Chronotoxicity of Sodium Valproate in Pregnant Mouse and Embryo

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ABSTRACT—The influence of dosing time on the embryotoxicity of sodium valproate (valproic acid, VPA) was investigated in ICR mice under a light-dark (12 : 12) cycle. A significant circadian rhythm was shown for VPA-induced embryotoxicity, with the highest value at 1700 and the lowest at 0100. A similar pattern of rhythm was also shown for VPA-induced toxicity in pregnant and nonpregnant mice. No significant dosing time-dependent difference between injection at 1700 and 0100 was demonstrated for VPA concentrations in the embryo, plasma and brain. The rhythm in the embryotoxicity seems to be related to the rhythm in the sensitivity of the embryo and dam to the drug. Embryotoxicity and VPA concentrations were significantly higher on gestational day 13 than day 7. The pharmacokinetics of VPA contribute, at least partly, to the gestational stage-dependent embryotoxicity of VPA. The timing of drug administration (i.e., gestational stage and circadian phase) appears to be essential for studies on the embryotoxicity of VPA in mice.

Keywords: Sodium valproate, Embryotoxicity, Chronotoxicity

About 30 years ago, the thalidomide disaster shocked the public into an awareness of the potential hazards of drugs and environmental agents to the unborn human being. Embryotoxicities induced by a drug in animals are not only dependent on the dosage, the dosing route, and the dosing schedule, but also on the dosing time. The ingestion of some drugs during pregnancy produce embryotoxicity that is gestational stage- and circadian phase-dependent. These drugs include glucocorticoids, hydroxyurea, 5-fluorouracil, cyclophosphamide and ethanol and so on (1). The mechanism underlying the circadian rhythm on drug susceptibility has been discussed from the viewpoints of the sensitivity of living organisms to drug and the pharmacokinetics. However, many reports have focused only on the dose-response or dose-concentration relationship.

Sodium valproate (valproic acid, VPA) is a widely used antiepileptic drug. A significant circadian rhythm is demonstrated for the ES (electroshock seizure) threshold, plasma VPA concentrations and brain VPA concentrations after administration of VPA at a dose of 600 mg/kg (2). The rhythm in VPA concentrations nicely corresponds to the rhythm in the ES threshold. From the viewpoint of the sensitivity of living organisms to drug, the relationship between the brain VPA concentration and the ES threshold was not different between the light phase and the dark phase. Therefore, the mechanism underlying the circadian rhythm of the ES threshold can be explained mainly by the rhythm in VPA absorption. On the other hand, a significant circadian rhythm of acute toxicity is also demonstrated in mice given 1200 mg/kg of VPA by intraperitoneal injection, although no significant circadian rhythm is demonstrated for plasma and brain VPA concentrations after intraperitoneal injection with a high dose of VPA (3). The rhythm of mortality is due to the rhythm in the sensitivity of the central nervous system to the drug. Furthermore, a significant circadian rhythm is found for VPA clearance and volume of distribution after a low dose of VPA (4). However, no information is available, to date, on the influence of dosing time on VPA embryotoxicity, although VPA is known to be teratogenic in experimental animals (5, 6) and may induce neural tube defects in humans (7).

This study was designed to examine the influence of the timing of administration in the gestational stage and circadian phase on VPA embryotoxicity. The underlying mechanism of the circadian drug susceptibility rhythm was investigated from the viewpoints of the sensitivity of living organisms and the pharmacokinetics of drugs. The relationship between the rhythm of acute toxicity in preg-

nant mice or nonpregnant mice and the rhythm in embryos were investigated. The treatment of the animals was based on the Guidelines for Animal Experiments at the Ehime University School of Medicine.

MATERIALS AND METHODS

Animal and mating schedule

Male and female ICR albino mice (5-weeks-old) were purchased from Clea, Inc. (Tokyo). Mice were housed 10 per cage in a standardized light-dark cycle (lights on at 0700, off at 1900) at a room temperature of 24±1℃ and a humidity of 60±10% with food and water available ad libitum. After a 1-week acclimatization to the laboratory conditions, one female was caged with one male for 2 hr under one of six different mating regimens (0800−1000, 1200−1400, 1600−1800, 2000−2200, 2400−0200 or 0400−0600 hr). Two-hour timed matings at different circadian phases were used to obtain embryos of the same chronological age for the drug treatment. The regimens were repeated up to three times during one week to achieve the necessary number of successful matings. The presence of a vaginal plug was taken to indicate day 0 of gestation. Pregnant animals were housed 10 per cage.

