CHAPTER III

RESULTS

Comparison of Swiss Albino Mouse Embryonic Development *in Vivo* and *in Vitro*

Detailed results of the extent of mouse embryonic development *in utero* and *in vitro* are shown in table 1. From embryonic age day 8 to 10, mouse enbryos develop from the neurular stage with 3 - 5 somites to hindlimb bud stage with 30 - 35 somites. In culture, the development of mouse embryo over 48 hours period showed extensive development and differentiation (figure 1 B). Morphologically, they were similar to embryos maintained *in vivo* (figure 1 A). But they have slightly smaller crown-rump and head lengths, reduced somite count, reduced DNA content and smaller morphological score points; each being about 90 % of the *in vivo* state. This slowing in growth of mouse embryos in culture is probably related to the absence of a functional allantoic placenta and results from the failure of the ectoplacental cone to develop normally *in vitro* (New, 1978). Despite the slowing in growth of cultured mouse embryos, normal development of the yolk sac was apparent.

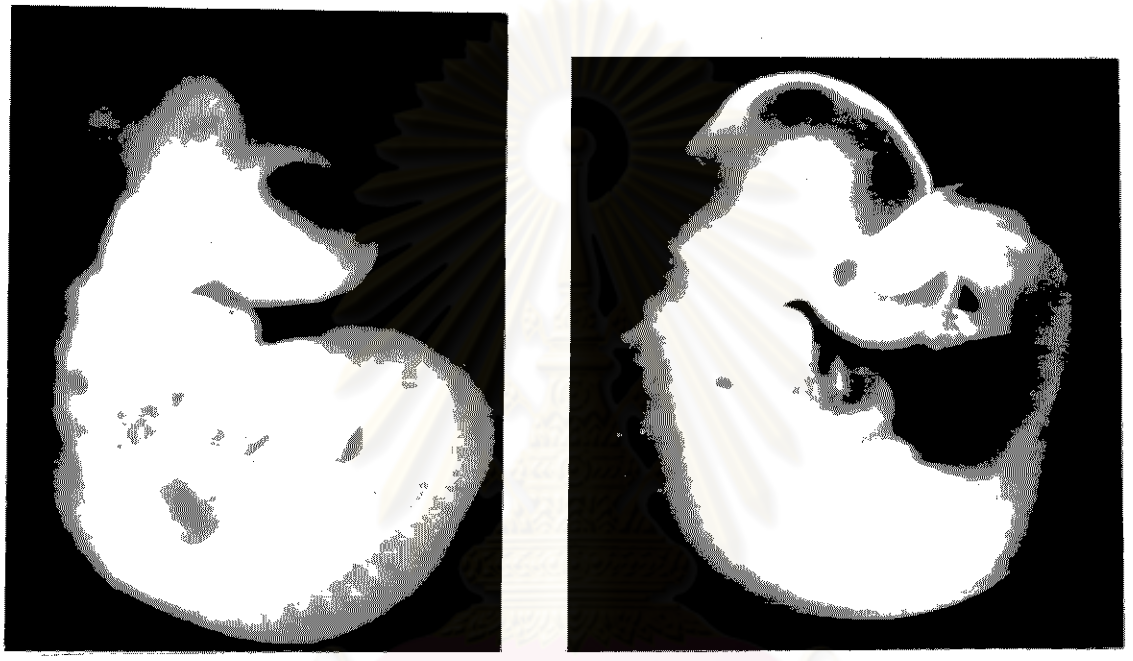
Table 1. Comparison of Swiss Albino Mouse Embryonic Development *in Vivo* and *in Vitro*

Development parameter [c]	Mouse embryonic development	
	in vivo [a]	*in vitro* [b]
	n = 47	n== 50
Yolk sac: Diameter (mm.)	5.10 ± 0.21	5.11± 0.09
DNA (ug)	10.35 ± 1.37	9.94± 0.17
Embryo: Somites	35.91 ± 1.13	33.26± 0.80 **
Crown rump length (mm.)	4.86 ± 0.02	4.34 ± 0.07 **
Head length (mm.)	2.47 ± 0.03	2.23 ± 0.04 **
DNA (ug)	50.10 ± 0.81	46.27± 0.72**
Morphological score	44.52 ± 0.52	43.65 ± 0.56 **

[a]Embryos were explanted and examined at embryonic age 10 days achieved by intrauterine development.

[b]Embryos were explanted at embryonic age 8 days and cultured *in vitro* for 48 hours.

[c]Figures given represent mean ± S.D. ** Significantly different from *in vivo* at $P < 0.01$



A B

Fig. 1. (A) Photomicrograph of unfixed *in utero* embryo of 33 somites, developmental age 10 days; (B) Photomicrograph of unfixed embryo cultured for 48 hours *in vitro*, developmental age 10 days. Embryo was explanted at embryonic age 8 days and cultured *in vitro* for 48 hours. Magnification = x 20

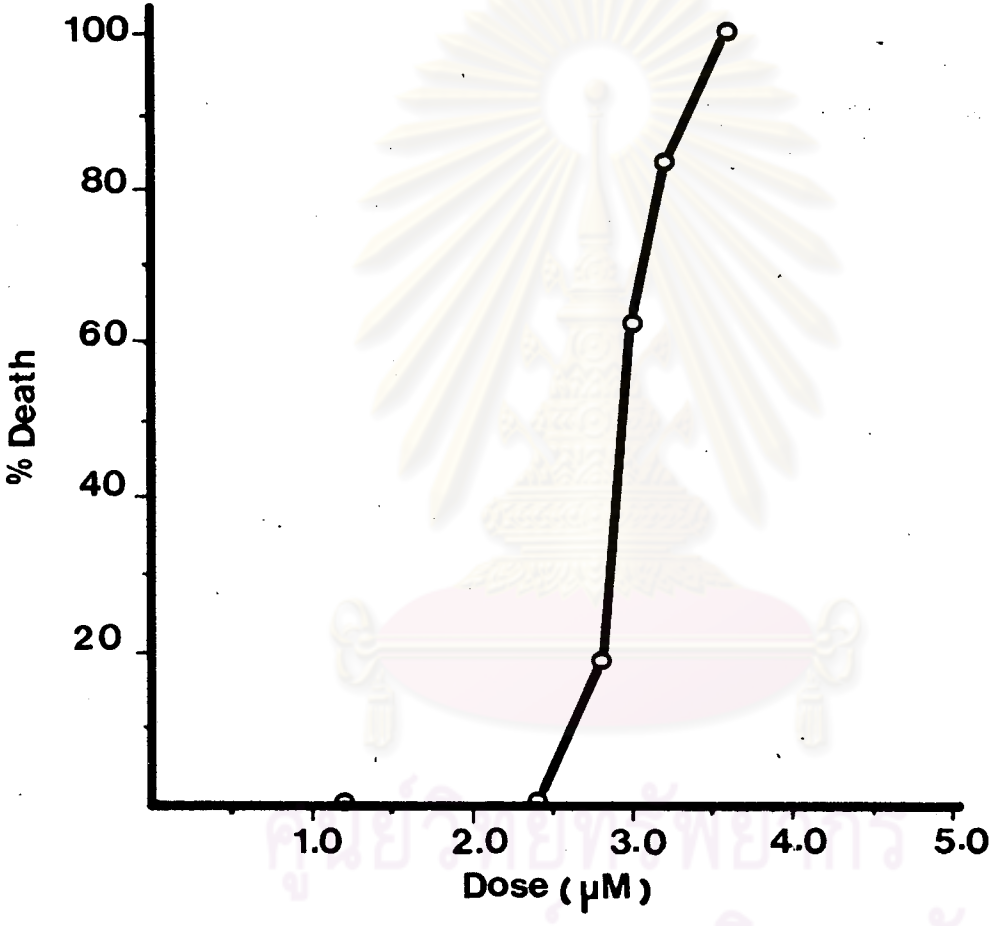
The Dose Response Curve for Embryolethality of Cadmium Chloride in Mouse Embryos Cultured *in Vitro*

