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[Original Article]

Fetal Vertebral Anomalies Induced by Calcium Channel Blockers in Rats

Yoshiki BAN and Masao HORIMOTO

The present study aimed at examining skeletal anomalies of fetuses induced by verapamil, calcium channel blocker, in rats, and comparing the skeletal morphological features and locations to those by another calcium channel blocker of diltiazem. Verapamil at 30 mg/kg or diltiazem at 90 mg/kg were given by a single intraperitoneal injection to pregnant rats on gestation day 11, and cesarean section was conducted and fetuses were obtained on gestation day 21. Fetal skeletons were stained with Alizarin red S and Alcian blue, and examined for the skeletal anomalies. Verapamil and diltiazem increased the incidences of fetuses with skeletal anomalies in the thoracic, lumbar, sacral or caudal vertebrae and the types of anomalies included absent, fused, misshapen and/or split shapes. The affected vertebrae by verapamil or diltiazem were observed from late number of thoracic vertebrae to early number of caudal vertebrae. These results indicate that verapamil-induced vertebral skeletal anomalies were similar in morphological features and locations to those by diltiazem, suggesting that the fetal vertebral anomalies might be common by basic pharmacological properties of verapamil and diltiazem in rats.

Key Words: Calcium channel blocker, Diltiazem, Fetus, Rats, Verapamil, Vertebral anomaly

Introduction

Calcium channel blockers (CCBs) are being widely used for the treatment of cardiovascular diseases [1) 2)]. However, CCBs have embryolethality and teratogenic potentials, as indicated by digital, cardiovascular or skeletal anomalies in experimental animals. Nifedipine of the dihydropyridines and diltiazem (DIL) of the benzothiazepines induced embryo/fetal deaths, and digital and skeletal anomalies in rats [3) 4) 5)]. A single intraperitoneal injection of DIL produced stage-specific embryolethality, and skeletal and digital anomalies were observed between gestational days (GD) 11 and 14 in rats [5)]. At 80 mg/kg, there was 100% of resorptions following the single injection on GD 12, and the skeletal or digital anomalies were observed in surviving fetuses following the single injection on GD 11, or GD 13 or 14, respectively. The skeletal anomalies include lumbar, sacral and caudal vertebral anomalies with incidence of 45.6% by treatment of DIL on GD 11.

Verapamil (VER), calcium channel blocker, is another class of the phenylalkylamines, but there have been few reports that indicate VER induced embryolethality and teratogenic potential in rats.

In the present study, we focus on the vertebral skeletal anomalies in fetuses and examine whether VER induce the vertebral anomalies which were similar to those induced by DIL. The basic pharmacological properties of VER and DIL have similarly relevant inhibitory effects on AV node (inhibition of supraventricular tachycardia) [6)]. VER or DIL was given by a single intraperitoneal injection to pregnant rats on GD 11 and the results were compared each other.

To whom all correspondence; Yoshiki BAN
yban@cis.ac.jp
Department of Animal Risk Management, Faculty of Risk and Crisis Management, Chiba Institute of Science
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Materials and Methods

Animals

Female Slc:SD rats at approximately 8 weeks of age were purchased from Japan SCL Inc. (Shizuoka Pref., Japan), and were housed in an animal room where the temperature (22 ± 2°C), the relative humidity (55 ± 10%) and the light and dark cycle (12 hr each) were controlled. They were allowed to have free access to Rodent Chow (Labo MR Standard, Nosan corporation) and tap water. Females at 10 weeks of age were mated overnight with mature male breeders of the same strain. The day on which vaginal plugs and/or sperms were found was designated as GD 0. Mated females were randomly divided into groups of 6 to 7 females in the VER and DIL experiments.

The research protocol, including all experimental procedures

involving animals, was approved by the animal experimentation committee of Chiba Institute of Science (18-20).

Experimental design and compound treatment

Verapamil hydrochloride and diltiazem hydrochloride (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) was dissolved in distilled water before treatment. The dosing volume for all females was 5 mL/kg. VER or DIL was given by a single intraperitoneal injection to 6 to 7 females each on GD 11 at 30 mg/kg, or 90 mg/kg, respectively. The dose levels of VER were determined based on the results of preliminary study (data not shown), and 100 mg/kg was maternal lethal dose (3 of 5 pregnant females were dead). The dose levels of DIL were referred to Ariyuki's report [5]. Seven females each in the control groups in VER and DIL experiments received the distilled water on GD 11 as the same manner as the treatment groups. All females were observed for physical signs on GD 0, and daily from GD 11 to 21. Body weights and food consumption were measured daily from GD 0 to 21. Mated females were cesarean-sectioned on GD 21 and the uterus of each female was examined to determine pregnancy status. Uterine implants were counted and each was classified as a live fetus, dead fetus and resorptions. All live fetuses were euthanized by hypothermia. After the evisceration, fetal skeletons were stained with Alizarin red S and Alcian blue, and examined for the skeletal anomalies.

