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Short communication

Germ cell mutagenicity of γ-ethyl-γ-phenyl-butyrolactone (EPBL) detected in the CF1 mouse-dominant lethal study

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Abstract

Female and male CF1 mice weighing 25–30 g were given 0, 50, 100 or 200 mg/kg of γ-ethyl-γ-phenyl-butyrolactone (EPBL) for 5 days intraperitoneally. In the male-dominant lethal phase, males treated with EPBL were mated with untreated females following a 7-day mating schedule with three consecutive mating events. In the female-dominant lethal phase, females treated with EPBL were caged with untreated males. The above dosages and schedule treatments were used. The incidence of pregnancy of females mated on days 1–7 and 8–14 after males were given 200 mg/kg of EPBL and of females given 200 mg/kg when mated to untreated males was decreased. Upon examining surgically exposed uteri and ovaries of pregnant females during the first phase, on gestation days 13–15, an increased incidence of pre-implantation losses with 200 mg/kg of EPBL and an increased incidence of post-implantation losses with 100 and 200 mg/kg was observed. In addition, an increased frequency of pre- and post-implantation losses was seen in females treated with 200 mg/kg. These results support the conclusion that EPBL is a germ cell mutagen and its effects are more pronounced during the post-meiotic stage.

Keywords: Dominant lethal; Germ cell mutagenicity; γ-Ethyl-γ-phenyl-butyrolactone

1. Introduction

An important concern in toxicology is the identification of genetic damages induced by exposure to chemical stimuli (Shelby et al., 1991). Recently, there has been an increasing awareness of the genotoxic potential of a wide variety of

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drugs the human population is exposed to (Odeigah, 1997).

γ-Ethyl-γ-phenyl-butyrolactone (EPBL; Fig. 1) is a new synthetic oral anticonvulsant and hypnotic agent (Vega-Díaz et al., 1992). This drug results from a structural reaction between γ-hydroxy-γ-ethyl-γ-phenylbutyramide (HEPB), an anticonvulsant drug, and γ-butyrolactone (GBL), a convulsant/anticonvulsant drug (Vega-Díaz and Vega-Rasgado, 1991).

HEPB had no teratogenic effects on rats (Salazar et al., 1989) or mice (Chamorro et al., 1994). However, GBL caused decreased placental weights and increased fetal weights in rats (Kronevi et al., 1988). Also, slight teratogenic effects or anomalies were seen after intraperitoneal EPBL was given to mice throughout the organogenic period (Rodríguez, 1994). However, the reproductive performance and development of F_2 progeny was not affected (Chamorro et al., 2002) when the drug was given during spermatogenesis and ovulation to male and female mice, respectively.

In this study, and as a part of a program to develop indications for the safe and effective use of EPBL as an anti-epileptic and/or hypnotic drug, we studied the response of male and female mice to the dominant lethal effects of this drug. The effects on testicular weights and accessory sex organ weights were studied and a sperm examination was performed to ascertain gonadal effects that might be undetectable in the dominant lethal test.

2. Materials and methods

2.1. Animals, diet and animal maintenance

Adult CF1 male and female mice (25–30 g) supplied by the animal house of the Instituto Nacional de Virología, S.S.A., Mexico City, were housed in polypropylene cages. Each male mouse was housed in one cage while two females were housed in every cage. They were fed standard pellet diets (lab Rodent Diet 5001, PM Nutrition International, Richmond, IN) and drinking water was given ad libitum. The temperature and relative humidity were kept at 24±2 °C and 60±15%, respectively, with 12 h light and darkness periods (08:00 A.M. and 08:00 P.M.). Animals were randomized into different groups by a computer-generated program and acclimatized to the test room at least 1 week prior to the beginning of the experiments. The care and use of animals followed the Scientific Research Committee regulations of our Institute.

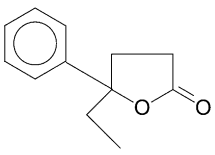

Fig. 1. γ-Ethyl-γ-phenyl-butyrolactone (EPBL).

2.2. Test substance

EPBL, the test substance, was prepared by one of the members in our team, following the Vega-Díaz and Vega-Rasgado (1991) method as identified by NMR analysis. The drug was dissolved in corn oil at 0, 50, 100 and 200 mg/10 ml concentrations immediately prior to its administration by intraperitoneal injection at a dosing volume of 1 ml/100 g of body weight. The control group was given the vehicle only. Animals were treated at approximately 09:00 A.M.

2.3. Dominant lethal assay

There were two phases to this study. In the male-dominant lethal phase, the modified method by Epstein et al. (1972) was used. Groups were made by 12 males. Each group was given a treatment dose or the control dose. Intraperitoneal doses were as follows: 0 (control), 50, 100 and 200 mg/kg, with a short five daily injection treatment. The maximum dose was selected based on an earlier pharmacological study where 200 mg/kg or more were given to mice, resulting in momentary loss of the righting reflex as a sign of neurotoxicity (Vega-Díaz et al., 1992). Doses accounted for about 5, 10 and 20 times the expected maximum human dosage. Animals were observed every day for clinical and behavioral signs of toxicity.

