CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemicals

Absolute alcohol AR: E. Merck, Germany.

Acetone : May & Baker Ltd., England.

Alcian blue : Sigma, U.S.A.

Alizarin red S : Sigma, U.S.A.

Cadmium chloride : Merck Brandenberger AG, Switzerland or BDH, England.

Glycerin : The Government Pharmaceutical Organization, Thailand.

Potassium hydroxide pellets : J.T. Baker Chemical Co., N.J., U.S.A.

2. Media and Buffer

Phosphate buffered saline (PBS), pH 7.4 : Oxoid, Switzerland

Pure male rat serum : Freshly prepare.

Trowell's T-8 medium : Gibco, U.S.A.

3. Animals

Füllinsdorf albino rats : Füllinsdorf, Switzerland.

Swiss albino mice : Füllinsdorf, Switzerland or Mahidol University, Salaya, Thailand.

4. Apparatus

Culture bottles : 30 ml pyrex bottles with a silicone rubber bungs were used as culture bottles.

Roller apparatus : Rollacell, Model RC-41, New Brunswick Scientific Co., Inc., New Jersey, U.S.A.

Incubator : Hotpack, Model 316580, Hotpack Co., U.S.A.

Methods

1. Feeding and Housing of Animals

Animals were maintained on commercial laboratory food and tap water which were allowed *ad libitum*. They were housing in control room at ambient temperature and under conventional conditions.

2. Mating Procedure

The dark cycle was routinely maintained between 18.00 pm. and 6.00 am. (12 hours). Two female mice (age : approximately 10 weeks, weight : 25-30 g) were housed overnight with one male (age :approximately 10 weeks, weight : 30-35 g) of the same stock. Successful copulation was verified on the next morning by the presence of a vaginal plug and this was designated day 1 of pregnancy. Midpoint of the previous dark cycle (24.00 hours) was designated as embryonic age 0. The pregnant mice were kept singly in metal-box cages on sawdust bedding. Only animals which had gained 2 g or more by day 8 of gestation were selected to ensure that they were pregnant. By using these criteria, greater than 95 % of selected animals were found to be pregnant at necropsy on day 18.5 of pregnancy.

3. Preparation of Culture Medium

The culture medium consisted of pure male rat serum. The medium was prepared by the method previously described by New *et al*. (1976) with some modifications. Blood was withdrawn from the dorsal aortas of Fullinsdorf albino rats, and immediately centrifuged at 4 c (3,000 rpm) for 10 min. The serum was then decanted and thawed, heat-inactivated at 56 c for 40 min. prior to use.

4. Explantation of Mouse's Embryo

Mouse conceptuses were explanted on day 8 of gestation. The dam was killed by cervical dislocation, and uterus was rinsed in Trowell's T-8 medium and placed in a second dish of Trowell's T-8 medium in which it was torn open along the anti-mesometrial side with fine scissor. The conceptuses, which were embeded in a mass of decidua, were cut away from the uterine wall with a fine scissor, using a pair of fine forceps to steady the uterus . The conceptuses were transferred to a dish of fresh Trowell's T-8 medium, which was examined under stereomicroscope.

Two pairs of watchmaker's forcep were used to remove the decidua. Start tearing from the blunt or whiter end of the conceptus, and work up towards the sharper end. All of the maternal decidua and Reichert's membrane were removed. The embryo which is suitable to be used for *in vitro* studies must have the intact yolk sac, amnion sac and ectoplacental cone. Embryo which did not meet these criteria was excluded.

5. Experimental Part I : *In Vitro* Studies

5.1 Assessment of Mouse's Embryonic Development *in Utero*

Procedure

Female mice were mated as previously described in section 2. The pregnant mice were kept singly in metal-box cages in a room under controlled environmental conditions (Temperature 25 ± 0.5 c, relative humidity 60 ± 5 %, 12-hours light-dark sequences). They were fed on commercial laboratory diet and tap water *ad libitum*. On day 10 of gestation, the dam was sacrificed and the uterus was removed, rinsed with Trowell's T-8 medium and transferred into a second dish of the medium

in which it was torn open along the anti-mesometrial side. The conceptuses were cut away from the uterine wall and transferred into the third dish of the medium which was placed under stereomicroscope. Two pairs of watchmaker's forcep were used to remove all of the meternal decidua and Reichert's membrane. The embryos with intact ectoplacental cones and yolk-sacs were then transferred to a dish of PBS solution and examined under a stereomicroscope. Growth was estimated by crown-rump and head measurements together with DNA determinations by the methods of Giles and Myers (1965). The nature and degree of differentiation and development of each embryo were evaluated by examining a number of morphological features; which included yolk sac vasculature; chorioallantois; flexion; heart; fore-, mid-and hindbrain; otic, optic and olfactory systems; branchial bars; mandibular and maxillary processes; fore- and hindlimb buds and somite formation. The degree of differentiation was evaluated by counting somite numbers and using the morphological score table described by Brown and Fabro (1981). Statistics were presented as mean $\pm$ standard deviation.

