AATEX 3, 17–27 (1995)

Cardiovascular Malformations Induced by Caffeine and Phenobarbital in Chick Embryos

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Summary

The usage of chick embryos for studying the mechanism of cardiovascular malformations caused by chemicals was studied. Caffeine (CA), well known as a teratogen for the cardiovascular system of chick embryos, was administered at the dose of 3.0 mg per egg to chick embryos from two different breeds between incubation day 2 (ID2, H-H stage 14) and 5 (H-H stage 27). The eggs were incubated until ID12 to examine the cardiovascular malformation. Treatment at H-H stage 19 (ID3) was highly lethal and treatment at this stage and at stages 23–24 (ID4) induced a high degree of cardiovascular malformation in the embryos from both breeds. The embryotoxicity caused by CA in the two different breeds similarly varied with the developmental stage of treatment. Phenobarbital (PB), also a teratogen, was administered to chick embryos during H-H stages 22–24 at the doses of 1.13, 2.25 and 4.5 mg/egg to compare its embryotoxicity with that caused by treatment with CA. PB at the dose of 2.25 mg/egg or more was highly lethal and induced cardiovascular malformation. Administration of 13 μg of adenosine (AD) before CA and PB treatment reduced the lethality of PB but the effects of CA and teratogenicity of PB were not changed. However, in cultured cardiac cells at H-H stages 23–24 the frequency of spontaneous cell beating increased by treatment with 5 and 10 μM CA and decreased by that with 20 μM CA and those effects were reduced by co-administration of 0.5 μM AD. PB at the concentration of 6 μM or more reduced the frequency of cell beating and its effect was not changed by co-administration of 0.5 μM AD. Th responses to CA and PB treatment in whole chick embryos and cultured cardiac cells were different. We believe that chick embryos can produce reproducible data when the developmental stage is precisely judged and the interactions of some chemicals on whole embryos and selected tissue or cells can be easily examined, so that they are very convenient to explore the mechanism of teratogenicity of chemicals.

Introduction

Chick embryos have been studied to determine their value as a screening material for embryotoxicity (1), and generally show high sensitivity and respond more than rodents (2). However, chick embryos have not been accepted as a standard tool for developmental toxicity potential testing of chemicals, because the chick embryo is not a mammal and has not been methodologically established for use in embryotoxicity screening tests, for example, the method of application similar to that intended for human usage, the age of embryos, the difference of sensitivity between breeds and the sufficient number of embryos to allow meaningful interpretation of the data. These problems have been studied (3–6), but have not been resolved.

The development of chick embryos has been extensively studied (7) and these embryos are regarded as very convenient material for teratological testing, the easy access to embryos being especially important. The developing cardiovascular system of the chick embryo has been well described (8, 9) and frequently used to study cardiovascular malformation induced by various chemicals (10–15) and by mechanical interference with blood flow patterns (16–19) during early morphogenesis. These studies indicate that chick embryos are very convenient material to examine the pathogenesis of cardiovascular malformation.

In this study, we examined the usage of chick embryos for pathogenetic studies in cardiovascular malformation caused by chemicals. We used caffeine (CA) and phenobarbital (PB), because they are known to induce cardiovascular malformation in mice (20), rats (21) and chick embryos (22) and because the effects of CA on the cardiovascular function of chick embryos have been reported (23). We examined the embryotoxicity of CA and the critical stage of lethality and teratogenicity by treatment at between ID3 (H-H stage 14) and ID5 (H-H stage 27) in two different breeds. One breed had been established as an experimental animal, White Leghorn, and the other was established not for experimental materials but for poultry, an incrossbred from a local farm. Then the differences in embryotoxicity of CA and PB in chick embryos were examined in ovo and the effects of CA and PB on cultured embryonic cardiac cells were examined to explore the pathogenesis of cardiovascular malformation caused by those chemicals.