Before attempting detailed studies on the effects of cadmium chloride on cultured mouse embryos. The relationship of embryonic mortality and doses of cadmium chloride was investigated. Dose of cadmium chloride was varied and the number of dead embryo was examined. The embryolethality curve of cadmium chloride in mouse embryos cultured *in vitro* as shown in figure 2, when increasing the dose of cadmium chloride (1.20 to 3.60 μM) embryonic mortality was increased. Cadmium chloride at a concentration of 2.40 μM had no effect upon the survival of mouse embryos; doses higher than 2.40 μM showed to increase the embryonic mortality rate. Thus a concentration of 2.40 μM of cadmium chloride seem to be the threshold dose level for embryolethality in postimplanted mouse embryos which are cultured *in vitro*. This threshold dose level may not be precise, but is relebant to establish the dose levels for the main study.

Effects of Cadmium Chloride on the Development of 8 Days Mouse Embryos *in Vitro*

Detailed results of the effects of cadmium chloride on various developmental parameters of the cultured mouse embryos are summarized below.

Figure 2 The embryonic lethality curve of cadmium chloride in 8 days mouse embryos maintained in whole-embryo culture for 48 hrs. Each data point (.) represents the percentage of mortality from 2-4 experiments.



A. Total DNA Content of Embryos

Total DNA content of embryos cultured *in vitro* for 48 hours in the presence of various concentrations of cadmium chloride are presented in table 1 of the appendix. As shown in figure 3, cadmium chloride decreased the final embryonic DNA content in all dosage groups. At the lowest dose studied, the total DNA content of the cultured mouse embryos was slightly but significantly ($P < 0.01$) lower than the controls.

B. Yolk Sac Diameter, Crown - Rump Length and Head Length of Embryos.

The average values $\pm$ S.D. of the yolk sac diameter, crown - rump length and head length of embryos cultured *in vitro* for 48 hours in the presence of various concentrations of cadmium chloride are presented in table 2 - 4 of the appendix. As shown in figure 4, growth inhibition, as determined by yolk sac diameter, crown - rump and head lengths, became obvious in embryos exposed to 1.0 - 3.0 μM of cadmium chloride.

C. Morphological Score and Number of Somites

The morphological score - table of Brown and Fabro is presented in table 5 of the appendix. The average values $\pm$ S.D. of morphological score points and somite numbers of mouse embryos cultured *in vitro* for 48 hours in the presence of various concentrations of cadmium chloride are presented in table 6 and 7 of the appendix.

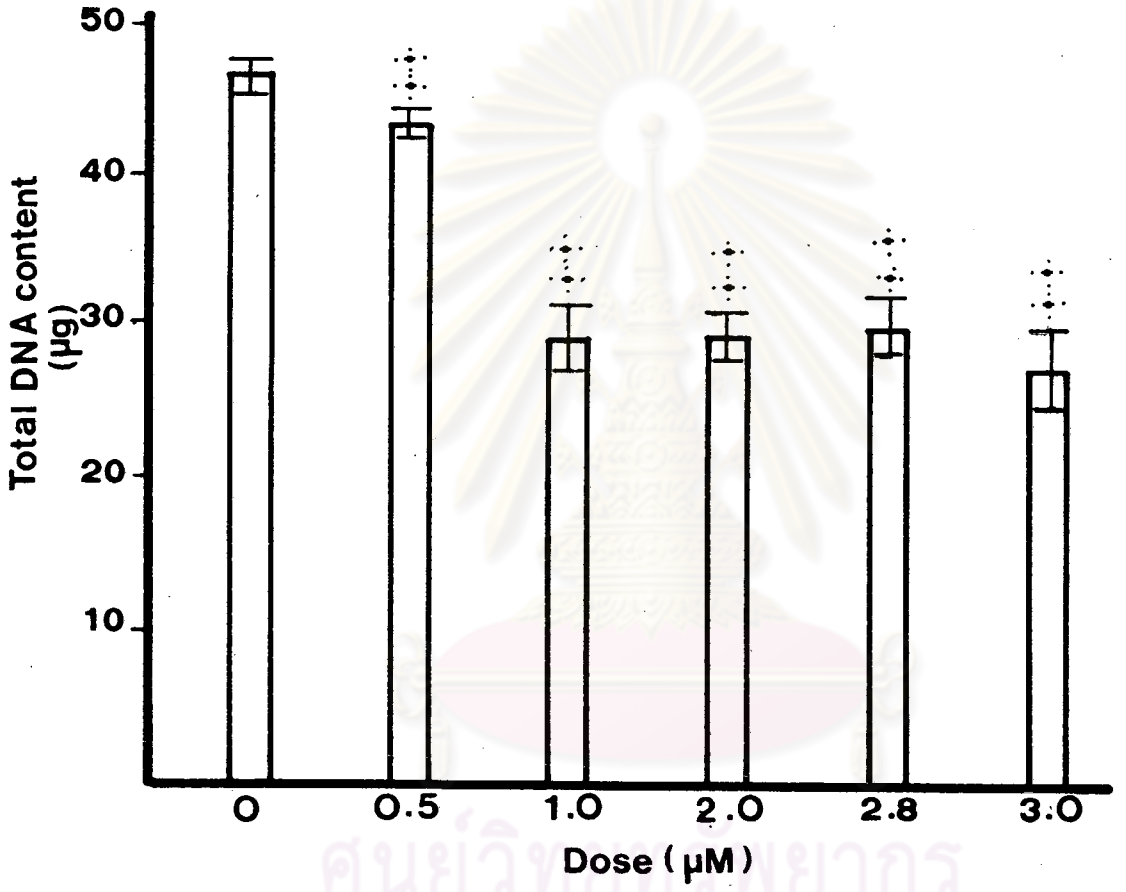


Figure 3 The relationship between doses of cadmium chloride and total DNA contents of embryos maintained in whole-embryo culture for 48 hrs. Each data point (I) represents mean ± S.D. Values significantly different from controls are indicated with two asterisks ($P < 0.01$)