5.2 Assessment of Mouse's Embryonic Development *in Vitro*

Procedure

Mouse conceptuses were explanted on day 8 of gestation as described in section 4. The embryos (two per bottle) were transferred into culture bottles containing heat-inactivated male rat serum (5 ml/bottle) which prepared as stated in section 3. The culture medium was gasses with a mixture of 5 % O_2, 5 % CO_2 and the balance N_2 for 20 min before use. All operations were carried out aseptically and no antibiotics were used throughout the studies.

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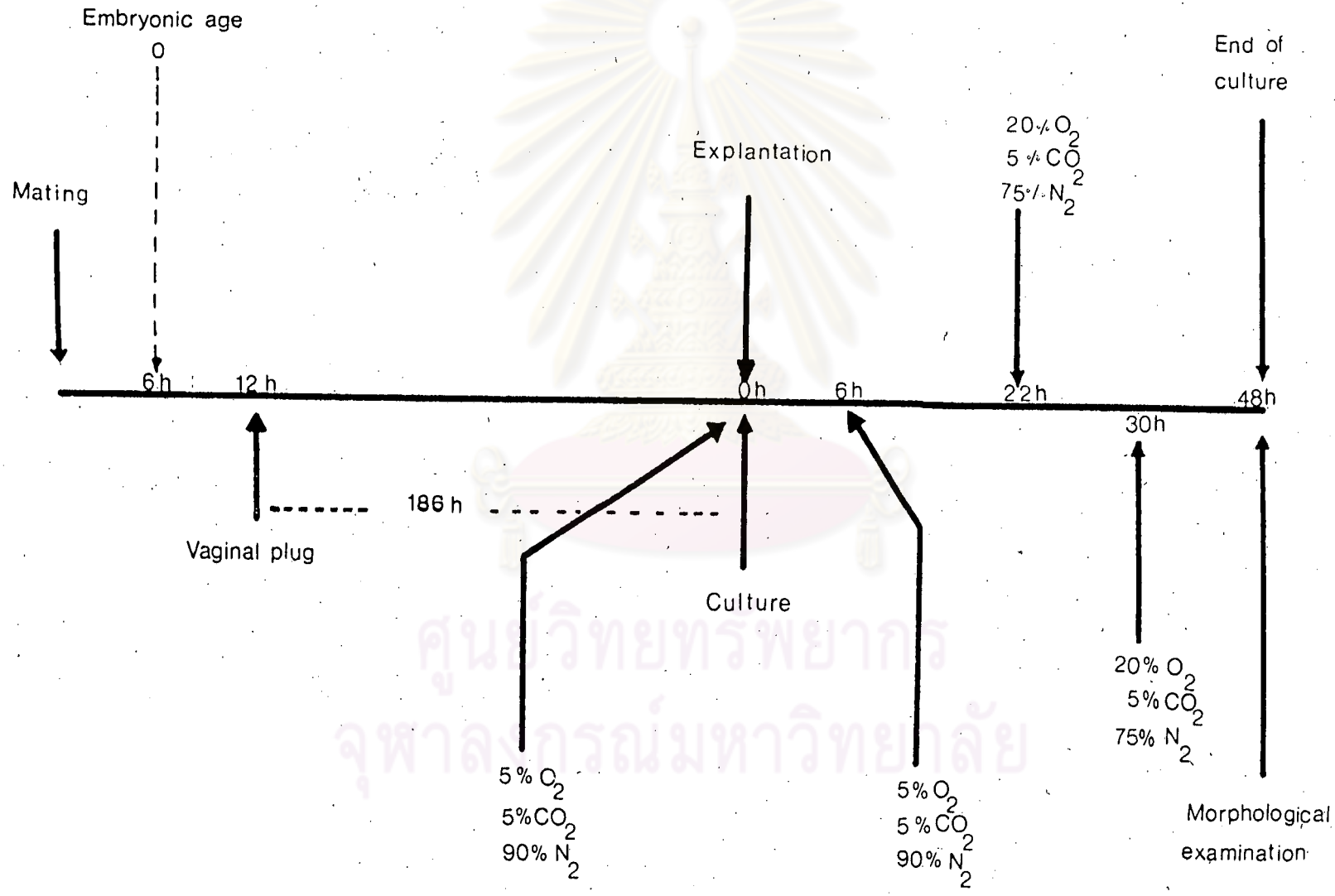