Statistical analyses

Statistical significance for the numbers of resorptions and dead fetuses/litter, % of postimplantation loss, the number of live fetuses/litter, placental and live fetal weights, and sex ratio between the control and each treatment group was analyzed by Turkey-Kramer test. Wilcoxon test was applied to compare the incidences of skeletal anomalies between the control and each treatment group. The significant levels were 1% or 5% (two-sided).

Results

Maternal examination

No maternal deaths occurred in VER and DIL experiments. Slight decreased activity or cool in touch were observed on the VER or DIL treatment day but were not observed next day and thereafter. Maternal body weights and food consumption were temporally and slightly decreased next day of treatment but recovered thereafter.

Cesarean-section examination

VER experiment: There were increases in % of postimplantation losses (16.9%) at 30 mg/kg. Two litters had high incidence of postimplantation losses (46.7% each) but the incidences of other 4 litters (0% in 3 litters and 8.3% in 1 litter) were similar to controls (7.3% in average). The average number of live fetuses/litter at 30 mg/kg was slightly decreased (11.2 in average, compared to 12.6 in controls). In the live fetuses, there were slight decreases in the average fetal body weights (9% and 12% below controls in males and females, respectively) at 30 mg/kg, although there were no statistical differences. The

Table 1. Cesarean-section Findings

Groups	Verapamil (mg/kg)		Diltiazem (mg/kg)	
	Control (0)	30	Control (0)	90
No. females examined	7	6	7	7
No. non-pregnant females	2	0	0	0
No. pregnant females	5	6	7	7
No. implants/litter	13.8 ± 3.4[a]	13.7 ± 2.2	13.9 ± 2.0	14.0 ± 1.6
No. resorptions and dead fetuses/litter	1.2 ± 1.3	2.5 ± 3.5	0	1.1±1.1[*]
Postimplantation loss/litter (%)[b]	7.3 ± 7.6	16.9 ± 23.2	0	7.8 ± 7.3[*]
No. of live fetuses/litter	12.6 ± 2.3	11.2 ± 3.2	13.9 ± 2.0	12.7 ± 1.3
Live fetal weights/litter (g)				
Males	5.20 ± 0.48	4.73 ± 0.83	5.08 ± 0.26	4.80 ± 0.25
Females	4.98 ± 0.30	4.40 ± 0.72	4.78 ± 0.21	4.58 ± 0.35
Placental weights (g)	0.42 ± 0.01	0.40 ± 0.09	0.40 ± 0.40	0.37 ± 0.04

[a]: Values are given as mean ± SD (based on litter mean).

[b]: (No. of resorptions and dead fetuses/number of implants) x 100

[*]: $p \leq 0.05$

Table 2. Incidence of skeletal anomalies in fetuses

Groups	Verapamil (mg/kg)		Diltiazem (mg/kg)	
	Control (0)	30	Control (0)	90
No. of fetuses examined (no. of maternal animals)	63 (5)	67 (6)	85 (7)	89 (7)
Malformations				
Incidences of fetuses with malformations (%)	1.3 ± 3.0[a]	58.3 ± 46.7[*]	1.0 ± 2.6	69.0 ± 15.6[**]
Cervical vertebrae: split	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 2.6	1.3 ± 3.4
Thoracic vertebrae: absent, fused, misshapen, split	0.0 ± 0.0	18.8 ± 40.1[*]	0.0 ± 0.0	0.0 ± 0.0
Lumbar vertebrae: absent, fused, misshapen, split	0.0 ± 0.0	42.6 ± 42.3[*]	0.0 ± 0.0	44.3 ± 11.6[**]
Sacral vertebrae: absent, fused, misshapen	0.0 ± 0.0	29.5 ± 30.8[*]	0.0 ± 0.0	32.2 ± 22.3[**]
Caudal vertebrae: fused, misshapen	0.0 ± 0.0	13.6 ± 13.9[*]	0.0 ± 0.0	9.5 ± 10.0
Variations				
Incidences of fetuses with variations (%)	1.7 ± 3.7	8.5 ± 4.7[*]	23.1 ± 15.9	15.3 ± 13.1
Bipartite ossification of thoracic vertebrae	1.7 ± 3.7	8.5 ± 4.7[*]	1.5 ± 3.7	9.2 ± 12.1
Short supernumerary rib	0.0 ± 0.0	0.0 ± 0.0	23.1 ± 15.9	6.1 ± 9.3

[a]: Values are given as mean ± SD (based on litter mean).
[*]: $p \leq 0.05$ [**]: $p \leq 0.01$

average placental weights at 30 mg/kg were comparable to those in controls (Table 1).