Twenty-four untreated virgin females were randomly allocated to treated males and placed in

their cages at a two females to one male ratio. Cohabitation occurred for 3 consecutive weeks post-injection (with two virgin females each week), on days 1–7, 8–14, and 15–21. Copulations during these time periods involved spermatozoa, late spermatids and early spermatids, respectively (Nagao, 1987). Females were killed by cervical dislocation 13–15 days after the mid-point of each cohabitation week. A laparatomy was performed to expose the uteri and ovaries of each female and the fetuses were evaluated. The number of pregnant females, *Corpora lutea*, implantation sites, resorptions and deaths were recorded. All females showing implants in their uterus were rated as fertile.

In the female-dominant lethal phase, 10 untreated males were mated with 20 treated females (1:2) in each group, using the above dosages, treatment regimens, times of cohabitation and kill days.

In both study phases, the frequency of pre- and post-implantation losses was estimated. Data were analyzed for statistical significance against their respective controls by using the chi-square test. Results were considered as significant when $P < 0.05$.

In addition, four groups with 10 males each were killed on weeks 2, 4, 6 or 8 of treatment (controls and two high dose groups). Immediately after being killed, the distal portion of the right epididymis and the vas deferens were dissected by cutting the middle tail section and immersing it in 1 ml of saline, to be first stored at a 37 °C. Tissues were then perfused by inserting a syringe with an additional 1 ml of the same solution, in the previously opened vas deferens. After the semen suspension was stirred and thoroughly mixed by repeated pipeting, the concentration, motility and morphology of sperm from individual animals were studied as described by Albert and Roussel (1983). Results were presented as percentages. Finally, in animals having completed the 8-week treatment, the left testis, epididymis and seminal vesicle were weighed. Testes histology was not completed. Data on sperm morphology, motility and epididymal sperm counts were analyzed by using the Mann–Whitney *U*-test and data on sex organ weights were analyzed with Student's *t*-test. A probability of < 0.05 was assumed to indicate a significant difference.

3. Results

In the treatment group where 200 mg/kg were given intraperitoneal daily for 5 consecutive days, some male and female mice showed momentary ataxia. One male mouse out of 12 died after the fourth dose. However, the autopsy revealed no apparent toxic effects. No effects were seen with 50 or 100 mg/kg. In the female-dominant lethal test, one animal in the mid-dose group died after dosing. Death occurred 8 days after the last dose.

The results from the dominant lethal assay in male mice are summarized in Table 1. The percentage of pregnant control females varied between 88 and 100% and between 63 and 100% in experimental groups at different mating intervals and doses. Statistically significant decreases in fertility occurred at the 200 mg/kg dose after the 1–7- and 8–14-day mating interval. On all mating intervals, the frequency of pre-implantation losses was increased with the high 200 mg/kg dose and the frequencies of post-implantation losses were increased for the mid- and high-dose groups. The frequency of post-implantation losses was also increased for the mid- and high-dose groups on all days after the mating interval. The mouse germ cells most sensitive to EPBL were early spermatids.

The female-dominant lethal test phase is summarized in Table 2. Administration of EPBL had no perceptible effects on the incidence of pregnancies at 50 and 100 mg/kg. However, at a 200 mg/kg dose, the fertility rate decreased to 55%. At this dose, pre- and post-implantation losses increased significantly.

As shown in Table 3, the examination of semen of males 2 and 4 weeks after being given 200 mg/kg revealed decreased sperm concentrations. Percentage of reduced sperm motility was seen to increase 2 weeks after completing the treatment at 100 and 200 mg/kg. However, semen morphology was normal with intact acrosome, mid-piece and principal piece. There were no statistical differences for terminal body weight, testis, epididymis or seminal vesicle weights (Table 4).

Table 1
Frequencies of pre- and post-implantation losses among the conceptuses of male mice treated with EPBL, mated to untreated females

Dose (mg/kg)	No. of male mice treated	Mating interval (days)	No. of mated females	No. of pregnant females (%)	Total *C. lutea* (A)	Total implants (B)	Frequency of pre-implantation losses[a]	Dead implants (C)	Frequency of post-implantation losses[b]
0	12	1–7	24	24 (100)	314	288	8.3	23	8.0
		8–14	24	22 (91.7)	276	250	9.4	23	9.2
		15–21	24	21 (87.5)	270	235	12.9	17	7.2
50	12	1–7	24	20 (83.3)	277	251	9.4	27	10.7
		8–14	24	23 (95.2)	285	255	10.5	21	8.2
		15–21	24	24 (100)	351	307	12.5	19	6.2
100	12	1–7	24	20 (83.3)	260	230	11.5	55	23.9**
		8–14	24	20 (83.3)	265	233	12.1	51	21.5**
		15–21	24	21 (87.5)	266	228	14.3	39	17.1**
200	12	1–7	24	16 (66.7)	198	163	17.6**	62	38.0**
		8–14	24	15 (62.5)	186	140	25.0**	42	30.6**
		15–21	24	21 (87.5)	298	202	32.2**	42	20.8**

[a] $A-B/A \times 100$.
[b] $C/B \times 100$. Differ significantly from the control value at corresponding week: $P < 0.05$.
** Differ significantly from the control value at corresponding week: $P < 0.01$.