Embryos were cultured in pure rat serum for a period of 48 hrs. Throughout this culture period, the culture bottles were continuously rotated at 11 rpm. on a roller apparatus and maintained at 37.5±0.5 c in an incubator. Gassing conditions were 5 % O_2, 5 % CO_2, 90 % N_2 at the start of the experiment, the initial 5 % O_2 was maintained by flushing with 5 % O_2 for 4 min at 6 hr. At 22 and 30 hr, the culture were flushed with 20 % O_2 for 4 min. All times, these gas mixtures also contained 5 % CO_2 with balance N_2.

At the end of the culture, the developing intact embryos were transferred to PBS solution and examined under a stereomicroscope. Embryos were considered for evaluation when evidence of growth and differentiation and the presence of heart beats were apparent. Growth was estimated by crown-rump and head measurements together with DNA determinations. The nature and degree of differentiation and development of each embryo were evaluated by examining a number of morphological features, which included embryonic flexion; heart; telen-,mesen-and rhombencephalon; otic, optic and olfactory system; branchial bars; mandibular and maxillary processes; fore-and hindlimb buds; and somites formation. The degree of differentiation was also evaluated by counting the somites and by using a modification of the morphological score-table of Brown and Fabro (1981). Statistics were presented as mean± standard deviations, and significances were evaluated using Z-test.

5.3 Determination of the Embryonic Lethality Doses of Cadmium Chloride

Procedure

A 0.03957 mg amount of cadmium chloride was dissolved in 60 ml pure male rat serum. The initial concentration of cadmium

chloride was 3.6 μM (6.595×10^{-4} mg.ml^{-1}) Pipeted 10.67, 10.005, 9.459, 8.004, 4.002 and 0.00 ml of the initial solution into a sterile conical flask, diluted to 12 ml with pure rat serum. The final concentrations of cadmium chloride in rat serum were 3.2, 3.0, 2.8, 2.4, 1.2 and 0.00 μM respectively.

The method used for determining the embryonic lethality doses of cadmium chloride was essentially the same as that employed in section 5.2. The principal deviation was that the embryos were cultured in the rat serum containing various concentrations of cadmium chloride for a period of 48 hrs. At the end of experiment, the number of live and dead embryos were evaluated. Embryos were considered alive when evidence of growth and differentiation and the presence of heart beats were apparent. The embryonic lethality curve was obtained by percent death versus concentration plots.

5.4 Assessment of Embryotoxic Potential of Cadmium Chloride *in Vitro*

The embryonic lethality curve of cadmium chloride was used as the basis for selecting doses for embryotoxic evaluation. Eventually a dose of 3.0 μM was selected as the highest dose.

Procedure

A 0.03957 mg amount of cadmium chloride was dissolved in 60 ml pure male rat serum. The initial concentration of cadmium chloride in the serum was 3.6 μM (6.595×10^{-4} mg.ml^{-1}) Pipetted 10.005, 9.459, 6.674, 3.337, 1.668 and 0.00 ml of the initial solution into a sterile conical flask, diluted to 12 ml with pure rat serum. The final concentrations of cadmium chloride in rat serum were 3.0, 2.8, 2.0, 1.0, 0.5 and 0.00 μM respectively.

The embryotoxic effects of cadmium chloride were examined by using the technique of culturing postimplantation embryos. The procedures for culturing embryos, culture conditions and evaluation of cultured embryos were essentially the same as described in section 5.2. The principal deviation was that embryos were cultured in rat serum containing various concentrations of cadmium chloride prepared as stated above.

Statistics were presented as mean ± standard deviation, and significances were evaluated using analysis of variance and Newman-Keuls test.

6. Experimental Part II : *In Vivo* Studies

6.1 Determination of Adult Lethality Doses of Cadmium Chloride

Lethality was estimated in pregnant mice following single IP administration of cadmium chloride. Mortalities were recorded for up to 10 days after the injection.