DIL experiment: There was slight increase in the postimplantation loss (7.8%) at 90 mg/kg. The average number of live fetuses/litter at 90 mg/kg was slightly decreased (12.7 in average, compared to 13.9 in controls). The average fetal body and placental weights at 90 mg/kg were comparable to those in controls (Table 1).

Skeletal Examinations

VER experiment: There were increases in the incidences of fetuses with skeletal malformations in the thoracic, lumbar, sacral and caudal vertebrae including absent, fused, misshapen and/or split shapes at 30 mg/kg. The incidences of the thoracic, lumbar, sacral and caudal vertebral malformations were 18.8%, 42.6%, 29.5% and 13.6%, respectively, compared to controls (0% each). The incidences of fetuses with variations of bipartite ossification of thoracic vertebrae had slightly high (8.5%, compared to 1.7% in controls) at 30 mg/kg (Table 2).

DIL experiment: There were increases in the incidences of fetuses with skeletal malformations in the lumbar, sacral and caudal vertebrae at 90 mg/kg. The vertebral malformations included absent, fused, misshapen and/or split shapes. The incidences in the lumbar, sacral and caudal vertebral malformations at 90 mg/kg were 44.3%, 32.2% and 9.5%, respectively, compared to controls (0% each). The incidences of fetuses with variations of bipartite ossification of thoracic vertebrae had slightly high (9.2%, compared to 1.5% in controls) at 90 mg/kg (Table 2).

Discussion

A single intraperitoneal injection of VER at 30 mg/kg or DIL at 90 mg/kg on GD 11 induced slightly high incidences of postimplantation losses (16.9% and 7.8%, respectively), and the surviving fetuses (11.2 and 12.7/litter in average, respectively) had vertebral anomalies (Table 1). The incidences of fetuses with skeletal malformations by VER or DIL were 58.3% or 69.0%, respectively, and included the absent, fused, misshapen and/or split shapes in the thoracic, lumbar, sacral or caudal vertebrae. VER and DIL also induced skeletal variations of bipartite ossification of thoracic vertebral centrum with incidences of 8.5% and 9.2%, respectively (Table 2). Skeletal malformations of cervical vertebrae and variations of short supernumerary rib were observed in DIL experiment with low incidences of controls and was not considered by DIL (Table 2). Our results in DIL experiment was almost in agreement with that in Ariyuki's report [5]. A single intraperitoneal injection of DIL on GD 11 at 80 mg/kg induced 30.4% of resorptions and skeletal malformations (incidence of 45.6%) in the lumbar, sacral and caudal vertebrae [5].

Figure 1 shows the distribution of number of fetuses with thoracic, lumber, sacral or caudal vertebral malformations in VER and DIL experiments. In VER experiment, the vertebral malformations were distributed in 18 vertebrae from thoracic vertebra number 9 (T9) to caudal vertebra number 3 (C3) with

peak at lumbar vertebral number 5 (L5). In DIL experiment, the vertebral malformations were distributed in 13 vertebrae from lumber vertebra number 1 (L1) to C3, with peak at L6.