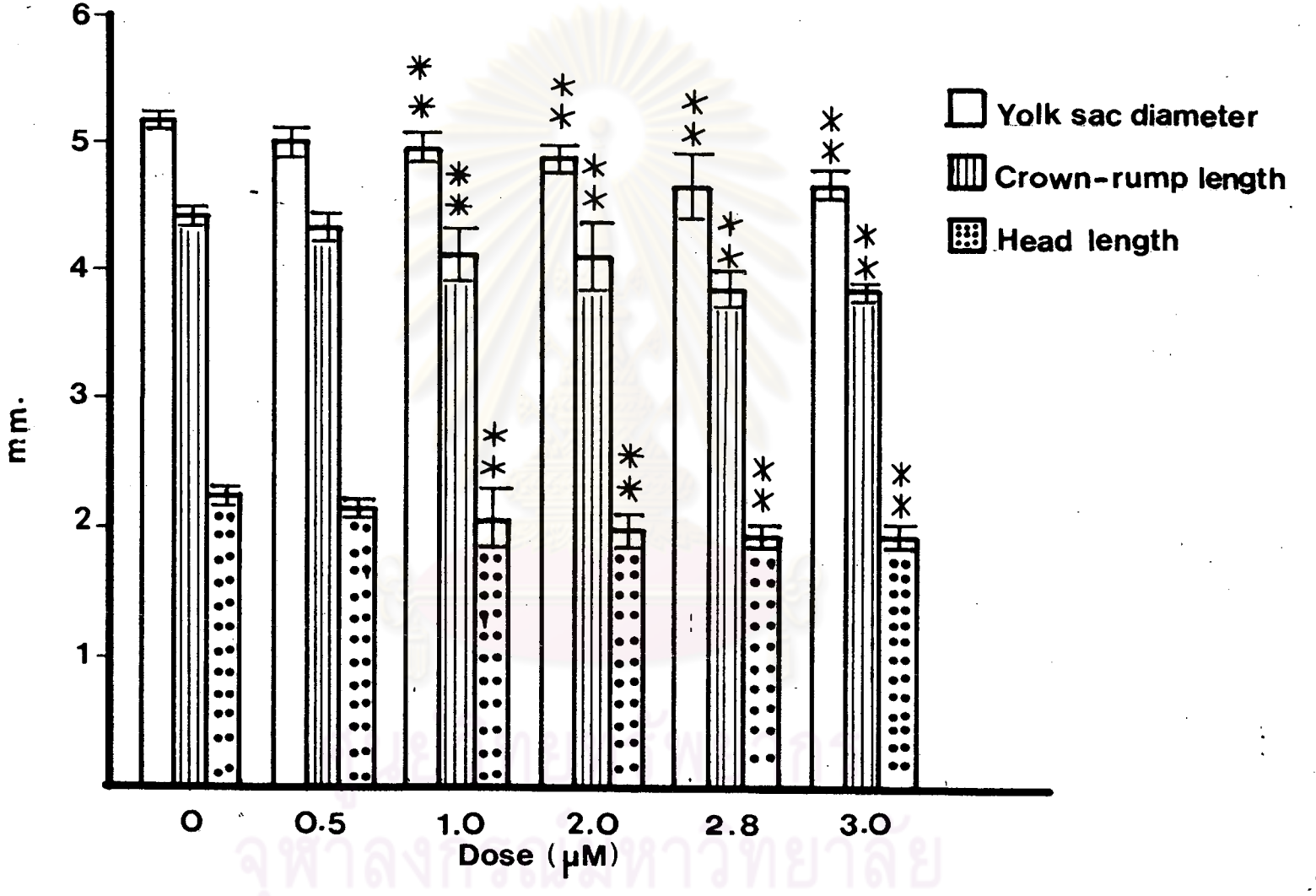


Figure 4 The relationship between doses of cadmium chloride and yolk sac diameters, crown-rump lengths and head lengths of embryos maintained in whole-embryo culture for 48 hours. Each data point (I) represents mean ± S.D. Values significantly different from control are indicated with two asterisks ($P < 0.01$)

Somite counts and morphological score estimations of cadmium chloride treated mouse embryos showed slightly retarded in differentiation (Figure 5). This developmental retardation became obvious when the concentration of cadmium chloride was 1.0 μM or greater.

D. External Morphology

Results of morphological assessment of cultured mouse embryos are presented in table 8 of the appendix. External malformations of cultured mouse embryos were identified at the cadmium chloride concentration levels of 2.8 and 3.0 μM. These external malformations were often associated with irregular fusion of the telencephalic and mesencephalic regions, and stunted telencephalic hemisphere. Non - closure of the cranial neural tube was well demonstrated (figure 6).