Reagents and drugs

Sodium valproate was purchased from Wako Pure Chemical Industries (Osaka) and used at a dose of 900 mg/kg for acute toxicity in nonpregnant mice, 600 mg/kg for acute toxicity in pregnant mice, 550 mg/kg for embryotoxicity and 500 mg/kg to determine the pharmacokinetics. The drug was dissolved in sterilized physiological saline to yield appropriate concentrations of 900 mg, 600 mg, 550 mg and 500 mg/10 ml, respectively. Administration was performed by subcutaneous injection with a 23-gauge needle that was connected to a 0.5-ml syringe. The administered volume of drug solutions was 10 ml/kg. The injections were made by a single person at each injection time and stage of gestation. The injection was performed subcutaneously so that the injection needle does not enter the uterine and fetal tissues. Also, the oral route was not used, because this method exhibited a significant circadian rhythm and a large variation at the absorption phase after an oral dose of VPA (2). The dosage of VPA was chosen, respectively, based on the LD_{50}'s in nonpregnant mice (900 mg/kg), pregnant mice (600 mg/kg) and embryo (550 mg/kg). Different dosages were used for pregnant mice and embryos because we observed that there were no dead pregnant mice at 550 mg/kg of VPA and no live embryos at 600 mg/kg of VPA. A 500 mg/kg dose of the drug was used for the pharmacokinetic study, since at dosages over 500 mg/kg, it is harder to follow the VPA concentrations because of dead embryos. Diphenyl and other reagents, analytical grade, were purchased from Wako.

Determination of VPA concentrations

The VPA concentrations in the plasma, whole brain and embryo were determined by gas-liquid chromatography (GLC) (GC-9A type gas chromatograph; Shimadzu, Kyoto) with a FID detector according to the procedure developed by Löscher with slight modifications (8). One hundred microliters of 12 N hydrochloric acid and 100 μl chloroform containing diphenyl as an internal standard (30 μg/ml) were added to 100 μl plasma in a 400-μl tube. The whole brain or whole embryo were homogenized in a 10-ml test tube in a mixture of 4 ml saline, 1 ml 12 N hydrochloric acid and 1 ml chloroform containing diphenyl as an internal standard (150 μg/ml). After the homogenates and plasma mixture were shaken vigorously for 10 min, they were centrifuged 3000 rpm for 10 min, and the water layer was removed. A 2-μl aliquot of the organic layer was applied into a gas-liquid chromatograph with a 2 m×3 mm glass column packed with 5% FFAP on 80/100 Gas-Chrom Q (Japan Chromato Kogyo, Tokyo). The column temperature was maintained at 170℃, and the injection and detection temperature were kept at 250℃. Nitrogen, hydrogen and air flows were 30, 30 and 300 ml/min respectively. Quantitation was achieved by comparing peak area ratios (VPA to diphenyl). The coefficient of variation for the assay error was less than 7% for GLC as exemplified by Löscher (8).

Circadian rhythm of mortality in embryos and pregnant and nonpregnant mice

Groups of pregnant mice, each on gestational day 7 or 13, were subcutaneously injected with 550 mg/kg VPA at one of six times: 0900, 1300, 1700, 2100, 0100 and 0500. On gestational day 18, the pregnant mice were sacrificed. The number of live, dead, resorbing conceptuses and external abnormalities in embryos were recorded. Other groups of pregnant mice, each on gestational day 13, were subcutaneously injected with 600 mg/kg VPA at one of the six times described above. Groups of nonpregnant mice were subcutaneously injected with 900 mg/kg VPA at one of the six times described above. Mice were returned to their home cages after VPA injection. VPA-induced mortality was observed for 24 hr after VPA injection. Dead mice were removed at each observation.

Influence of dosing time on VPA concentrations in plasma, brains and embryos

Groups of 5 pregnant mice on gestational day 7 or 13 each or groups of 5 nonpregnant mice each were subcutaneously injected with 500 mg/kg VPA on two occa-

sions, in the latter half of the light phase (1700) or in the middle of the dark phase (0100). Mice were returned to their home cages after VPA dosing. Blood samples of 300 μl each were drawn by orbital sinus collection using micropipets (Drummond Scientific, Broomall, PA, USA) at 0.5, 2, 4 and 6 hr from the dams or 0.5 and 2 hr from nonpregnant mice after VPA injection. The plasma samples were obtained after centrifugation at 3000 rpm for 10 min (KN-70; Kubota, Tokyo) and then stored at −20℃ until assayed. Brains and embryos were obtained from dams at 0.5, 2, 4, 6 hr after VPA injection. Brains were obtained from nonpregnant mice at 0.5 and 2 hr after VPA injection. Then brain and embryo were stored at −20℃ immediately after completing dissection and weighing.

