosulfan insecticide. Comparable results were obtained by Tian *et al.* (2005) and Farag *et al.* (2006) who recorded reduced maternal body weights accompanied by lowered fetal weights and increased number of resorptions in rats exposed to chlorpyrifos and dimethoate insecticides.

As expected, exposure to EMB at doses of 4.4, 8.8 or 17.6 mg kg⁻¹ had no considerable effect on the number of implantation sites (Table 1) as the exposure to the insecticide begins after implantation (GD 6). There was no resorption among the control group or in the group treated with EMB at 4.4 mg kg⁻¹ from the GD 6 to the GD 15. Exposure to 4.4 mg kg⁻¹ had no significant effect on the numbers of viable (11.7±1.25) and dead (1.2±0.63) fetuses, compared to 13.1±0.73 and 0.1±0.31, respectively in the controls. Numbers of viable fetuses were found to be significantly decreased (6.6±2.01 and 1.2±1.31, respectively) while the dead fetuses were significantly increased (2.7±0.48 and 0.8±1.03, respectively) in dams exposed to 8.8 and 17.6 mg kg⁻¹ of EMB (Table 1). It may be assumed that injury to cells and organ system due to insecticide exposure caused embryo lethality. The rates of resorbed fetuses maternally treated with EMB at doses of 8.8 and 17.6 mg kg⁻¹ during the period of organogenesis were 29 and 84.73%, respectively. Complete resorption of all embryos also occurred in a few dams exposed to 17.6 mg kg⁻¹ (Fig. 2). Placental transfer of EMB to fetuses during pregnancy may be one of the reasons which led to the increased numbers of resorption in the treated groups. In confirmation with the present suggestion, many reports

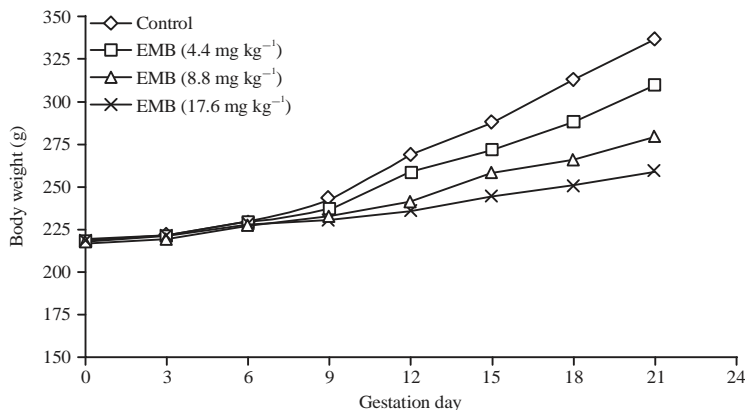


Fig. 1: Effect of EMB exposure on maternal body weight



Fig. 2: Fetal resorption in uterine horns of pregnant rats exposed to EMB at 17.6 mg kg⁻¹ from GD 6 to GD 15

Table 1: The fetal morphological changes following oral administration of EMB to pregnant rats from GD 6 to GD 15, n = 10 pregnant rats

Parameters	Control	EMB		
		4.4	8.8	17.6
No of implantation sites	13.2±0.91	12.9±1.10	13.1±1.85	13.1±0.99
Total No of viable fetuses	131	117	66	12
No. of viable fetuses	13.1±0.73 (99.24%)	11.7±1.25 (90.70%)	6.6±2.01* (50.38%)	1.2±1.31** (9.16%)
No. of dead fetuses	0.1±0.31 (0.76%)	1.2±0.63 (9.30%)	2.7±0.48** (20.61%)	0.8±1.03 (6.11%)
Number of resorbed fetuses	0.0±0.00 (0.00%)	0.0±0.00 (0.0%)	3.8±0.78** (29.00%)	11.1±1.44** (84.73%)
Post-implantation death (5)	2.87±3.56	16.91±7.45	50.60±7.55**	91.15±9.64**
Mean fetal body weight (g)	4.21±0.21	3.45±0.28	2.17±0.16**	1.14±0.28**
Mean fetal body length (cm)	4.10±0.15	3.62±0.19	2.54±0.19**	1.64±0.20**
Placenta weight (g)	1.46±0.03	1.09±0.03	0.78±0.02**	0.37±0.01**
Morphological malformations (%)	0.00	8.74	34.85	100.0

