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Effects of Phenobarbital Pretreatment on Ethyl Carbamate-induced Embryotoxicity in Rats

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ABSTRACT *: Ethyl carbamate (EC) is a potent teratogen in rodents and is present at low concentration in fermented foods and alcohol beverages. It has been well hypothesized that some metabolic products are responsible for the teratogenic effects of the compound. In the present study, the effects of phenobarbital (PB) on EC-induced embryotoxicity were investigated in SD rats. Six groups were constructed: EC 300 (EC 300 mg/kg/day), EC 600 (EC 600 mg/kg/day), EC 600+PB (EC 600 mg/kg/day and PB 80 mg/kg/day), PB (PB 80 mg/kg/day), DR (dietary restriction, 8 g/day/rat) and a control group. Rats of the EC 600+PB group were pretreated with phenobarbital intraperitoneally for three days to induce cytochrome P450 enzymes, followed by oral administration of EC for two consecutive days. The incidence of fetal deaths in the EC 600+PB group was higher than that of the EC 600 group(42.7 vs. 14.3%). The incidence of fetal malformations in the EC 600+PB group was higher than that of the EC 600 group (external; 7.0 vs. 4.1%, visceral; 31.4 vs. 11.3%, skeletal; 11.1 vs. 6.5%). There was no embryotoxicity in the control, EC 300, PB and DR groups. These results show that the pretreatment with phenobarbital augments EC-induced embryotoxicity in rats, indicating an evidence that metabolic activation by cytochrome P450 may be the major pathway of EC to its embryotoxic forms.*

Key Words *: Phenobarbital, Ethyl carbamate, Embryotoxicity, Rats.*

I. INTRODUCTION

Ethyl carbamate (urethane, EC) has been used in human medicine as an antineoplastic agent and an anaesthetic for laboratory animals. It has also been used in industry as a chemical intermediate in the preparation and modification of amino resins and as a solubilizer and cosolvent for pesticides (IARC, 1974). It has been reported that EC may be produced accidentally at low levels as a result of the reaction of ethanol with carbamyl phosphate in fermented foods and alcoholic beverages (Miller and Miller, 1983). EC has been found in brandy, wine, Japanese sake, ale, liqueurs, soy sauce, yogurt, olives, bread, etc. at concentrations ranging from 0.6 to 192 μg per liter (Ough, 1976; Canas *et al.*, 1989). EC is well known to be carcinogenic in versatile animals and human (IARC, 1974) and teratogenic in rats and mice(Takaori *et al.*, 1966; Nishimura and Kuginuki, 1958).

EC is mainly metabolized to ethanol, CO_2, and NH_3 in the liver (Nomeir *et al.*, 1989). Minor metabolic pathways include C-oxidation, which may play an important role in the mutagenic action of EC. Dahl *et al.* (1978, 1980) proposed that metabolic activation of EC by cytochrome P450 may result in the formation of vinyl carbamate and subsequent epoxidation to vinyl carbamate epoxide, a highly reactive metabolite, and vinyl carbamate is more potent than its parent compound in its carcinogenicity.

Although the role of metabolism by cytochrome P450 in EC-induced carcinogenesis has been well established, an extensive study on the possible role of metabolism in EC-induced embryotoxicity has not been attempted. Recent reports have provided strong evidence that the oxidation of both EC and vinyl carbamate to form 1, N^6-ethenoade-

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nosine is catalysed by cytochrome P450 IIE1 with quite different affinities (Guengerich *et al.*, 1991; Guengerich and Kim, 1991).

The objective of our present study was to investigate the possible role of metabolism by cytochrome P450 in EC-induced embryotoxicity in SD rats, because the embryotoxicity altered by EC-metabolite was not studied extensively.