Statistical analyses

Analysis of variance (ANOVA) and Schéffe's test were used for the statistical analysis of VPA concentration. The χ^2-test for k independent samples and two independent samples were used for the mortality. A probability level of <0.05 was considered to be significant.

RESULTS

Circadian rhythm of mortality in embryos and pregnant and nonpregnant mice

A significant circadian rhythm was demonstrated for the mortality of day-18 embryos after an injection of VPA (550 mg/kg, s.c.) to pregnant mice on gestational day 7 or 13 ($P<0.05$, $P<0.01$ respectively, Fig. 1). The highest mortality was found in mice injected with the drug at 1700, toward the latter half of the light phase. The lowest was found in mice injected with the drug at 0100, the middark phase. Mice injected with the drug on gestational day 13 showed a significantly higher value in the mortality of day-18 embryos as compared with mice injected with the drug on gestational day 7 irrespective of dosing time ($P<0.01$, respectively, Fig. 1). Although no exencephaly was found in mice injected with the drug on gestational day 13, about 10% of the total embryos in mice injected with the drug on gestational day 7 showed exencephaly.

There was a significant circadian rhythm in the mortality of dams after an injection of VPA (600 mg/kg, s.c.) to pregnant mice on gestational day 13 ($P<0.01$, Fig. 2). The mortality was found only in the mice injected with the drug at 1700.

A significant circadian rhythm was demonstrated for the mortality of nonpregnant mice after an injection of VPA (900 mg/kg, s.c.) ($P<0.01$, Fig. 3). The highest mortality was found in mice injected with the drug at 1700, whereas the lowest was found in mice injected with the drug at 0900 and at 0100.

Influence of dosing time on VPA concentrations in embryo

No significant difference of VPA concentrations in embryos at 0.5, 2, 4 and 6 hr after injection of VPA at 500

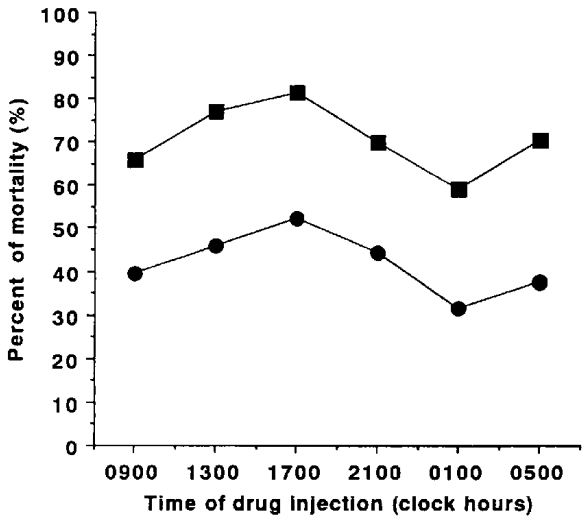

Fig. 1. Circadian rhythm of mortality in day-18 embryo after an injection of VPA (550 mg/kg, s.c.) to pregnant mice on gestational days 7 and 13. Results shown are each from 10 observations of dams. ●: injection on day 7, ■: injection on day 13. A significant circadian rhythm was demonstrated (day-7 group: $P<0.05$, day-13 group: $P<0.01$). The value of the day-13 group was significantly different from that of the day-7 group at the level of $P<0.01$ at each dosing time.

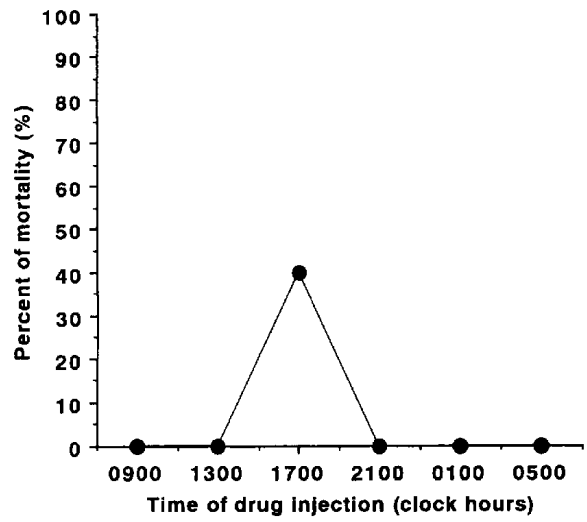

Fig. 2. Circadian rhythm of mortality in dams after an injection of VPA (600 mg/kg, s.c.) to pregnant mice on gestational day 13. Results shown are each from 10 observations. There was a significant circadian rhythm ($P<0.01$).