Materials and Methods

Experiment I

Fertilized chicken eggs were obtained from two sources; one was White Leghorn, which has been established as an SPF experimental animal, from Nippon Institute for Biological Science (NIBS, tokyo), and the other was an incrossbred, consisting mainly of the Hy-Line breed from a local farm. In total, we used 607 fertile eggs from NIBS and 306 from the local farm. Eggs obtained from both sources were incubated at 38 °C in a highly humidified atmosphere. On ID2, after 4 ml of albumen was extracted with a sterilized needle and syringe, a window was made in the shell to facilitate latr treatments. It was then sealed with cellophane tape under sterile conditions. All embryos were staged according to Hamburger and Hamilton (24) at the time of treatment.

In preliminary experiments, chick embryos from the local farm were treated with 1, 2.5, 5, or 10 mg of CA/egg at H-H stages 19–21 (ID3) by the same method described later. Viability was checked daily and the heart and great vessels were observed under stereomicroscope on ID10. Treatment with 5 mg or more of CA/egg was highly lethal, and treatment with 2.5 mg or less was not sufficient to induce a high degree of caridovascular malformation. We therefore selected 3 mg of CA/egg as the dose to cause cardiovascular malformations in chick embryos.

Between H-H stage 14 (ID2) and 27 (ID5), 32–47 embryos per stage from NIBS and 13–26 per stage from a local farm were treated with a single dose of CA (Sigma Co., St. Louis, MO). To the surface of the exposed vitelline membrane in a volume of 0.2 ml warmed at 38 °C was applied 3 mg of CA dissolved in saline, using a Hamilton syringe through the window in the shell. The eggs were then sealed and incubated again until ID12 at which stage the cardiovascular system is usually established in chick embryos. Control embryos from both breeds were treated with 0.2 ml of saline at H-H stage 19 (ID3).

On ID12, embryos were extracted from the shell and perfused with saline and 10% formalin in a neutral buffer through the left ventricle by a 24 G needle; subsequently they were fixed in Bouin's solution for more than a week. After external examination of the fixed

embryos, the great vessels were observed by thoracolaparotomy. The heart was removed and microdissected to examine for intracardiac malformation.

Experiment II

Fresh fertile eggs, obtained from the same local farm as for experiment I, were used.

To compare the teratogenic effects of CA and PB on cardiovascular organogenesis in the chick, 3 mg of CA/egg and 1.13, 2.25, or 4.5 mg of PB (Maruishi Pharmachemical Co., Ltd., Tokyo) per egg was administered to chick embryos at a stage between H-H stage 22–24 (ID3 or 4) at which treatment with 3 mg of CA showed lower lethality and greater teratogenic effects on the cardiovascular system of chick embryos than treatment at other developmental stages in experiment I. The dose of PB was determined by reference fo previous data (25). Embryos were administered either chemical and examined for cardiovascular malformation by the same method as that described in experiment I. Some embryos were treated with 13 μg of AD (Wako Pure Chemical Industries, Ltd., Osaka) per egg before either CA or PB treatment to examine alteration of the effects of CA and PB on cardiovascular morphogenesis. The dose of AD was selected as the dose expected to reduce the embryotoxicity of 3.0 mg of CA per egg (26).

Experiment III

To determine the effects of CA and PB on the embryonic cardiac cell beating *in vitro*, cardiac cells were prepared from chick embryos at H-H stage 23 and 24 (ID4), using the method of Goshima (27) with some modification. Hearts were excised and transferred into Ca^{2+}-free and Mg^{2+}-free Hanks' solution and ventricles only were trimmed and minced. Small pieces of ventricle were then transferred to a flask with 3 ml Hanks' solution containing 0.1% trypsin. The entire trypsinization procedure was carried out at 37 °C with a magnetic stirrer for 8–10 minutes. The supernatant was decanted into centrifuge tubes to which ice-cold Hanks' solution was then added. The collected supernatant was then centrifuged at 250 g for 8 min. The cells were washed and rinsed twice with fresh Hanks' solution, after which they were seeded into Petri dishes (35–mm diameter) and incubated in Eagle MEM supplemented with 20% calf serum at 37 °C under an atmosphere of 5% Co_2 and 95% air. After an hour, when fibroblast-like cells had attached to the surfaces of the dishes, supernatants containing ventricle cells were aspirated and seeded into 96–well Falcon micro plates, at a density of 1×10^4 cells per well. These procedures were performed under sterile conditions.