II. MATERIALS AND METHODS

1. Animal maintenance and mating procedure

Sprague-Dawley rats (Korea Research Institute of Chemical Technology, Toxicology Center Breeding Facility) were kept under SPF (specific pathogen free) conditions at a constant day/night cycle (light; 7 to 19h). Standard laboratory rodent diet (Jeil Feed Company, Daejeon, Korea) and sterilized tap water were available *ad libitum*. For mating, two females were placed into the cage of one male overnight and the first 24h period following the mating procedure was designated as day 0 of pregnancy if sperm were detected.

2. Test substances

The test agent ethyl carbamate (EC) was supplied by Sigma Chemical Company(St. Louis, MO, USA), and phenobarbital (PB) was purchased by Daehan Pharmaceutical Company (Seoul, Korea). EC was dissolved in distilled water and PB was dissolved in normal saline before administration. The molecular weight of EC was 89.1 and that of PB was 254.25. The chemical structures of EC and PB are shown in Fig. 1.

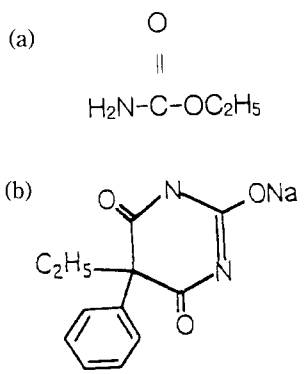

Fig. 1. Chemical structures of ethyl carbamate(a) and phenobarbital(b).

3. Drug treatment

PB was injected intraperitoneally from days 5 to 7 of gestation. And EC was administered orally by gavage to rats from days 8 to 9 of gestation. The application volume was calculated according to the body weight on day 5 or day 8 of gestation.

4. Experimental groups

Six groups were constructed : EC 300 (EC 300 mg/kg/day), EC 600 (EC 600 mg/kg/day), EC 600+ PB (EC 600 mg/kg/day and PB 80 mg/kg/day), PB (PB 80 mg/kg/day), DR (dietary distriction, 8g/day/rat) and a control group.

5. Observation of animals

Dams were observed for signs of intoxication, body weight change, food consumption, and autopsy findings. Pregnant females were subjected to Caesarean section on day 20 of pregnancy. The implantation sites, corpora lutea, living fetuses, dead fetuses, and resorptions were numbered and registered. All living fetuses were weighed, sexed, and evaluated for externally visible abnormalities. Alternate fetuses were selected for either skeletal or visceral examination(Wilson, 1965; Nishimura, 1974).

6. Statistical analysis of data

Variables such as maternal body weight, implantation rate, and number of live fetuses were evaluated by ANOVA test, followed by Dunnett's or Scheffe's test. Fetal deaths were analyzed by Kruskal-Wallis test. The sex ratio was analyzed using a χ^2-test. Fetal body weight was analyzed separately for each sex using Dunnett's or Scheffe's test. The incidence of fetal anomalies in treated groups was compared with that of controls by Kruskal-Wallis test, followed by Dunnett's or Scheffe's test. A difference was considered statistically significant at $P<0.05$.

III. RESULTS

1. Effects on dams

Clinical signs observed in dams of the EC 600, EC600+PB and PB groups are shown in Table 1. The characteristic toxic signs were decreased activity, weakness and dark-red vaginal discharge. No clinical signs or abnormal behaviour were observed in dams of the EC 300 and DR groups. The maternal body weights of the EC 600, EC 600+PB and DR groups were significantly lower, compared with those of the control group. And dams' body weights of the EC 600+PB group differed sig-

Table 1. Clinical findings of dams treated with ethyl carbamate alone or in combination with phenobarbital.

