

A Calcium Agonist, Bay k 8644, Suppresses the Embryotoxic Effects Induced by Dihydropyridines Calcium Channel Blockers in Cultured Rat Embryos

Yoshiki BAN and Takashi MAKITA

Department of Veterinary Anatomy, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan

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ABSTRACT. Day 9 rat embryos were exposed to 1,4-dihydropyridine calcium channel blockers; nifedipine (NIF), nicardipine (NIC) or nitrendipine (NIT), for 48 hr in the whole embryo culture system. There were dose-dependent growth retardation and abnormalities, predominantly in cardiovascular system. The three compounds exhibited very similar pattern of dysmorphogenic effects, but the potency of these compounds were quantitatively different. The incidences of embryos with the abnormalities were 100%, 100% and 85% following either exposure of NIF, NIC or NIT at concentration of 300, 8 and 15 μ M, respectively. This study was to investigate whether these blocker-induced embryotoxicity was due to calcium channel blocking properties themselves in the embryos. Day 9 rat embryos were co-exposed to 1,4-dihydropyridine calcium channel agonist, Bay k 8644 (BAY) and each calcium channel blocker under the same culture condition. The retarded embryonic growth induced by 200 or 300 μ M of NIF, 8 μ M of NIC and 15 μ M of NIT nearly or completely ameliorated when embryos were co-exposed with BAY at one-third or half concentration of each calcium channel blocker. Supplementation of BAY reduced the incidence of abnormalities by NIF-, NIC- and NIT-alone. These results suggested that one of mechanisms for embryotoxicity induced by calcium channel blocker was directly related to channel blocking property of the chemicals.

— KEY WORDS: Bay k 8644, calcium channel blocker, culture, embryo, rat.

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Calcium channel blockers [10, 24] are being widely used for the treatment of cardiovascular diseases. However, some calcium channel blockers show prenatal effects in rats [1, 8, 21, 22, 25]. In our previous study [2], nifedipine (NIF), diltiazem (DIL) and verapamil (VER) caused dose-dependent reductions in embryonic heart rates, growth retardation and morphological abnormalities which were related to circulation defects, when day 11 rat embryos were cultured for 20 hr with these chemicals. However, no such the abnormalities were observed following co-exposure of each calcium channel blocker with Bay k 8644 (BAY), a calcium channel agonist, indicating that one of mechanisms for embryotoxicity induced by calcium channel blockers was directly related to the calcium channel blocking properties, resulting in subsequent death and dysmorphogenesis in developing rats [3].

Embryonic mortality increased following treatment of NIF during the organogenesis in rats, and the critical period was determined between gestational days 7 and 10 [8]. A single treatment of DIL produced stage-specific embryotoxicity, and significant increases in embryonic deaths and morphological defects between gestational days 11 and 14 were reported in rats [1]. We examined that critical period for embryotoxicity in rats was determined as gestational days 11 and thereafter, when a single treatment of VER was made to rats on various gestational days. Thus the critical period for embryotoxicity by NIF is different from those of DIL and VER. The aim of the present study was not only to evaluate the direct effects of NIF on cultured rat embryos during the critical period for embryotoxicity but to investigate the role of pharmacological action in the embryotoxic outcomes. Nicardipine (NIC) and nitrendipine

(NIT) were also investigated whether the observed embryotoxicities were basically class effects for dihydropyridines calcium channel blockers. The whole embryo culture system [13] was employed since the method allows observation of the direct effects of drug on the embryonic morphogenesis, and it also eliminates maternal factors as well as environmental influences [5]. Embryos were co-exposed to either NIF, NIC or NIT and BAY, and examined whether the embryotoxic effects of each calcium channel blocker are suppressed. BAY has positive inotropic and vasoconstrictor actions through the activation of voltage-dependent calcium channel in contrast to the negative inotropic and vasodilator effects of NIF, even though BAY belongs to the dihydropyridines and is similar in structure to NIF [19, 20].

MATERIALS AND METHODS

Animals: Sprague-Dawley rats of the Crj; CD(SD) strain at approximately 11 weeks of age purchased from Charles River Japan Inc. were kept in an animal room where the temperature ($22 \pm 2^\circ\text{C}$), the relative humidity ($55 \pm 10\%$) and the light and dark cycle (12 hr each) were controlled. They were allowed to have free access to Purina Rodent Chow and tap water. Females were mated overnight with mature male breeders of the same strain. The day on which a vaginal plugs were found was designated as gestational day (GD) 0.