Significantly different from control group at *p<0.05 and **p<0.01, EMB: Emamectin benzoate

established the penetration of insecticides through the placental barrier (Saxena *et al.*, 1981; Dewan *et al.*, 2013). Both doses (8.8 and 17.6 mg kg⁻¹) of EB increased the percentage of post-implantation loss to 50.60 and 91.15%, respectively (Table 1).

The placenta is a transient structure that establishes a functional connection between dam and fetus. This matchless structure transfers all the nutrients required for development, eliminates fetal metabolic waste and synthesizes hormones essential to pregnancy (Desforges and Sibley, 2010). The

placenta contains highly specialized trophoblastic cells that form a barrier between the maternal uterus and the fetus. In our experiments, the placental weight of rats exposed to EMB at 8.8 and 17.6 mg kg⁻¹ exhibited a significant decrease (0.78±0.02 and 0.37±0.01 g, respectively) compared to the controls (1.46±0.03 g). This is in agreement with the study Houghton *et al.* (2000) that showed a decrease of placental weight parallel to the decrease of fetal body weight. A smaller placenta probably has a lower blood flow, resulting in significant fetal hypoxia that may lead to retarded intrauterine growth (Houghton *et al.*, 2000). The marked reduction in placental weight in animals exposed to EMB possibly influences its nutritive role, leading to resorption or at least growth retardation. Among the treatment doses, results from treatment with EMB at 4.4 mg kg⁻¹ showed no effect on embryo placental weight (Table 1).

It is well known that insecticides cross the placental barrier and can induce some changes in the development of placental structures. Drug or chemical-induced placental injury subsequently result in fetal growth retardation, resorption or teratogenicity (El Ghareeb *et al.*, 2015). A dose-dependent decline in fetal weight and length was observed in all the experimental groups. This correlates well with the decrease in maternal weight. Decreases in fetal body weight and length are sensitive and precise indicators for growth retardation. The mean of fetal body weight and length

on GD 21 were recorded in Table 1. In the present study, treatment with EMB at doses of 4.4, 8.8 and 17.6 mg kg⁻¹ caused fetal growth retardation indicated by reduction of both body weight (3.45±0.28, 2.17±0.16 and 1.14±0.28 g, respectively) and length (3.62±0.19, 2.54±0.19 and 1.64±0.20 cm, respectively) of the fetuses. The intrauterine development in mammals is the period of active cell proliferation and differentiation and it is highly sensitive to chemical insults. A number of effects, ranging from growth retardation to severe organ anomalies and functional defects have been reported to result from chemical exposure to embryos (Garber, 1989). The significantly reduced weight gain of rats exposed to high doses of EMB could indirectly related to the high incidence of resorptions and reduced litter weight. Pregnant dams exposed to EMB at 4.4, 8.8 and 17.6 mg kg⁻¹ showed fetuses with external malformations, with rates of 8.74, 34.85 and 100%, respectively (Table 1). The majority of the fetuses showed growth retardation (Fig. 3).

Table 2 presents the percentage of visceral alterations that occurred in fetuses exposed to EMB. Cross sections through the fetal body revealed a few visceral anomalies. Major anomaly noted in the head region was hydrocephaly (Fig. 4), which was represented by enlargement of the cerebral ventricles. The occurrence of hydrocephaly was 12.67, 54.54 and 100% in fetuses exposed to EMB at 4.4, 8.8 and 17.6 mg kg⁻¹, respectively. Fluid retention in the brain is the speculated reason for this abnormality. This is in concomitance with the results obtained by Sakata-Haga *et al.* (2004) and