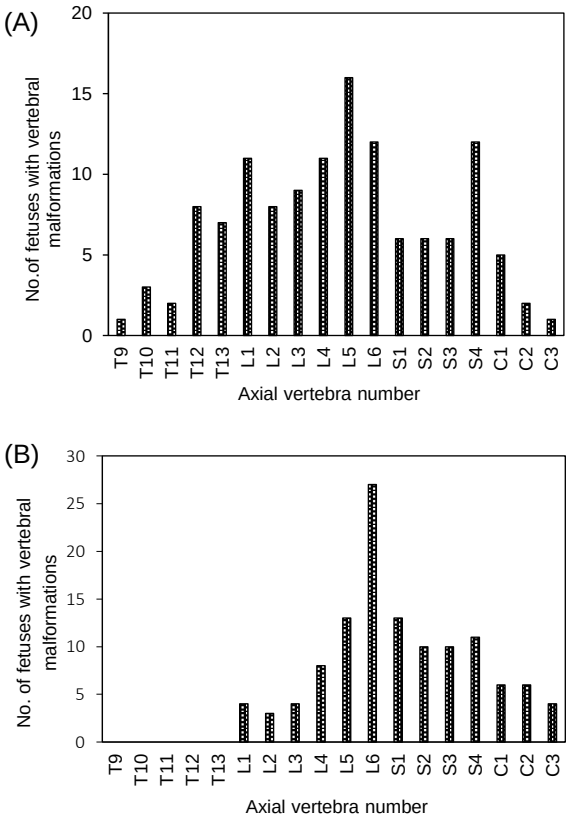

Fig. 1 Distribution of number of fetuses with thoracic, lumber, sacral or caudal vertebral malformations in VER (A) and DIL (B) experiments. T: Thoracic vertebra, L: Lumbar vertebra, S: Sacral vertebra, C: Caudal vertebra

Comparison of distribution pattern of malformed vertebrae induced by VER or DIL shows almost similar but VER also affected to lower thoracic levels from T9 to T13. One of 5 litters at 30 mg/kg of VER had malformed vertebrae between T9 and S4, but another 4 litters had malformed vertebrae between L1 and C3, which were similar distribution pattern to DIL experiment that observed between L1 and C3. This variation among litters in VER experiment may be related to differences of development stage at the timing of treatment of VER or compound retention to developing embryos.

One of possible mechanisms for embryolethality and skeletal anomalies induced by VER or DIL has been thought to be related to circulation defects. Gestational day 11 rat embryos were cultured for 24 hours in the presence of VER or DIL. Reduction of embryonic heart rates, retardations of embryonic growth and morphological abnormalities were observed in dose-dependence manner. The embryotoxicity, including embryolethality and malformations, seen *in vivo* studies might be partly due to circulation defects, and the hypoxic condition resulting from the decreased heart rates thought to be caused for the embryonic morphological changes [7) 8)].

Morphological alterations and inferior growth were attributed to a hypoxic condition induced by reduced embryonic heart rates. It is likely that developing embryonic cells are not supplied enough oxygen due to the decreased heart rates. There are reports that described a negative chronotropic effect during embryogenesis by hypoxia [9) 10)]. It is well established that embryos require oxygen for normal growth and development. As gestation proceeds, there is increased dependence on cellular oxidative phosphorylation to meet energy demands of growth and development [11)]. There are reports that the effects of oxygen concentration on embryonic morphogenesis in cultured rat embryos and hypoxic condition caused cell degeneration and necrosis [12) 13)]. Thus, the hypoxic condition resulting from the decrease of the heart rate might be a cause for the embryonic deaths or morphological changes.

The skeletal defects were characterized by a clear-cut head to tail shift of defects as the developmental stage of induced oxygen deficiency advanced in rats [14)]. In addition to external stage-specific effects, episodes of hypoxia have been reported to cause a variety of internal malformations and skeletal defects in mice, rats, and rabbits [10) 15)]. Considering that developing embryos exposed to VER or DIL induced hypoxic condition resulting from decreased heart rates or circulation defects in embryos [7) 8)], skeletal anomalies observed in the present study might be related to the hypoxic conditions by VER or DIL. Bradycardia and arrhythmia induced by phenytoin resulted in severe embryonic hypoxia during the IKr susceptible period, supporting the idea of an IKr-arrhythmia-hypoxia–related teratogenic mechanism. Treatment of phenytoin resulted in strong immunostaining for hypoxia marker in embryonic tissues on GD 10 and marked increase in hypoxia staining with increasing phenytoin dose at this stage of embryonic development [16)]. Stage specific skeletal defects induced by almokalant (potent IKr-blockers) were reported in rats [17)]. The skeletal defects were observed on the thoracic and upper lumbar level, on lower thoracic and lumbar level, and on the sacral and caudal level following a single treatment of this compound on GD 10, 11 and 12, respectively, and the types of malformation were absent or partial ossification of vertebral centra and arches. Thus, the skeletal defects were characterized by head to tail shift of defects as developmental stage. Although our experiments were designated by a single treatment on GD 11, this stage of development around GD 11 seems to be induced lower thoracic to upper caudal vertebrae. The ranges or variations of affected sites

in individual fetuses affected may be related to compound retention to developing individual embryos at timing of treatment.

In conclusion, VER induced the vertebral skeletal anomalies following a single intraperitoneal injection on GD 11 at dose levels of embryolethality, and were similar in morphological features and locations to those by DIL, suggesting that the skeletal anomalies might be common by basic pharmacological properties of VER and DIL in rats.

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