Embryo culture: Females were euthanized by carbon dioxide asphyxiation on GD 9. The uterus was excised and all individual implantation sites were placed in a sterile dish containing Hank's balanced salt solution maintained at 38°C .

The decidua was dissected and the Reichert's membrane was removed from visceral yolk sac under a dissecting microscope. Two embryos were placed in a glass culture bottle that contained 2 ml male rat serum and 1 ml Hank's balanced salt solution, and then cultured for 48 hr at 38°C. Rat serum was obtained from immediately centrifuged blood and was heat-inactivated at 56°C for 30 min. Culture bottles containing the embryos and media were rotated at 20 rpm and gassed with water-saturated mixtures of 5% O₂, 5% CO₂ and 90% N₂ for the first 17 hr, 20% O₂, 5% CO₂ and 75% N₂ for the next 9 hr, and 40% O₂, 5% CO₂ and 55% N₂ for the last 22 hr. At termination of the culture, the yolk sac diameter, head length and crown-rump length were measured using a micrometer disc in a focusing eyepiece. The number of somites was counted. Each embryo was examined for morphological abnormalities and the morphological score [4] was applied to assess the embryonic development.

Exposure to chemicals: NIF, NIC, NIT and BAY were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries). Embryos in control groups were exposed to DMSO (maximum concentration of 0.27%, v/v) in the same regimen as the chemical exposure groups. The control vehicles had no influence on embryonic growth and the gross morphology under the current culture condition. In Experiment-1, embryos were exposed to NIF, NIC or NIT at final concentration of 100, 200 and 300 µM, 4, 6 and 8 µM, and 5, 10 and 15 µM, respectively in order to examine for dose-dependent embryotoxicity by each calcium channel blocker. High doses for each calcium channel blocker were determined as expected minimum doses to produce 100% effect on morphogenesis under current culture

condition based on the results from preliminary studies. All embryos were dead following exposure of 400 µM of NIF, 20 µM of NIC or 100 µM of NIT in the preliminary studies. In Experiment-2, each calcium channel blocker was simultaneously exposed with BAY in the media to investigate the suppressive effect of BAY on embryotoxicity of each calcium channel blocker. The following co-exposure groups were selected; (i) NIF, 200 µM, and BAY, 100 µM, (ii) NIF, 300 µM, and BAY, 100 µM, (iii) NIC, 8 µM, and BAY, 4 µM, (iv) NIT, 15 µM, and BAY, 7.5 µM. In addition to the co-exposure groups, DMSO control, the corresponding BAY or calcium channel blocker alone groups were examined in each study.

Statistics: Statistical significance between chemical-exposure and the control groups on morphological parameters was determined based on an analysis of variance using a least significant difference (LSD test) procedure. Non-parametric data for percentage of embryos with abnormalities between them were analyzed using a Chi-square test. To evaluate whether BAY ameliorates the effect of calcium channel blocker, the differences between each calcium channel blocker alone and the corresponding BAY co-exposure groups were analyzed using the same statistical methods. Significant differences were determined at a confidence level of $p \leq 0.05$.

RESULTS

Experiment-1: The effect of each calcium channel blocker on growth parameters and morphogenesis is given in Table 1. There were dose-dependent growth retardation after exposure of NIF, NIC or NIT. Significant ($p \leq 0.05$)

Table 1. Morphological data (mean \pm standard error) and incidences of abnormalities following *in vitro* exposure of nifedipine, nicardipine and nitrendipine from gestational days 9 to 11