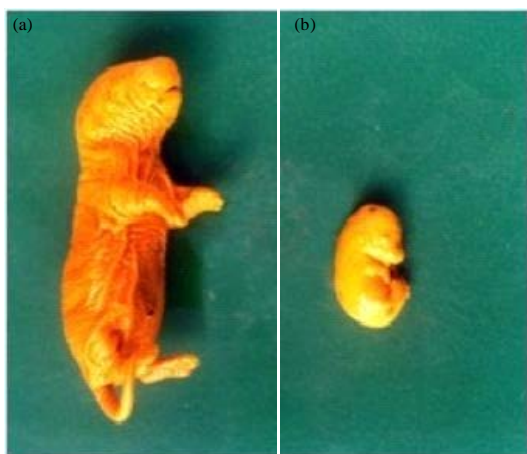


Fig. 3(a-b): Growth retardation (a) Control and (b) Malformed in a fetus obtained from pregnant rats exposed to EMB at 17.6 mg kg⁻¹ from GD 6 to GD 15

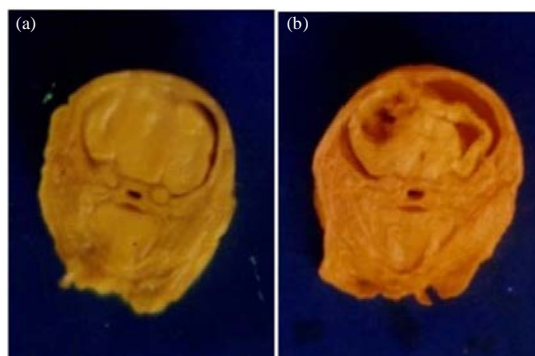


Fig. 4(a-b): Hydrocephaly in a fetus (a) Control and (b) Malformed obtained from pregnant rats exposed to EMB at 17.6 mg kg⁻¹ from GD 6 to GD 15

Table 2: Types and incidence of visceral anomalies in fetuses from pregnant rats exposed to EMB from GD 6 to GD 15, n = 10 pregnant rats

Groups	Malformed fetuses		Malformation occurrence						
	No. of malformed	Total survivors	%	Hydrocephaly		An ophthalmia		Lung hypoplasia	
				No.	%	No.	%	No.	%
Control	0	85	0	0	0	0	0	0	0
EBM (4.4 mg kg ⁻¹)	14	71	19.72	9	12.67	0	0	0	0
EBM (8.8 mg kg ⁻¹)	29	44	65.91	24	54.54	21	47.73	20	45.45
EBM (17.6 mg kg ⁻¹)	8	8	100	8	100	4	50	5	62.5

Malformation occurrence %: Percent of malformation in relation to the total number of examined fetuses, EMB: Emamectin benzoate

Table 3: Types and incidence of skeletal anomalies in fetuses from pregnant rats exposed to EMB from GD 6 to GD 15, n = 10 pregnant rats

Groups	Malformed fetuses		Malformation occurrence												
			Impairment of skull ossification		Hypoplasia of metacarpal bones		Hypoplasia of fore limb digits		Hypoplasia of metatarsal bones		Hypoplasia of hind limb digits		Absence of caudal vertebrae		
	No. of malformed	Total survivors	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Control	0	43	0	0	0	0	0	0	0	0	0	0	0	0	0
EMB (4.4 mg kg ⁻¹)	9	36	25.00	7	19.44	0	0	7	19.44	0	0	6	16.66	2	5.55
EBM (8.8 mg kg ⁻¹)	15	22	68.18	12	54.54	7	31.82	11	50.00	7	31.82	8	36.36	6	27.27
EMB (17.6 mg kg ⁻¹)	4	4	100	4	100	3	75	4	100	3	75	4	100	2	50

Malformation occurrence %: Percent of malformation in relation to the total number of examined fetuses