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of observations	11	11	11	9	12	12
No. of dams with clinical signs(%)	0	0	6(55)	9(100)	9(75)	0
Decreased activity	0	0	1	3	9	0
Decreased activity and weakness	0	0	5	3	0	0
Decreased activity and dark-red vaginal discharge	0	0	0	2	0	0
Decreased activity, weakness	0	0	0	1	0	0

Table 2. Mean body weights of dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of dams	11	11	11	9	12	12
Gestation						
Day 0	243.4±23.8	242.6±20.1	235.3±20.8	233.6±13.4	242.7±26.9	238.4±15.7
Day 6	279.7±26.5	282.2±19.8	270.7±19.6	255.5±11.6	267.1±24.3	274.4±13.5
Day 8	288.8±24.2	276.3±20.6	278.0±21.1	256.6±12.8**,[a]	275.2±22.1	280.4±15.7
Day 12	309.0±25.2	295.9±17.7	265.6±25.6**	239.6±20.5**,[a]	297.5±22.3	236.2±14.0**
Day 15	329.1±27.2	319.0±17.2	297.9±27.0*	268.1±27.7**,[a]	316.4±26.3	285.2±17.0**
Day 20	401.2±29.2	402.8±19.6	377.3±33.7	320.1±62.5**,[a]	384.3±25.6	370.2±22.1**

Each value represents the Mean±S.D. * and ** indicate signifcant difference at P<0.05 and P<0.01, when compared with the control group. [a] indicates signifcant difference at P<0.05, when compared with the EC600 group.

Table 3. Caesarean section data of dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of dams	11	11	11	9	12	12
Corpora lutea(Mean±SD)	16.5±2.3	17.0±2.4	17.0±2.6	16.7±2.5	15.8±1.9	16.8±2.1
Implantations(Mean±SD)	15.8±2.1	16.1±1.9	15.6±3.2	13.8±3.8	13.4±2.6	15.5±2.5
% to corpora lutea	96.3±3.9	95.8±2.8	91.4±9.6	82.5±18.3	84.7±11.8	91.8±8.6
Fetal deaths(Mean±SD)						
(resorptions+dead fetuses)	1.2±2.1	0.9±1.4	2.2±2.2	5.9±6.3**	0.9±1.1	1.9±3.4
% to implantation	8.1±16.1	5.2±7.8	14.3±14.2	42.7±44.5**	6.9±8.1	13.5±23.0
Resorptions Total	13	10	24	63	11	23
Early	13	9	23	63	10	23
Late	0	1	0	0	1	0
Dead fetuses	0	0	1	0	0	0
Live fetuses(Mean±SD)	13.3±5.3	15.3±1.6	13.5±3.5	7.9±7.1*	12.5±2.7	13.6±4.5
% to implantations	91.9±16.1	94.8±7.8	91.3±9.7	57.3±44.5*	93.1±8.1	86.5±23.0
Sex Ratio(male/female)	0.94(78/83)	1.21(92/76)	0.98(92/94)	1.37(41/30)	1.00(75/75)	1.01(81/80)
Body weights of live fetuses						
Male (Mean±SD)	3.4±0.2	3.3±0.2	3.4±0.3	3.3±0.3	3.5±0.7	3.3±0.3
Female(Mean±SD)	3.3±0.3	3.1±0.2	3.2±0.2	3.1±0.3	3.4±0.6	3.2±0.3

* and ** indicate signifcant difference at P<0.05 and P<0.01, when compared with the control group.

Table 4. External findings in fetuses from dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of fetuses examined	161	167	148	71	150	161
No. of fetuses with malformations(%)	1(0.62)	0	6(4.1)	5(7)*	1(0.67)	0
Oligodactyly	0	0	1	0	0	0
Scoliosis	0	0	1	0	0	0
Hematoma	0	0	0	1	0	0
Gastroschisis	1	0	1	0	0	0
Acaudate	0	0	1	1	0	0
Kinky tail	0	0	4	0	0	0
Vestigial tail	0	0	2	3	1	0

* indicates signifcant difference at $P<0.05$, when compared with the control group.