Procedure

The doses 8, 7, 6, 5, 4.5, 4.2 and 4.0 $mg\,kg^{-1}$ were used. Cadmium chloride was dissolved in normal saline so as to yield a volume of 0.2 $ml\,kg^{-1}$ body weight at all dose levels. The pregnant mice were randomly allocated to the dose groups so that there were 20 females in each group. The females were treated with a single dose of cadmium chloride on day 8.5 p.c. (post coitus) and checked daily for viability. The findings were recorded for up to 10 days after the injection. The adult lethality curve was obtained by percent death versus concentration plots.

6.2 Assessment of the Embryotoxic Potential of Cadmium Chloride in Vivo

Adult lethality curve of cadmium chloride was used as the basis for selecting doses for embryotoxic evaluation. Eventually a dose of 4 $mgkg^{-1}$ dissolved in 0.2 ml normal saline and injected intraperitoneally was selected. Embryonic days 7-9 were chosen for this study because it has been suggested from the in vitro studies that the abnormal prenatal development may occur only after cadmium exposure in the early phase of the organogenetic period.

Test Procedure

Mated females were randomly allocated to the controls, 7.0, 7.5 and 8.5 days groups so that there were 20 females in each group. The experimental groups were injected with cadmium chloride on days 7.0 p.c. or 7.5 p.c. or 8.5 p.c. The majority were injected at 12.00 a.m., but for day 7 some were injected at 12.00 p.m. The control animals were injected with 0.2 $mlkg^{-1}$ normal saline. Individual body weights were taken on days 0, 7, 7.5, 8.5 and 18.5 p.c. Those obtained on days 0 and 18.5 p.c. were evaluated. The dams were recorded daily for viability and clinical signs.

Examinations

The dams were killed by cervical dislocation on day 18.5 p.c., that is, 0.5 day before expected delivery, and examined immediately. Their inner organs were examined macroscopically and histologically if considered necessary. Uterine examination consisted of checking the number of live and dead fetuses and the number of fetal and embryonic resorption sites.

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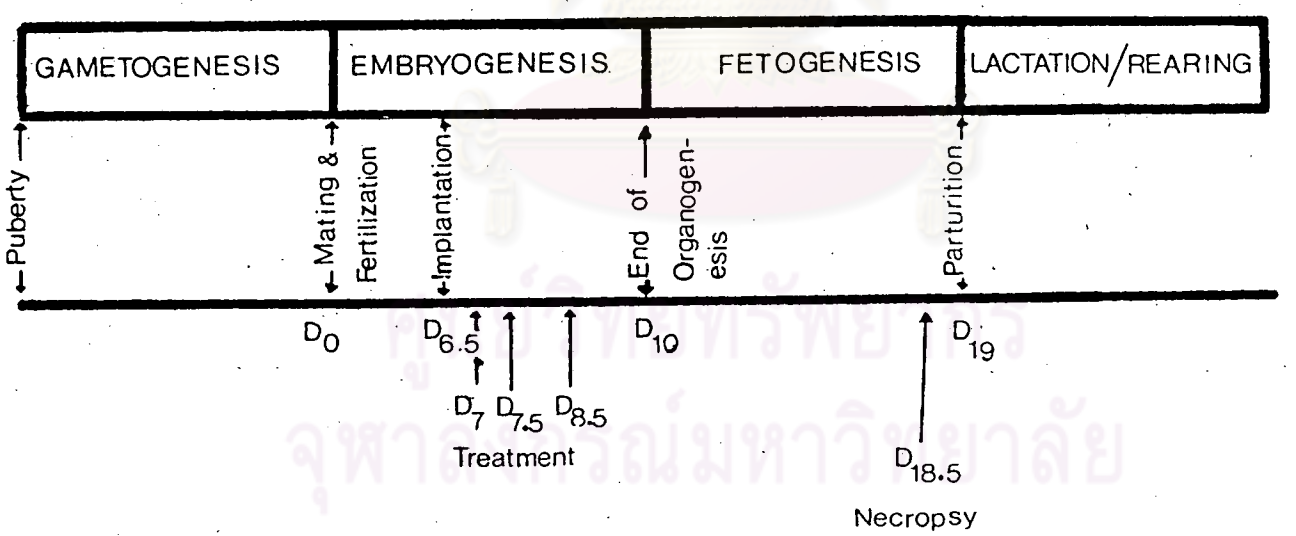
GAMETOGENESIS
EMBRYOGENESIS
FETOGENESIS
LACTATION/REARING
Puberty
Mating & Fertilization
Implantation
End of Organogenesis
Parturition
D0
D6.5
D10
D19
D7
D7.5
D8.5
Treatment
D18.5
Necropsy

Fetuses, which were delivered by cesarean section, were individually weighed, with and without placenta, examined for external and internal anomalies. Heart and kidneys of every fetus were fixed in alcohol. For closer examination heart was cut through the septum and ventricles, and kidneys through the pelvis.