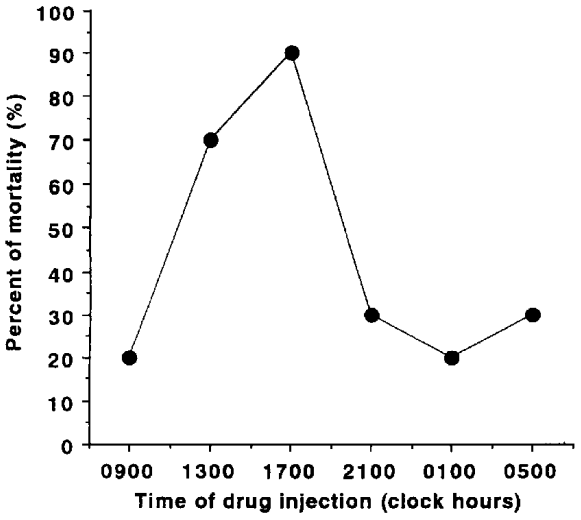

Fig. 3. Circadian rhythm of mortality in nonpregnant mice after an injection of VPA (900 mg/kg, s.c.). Results shown are each from 10 observations. A significant circadian rhythm was demonstrated ($P<0.01$).

mg/kg on gestational day 7 or 13 were demonstrated between injection at 1700 and 0100 (Fig. 4). Mice injected with the drug on gestational day 13 showed a significantly higher VPA concentration in embryos at 2, 4 and 6 hr after injection as compared with mice injected with the drug on gestational day 7 irrespective of dosing time ($P<0.01$, respectively, Fig. 4). VPA concentrations of embryos in mice injected with the drug on gestational day 7 reached a peak within 0.5 hr after injection and then rapidly decreased. On the other hand, those in mice injected with the drug on gestational day 13 increased gradually up to 4 hr after injection and remained at high levels at 6 hr after injection.

Influence of dosing time on VPA concentrations in plasma and brain

In pregnant mice, no significant difference of VPA concentrations in plasma and brain after injection of VPA at 500 mg/kg on gestational day 7 or 13 were demonstrated between injection at 1700 and 0100 (Figs. 5 and 6). Mice injected with the drug on gestational day 13 showed a significantly lower VPA concentration at 0.5 hr (in plasma and brain) and 2 hr (in brain) after injection as compared with mice injected with the drug on gestational day 7 irrespective of the dosing time ($P<0.01$, respectively, Figs. 5 and 6). On the contrary, mice injected with the drug on gestational day 13 showed a significantly higher VPA concentration in the plasma and brain at 4 and 6 hr after injection as compared with mice injected with the drug on gestational day 7 irrespective of the dosing time ($P<0.01$, respectively, Figs. 5 and 6). VPA concentrations in mice injected with the drug on gestational day 7 reached a peak within 0.5 hr after injection and then rapidly decreased. On the other hand, those in mice in-

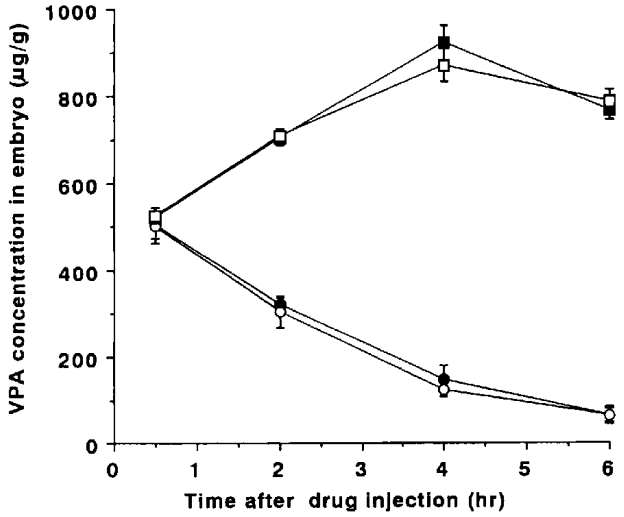

Fig. 4. Influence of dosing time on VPA concentration in embryos after an injection of VPA (500 mg/kg, s.c.) to pregnant mice on gestational days 7 and 13. Each point represents the mean±S.E.M. of twenty mice. ●: injection at 1700 on day 7, ○: injection at 0100 on day 7, ■: injection at 1700 on day 13, □: injection at 0100 on day 13. The value of the day-13 group was significantly different from that of the day-7 group at the level of $P<0.01$ at 2, 4 and 6 hr after injection irrespective of dosing time.