The cells were then incubated for 3 days at 37 °C. After the cells were confirmed spontaneously beating in each well, one cell which appeared to be rhythmically beating was selected for counting cell beats from each well. Cell beating was counted for 15 sec on three occasions, that is, before treatment, and 1 and 10 min after the addition of the chemicals. Cell beating was determined in ten cells (from 10 wells) for each treatment. These procedures, i.e., confirming and counting cell beating, were performed under an inverted microscope; a hot plate maintained the temperature at 37 °C. CA was added into each well at a concentration of 5, 10, or 20 μM and PB was added at 6, 12, or 24 μM into each well. Some wells were added AD at 0.5 μM before treatment of CA or PB. The chemicals wrc dissolved in Eagle MEM and the final concentration was adjusted to a volume of 0.2 ml in each well. In control wells, saline was added to adjust the medium volume to be 0.2 ml in total.

Results

Experiment I

Tables 1 and 2 summarize the results.

Some embryos had external malformations, i.e., exencephaly, microphthalmia, ectopic cardia, or omphalocele; however, the fre-

Table 1. Lethality and cardiovascular malformation index in White Leghorn

Treatment						Caffeine 3.0
Stage at treatment	14	15	16	17	18	19
Number of eggs	35	41	39	40	47	39
Number of dead embryos (%: a)	7(20.0)	6(14.6)	7(17.9)	6(15.0)	8(17.0)	19(48.7)
Number of live embryos	28	35	32	34	39	20
Number of embryos with external malformations (%: b)	2(7.1)	1(2.9)	2(6.3)	3(8.8)	2(5.1)	1(0.5)
Number of embryos with cardiovascular malformations (%: b)	11(39.3)	7(20.0)	7(21.9)	5(14.7)	6(15.4)	16(80.0)
Number of embryos with VSD (%: b)	6(21.4)	5(14.3)	4(12.5)	2(5.9)	3(7.7)	14(70.0)
VSD alone	3	2	1	2	2	10
Number of embryos with great vessel malformations (%: b)	8(28.6)	5(14.3)	6(18.8)	3(8.8)	4(10.3)	6(30.0)
Hypoplasia of aorta	0	0	0	0	0	0
Defects of aorta	1	1	2	1	0	0
Hypoplasia of pulmonary artery	4	0	1	0	1	6
Defects of pulmonary artery	2	2	1	0	3	0
Hypoplasia of brachiocephalic artery	0	0	0	0	0	0
Defects of brachiocephalic artery	3	2	2	2	1	1
Persistence of left 4th aortic arch	0	1	0	0	0	0

a: Number of dead embryos/number of eggs treated.
b: Number of embryos with malformations/number of live embryos.