Groups	Control	Nifedipine			Nicardipine			Nitrendipine		
		100 µM	200 µM	300 µM	4 µM	6 µM	8 µM	5 µM	10 µM	15 µM
No. of embryos examined	40	20	20	20	20	20	20	20	20	20
Yolk sac diameter (mm)	4.39 \pm 0.04	4.41 \pm 0.09	4.34 \pm 0.05	4.20 \pm 0.06 ^{b)}	4.45 \pm 0.05	4.21 \pm 0.06 ^{b)}	4.13 \pm 0.05 ^{b)}	4.31 \pm 0.06	4.20 \pm 0.07 ^{b)}	4.13 \pm 0.05 ^{b)}
Head length (mm)	1.94 \pm 0.02	1.92 \pm 0.06	1.57 \pm 1.57 ^{b)}	0.96 \pm 0.05 ^{b)}	1.87 \pm 0.03	1.53 \pm 0.08 ^{b)}	1.11 \pm 0.07 ^{b)}	1.87 \pm 0.03	1.53 \pm 0.08 ^{b)}	1.34 \pm 0.08 ^{b)}
Crown-rump length (mm)	3.78 \pm 0.03	3.88 \pm 0.08	3.29 \pm 0.15 ^{b)}	2.42 \pm 0.07 ^{b)}	3.77 \pm 0.04	3.35 \pm 0.12 ^{b)}	2.81 \pm 0.10 ^{b)}	3.72 \pm 0.06	3.27 \pm 0.12 ^{b)}	2.96 \pm 0.11 ^{b)}
No. of somites	25.6 \pm 0.2	25.2 \pm 0.3	23.8 \pm 0.6 ^{b)}	20.7 \pm 0.6 ^{b)}	25.4 \pm 0.3	24.0 \pm 0.4 ^{b)}	20.8 \pm 0.5 ^{b)}	25.4 \pm 0.3	23.5 \pm 0.6 ^{b)}	21.7 \pm 0.6 ^{b)}
Morphological score	39.5 \pm 0.1	38.8 \pm 0.5	35.7 \pm 1.1 ^{b)}	29.9 \pm 0.5 ^{b)}	39.5 \pm 0.1	37.2 \pm 0.8 ^{b)}	31.6 \pm 0.6 ^{b)}	39.5 \pm 0.1	36.5 \pm 0.9 ^{b)}	33.9 \pm 1.0 ^{b)}
No. (%) ^{a)} of embryos with abnormalities	2 (5)	3 (15)	11 (55) ^{b)}	20 (100) ^{b)}	1 (5)	11 (55) ^{b)}	20 (100) ^{b)}	2 (10)	10 (50) ^{b)}	17 (85) ^{b)}
No. (%) of embryos with:										
Altered yolk sac circulation	0	2 (10)	11 (55)	20 (100)	0	8 (40)	18 (90)	0	8 (40)	17 (85)
Altered umbilical vessel circulation	0	0	8 (40)	20 (100)	1 (5)	9 (45)	17 (85)	0	8 (40)	16 (80)
Failure of turning	2 (5)	1 (5)	1 (5)	0	0	0	0	2 (10)	0	0
Enlarged cardiac tube	0	0	2 (10)	10 (50)	0	0	3 (15)	0	2 (10)	4 (20)
Enlarged pericardium	0	0	1 (5)	9 (45)	0	3 (15)	10 (50)	0	5 (25)	3 (15)
Open posterior neuropore	0	0	0	0	0	0	1 (5)	0	1 (5)	0
Craniofacial abnormalities	0	0	3 (15)	10 (50)	0	2 (10)	11 (55)	0	7 (35)	12 (60)
Irregular neural tube	0	0	3 (15)	14 (70)	0	6 (30)	15 (75)	0	4 (20)	9 (45)
Hyperemia	0	0	0	1 (5)	0	2 (10)	1 (5)	0	1 (5)	2 (10)

a) (No. embryos with morphological abnormalities/no. embryos examined) \times 100.

b) Statistically significant ($p \leq 0.05$) from control group.

decreases in yolk sac diameter, head length, crown-rump length, number of somites and/or morphological score were observed in a concentration dependent manner. The incidences of embryos with abnormalities were dose-dependently increased ($p \leq 0.05$) in groups exposed to 200 and 300 μM of NIF, 6 and 8 μM of NIC, and 10 and 15 μM of NIT. Types of morphological abnormalities produced were predominantly altered circulation; the surface of yolk sac was irregular, and the vitelline and umbilical vessels were not prominent (Fig. 1-B). Some embryos showed enlargement of pericardium and cardiac tube, craniofacial abnormalities (irregular shape and/or small size), and irregular neural tube (Fig. 1-E).