Table 5. Visceral findings in fetuses from dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of fetuses examined	78	80	71	35	72	77
No. of fetuses with malformations	0	0	0	0	0	0
No. of fetuses with variations(%)	2(2.6)	6(7.5)	8(11.3)*	11(31.4)**,[a]	4(5.6)	6(7.8)
Thymic remnant in the neck	1	3	2	3	0	2
Dilatation of the renal pelvis	0	0	1	2	0	0
Dilatation of the ureter	1	3	5	8	4	4
Malposition of the kidney	0	0	0	1	0	0

* and ** indicate signifcant difference at $P<0.05$ and $P<0.01$, when compared with the control group.
[a] indicates signifcant difference at $P<0.05$, when compared with the EC600 group.

nificantly from those of the EC 600 group(Table 2).

2. Effects on fetuses

Significant differences were observed in fetal deaths and litter size of the EC 600+PB group, compared with those of the control group (Table 3).

Fetuses of the EC 600 and EC 600+PB groups showed external malformations, including oligodactyly, scoliosis, hematoma, gastroschisis, acaudate, kinky tail and vestigial tail (Table 4). The incidence of external malformations in the EC 600+PB group was significantly higher than that of the control group. The results of visceral examination of the fetuses are shown in Table 5. The incidence of variations in the EC 600 and EC 600+PB groups differed significantly from that of the control. Thymic remnant in the neck, dilatation of the renal pelvis, dilatation of the ureter were observed in the fetuses of the EC 600 and EC 600+PB groups. The incidence of visceral variations in the EC600+PB group was significantly higher than that of the EC 600 group.

The results of skeletal examination of the fetuses are shown in Table 6. Fetuses of the EC 600 and EC 600+PB groups showed skeletal malformations, including shortened 13th rib, absence of the thoracic or lumbar vertebral body, and absence of the lumbar or sacral vertebrae. The incidence of skeletal malformations in the EC 600+PB group was significantly higher than that of the control group. The incidence of skeletal variations of the EC 600 and EC 600+PB groups compared well with the control. The incidence of skeletal retardations in the EC 600+PB group was significantly higher than that of the control group. Dumbbell-shaped thoracic vertebral body was observed in all groups. Retarded ossification of the sternebrae was ob-

Table 6. Skeletal abnormalities in fetuses from dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of fetuses examined	83	87	77	36	78	84
No. of fetuses with malformations(%)	1(1.2)	3(3.4)	5(6.5)	4(11.1)*	0	0
Fused sternebrae	0	1	0	0	0	0
Absence of thoracic vertebral body	0	0	2	0	0	0
Shortened 13th rib	1	2	4	2	0	0
Absence of lumbar vertebral body	0	0	2	0	0	0
Absence of lumbar vertebrae	0	0	0	1	0	0
Absence of sacral vertebrae	0	0	0	2	0	0
No. of fetuses with variations(%)	2(2.4)	3(3.4)	2(2.6)	4(11.1)	3(3.8)	2(2.4)
Asymmetric sternebrae						
Cervical rib	0	0	0	0	1	0
Lumbar rib	1	1	1	4	1	2

* indicates signifcant difference at $P<0.05$, when compared with the control group.

Table 7. Rate of ossification in fetuses from dams treated with ethyl carbamate alone or in combination with phenobarbital

Parameter	Groups					
	Control	EC300	EC600	EC600+PB	PB	DR
No. of fetuses examined	83	87	77	36	78	84
No. of fetuses with retardations(%)	2(2.4)	3(3.4)	7(9.1)	6(16.7)**	4(5.1)	4(4.8)
Cleaved sternebrae	0	0	2	0	1	0
Bicentric thoracic vertebral body	0	2	1	1	1	1
Bicentric lumbar vertebral body	0	0	2	0	0	0
Bicentric sacral vertebral body	0	0	0	0	0	1
Dumbbell-shaped thoracic vertebral body	2	2	2	5	3	2
Dumbbell-shaped lumbar vertebral body	0	0	1	0	0	0
Hemivertebrae(lumbar)	0	0	1	0	0	0
No. of ossification centers(Mean±SD)						
Sternebrae	5.3±0.5	4.7±0.6	4.3±0.8**	3.7±0.8**	4.9±0.7	5.1±0.5
Metacarpals in both forelimbs	6.8±0.7	6.3±0.5	6.6±0.8	6.1±0.2	6.9±0.8	6.7±0.8
Metatarsals in both hindlimbs	8.0±0.1	7.9±0.2	7.8±0.4	7.9±0.2	7.9±0.2	8.0±0.1
Sacral and caudal vertebrae	7.5±0.4	7.3±0.3	7.0±0.9	6.9±0.7	7.6±0.5	7.4±0.4