Table 2. Lethality and malformation index in an incrossbred chick embryos

Treatment						Caffeine 3.0
Stage at treatment	14	15	16	17	18	19
Number of eggs	26	22	17	20	17	13
Number of dead embryos (%: a)	4(15.4)	3(13.6)	3(17.6)	4(20.0)	3(17.6)	7(53.8)
Number of live embryos	22	19	14	16	14	6
Number of embryos with external malformations (%: b)	2(9.1)	1(5.3)	2(14.3)	1(6.3)	1(7.1)	0(0.0)
Number of embryos with cardiovascular malformations (%: b)	6(27.3)	4(21.1)	3(21.4)	2(12.5)	2(14.3)	6(100.0)
Number of embryos with VSD (%: b)	3(13.6)	2(10.5)	1(7.1)	2(12.5)	2(14.3)	5(83.3)
VSD alone	1	1	1	1	0	4
Number of embryos with great vessel malformations (%: b)	5(22.7)	3(15.8)	2(14.3)	1(6.3)	2(14.3)	2(33.3)
Hypoplasia of aorta	0	0	0	0	0	1
Defects of aorta	1	1	1	0	0	0
Hypoplasia of pulmonary artery	0	0	0	0	0	0
Defects of pulmonary artery	2	1	1	0	0	0
Hypoplasia of brachiocephalic artery	0	0	0	0	1	0
Defects of brachiocephalic artery	1	0	1	1	0	0
Persistence of left 4th aortic arch	1	2	0	0	1	0

a: Number of dead embryos/number of eggs treated.
b: Number of embryos with malformations/number of live embryos.

chick embryos obtained from NIBS treated with caffeine

mg/egg								Control
20	21	22	23	24	25	26	27	19
41	43	42	39	41	35	33	32	61
23(56.1)	22(51.2)	14(33.3)	11(28.2)	12(29.3)	7(20.0)	6(18.2)	4(12.5)	8(13.1)
18	21	28	28	29	28	27	28	53
2(11.1)	2(9.5)	1(3.6)	3(10.7)	2(6.9)	0(0.0)	1(3.7)	2(7.1)	4(7.5)
13(72.2)	6(28.6)	11(39.3)	17(60.7)	15(51.7)	6(21.4)	7(25.9)	5(17.9)	6(11.8)
11(61.1)	4(19.0)	10(35.7)	16(57.1)	13(44.8)	2(7.1)	2(7.4)	1(3.6)	4(7.5)
8	2	7	11	7	1	0	0	2
5(27.8)	4(19.0)	4(14.3)	6(21.4)	8(27.6)	5(17.9)	7(25.9)	5(17.9)	4(7.5)
2	2	1	2	3	1	1	0	1
0	0	0	1	0	0	0	0	0
5	2	5	4	5	5	5	4	2
0	0	0	1	0	0	0	0	1
0	0	0	0	0	0	1	2	1
0	0	1	0	1	0	0	0	0
0	0	1	1	1	0	0	0	0

obtained from a local farm treated with caffeine

mg/egg								Control
20	21	22	23	24	25	26	27	19
19	19	24	17	14	15	15	14	54
8(42.1)	8(42.1)	3(12.5)	2(11.8)	2(14.3)	1(6.7)	2(13.3)	3(21.4)	6(11.1)
11	11	21	15	12	14	13	11	48
2(18.2)	1(9.1)	1(4.8)	2(13.3)	1(8.3)	1(7.1)	1(7.7)	2(18.2)	5(10.4)
3(27.3)	4(36.4)	10(47.6)	10(66.7)	8(66.7)	4(28.6)	3(23.1)	2(27.3)	7(14.6)
2(18.2)	2(18.2)	9(42.9)	8(53.3)	7(58.3)	1(7.1)	1(7.7)	1(9.1)	4(8.3)
2	2	7	7	5	0	0	0	1
1(9.1)	2(18.2)	3(14.3)	3(20.0)	3(25.0)	4(28.6)	3(23.1)	3(27.3)	6(12.5)
0	1	2	0	1	2	2	2	1
0	0	0	1	0	0	0	0	0
0	0	0	0	2	3	2	1	2
0	1	0	0	0	1	0	0	1
0	0	0	0	0	1	0	0	1
0	0	0	0	0	0	0	0	1
2	0	0	2	1	0	0	0	2

quency of external malformation was the same level as in each control of two breeds. The malformation in the heart and great vessels were: ventricular septal defect (VSD), hypoplasia (or defect) of the aorta (AA) and pulmonary artery (PA), persistence of the left 4th aortic arch (L4AA), hypoplasia of the brachiocephalic artery (BA), and complications of these abnormalities. VSD was the most frequently observed in both breeds.