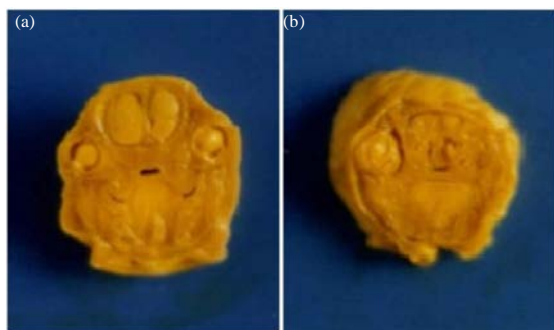


Fig. 5(a-b): Unilateral anophthalmia in a fetus (a) Control and (b) Malformed obtained from pregnant rats exposed to EMB at 17.6 mg kg⁻¹ from GD 6 to GD 15

Sitarek (2004) with other teratogenic agents. Ophthalmic malformations including unilateral anophthalmia (Fig. 5) were also observed in fetuses of the treatment groups. The percentage of anophthalmia was 47.73 and 50.0% in the groups treated with EMB at 8.8 and 17.6 mg kg⁻¹, respectively. Hoogenboom *et al.* (1991), Ozeki and Shirai (1998) and Iyer *et al.* (1999) also reported these congenital eye abnormalities in rats and mice. The fetuses exhibited pulmonary hypoplasia as seen by small-sized lungs of 8.8 mg kg⁻¹ dose (45.45%) and 17.6 mg kg⁻¹ dose (62.50%) groups (Table 2).

Table 3 illustrated the total number of fetuses with skeletal anomalies. Fetuses of mothers treated with EMB during the period of organogenesis have exhibited several skeletal alterations as compared with control ones (Fig. 6). Skeletal anomalies showed a dose related response. The percentages of fetuses with skeletal abnormalities were 0, 25, 68.18 and 100 for the control and the 4.4, 8.8 and 17.6 mg kg⁻¹ EMB groups, respectively. Bones of the skull of 21 days old fetuses maternally treated with low dose of EMB (4.4 mg kg⁻¹) from the 6th to the 15th day of gestation, showed mild degree of lack of ossification of frontal, parietal, interparietal, squamosal and occipital bones of the skull (Fig. 4). Hypoplasia of the phalanges of fore and hind limbs and absence of caudal vertebrae were also noticed.

As shown in Table 3, fetuses whose dams were exposed to EMB at doses of 8.8 and 17.6 mg kg⁻¹ revealed severe



Fig. 6: Impairment of skull ossification, hypoplasia of metacarpal and metatarsal bones, hypoplasia of fore limb and hind limb phalanges and absence of caudal vertebrae in a fetus obtained from pregnant rats exposed to EMB at 17.6 mg kg⁻¹ from GD 6 to GD 15

retardation of ossification of skull bones (54.54 and 100%, respectively), hypoplasia of metacarpal and metatarsal bones (31.82 and 75%, respectively), hypoplasia of the phalanges of fore and hind limbs (36.36 and 100%, respectively) and absence of caudal vertebrae (27.27 and 50%, respectively). Moreover, less ossification of the bones of the pelvic girdle (ilium, ischium and pubis) and the bones of the hind limbs (femur, tibia and fibula) were also seen. The fore and hind limbs not only showed lower degree of ossification but also became shorter as compared with the corresponding bones of the control fetuses. The reduced ossification of fetal skeletons may be a probable reason for decrease in fetal weight. Similar relationship between reduced fetal body weights and retarded ossification of the skeleton has been reported by Murray *et al.* (1979) following exposure to carbaryl in rabbits and mice.

Incomplete ossification of most bones may be related to the effect of insecticide on calcium metabolism and/or bone morphometry by reducing the supply of calcium and magnesium ions to the growing fetus thereby inducing retardation in the bone development (Andrews and Gray, 1990). Moreover, the delay in ossification of the skeletal system may be associated to the delay in fetal growth, as indicated by the reduced fetal weights in the high dose group of the present study. Welsch and Morgan (1985) have also related poor ossification of the skeletal system to growth retardation of the fetuses.

CONCLUSION

In conclusion, the occurrence of morphological, visceral and skeletal malformations in fetuses exposed to EMB was dose dependent and was more severe in the group exposed to the dose of 17.6 mg kg⁻¹. Accordingly, exposure to EMB during pregnancy should be avoided or if necessary, their doses must be decreased.

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