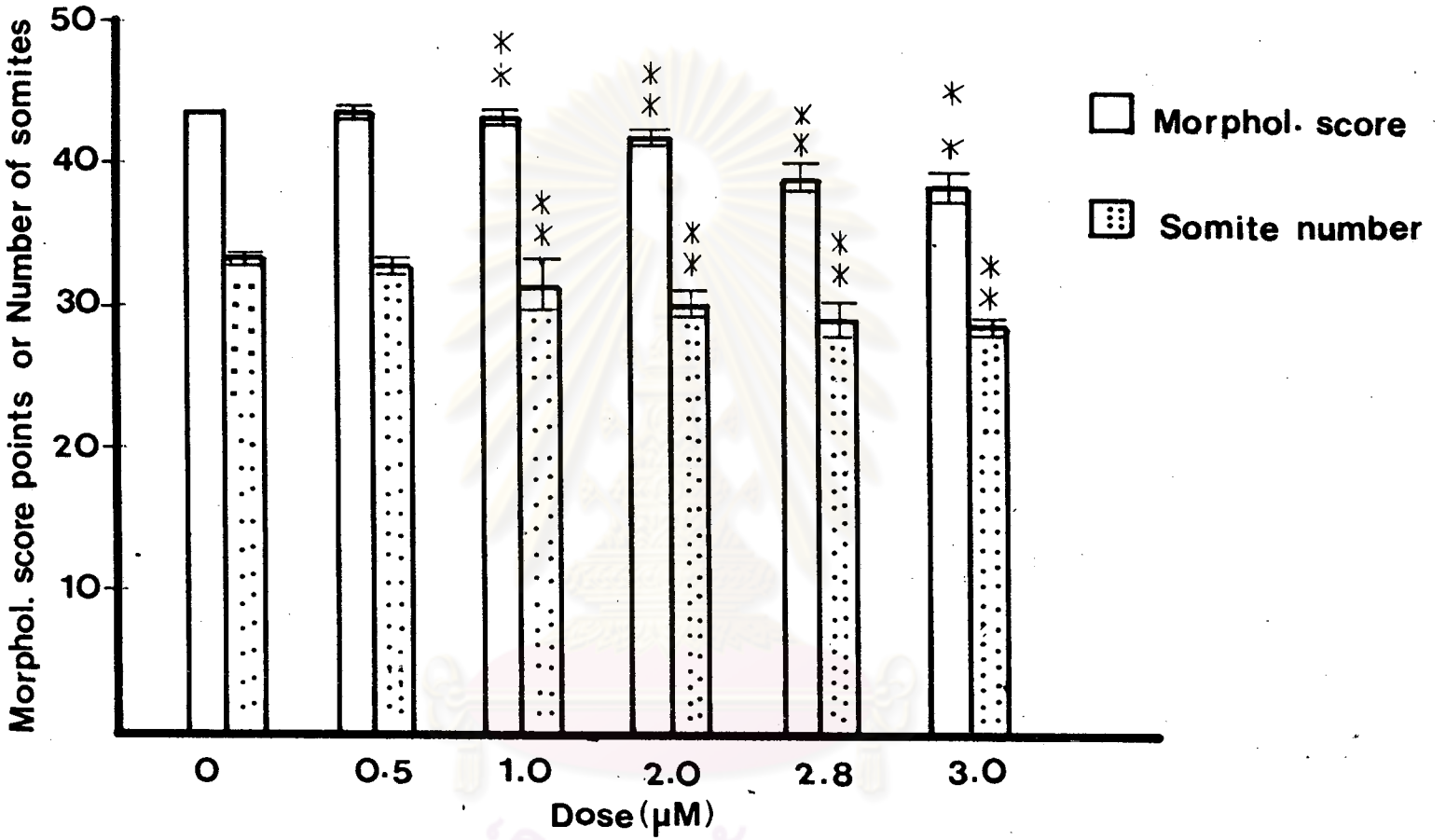


Figure 5 The relationship between doses of cadmium chloride and number of somites and morphological score points of embryos maintained in whole-embryo culture for 48 hrs. Each data point (I) represents mean ± S.D. Values significantly different from controls are indicated with two asterisks ($P < 0.01$)

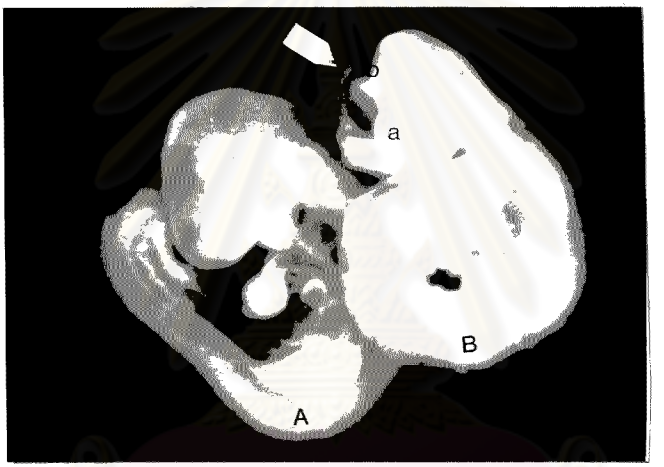


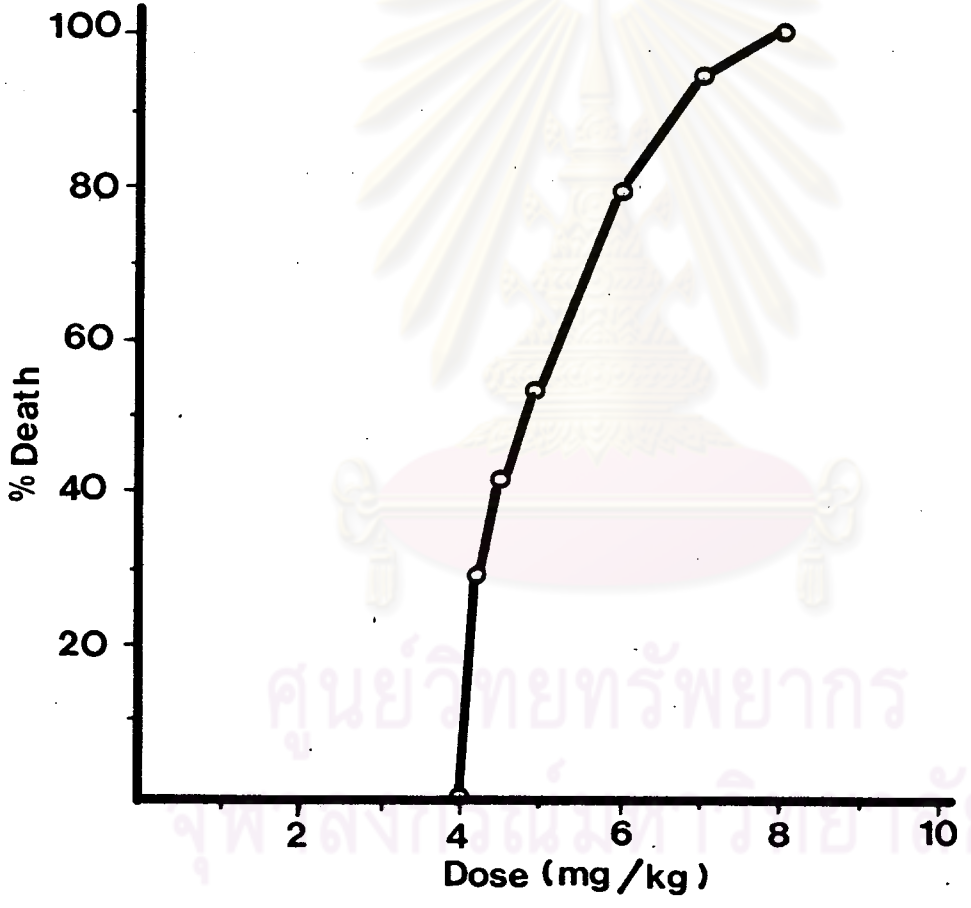
Figure 6. Photomicrograph of (A) Controlled embryo and (B) embryo treated for 48 hours with 3.0 μM cadmium chloride. Characteristic anomalies observed were: (a) stunted telencephalic regions; (b) open neural tube. Magnification = x 15

The Adult Lethality Curve of Cadmium Chloride

Before attempting detailed studies on the embryotoxic potential of cadmium chloride *in vivo*, the relationship between doses of cadmium chloride administered to female mice during organogenesis and mortality rate of maternal animals were investigated. Figure 7 showed a dose - response curve for adult lethality of cadmium chloride in pregnant mice. The mortality data of maternal animals are presented in table 9 of the appendix.