Table 2
Frequencies of pre- and post-implantation losses among the conceptuses of female mice treated with EPBL, mated to untreated males

Dose (mg/kg)	No. of mated females	No. of pregnant females (%)	Total *C. lutea* (A)	Total implants (B)	Frequency of pre-implantation losses[a]	Dead implants (C)	Frequency of post-implantation losses[b]
0	20	17 (85)	213	189	11.3	20	10.6
50	20	17 (85)	212	190	10.4	18	9.5
100	20	18 (90)	212	186	12.3	17	9.2
200	20	11 (55)	127	101	20.5*	33	32.6**

[a] A − B/A × 100.
[b] C/B × 100.
* Differs significantly from the control value: $P < 0.05$.
** Differs significantly from the control value: $P < 0.01$.

Table 3
Epididymal sperm concentration, motility and percentage of morphologically normal sperm in mice treated with EPBL intraperitoneal on 5 consecutive days

Dose (mg/kg)	Weeks			
	2	4	6	8
Sperm concentration ($\times 10^6$ ml^{-1})				
0	7.6±0.9	7.9±1.3	8.4±1.2	8.9±1.3
100	7.3±1.3	7.6±1.6	7.8±1.4	8.6±1.2
200	5.3±1.1*	5.0±1.3*	8.5±1.5	9.2±1.7
Reduced sperm motility (%)				
0	15.8± 3.8	16.2±3.3	15.3±2.9	16.6±2.5
100	22.1±3.9*	16.6±3.8	15.7±4.1	10.7±2.2
200	23.3±4.2*	18.1±4.4	14.6±2.6	14.3±4.5
Normal shaped sperm (%)				
0	69.6±3.6	67.4±4.2	64.3±4.7	70.6±5.3
100	70.3±4.5	69.5±3.6	66.6±5.0	71.1±4.6
200	68.7±3.8	68.4±5.3	71.7±4.8	68.6±4.6

* Differ significantly from the corresponding control value: $P < 0.05$.

4. Discussion

The decreased frequency of fertile mating vs. the control in experimental females mated on weeks 1 and 2 post-treatment at the highest dose level could have been related to pre-implantation losses due to decreased fertilization elicited by the impaired sperm concentration and motility (Odeigah, 1997). The results differ from those previously reported in a reproductive toxicity study on mice in which similar doses were used but fertility was not affected (Chamorro et al., 2002). However, each study had a different objective and animal model. With treated post-meiotic cells sampled on days 1–21 after completion of treatment, pre-implantation losses were significantly induced at 200 mg/kg. On the other hand, the frequencies of post-implantation losses in untreated female mice mated with EPBL-treated males showed a significant, dose-dependent increase over the corresponding control at 100 and 200 mg/kg in all three post-treatment periods. These results are consistent with those showing that most mutagens elicit their effects in post-meiotic germ cell stages (Adler and Anderson, 1994) and in the early weeks of the dominant lethal test (Bateman, 1966). The increase was less striking when the treated post-

Table 4
Final body and sex organ weight of male mice killed after the completion of 8 weeks of treatment with EPBL

Dose (mg/kg)	Terminal body weight	Testis (g)	Epididymis (mg)	Seminal vesicle (mg)
0	34.2±3.7	0.576±0.07	59.9±3.4	0.18±0.02
100	35.7±4.2	0.513±0.05	64.5±3.8	0.20±0.02
200	32.5±3.5	0.566±0.05	57.7±4.4	0.16±0.03

Values are mean±S.D.

meiotic cells were sampled on days 15–1, suggesting that different stages of germ cell maturation respond differently. The effect was largely due to post-implantation rather than pre-implantation losses, a more indicative sign of dominant lethality and representing a reliable measure of genetic damage (Short et al., 1977; Rao et al., 1994).

In conclusion, intraperitoneal administration of EPBL induced germinal mutations in the post-meiotic phase of gametogenesis in male mice as expressed by a dominant lethal effect when a short-term treatment was used.

In the female phase study, the duration of dosing and mating procedures were similar to those of males and the effects were also probably of the dominant lethal type induced in maturing oocytes indicating that certain chemicals may be mutagenic for both males and females. However, although maternal toxicity was not seen, the female-dominant lethal test should be supplemented by cytogenetic and/or other methods to confirm the genetic nature of the response derived (Holmstrom et al., 1993).

Quantitative and qualitative sex differences in dominant lethal testing have been reported (Katoh et al., 1990). Indeed, a few chemicals have been identified as female-specific (Sudman and Generoso, 1991), attributed to the chromosomal condensation states of the germ cells (Holmstrom et al., 1993).

Because adverse effects in dominant lethal studies are not conclusive for evidence of mutagenicity, further research is required to evaluate the effects of EPBL on gene mutation in mammalian cells in vitro as well as in whole animals in vivo.

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