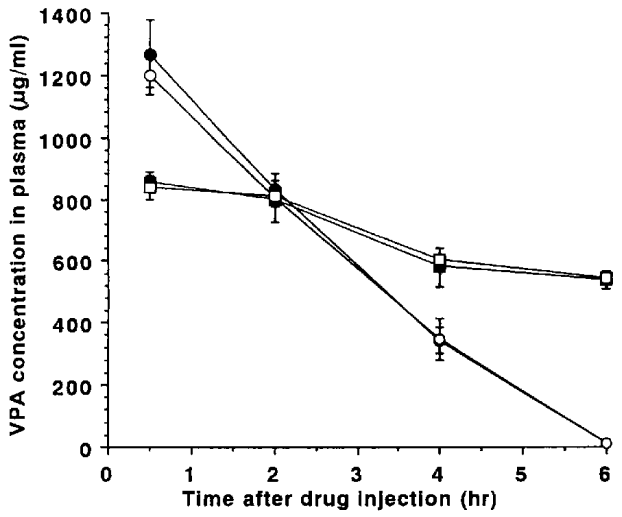

Fig. 5. Influence of dosing time on VPA concentration in plasma after an injection of VPA (500 mg/kg, s.c.) to pregnant mice on gestational days 7 and 13. Each point represents the mean±S.E.M. of five mice. ●: injection at 1700 on day 7, ○: injection at 0100 on day 7, ■: injection at 1700 on day 13, □: injection at 0100 on day 13. The value of the day-13 group was significantly different from that of the day-7 group at the level of $P<0.01$ at 0.5, 4 and 6 hr after injection irrespective of dosing time.

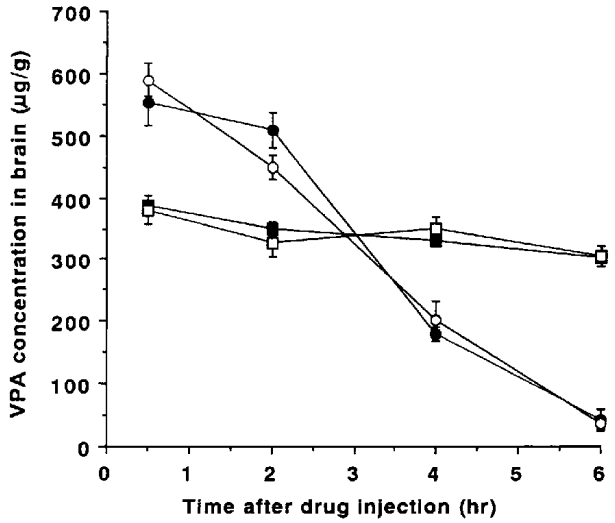

Fig. 6. Influence of dosing time on VPA concentration in brain after an injection of VPA (500 mg/kg, s.c.) to pregnant mice on gestational days 7 and 13. Each point represents the mean±S.E.M. of five mice. ●: injection at 1700 on day 7, ○: injection at 0100 on day 7, ■: injection at 1700 on day 13, □: injection at 0100 on day 13. The value of the day-13 group was significantly different from that of the day-7 group at the level of $P<0.01$ at 0.5, 2, 4 and 6 hr after injection irrespective of dosing time.

jected with the drug on gestational day 13 still remained at high levels at 6 hr after injection. In nonpregnant mice, no significant difference of VPA concentrations in the plasma and brain at 0.5 and 2 hr after injection of VPA at 500 mg/kg was demonstrated between injection at 1700 and 0100 (Table 1).

Table 1. Influence of dosing time on VPA concentration in plasma and brain after an injection of VPA (500 mg/kg, s.c.) to nonpregnant mice

Dosing time (clock hours)	Time after VPA injection 0.5 hr	2 hr
Plasma	(μg/ml)	
1700	1136.0±31.2	854.4±29.8
0100	1100.8±25.0	841.6±41.9
Statistical significance	NS	NS
Brain	(μg/g)	
1700	411.1±10.1	334.4±22.2
0100	400.5±14.5	336.1±17.5
Statistical significance	NS	NS

Each value represents the mean±S.E.M. of five mice.