* and ** indicate signifcant difference at $P<0.05$ and $P<0.01$, when compared with the control group.

served in the fetuses of the EC 600 and EC600+ PB groups (Table 7).

IV. DISCUSSION

It was previously reported that ethyl carbamate (EC) is teratogenic in mice and rats. According to the report of Nishimura and Kuginuki (1958), EC induced fetal malformations in the skeletal system, especially as the various digital malformations (syndactyly, adactyly etc.) and cleft palate, when EC at 1.5 g/kg was injected to pregnant mice intraperitoneally from days 9-12 of gestation.

Takaori *et al.* (1966) found various types of fetal malformations in the vertebrae and ribs, when EC at 1 g/kg was given to pregnant Wistar rats by gavage from days 8-9 of gestation. In our study, in which EC at 600 mg/kg was given to pregnant SD

rats by gavage from days 8-9 of gestation, we could find similar fetal malformations, but with the lower incidence. Explanations of the discrepancy might include the differences of rat strains, dosing forms, or dosing levels. Among these factors, the difference in the rat strain seems to be the most plausible.

The metabolism of EC by cytochrome P450 has been well documented. In earlier papers, alcohol and aldehyde dehydrogenases were considered to play a role in the metabolism of EC (Waddell *et al.*, 1989; Kuruta *et al.*, 1990). However now it is well known that alcohol-inducible cytochrome P450 IIE 1 metabolizes EC to vinyl carbamate, and ultimately to an epoxide which produces adducts with adenosine, cytosine and guanosine residues in DNA (Guengerich *et al.*, 1991; Guengerich and Kim, 1991). When the rate of 1, N^6-ethenoadenosine formation was studied in human liver microsome, the rate from EC was demonstrated to be much slower than from vinyl carbamate, indicating that once EC is metabolized to vinyl carbamate, the adduct is formed relatively easily (Guengerich and Kim, 1991).

It has been hypothesized that some metabolic products are responsible for the teratogenic effects of EC (Takaori *et al.*, 1966). Even though the role of metabolic activation in chemical-induced toxicity has been addressed in numerous reports, the possible involvement of metabolism in the production of embryotoxic metabolites has not been studied extensively.

According to the results of our study there was an increased incidence of fetal deaths and malformed fetuses in the EC 600+PB group, compared with that of the EC 600 group. The embryotoxicity induced by EC was potentiated by the pre-induction of cytochrome P450 by phenobarbital (PB).

PB mainly induces P450 IIB enzymes such as P 450 IIB1 and P450 IIB2 in rat liver (Guengerich *et al.*, 1982). However, EC has been characterized to be selectively metabolized by P450 IIE1 to vinyl carbamate which is very toxic (Guengerich and Kim, 1991). Based on these, the potentiation of EC embryotoxicity by PB in these studies would be explained if PB induces P450 IIE1 expression as well as P450 IIBs in rats. Although we don't have any clues for these yet, the possibilities should be studied. In support of the possibility, Jeong *et al.* (1996) observed that the pretreatment of BALB/c mice with phenobarbital significantly induced P450 IIE1-selective enzyme activity, as well as P450 IIB-selective monoxygenase.

Taken together, the conclusion that PB pretreatment increases the embryotoxicity induced by EC would suggest that the metabolism by cytochrome P450 may be involved in EC embryotoxicity in rats.

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