Experiment-2: Table 2 shows the results of embryonic growth parameters and the incidence of morphological abnormalities following either exposure of calcium channel blocker alone, BAY alone, or co-exposure of calcium channel blocker and BAY. There were no treatment-related growth retardation and morphological abnormalities at concentration of 4, 7.5 and 100 μM of BAY. The embryonic growth in the group co-exposed to 200 μM of NIF and 100 μM of BAY was comparable to that of the control group. There were slight but significant ($p \leq 0.05$) decreases in embryonic growth parameters following co-exposure of 300 μM of NIF and 100 μM of BAY. The incidences of abnormalities in 200 and 300 μM of NIF groups were significantly ($p \leq 0.05$) reduced from 67 to 12% and from 97 to 69%, respectively by co-exposure of 100 μM of BAY. Supplementation of BAY nearly or completely ameliorated the growth retardation and morphological abnormalities

induced by NIC- and NIT-alone, respectively. The embryonic growth was slightly delayed and the incidence of abnormalities were very low (12.5%) in a combination of 8 μM of NIC and 4 μM of BAY. There were no significant ($p > 0.05$) differences from controls in embryonic growth parameters, and no morphological abnormalities were observed in the group co-exposed to 7.5 μM of BAY and 15 μM of NIT (Fig. 1-C and 1-F).

DISCUSSION

When rat embryos were cultured from GD 9 for 48 hr, NIF, NIC and NIT obviously elicited dose-dependently growth retardation and morphological abnormalities. These three compounds exhibited very similar pattern of dysmorphogenic effects, although NIF was less potent than others under the current exposure conditions. The dysmorphogenic features predominantly consisted of alteration in the yolk sac and embryonic circulation, enlargement of the cardiac tube or pericardium, craniofacial abnormalities and irregular neural tube. Stein *et al.* [23] examined embryotoxicities of six calcium channel blockers, including NIF and NIT, and suggested that breakdown of yolk sac circulation may drastically affect embryonic development.

In vivo, the yolk sac of the rat embryo acquires a blood circulation and probably becomes an important organ of O_2/CO_2 exchange at about the 10-somite stage (10.5-days). The allantoic placenta acquires a blood circulation at about the 17-somite stage (11-days), and becomes a second organ of

Table 2. Morphological data (mean \pm standard error) and incidences of abnormalities following a single exposure of Bay k 8644 or calcium channel blockers and simultaneous exposures of Bay k 8644 and calcium channel blockers from gestational days 9 to 11

Groups	No. of embryos examined	Yolk sac diameter (mm)	Head length (mm)	Crown-rump length (mm)	No. of somites	Morphological score	% embryos with abnormalities ^{a)}
Control	30	4.37 \pm 0.05	1.97 \pm 0.02	3.87 \pm 0.04	25.1 \pm 0.2	39.4 \pm 0.2	10.0
BAY (100) ^{b)}	30	4.37 \pm 0.05	1.85 \pm 0.03	3.79 \pm 0.04	25.5 \pm 0.2	39.5 \pm 0.1	3.3
NIF (200)	36	4.39 \pm 0.05	1.60 \pm 0.06 ^{c)}	3.42 \pm 0.09 ^{c)}	23.4 \pm 0.4 ^{c)}	36.3 \pm 0.7 ^{c)}	66.7 ^{c)}
NIF (300)	30	4.31 \pm 0.05	0.99 \pm 0.04 ^{c)}	2.62 \pm 0.06 ^{c)}	20.0 \pm 0.3 ^{c)}	30.0 \pm 0.4 ^{c)}	96.7 ^{c)}
NIF (200)+BAY (100)	34	4.47 \pm 0.04	1.88 \pm 0.03 ^{d)}	3.84 \pm 0.04 ^{d)}	25.3 \pm 0.2 ^{d)}	39.5 \pm 0.1 ^{d)}	11.8 ^{d)}
NIF (300)+BAY (100)	36	4.23 \pm 0.05 ^{c)}	1.71 \pm 0.03 ^{c)d)}	3.56 \pm 0.06 ^{c)d)}	24.0 \pm 0.3 ^{c)d)}	37.5 \pm 0.5 ^{c)d)}	69.4 ^{c)d)}
Control	32	4.39 \pm 0.04	1.91 \pm 0.03	3.85 \pm 0.03	25.2 \pm 0.2	39.2 \pm 0.1	3.1
BAY (4)	32	4.47 \pm 0.05	1.96 \pm 0.03	3.91 \pm 0.04	25.4 \pm 0.2	39.3 \pm 0.1	6.3
NIC (8)	30	4.17 \pm 0.05 ^{c)}	1.10 \pm 0.04 ^{c)}	2.85 \pm 0.05 ^{c)}	20.3 \pm 0.4 ^{c)}	31.5 \pm 0.4 ^{c)}	100 ^{c)}
NIC (8)+BAY (4)	32	4.36 \pm 0.04 ^{c)}	1.79 \pm 0.04 ^{c)e)}	3.70 \pm 0.05 ^{c)e)}	24.5 \pm 0.2 ^{c)e)}	38.8 \pm 0.3 ^{c)}	12.5 ^{c)}
Control	34	4.40 \pm 0.04	1.88 \pm 0.02	3.80 \pm 0.04	25.9 \pm 0.1	39.5 \pm 0.1	5.9
BAY (7.5)	32	4.39 \pm 0.04	1.88 \pm 0.02	3.79 \pm 0.03	25.9 \pm 0.2	39.5 \pm 0.1	0
NIT (15)	34	4.14 \pm 0.04 ^{c)}	1.26 \pm 0.06 ^{c)}	2.89 \pm 0.08 ^{c)}	22.0 \pm 0.4 ^{c)}	33.6 \pm 0.6 ^{c)}	85.3 ^{c)}
NIT (15)+BAY (7.5)	34	4.46 \pm 0.04 ^{f)}	1.89 \pm 0.02 ^{f)}	3.84 \pm 0.03 ^{f)}	25.9 \pm 0.2 ^{f)}	39.5 \pm 0.1 ^{f)}	0 ^{f)}