DISCUSSION

There was a significant circadian rhythm in the mortality of embryos, although no significant dosing time-dependent difference of VPA concentrations in embryos was demonstrated. Therefore, the mechanism underlying the chronotoxicity of embryos seems to be due to the rhythm in the sensitivity of the embryo to the drug. Embryogenesis involves periods of intense biosynthetic activity, including the synthesis of nucleic acids, cytoplasmic proteins, cell surface proteins and lipids. Most of these factors may be affected by circadian oscillation, since there is a circadian rhythm of DNA synthesis in a wide variety of organs (9). The supplement of nutrition from dam to embryo may vary depending on time, since circadian variations in uterine and umbilical blood flow have been reported (10). These could explain why drugs are observed to be severely embryotoxic at one time and mildly toxic at another. Circadian hormonal interactions among the mother, embryo and amniotic fluid have been detected (11). However, it is difficult to separate out the influence of maternal circadian rhythms from varying sensitivity of the embryo. Therefore, it seems reasonable to assume that both maternal and embryonic factors are involved.

A significant circadian rhythm was also demonstrated for the mortality in pregnant mice and nonpregnant mice, although there was no significant dosing time-dependent difference of VPA concentrations in the plasma and brain. Therefore, the rhythm of mortality in both types of mice seems to be due to the rhythm in the sensitivity of living organisms. Since the rhythm of toxicity in nonpregnant mice corresponded to the rhythm of toxicity in the embryo and dam, it might be related to the rhythm of toxicity in both.

The site of VPA action is presumably associated with the inhibitory central nervous system including the γ-aminobutyric acid (GABA) system (12). Although little is known about the effects of an overdose, coma has been rarely noted in patients with VPA. Naloxone has been demonstrated to antagonize the central depressant effect induced by VPA (13) and that by GABA in the brain (14). The rhythm for several biogenic amines in the brain also plays a role in the rhythm in the toxicity of drugs acting on the autonomic and central nervous systems (15). The central inhibitory drugs such as barbiturates are generally known to affect respiration and body temperature besides producing sedation and hypnosis (16). Respiratory and thermoregulatory function show circadian rhythm associated with the autonomic nervous system (17, 18). Therefore, the factors described above may be related to the chronotoxicity induced by VPA in nonpregnant and pregnant mice.

The present study also showed the gestational stage-dependent embryotoxity of VPA. The mechanism may be

determined by the pharmacokinetics of VPA, but also by the sensitivity of living organisms to the drug. First, there was a significant gestational stage-dependent VPA concentration in embryos and pregnant mice. It showed a time course of maternal and embryonal concentrations with an early peak concentration followed by rapid elimination in the mice injected on day 7 and sustained concentrations in the dam injected on day 13 producing a progressive rise in concentrations in the embryo. The sustained release from the third space, embryo and uterus, to the dam may contribute to the differences of concentrations in the dam and embryo between injections on day 7 and day 13 of gestation, because the delay of VPA elimination from the plasma and brain in the dam is closely reflected in the VPA concentration in the embryo. Secondly, the developing mouse embryo was more susceptible to the dysmorphogenic effects of VPA during early organogenesis, whereas the embryo was more sensitive to the embryotoxic effects during late organogenesis. Thus, it is natural that the sensitivity of living organisms to a drug varies depending on the gestational stage, the growth process of the embryo. Therefore, both the sensitivity of living organisms and the pharmacokinetics of drugs may contribute to the mechanism underlying the gestational stage-dependent embryotoxicity of the drug.

The present results suggest that the administration of VPA during pregnancy in mice can induce adverse effects in the embryo that are dependent on both the gestational stage and circadian phase. The circadian rhythm in embryotoxicity seems to be related to the rhythm in the sensitivity to the drug. On the other hand, the gestational stage-dependent embryotoxicity seems to be due to both the sensitivity to the drug and pharmacokinetics of the drug. Further investigations of the mechanisms at the molecular and cellular level are of pharmacological interest. However, to prevent epidemics of birth defects such as the thalidomide disaster, the findings of chronobiological study should be, at least, taken into account when screening new drugs for their teratogenicity. The choice of the most appropriate time of day for drug administration (i.e., the gestational stage and the circadian phase at which the drug is administered) may help to achieve rational chronotherapeutics of some drugs including VPA in certain experimental and clinical situations.

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