Both the lethality and the frequency of embryos with a malformation in the heart and great vessels varied according to the stage of treatment (Figure 1). In both breeds, the lethality was highest between H-H stages 19 and 21, while, the malformation rate had two peaks, one at H-H stage 19 and the other at around H-H stages 23–24. the chronlogical changes of lethality and malformation rate in the two different breeds were similar.

Figure 2 shows the changes in the frequency of VSD with/without great vessel malforma-

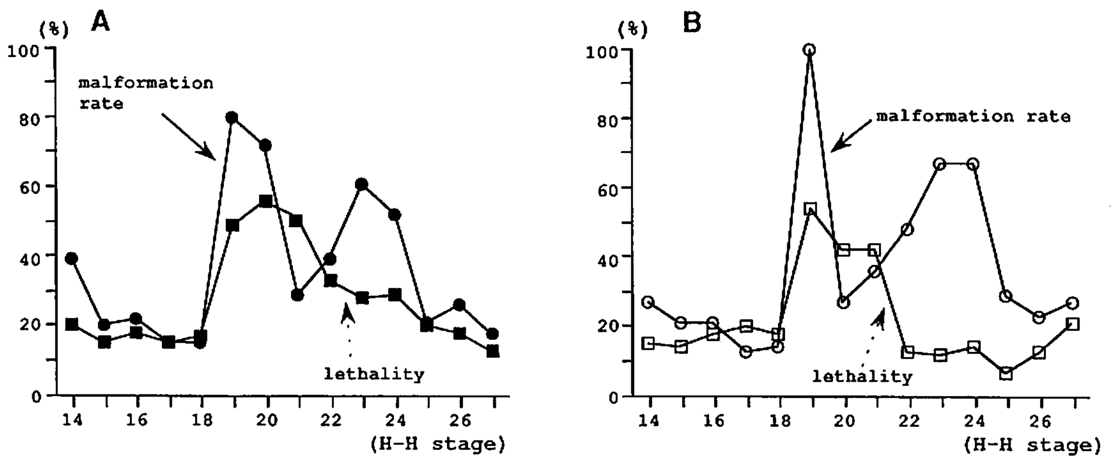

Fig. 1. Changes in lethality and cardiovascular malformation rate in chick embryos treated with caffeine (A: White Leghorn and B: an incrossbred)