As is evident, administration of cadmium chloride to pregnant mice during organogenesis at doses of 4.0, 4.2, 4.5, 5.0, 6.0, 7.0 and 8.0 mg kg^{-1} body weight resulted in an increase in mortality rate of maternal animals in a dose - related manner. At the highest dose of 8.00 mg kg^{-1}, mortality rate of the maternal animals reaches 100 %; and at the lowest dose of 4.00 mg kg^{-1}, mortality rate of the maternal animals decreases to 0.%. A dose of 4 mg. $CdCl_2$ $kg.^{-1}$, administered intraperitoneally, seem to be the threshold dose level for adult lethality in female mice during organogenesis.

Figure 7 The adult lethality curve of cadmium chloride in pregnant mice following single i.p. administration of cadmium chloride on day 8.5 of pregnancy. Each data point (.) represents the percentage of mortality from 2 experiments.



The Embryotoxic Potential of Cadmium Chloride *in Vivo*

A. Effects on the Dams

The effects of treatment with a single dose (4 mg. kg^{-1}) of cadmium chloride on days 7.0, 7.5 or 8.5 of gestation on the dams are presented in table 10 - 11 of the appendix. At the dose studied, no abortion or maternal deaths was observed. Maternal body weight gain during pregnancy was lower in 7.0 day group than in the control (figure 8). This finding is in accordance with the data of Baranski *et al.* (1982), who found a significant decrease in body weight gain of rats as a result of ingestion of cadmium chloride on days 7 - 16 of gestation.

B. Effects on the Fetuses

1. Observations Made at the Time of Caesarean Section.

Maternal animals, treated with a single dose (4 mg. kg^{-1}) of cadmium chloride on day 7.0, 7.5 or 8.5 of gestation, appeared normal and healthy with no postmortem abnormalities in the maternal tissues. Results of observation made at the time of caesarean section are presented in table 12 and 13 of the appendix. Reduction in the number of live fetuses per litter was found only in a group of pregnant mice receiving 4 mg. $CdCl_2$ kg^{-1} on day 7 of gestation. (Table 2) . Counts of litters with early of late resorptions were significantly different in 7.0 day group. The number of resorptions per litter was also significantly increased in this group of mice treated with a single dose of cadmium chloride on day 7.0 of gestation (Table 2).

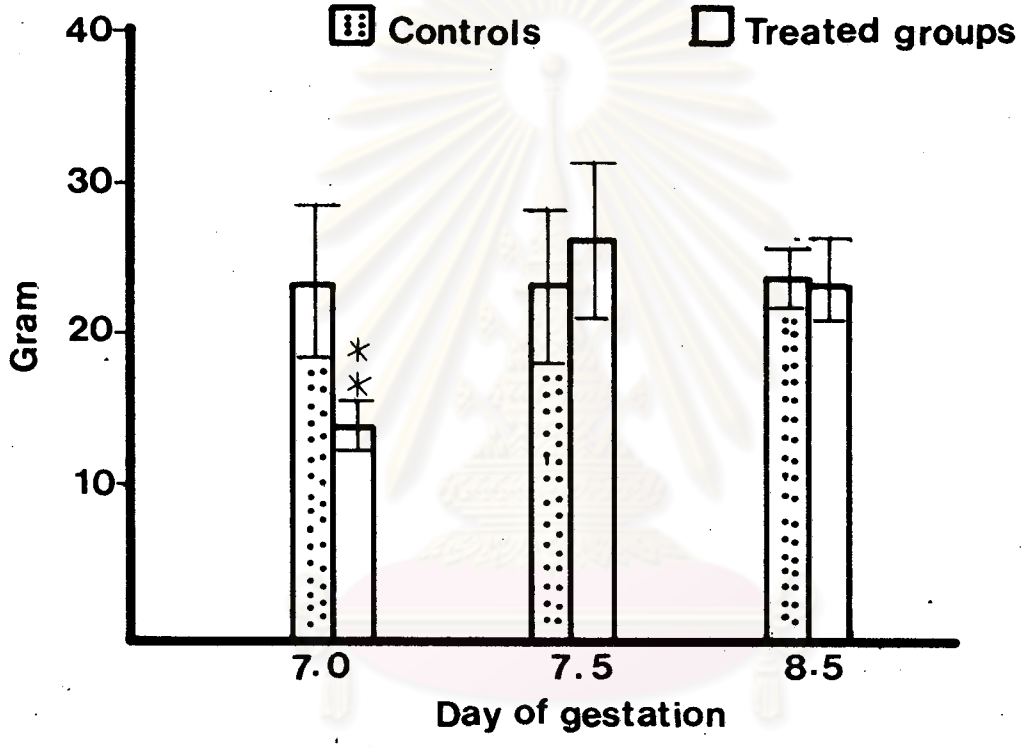


Figure 8 The relationship between day of first exposure to cadmium chloride and absolute weight gain of dams during the entire period of pregnancy. Each data point (I) represents mean $\pm$ S.D. Values significantly different from controls are indicated with two asterisks ($P < 0.01$).

Table 2. Pregnancy Outcome Following Intraperitoneal Administration of Cadmium Chloride to Pregnant Mice.

Treatment		Litter	Mean ± S.D.					
Day of Pregnancy	$CdCl_2$, i.p. (mg/kg)		Impl. Site	Viable Fet.	Dead Fet.	Resorption Embr.	Resorption Fet.	Post-impl Loss
7.0 - 8.5	Control	20	11.1 ± 2.8	10.6 ± 2.7	0	0.35±0.59	0.15 ± 0.49	0.5 ± 0.69
7.0	4.0	14	11.4 ± 1.1	4.14 ± 0.8**	0	7.29±1.27**	0.00 ± 0.0	7.29±1.27**
7.5	4.0	20	12.3 ± 2.1	10.9 ± 2.9	0	0.95±1.79	0.40± 0.6	1.35±1.98
8.5	4.0	20	11.1 ± 1.3	10.4 ± 1.1	0	0.60±0.82	0.05 ± 0.22	0.65±0.81

** Significantly different from the controls at $P < 0.01$ using one way Analysis of Variance couple with Newman-Keuls test.

Fetal crown-rump length in all experimental groups were significantly reduced (figure 9). Administration of cadmium chloride at a dose of 4 mg. kg^{-1} on days 7.0 or 7.5 of gestation significantly decreased fetal weight (figure 10). These findings are in accordance with the data of Samarawickrama and Webb (1981), who found that live fetuses from all cadmium chloride treated rats (1 - 1.58 mg. $CdCl_2$ $kg.^{-1}$ day^{-1} between the 8th and 15th day of gestation) were inactive and pale, smaller in size and weight than the controls.

Absolute weights of placenta in females treated with a single dose of cadmium chloride (4 mg. kg^{-1}) on days 7.0, 7.5 or 8.5 of gestation were significantly decreased (figure 10). This finding is in parallel to the report of Chiquoine (1965) which concluded that a single injection of cadmium chloride given to pregnant mice on any day from the sixth to the seventeenth of pregnancy results in localized necrosis of placenta and adjacent decidual tissue.