Note. Abbreviations: BAY, Bay k 8644; NIF, nifedipine; NIC, nicardipine; NIT, nitrendipine.

a) (No. embryos with morphological abnormalities/no. embryos examined) \times 100.

b) μM .

c) Statistically significant ($p \leq 0.05$) from the corresponding control group.

d) Statistically significant ($p \leq 0.05$) from the corresponding nifedipine alone group.

e) Statistically significant ($p \leq 0.05$) from nicardipine alone group.

f) Statistically significant ($p \leq 0.05$) from nitrendipine alone group.

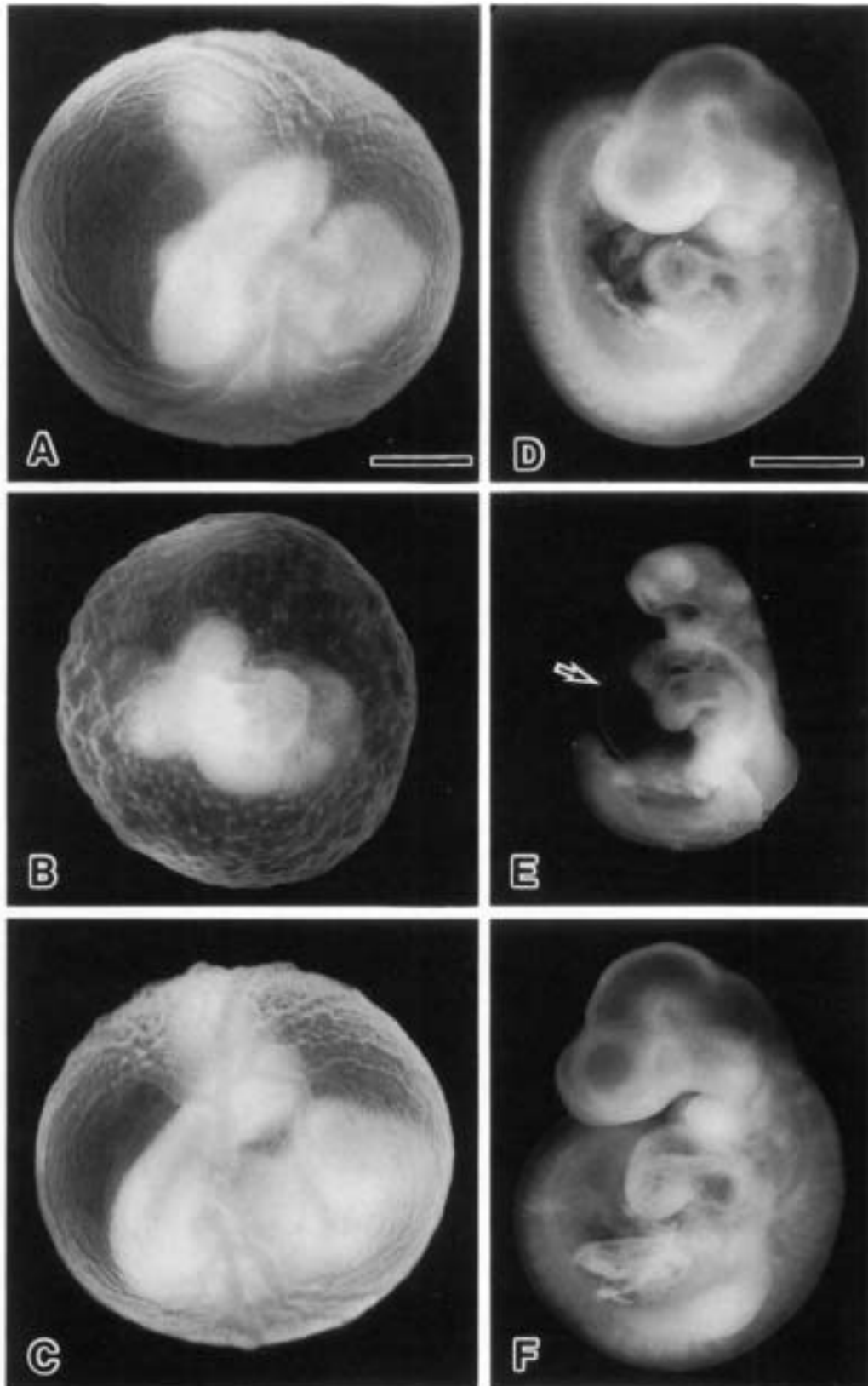


Fig. 1. Appearance of embryos cultured for 48 hr from gestational day 9: A; Control embryo with intact yolk sac: B; Yolk sac exposed to 15 μ M nitrendipine alone, showing the surface of yolk sac is irregular and the vitelline vessels are not prominent: C; Yolk sac co-exposed to 15 μ M nitrendipine and 7.5 μ M Bay K 8644,

respiratory exchange, taking over an increasing share as gestation continues. The yolk sac, but the allantoic placenta may be functional, and oxygen reach the embryo by direct diffusion or via the yolk sac blood circulation *in vitro* [13–15]. Osmoregulatory disturbances and hypervolemia produced by moderate hypoxia resulted in abnormal development [9]. Miki *et al.* [12] demonstrated that hypoxic conditions in cultured rat embryos caused retardation of embryonic growth and abnormal development, and the changes were due to cellular degeneration and necrosis in the neural tube, the somites and the mesenchyme. The yolk sac in culture is mainly responsible for the nutrition of cultured embryos [6, 7]. Chemically induced yolk sac dysfunction produced inhibition of histiotrophic nutrition and affected the embryonic growth and development [11, 16, 17]. Not all the embryonic growth retardation and dysmorphogenesis might not be attributable to the altered circulation, but all embryos with the abnormal development had altered circulation in this study. At low concentration of NIF, only altered yolk sac circulation without any other treatment-related abnormalities was evident. The embryonic alterations by calcium channel blockers may be caused by the resulting malnutrition related with disturbed yolk sac function.