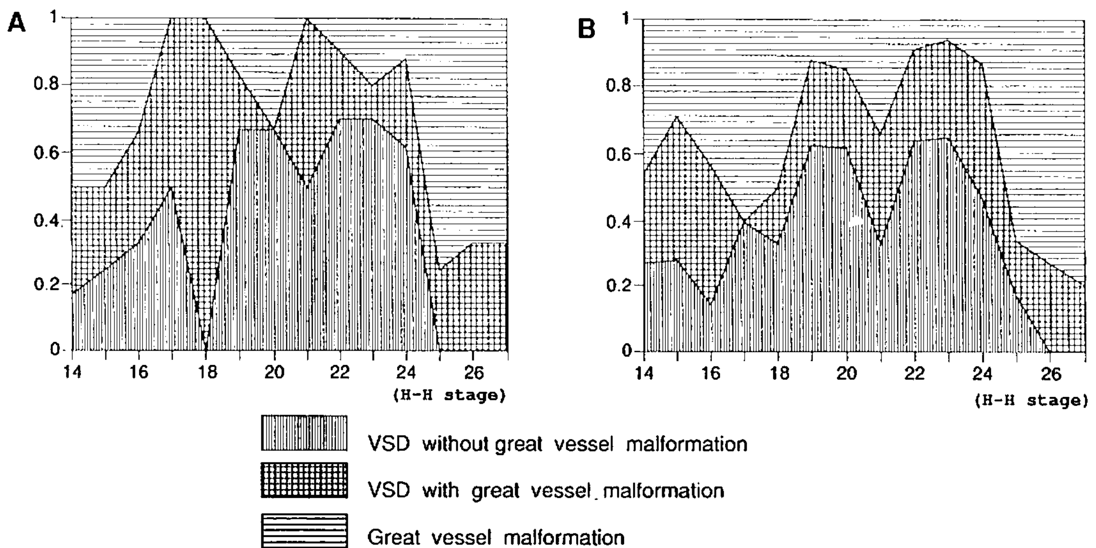

Fig. 2. Changes in the rate of VSD with/without great vessel malformation in chick embryos treated with caffeine (A obtained from NIBS and B obtained from a local farm)

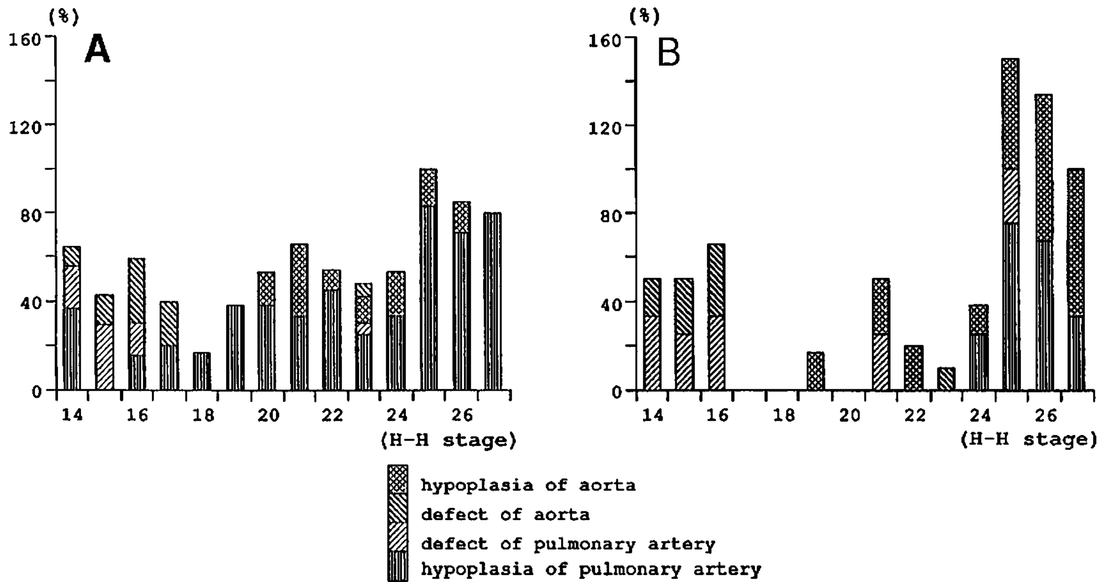

Fig. 3. Changes in the rate of great vessel malformation in chick embryos treated with caffeine (A obtained from NIBS and B obtained from a local farm)

tion. The frequency of VSD alone was lower in embryos treated after H-H stage 25, and highest at H-H stages 19 and 22–24 similar to the change of frequency of malformation mentioned above.

Figure 3 shows the rate of great vessel malformation, hypoplasia (or defect) of AA (and PA), persistence of L4AA, and hypoplasia of BA. In both breeds, the defect of AA and PA was observed mainly in embryos treated at earlier stages, while their hypoplasia was seen mainly at later stages.

There appeared to be little difference in the embryotoxic potential of CA and stage specificity of embryotoxicity between the chick embryos of the two breeds.

Experiment II

Table 3 shows the embryotoxic potential of CA and PB with or without AD.