2. Incidence of Gross and Skeletal anomalies.

The incidence of gross and skeletal anomalies are presented in table 3. The incidence of gross anomalies observed by external examination of fetuses was significantly greater than in the controls for the 7.0 and 7.5 days groups. A total of 42 abnormal fetuses were found among 58 live fetuses in the 7.0 day group; and a total of 103 abnormal fetuses were found among 218 live fetuses in the 7.5 day group. Only 2 abnormal fetuses were found among 208 live fetuses in the 8.5 day group. The most frequent external anomalies were exencephaly and open eye (figure 11). Other anomalies such as amelia (figure 12) were rare. These results clearly indicated that day 7 and

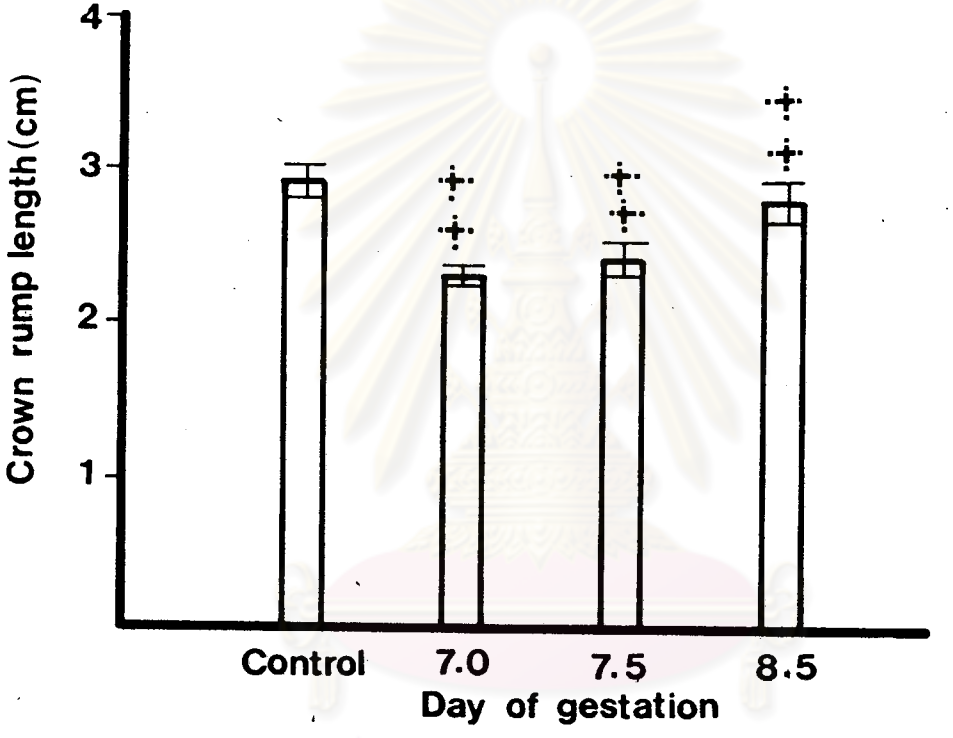


Figure 9 The relationship between the day of first exposure to cadmium chloride and crown-rump lengths of viable fetuses. Each data point (I) represents mean ± S.D. Values significantly different from controls are indicated with two asterisks.

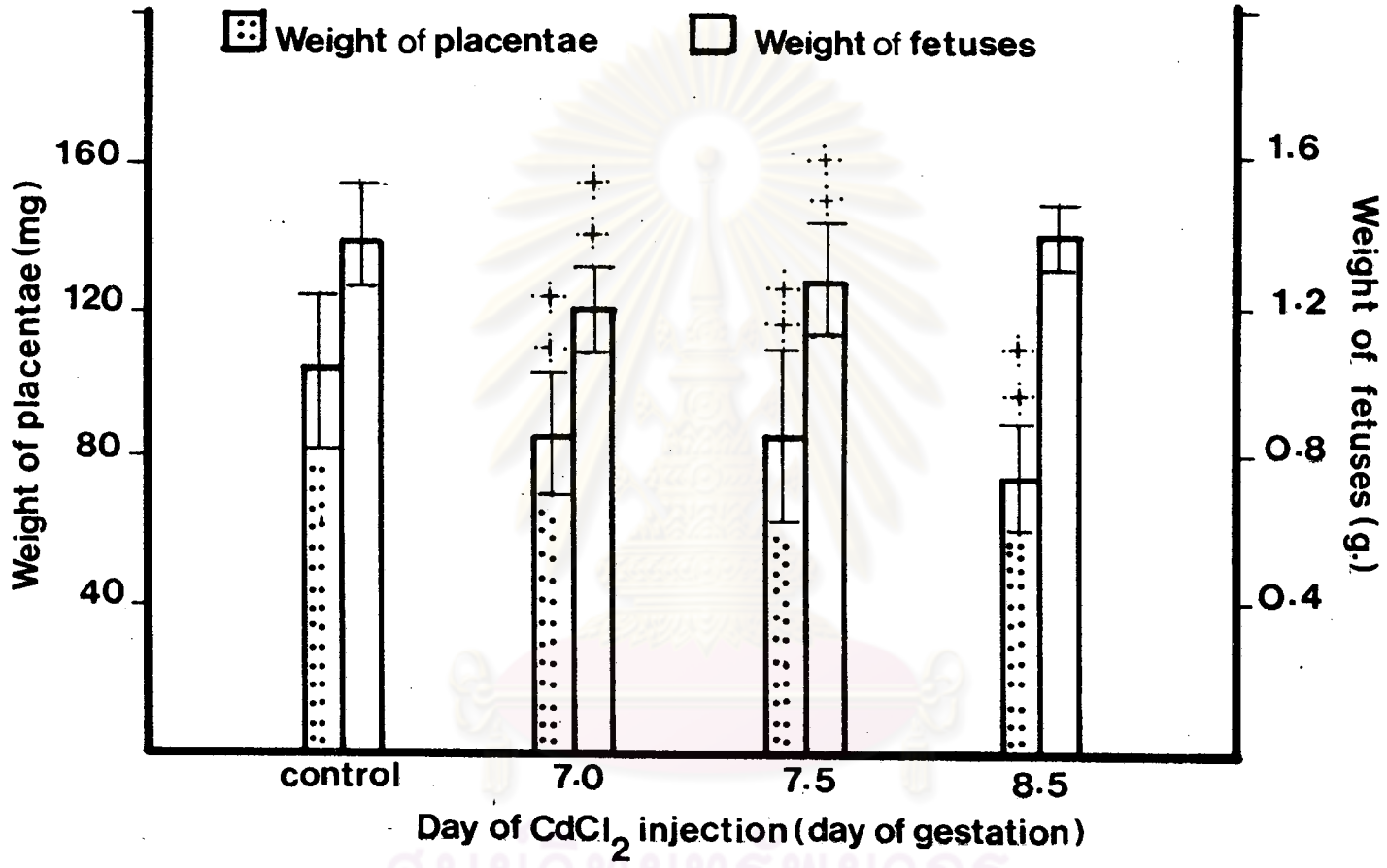


Figure 10 The relationship between day of first exposure to cadmium chloride and weight of fetuses and placentae. Each data point (I) represents mean $\pm$ S.D. Values significantly different from controls are indicated with two asterisks ($P < 0.01$).