The retarded embryonic growth, induced by NIF, NIC or NIT, was nearly or completely ameliorated when embryos were co-exposed with BAY at one-third or half concentration of each calcium channel blocker. Supplementation of BAY also reduced the incidence of abnormalities by NIF-, NIC- and NIT-alone. However, an addition of BAY at 100 μ M did not completely ameliorate the abnormal development seen at 300 μ M of NIF-alone. This explains that a high dose of NIF was more embryotoxic than those of NIC and NIT. Compared to the result of embryonic growth parameters at 8 μ M of NIC or 15 μ M of NIT, those of NIF at 300 μ M were more affected. BAY has a positive inotropic action and enhances the contractile state through the activation of voltage-dependent calcium channels. The positive chronotropic and inotropic effects of BAY were inhibited by NIF, and there is a strong structural similarity and a competitive antagonism between NIF and BAY in the isolated perfused guinea pig heart [19, 20]. Similar pharmacological responses were observed in cultured rat embryos in this experiment, suggesting that the effects were due to competitive antagonism between calcium channel blocker and BAY in the embryos. The calcium channel currents of the cardiomyocytes were present on a 12-day-old rat embryo [18], but the presence remains to be established prior to day 12. Our results indirectly suggested that functional calcium channels of embryos may be established in this stage of development.

In conclusion, these results suggested that one of

mechanisms for embryotoxicity induced by calcium channel blockers was directly related to pharmacological properties of the chemicals.

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showing normal blood circulation: D; Control embryo: E; Embryo exposed to 15 μ M nitrendipine alone, showing growth retardation, enlargement of pericardium (arrow): F; Yolk sac co-exposed to 15 μ M nitrendipine and 7.5 μ M Bay K 8644, showing identical growth with control embryo without any morphological abnormalities. Bar = 1 mm.

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