Chapter 3

Methods

1. Animals

Mature, 3 months old, virgin female Charles Foster rats (received from Ciba Research Center, Goregaon Bombay, India) weighing 220-270 gm. each were used in this study. The rats were housed in a temperature controlled (26 c) and artificial lighting cycle which alternated 12 hours of light (7 A.M.- 6 P.M.) and 12 hours of darkness (6 P.M.- 6 A.M.) animal room at Chulalongkorn Hospital. Excess laboratory chow diet (Zuellig's Gold Coin Mills) and tap water were given ad libitum. They were acclimatized to their new environment at least for 2 weeks prior to the start of mating.

2. Vaginal smears

Various stages of estrus cycles were determined by means of vaginal smear as described by Long & Evan (1922) and Tienhoven (1968). Vaginal smear of the rats, obtained by saline (0.85%) lavage. By using medicine dropper a small amount of saline is placed on a glass slide and examined at 100 X without staining. The stages in the estrous cycle were designated as

proestrus, estrus, metaestrus, and diestrus.

2.1 At proestrus, which is the time of rapid follicle growth, corpus luteum of the previous cycle has been regressed.The epithelium of the vagina become thick as a result of proliferation in its double layers. The uterus starts to distend with fluid as a result of high estrogen production. The stage is denoted by the presence of nucleated epithelial cells in the vaginal smear. The proestrus is about 12 hours duration and showed the sign of acceptance of the male at the end of this period.

2.2 Estrus At the early of this state, there is maximum estrogen production and ovulation occured. The uterus shows maximum distension with hyperemia. After ovulation estrogen level will decrease and uterine distension appears to subside rapidly. Only cornified cells are found in the vaginal smear and this stage of about 30 hours duration.

2.3 Metestrus The estrus stage progresses into the metestrus stage. Estrogen is very low and corpora lutea will be found in the ovary as a result of the last ovulation. This stage of about 6 hours duration is signified by the abundance of degraded cornified cells plus leukocytes in the vaginal smear.

2.4 Diestrus stage This stage, estrogen will

not be produced from the ovary and corpora lutea starts to degenerate. The uterus is small in size. The diestrus stage of about 60-70 hours is denoted by the presence of a lot of leukocytes and a few epithelial cells in the vaginal smear.

3. Vaginal Canalization

Female rats were examined daily for vaginal canalization starting from 28-30 days of age and afterwards.

4. Pregnancy and Lactation

Adult cycling female rats were mated with proven males. The day sperms were observed in the vaginal smear was designed as day 1 of pregnancy. The pregnant mother separated in an individual cage throughout pregnancy. The day of parturition was considered as day 1 of lactation.

5. Preparation of DMPA for injection

DMPA (vial 3 ml. containing 150 mg. of medroxy progesterone acetate) was diluted ten folds with DMPA's Vehicle.

6. Preparation of DMPA's Vehicle

Weigh Poly ethylene glycol 400 2.88 gm., Poly

sorbate 80 0.192 gm., Sodium Chloride 0.865 gm., Methyl paraben 0.173 gm., Propyl paraben 0.019 gm. by using electric balance. Mix together, add sterile distilled water to 100 ml. and keep in refrigerator.

7. Preparation of Chemical compounds

7.1 Fixatives

7.1.1 Bouin's fluid

Picric acid (saturated solution)	75 ml.
Formaldehyde (40%)	25 ml.
Glacial acetic acid	5 ml.

7.1.2 Sublimate formol

Mercuric chloride (saturated solution)	90 ml.
Formaldehyde (40%)	10 ml.

7.2 Reagents

7.2.1 Harris Hematoxylin

Hematoxylin	5 gm.
Ethyl alcohol (95%)	50 ml.
Aluminium ammonium sulphate	100 gm.
Distilled water	1000 ml.
Mercuric oxide (yellow)	2.5 ml.
Glacial acetic acid	50 ml.

Dissolve hematoxylin in 95% ethyl alcohol while grinding it in a mortar, the alum in the water (in a

large beaker) by aid of heat. Bring solution of alum almost to boil, cool slightly and add hematoxylin solution. Bring the mixture rapidly to boil, remove from heat and carefully add the mercuric oxide. Boil a minute or two until solution is a dark purple color. Remove from flame and cool rapidly by plunging into a basin of ice water. When cool, add glacial acetic acid. Filter before use.

7.2.2 0.5% Eosin

Eosin	0.5 gm.
Ethyl alcohol 95%	100 ml.

7.2.3 Schiff reagents

Basic fuchin	1 gm.
Distilled water	200 ml.

Boil for 5 minutes, cool to 50° c

Add 1 N HCl	20 ml.
Potassium metabisulphite	2 gm.

Shake during day and let stand overnight.

Add Norite	0.5 gm.

Shake and filter. Store in refrigerator when not in use the solution should remain colorless.

7.2.4 Orange-G

Orange-G	3 gm.
Distilled water	100 ml.

Make solution to pH 2 by adding hydrochloric acid.

7.2.5 1% Periodic acid

Periodic acid	1 gm.
Distilled water	100 ml.

7.2.6 Iron alum

Ferric ammonium sulphate	5 gm.
Distilled water	100 ml.