Table 3. Effects of Maternal Exposure to Cadmium Chloride (4mg / kg),Intraperitoneal Injection, on Gross External and Skeletal Morphology of Fetuses.

Morphological Feature [a]	Day of Pregnancy			
	7.0 - 8.5 Control	7.0	7.5	8.5
Number of litter examined	20 (212)	14 (58)	20 (218)	20 (208)
Exencephaly	0	13 (42)**	17 (103)**	0
Open eye	0	13 (23)**	17 (58)**	0
Lack of forelimb	0	0	0	1 (1)
Lack of hindlimb	0	0	0	1 (1)
Additional rib	5 (11)	3 (4)	3 (3)	3 (6)
Defective rib	0	0	8 (16)**	12 (38)**
Skull: retarded ossification	2 (2)	10 (36)	14 (116)**	12 (109)**
Defective vertebraes	0	0	0	2 (5)

Note: The maternal animals were sacrificed and examined on the 18.5th day of gestation.

[a]The number of fetuses is given in parenthesis.

**Significantly different from the control at $P < 0.01$ as determined by Fisher's exact test.

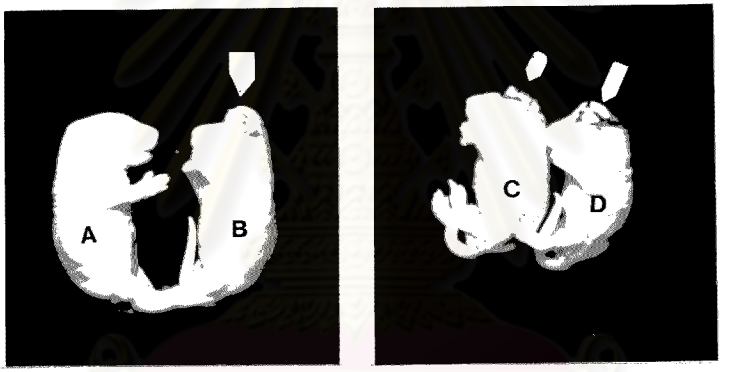


Figure 11 Lateral view of 18.5 days mouse fetuses from groups of pregnant mice treated with 4 mg $CdCl_2 kg^{-1}$ on days 7.0 or 7.5 of gestation:(A) Control (B) fetus with exencephaly (C & D) fetuses with exencephaly and open eyes.

Figure 12 Lateral view of 18.5 days mouse fetuses from a group of pregnant mice treated with 4 mg $CdCl_2 kg^{-1}$ on day 8.5 of gestation:(A) fetus which lack of forelimb; (B) fetus which lack of hindlimb.

7.5 were the most critical periods with the highest incidence (92 % and 85 %) of external anomalies.

The incidence of skeletal malformations was significantly higher in the 7.5 and 8.5 day groups than in the control. In the 7.5 day group 116 of 218 fetuses had skeletal malformations including fused ribs (figure 13), short ribs (figure 14), missing ribs (figure 15) and retarded ossification of skull (figure 16). In the 8.5 day group 109 of 208 fetuses had skeletal malformations including fused ribs, short ribs, missing ribs,bifurcated ribs (figure 17), rudimentary and flying ribs (figure 18) and retarded ossification of skull. In addition, two of 109 fetuses from the 8.5 day group had defective vertebraes including fused vetebral arches (figure 19) and hemivertebral arch (figure 20).

Injection of normal saline into control animals had no effect on fetal development. None of the fetuses from control group had exencephaly or eye deformities. Not surprisingly, 11 of 212 fetuses from control group had additional ribs and 2 of 212 had retarded ossification of skull. These relatively low background incidence of additional rib and retarded ossification of skull have been considered as sporadic malformations.

Postnatal Growth and Development of Fetuses Exposed to Cadmium Chloride *in Utero*

Detailed results of postnatal growth and development of mouse fetuses exposed to cadmium chloride *in utero* are summarized below

Figure 13 (A) Distal fused ribs. $CdCl_2$ 4 mg kg^{-1} on days 7.5 or 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 13 (B) Proximal fused ribs. $CdCl_2$ 4 mg kg^{-1} on day 7.5 or 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 13 (C) Central fused ribs $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 13 (D) Central & distal fused ribs. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 14 (A) Short rib. $CdCl_2$ 4 mg kg^{-1} on day 7.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Fifure 14 (B) Short rib. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 15 Missing rib. $CdCl_2$ 4 mg kg^{-1} on day 7.5 or 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 16 (A) Skull: normal ossification. Normal saline 0.2 ml kg^{-1} on day 7.5 or 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 16 (B) Skull: retarded ossification. $CdCl_2$ 4 mg kg^{-1} on day 7.5 or 8.5 of gestation. Magnification = x 40

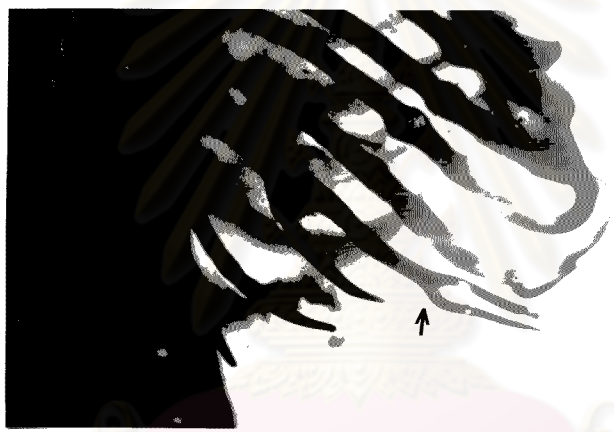


Figure 17 Bifurcated ribs. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 18 Rudimentary and flying rib. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 19 Fused vertebral arches. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

Figure 20 Hemivertebral arch. $CdCl_2$ 4 mg kg^{-1} on day 8.5 of gestation. Specimen is stained with alcian blue and alizarin red S. Cartilage stains blue and bone stains purple to red. Magnification = x 40

A. The Body Weight Development of Individual offsprings.

The weight curve of the offsprings from time of parturition till the 28th day after birth are presented in figure 21. Birth weight of offsprings from the experimental group did not differ significantly from the control. The body weight of offsprings from control group were significantly higher than the body weight of offsprings from experimental group on day 7th after birth. This indicated that the offsprings from control group gained their weights faster than those from experimental group during their first week of postnatal life. Body weight development of offsprings from both experimental and control groups did not differ from each other after the 14th day of postnatal life (figure 21).