In the embryos treated with 3.0 mg of CA alone, there was a high degree of malformation, mainly VSD, this being consistent with the results obtained in experiment I. The

Table 3. Embryotoxicity of caffeine and phenobarbital in chick embryos at stage 22 and 24

Agent	Caffeine		Phenobarbital						Control
Concentration; mg/egg	3	3	1.13	1.13	2.25	2.25	5	5	–
Co-treatment with adenosine 13 μg/egg	–	+	–	+	–	+	–	+	+
Number of eggs	35	22	21	19	23	23	23	20	35
Number of dead embryos (%: a)	6(17.1)	4(18.2)	5(23.8)	7(36.8)	17(73.9)	11(47.8)	22(95.7)	9(45.0)	7(20.0)
Number of live embryos	29	18	16	12	6	12	1	11	28
Number of embryos with cardiovascular malformations (%: b)	18(62.1)	11(61.1)	6(37.5)	2(16.7)	3(50.0)	6(50.0)	1(100.0)	8(72.7)	3(10.7)
VSD alone	12	7	5	1	3	5	1	7	2
VSD with vessel malformation	4	2	1	1	0	1	0	1	1
Vessel malformation alone	2	2	0	0	0	0	0	0	0

a: Number of dead embryos/number of eggs treated.
b: Number embryos with malformations/number of live embryos.

major great vessel malformation was hypoplasia of PA. In the embryos treated with PB alone, a high lethality was seen at a dose of more than 2.25 mg of PB/egg and the frequency of cardiovascular malformation was greater than that in the control at any dose. PB at the mid dose, 2.25 mg/egg induced a similar rate of malformation to that induced by treatment at 3.0 mg of CA. However, treatment with 13 μg of AD alone did not induce a high lethality or a higher degree of malformation than in the control.

Pre-treatment with 13 μg of AD immediately before 3.0 mg of CA treatment did not alter the lethality or malformation rate from that shown in the embryos treated with 3.0 mg of CA alone. Pre-treatment with 13 μg of AD before treatment with more than 2.25 mg of PB reduced the lethality when compared with treatment with the same dose of PB alone, but the lethality was not reduced at the 1.13 mg dose of PB. The malformation rate in the embryos treated with PB appeared to be slightly reduced by pretreatment with 13 μg of AD, but there was no clear dose dependency in this reduction.

Experiment III

Table 4 shows the effects on the frequency of embryonic cardiac cell beating of chick embryos in vitro produced by treatment of CA and PB without or with AD. Spontaneous cell beating was about 70 times per minute. The addition of CA at the concentration of 10 μM or less increased the frequency of beating at 1 and 10 min after treatment. The presence of 20 μM CA and the addition of any amount of PB reduced the frequency of beating at 1 min after treatment. PB at the concentration of 12 μM or more caused all the cells in the well to stop beating at 10 min after treatment. Treatment with 0.5 μM AD alone did not alter the frequency of cardiac cell beating at 1 and 10 min after treatment. Pre-treatment with 0.5 μM AD before treatment with any amount of CA did not alter the frequency of cell beating at 10 min after treatment, while pre-treatment with 0.5 μM AD before treatment with 12 μM PB stopped cell beating at 10 min after treatment as well as treatment with 12 μM and more of PB alone.

Discussion

We compared the reactivity to CA embryotoxicity potential in chick embryos from two different breeds in experiment I. One breed is well established as an experimental animal, White Leghorn from NIBS, and the other is an incrossbred from a local farm. The latter is much cheeper and the egg has a hard shell, which can facilitate experimental procedures, so that it will be more convenient material. The chick embryos from the two different breeds responded similarly to treatment of CA at the dosage of 3 mg/egg during H-H stage 14–27: for example, the critical stages of lethality and teratogenic-

Table 4. Changes in frequency of cell beating of chick embryonic

Agent	Caffeine					
Concentration: μM	5	5	10	10	20	20
Co-administration of 0.5 μM adenosine	−	+	−	+	−	+
Mean value of number of cell beating (min-max)						
before treatment	70(60–76)	72(64–72)	68(60–72)	70(60–76)	68(56–80)	70(64–76)
1 min after treatment	98(80–112)	74(68–76)	88(80–104)	64(52–80)	50(32–76)	68(52–76)
10 min after treatment	86(76–116)	70(60–76)	84(76–96)	62(48–76)	12(0–32)	68(48–76)