The eviscerated fetuses were stained with alcian blue and alizarin red according to the method of Mcleod (1980) and examined under a stereomicroscope at low magnification for the detection of skeletal anomalies.

Morphological Findings in the fetuses

All morphological deviations in the fetuses were recorded. They were grouped according to the most probable time of their initiation during intrauterine development:

-Retardations; originated in the late fetal phase of maturation,

-Minor anomalies; originated in the early fetal phase of organ differentiation and fetal growth,

-Major anomalies; originated in the organogenetic phase.

Statistical Evaluation

The following tests were used for statistical evaluation:

1. Fisher's two side exact-test when frequencies were compared,

2. The Analysis of Variance followed by Newman-Keuts test when the mean values were compared.

7. Experimental Part III : Postnatal Studies

It has been demonstrated in experimental part II that cadmium chloride can cause a variety of abnormalities in embryos of pregnant mice receiving 4 $mgkg^{-1}$ on days 7.0-8.5 of gestation. These abnormalities were often associated with skeletal and central nervous systems. Although gross deformities were frequently found at term, but those of subtler defects were not easily seen. This idea led to the proposal that functional consequences of the subtler defects which are not detectable in standard teratological examinations might appear postnatally. This part of study was undertaken to determine whether such functional impairments would be evident in postnatal studies of mice exposed *in utero* to cadmium chloride.

Procedure

Female mice were mated as previously described in section 2. The pregnant mice were kept singly in metal-box cages containing sawdust. They were fed on commercial laboratory diet and tap water *ad libitum*. Cadmium chloride dissolved in normal saline was injected intraperitoneally to 10 gravid mice at dose of 4 $mgkg^{-1}$ on day 8.5 of their pregnancy. Twenty control female mice received equivalent amounts of normal saline on the same days of gestation. Pregnant mice

were allowed to deliver normally and all litters were examined at birth. The litter size was standardized to 8 fetuses/litter to avoid the competition between the pups. All litter were wean at 24 days of age and housed in individual cages. Subjects were weighed and checked for the earliest time of physical landmarks appearance periodically from the time of birth until the conclusion of behavioral testing. In order to prevent undetectable behavioral disfunctions, testing was carried out at the earliest time that permits a majority of the control animals to meet the test criteria. Three types of parameters were investigated in this study :

(1) The weight development of individual offsprings.

(2) The earliest time of physical landmarks appearence e.g. ear and eye opening, testes descensus, vaginal opening and pinna detachment.

(3) Functional and behavioral tests, consisting of apical paradigms e.g. swimming, learning and memory tests. Evaluation of results was based on the proportion of animals that fail to meet a particular test criterion.

Swimming Tests

At 14 days of age the swimming ability of all offsprings was measured by the administration of 3 trials in a water filled (30 cm x 45 cm x 15 cm) aquarium. Swimming behavior of the offsprings were observed and recorded. Offsprings were considered to have normal swimming behavior when they can raise their heads, from the level of the ears, above the water and showed correct body coordination. The correct or proper body coordination can be declared only when the animal have straight body and all four feet move harmoniously while swimming.

Learning Tests

At 27 days of age all offsprings were tested for learning ability in the black box. The black box consisted of 22 x 28 x 18 cm rectangular box with black walls. The entry were joined by a 10 x 16 x 8 cm rectangular arm. At the end of the arm there was a grid floor connected to electric transformer which serve as a pain stimulus. Foot shock (1.25 mA, 60 Hz ac) was delivered to the grid floor through a manual switch. Noise stimulus was provided by a noise generator. A trial consisted of placing the offsprings on the entrance arm and switching the stimulus noise to frighten the animal. Entry into the black box resulted in the onset of shock, from this shock the animal will learn to avoid to enter the black box. The animal was tested until it did not enter into the black box and the number of entries were recorded. The highest and lowest values of control group were used as the basis for determining the number of animals that fail to meet the test criteria.

Memory Tests

At 34 days of age all offsprings were tested for memorial ability. The test procedure was essentially the same as that employed in learning tests. The principal deviation was that footshock was not delivered to the animal when it entered into the black box. Each animal was tested for 5 trials. The animal which entered into the black box more than 3 times in 5 trials was considered to have memory dificit. Number of animals that fail to meet this criterion were recorded. Values from control group were compared with the corresponding figures from the treated group. Differences from the control values were considered significant when $P < 0.01$.