B. Earliest Time of Physical Landmarks Appearance

Table 4 showed the day of physical Landmarks appearance of the growing offsprings. In addition, the absolute and relative number of the offsprings that failed to meet these test criteria are shown for both groups. Exposure of maternal animals to a single dose of cadmium chloride (4 mg. kg^{-1}) on day 8.5 of gestation had no significant effects on physical development in the offsprings. There was no difference in the percentage of the offsprings that failed to meet a particular test criterion in both control and experimental groups.

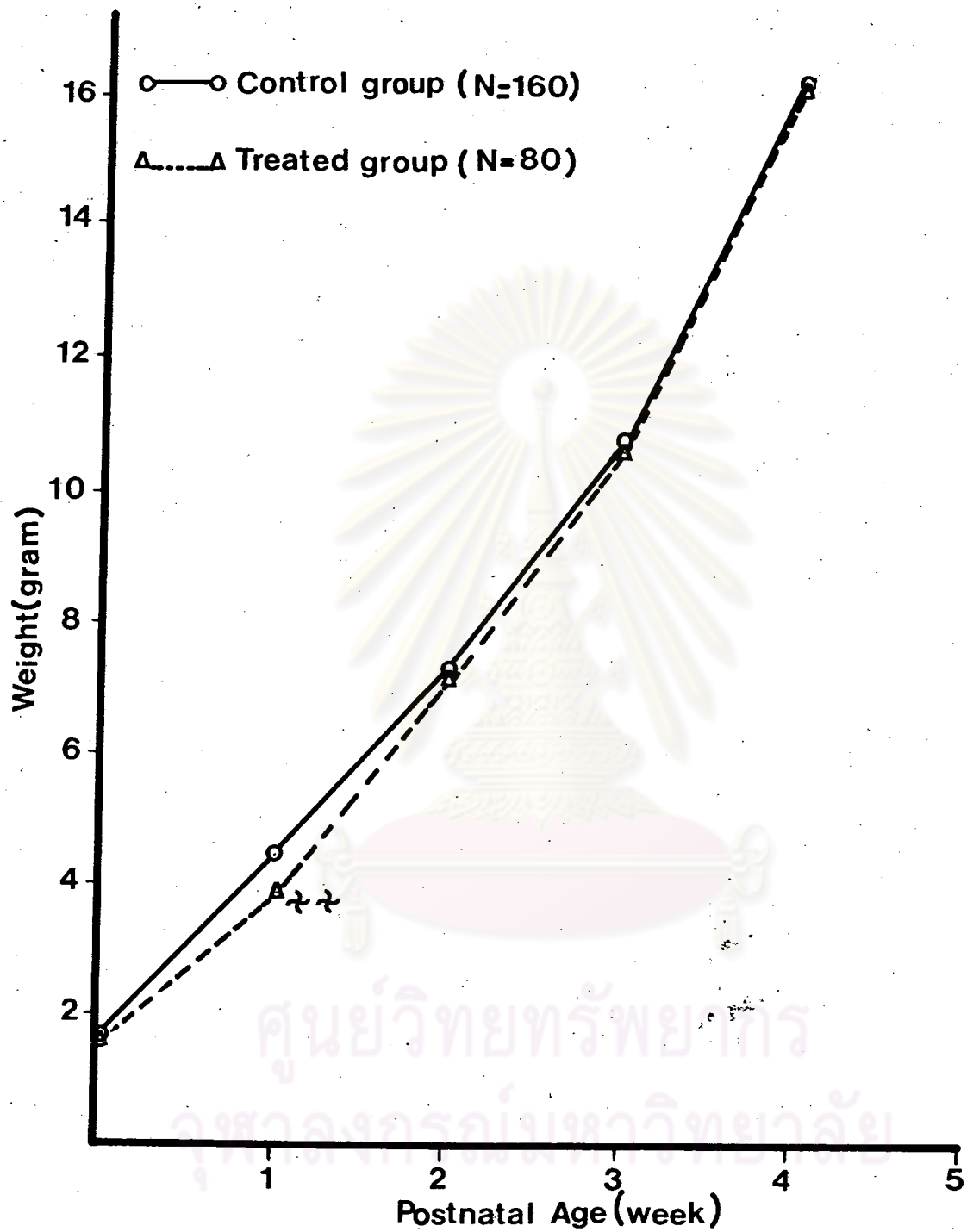


Figure 21 Postnatal weight development of the offsprings of mice exposed to cadmium chloride in utero on day 8.5 of gestation. Each data point represents mean weight. Value significantly different from control at $P < 0.01$ as determined by Z-test are indicated with two asterisks.

Table 4. First Appearance of Physical Features.[a]

Physical Features	Control Group				Treated Group			
	Number exam.	Day of first appearance	Affected offspring	% affected	Number exam.	Day of first appearance	Affected offspring	% affected
Pinna detachment	160(20)[b]	3	0	0	80(10)	3	0	0
Ear opening	160(20)	13	8	5	80(10)	13	3	3.75
Eye opening	160(20)	14	5	3.1	80(10)	14	2	2.50
Testes descensus	92(20)	25	3	3.3	38(10)	25	2	5.26
Vaginal opening	68(20)	35	5	7.4	42(10)	35	3	7.14

[a]Day of first appearance of physical features (Day after birth).

[b]Figures in parenthesis give number of litters examined.

C. Functional and Behavioral Tests

Table 5 showed the results of functional and beharioral tests. In addition, the absolute and relative number of those animals that failed to meet particular test criterion are shown for both groups. No changes in swimming attitude and learning behavior in the offsprings were obtained after the exposure of maternal animals to a single dose of cadmium chloride (4 mg. kg^{-1}) on day 8.5 of gestation. The percentage of the offsprings that failed to meet a particular test criterion did not differ significantly from each other in both control and experimental groups.

Table 5. The Results of Functional and Behavioral Tests.

Parameter	Day of testing	Control Group			Treated Group		
		Number exam.	Affected offspring	% affected	Number exam.	Affected offspring	% affected
Swimming	14	160(20)[a]	0	0	80(10)	0	0
Learning[b]	27	160(20)	26[c]	16.25	80(10)	14[c]	17.5
Memory[d]	34	160(20)	115[e]	71.88	80(10)	52[e]	65.0

[a]Figures in parenthesis give number of litters examined.

[b]Majority of the offsprings from control group entered the box between 2-4 times.

[c]The affected offsprings always enter the box even after the 20th trials.

[d]The offsprings were considered to have memory deficit when they entered the box more than 3 times in 5 trials.

[e]The affected offsprings entered the box 5 times in 5 trials.