Each mean value was calculated with data from 10 cells
Cardiac cells were prepared from H-H stage 23 and 24 chick embryos

ity and type of cardiovascular malformation caused by CA. Therefore, chick embryos of the incrossbred from the local farm were used in the subsequent experiments (II and III). The reproducibility of embryotoxicity to CA in the incrossbred was confirmed in experiment II. These findings showed that the eggs from the local farm were useful material for studying the embryotoxicity potential of CA. Chick embryos can be treated at a precise developmental stage that is well established, making it easy to determine the critical stage of the embryotoxicity of chemicals. The precise judgment of developmental stage at the time of treatment with chemicals might provide us more reproducible findings.

Treatment with PB at the dosages of 1.13 mg/egg or more showed high lethality and teratogenic effects on the developing cardiovascular system, when 3 mg of CA per egg was not lethal and induced a higher degree of cardiovascular malformation. These findings were consistent with the previous data (22, 25) in quality. The lethal effects of PB appeared to be reduced by prior administration of 13 μg of AD per egg, although the teratogenic potential of PB at that dosage level and of CA at 3 mg/egg during the H-H stage 22–24 was not changed by co-administration of 13 μg of AD. Considering the effect of PB on the cultured embryonic cardiac cells shown in experiment III, PB might have a cytotoxic potential on developing cardiac cells, result in functional disorder of the heart and lead mainly to the death of the embryo. Co-administration of 13 μg of AD, which had been expected as an effective dose to reduce the teratogenic potential of CA in ovo, did not reduce the lethality or teratogenicity of 3.0 mg of CA per egg, although effects of CA at 20 μM or less on cultured cardiac cells was reduced by treatment with 0.5 μM AD. The teratogenic potential of CA in chick embryos enhanced by co-administration of forskolin (28), a potent activator of adenylate cyclase, but reduced by co-administration of AD (26). Therefore; the mechanism of the teratogenic effects of CA on the cardiovascular system might be related to changes in the cAMP concentration in cardiac cells. The reason for the differences in the effect of co-administration of AD with CA between the present study and their study (26) could not be clarified.

Cardiovascular malformations have been suggested to be caused by cytotoxic effects on the development of cardiac cells or by alterations in embryonic hemodynamics (17, 19, 29). In mammals, the whole embryo culture method has been developed in rodents as a technique for direct application of substances. The technique is not easy to use routinely and there have been few reports on the functional changes in the cardiovascular system when the embryos were exposed to teratogens. The developmental stage of chick embryos can be judged precisely, they can be treated by direct application of chemicals and the responses to

cardiac cells treated with caffeine or phenobarbital with/without adenosine

Phenobarbital						Saline	
6	6	12	12	24	24	–	–
–	+	–	+	–	+	–	+
68(56–76)	70(60–76)	66(52–72)	68(60–72)	70(56–80)	70(60–76)	72(60–76)	70(64–76)
42(24–60)	68(52–80)	36(20–48)	40(20–54)	24(12–40)	18(4–24)	70(60–76)	68(60–76)
30(16–48)	66(48–76)	0	0	0	0	70(56–76)	70(60–76)

such treatments can be easily observed under a stereomicroscope. In the present study, the reproducibility of the teratogenic effects of CA on the cardiovascular system in chick embryos was shown by the use of precise judgement of the developmental stage. We recommend the use of chick embryos as an alternative in embryotoxicity studies to obtain data for exploring the pathogenesis of teratogenicity of chemicals.

Acknowledgment

We are grateful for the "Golden presentation" award at the 5th annual meeting of JSAAE.

Received: June 30, 1994; Accepted: October 30, 1994

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