8. Histology of mammary gland

After fixing mammary tissue in Bouin's fluid for 4 hours, tissues were washed in 70% alcohol. For dehydration, tissues were changed stepwise one hour each through 80% ethyl alcohol, 90% ethyl alcohol, 95% ethyl alcohol + n-butyl alcohol, n-butyl alcohol, n-butyl alcohol + xylene and cleared in xylene for another 1 hour. Then tissues were infiltrated with paraplast in oven (60-62° c). After embedding, tissues were sectioned serially with microtome at 6 micra in the horizontal plane and stained with Harris Hematoxylin and Eosin.

9. Whole mount technique for mammary gland

Left abdominal mammary glands were cut with skin and streched on cork board for 3 hours. Fix in Kahle's AFA fixative about 48 hours in refrigerator. The layer of subcutaneous fascia, containing the glands dissected

from the pelt by use of the blunt. Excess fat was removed by soaking in 2:1 chloroform methanol for 24 hours, followed by ether extraction for another 12 hours. The glandular tissues were stained with Hemtoxylin, followed by differentiation in 0.5% hydrochloric acid. The individual gland were taken up to the alcohol into xylene and mounted on slide in canada balsm.

10. Serial section of pituitary gland

After fixing in Sublimate formal fixative for 2 hours, pituitary gland were carefully dissected free of surrounded tissue and washed in running tap water for 2 hours. After dehydration, the pituitary glands were embed in paraplast in the same way as mammary tissue and were serially sectioned at 4 micra in the horizontal plane.

11. Staining procedure for pituitary gland

Pituitary sections were stained by the Periodic Schiff's reagent-iron Hematoxylin-Orange G technique as described by Elftman (1960) :

Deparafinized the tissue in xylene and gradually hydrated with ethyl alcohol until 70% ethyl alcohol. Staining procedures were then carried out as follows :

Lugol's Solution	3 min.
distilled water	3 min.

5% Sodium thiosulphite	5	min.
running tap water	10	min.
distilled water	3	min.
oxidized in Periodic acid	15	min.
Distilled water	5	change
Schiff's reagent	30	min.
running tap water	30	min.
Distilled water	1	min.
Orange-G I	10	min.
running tap water	1	min.
distilled water	3	change
Iron alum	2	min.
distilled water	3	change
Harris Hematoxylin I	30	sec.
Distilled water	5	change
Orange-G II	20	min.
running tap water	1	min.
Harris Hematoxylin II	30	sec.
Distilled water	5	change

Dehydrated and cleared in xylene. Mount in permount.

12. Pituitary cells count

Representative saggital sections from three levels of each gland were carefully chosen at approximately $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$ the distance through the gland. Using the light

microscope, at 400X magnification, cells were classified as either acidophiles, basophiles, or chromophobes and differential counts for 2,000 anterior pituitary cells from each of three levels were made in the same area around the middle part of the right wing of the adrenophpophysis as much as possible. (Figure 1)

Counts of three levels were averaged to give counts per unit area of each gland. Cell types populations in each treatment group were averaged to obtain a mean value per group and expressed as percentages of the total anterior pituitary cells with standard error.

Experimental designs

290 Rats were used in this study and were devided randomly into small groups each as follows :

1. Effect of DMPA on milk secretion

The effect of DMPA on milk secretion in lactating rats was determined by the increase in body weight of the suckling litters. Lactating rats were devided randomly into four subgroups of 12 animals each as follows :

group A : Lactating mother received DMPA (5 µg/gm. body weight) on day 9 of lactation.

group B : Control group injected vehicle of DMPA on day 9 of lactation.

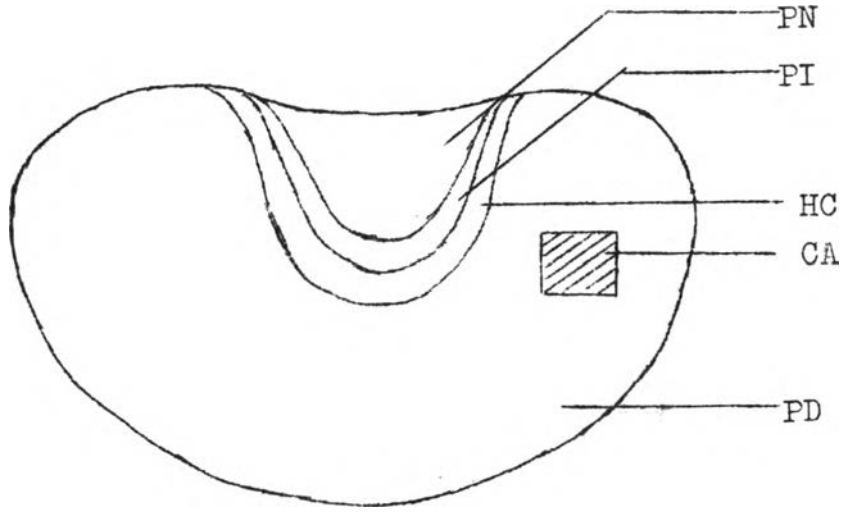


Figure 1 Diagram showed the cells counting area in anterior hypophysis

Abbreviations

HC = Hypophyseal Cleft

PD = Pars distalis

PI = Pars intermedia

PN = Pars nervosa

CA = Counting area

group C : Same as group A but litters were removed on the day of delivery.

group D : Same as group B but litters were removed on the day of delivery.

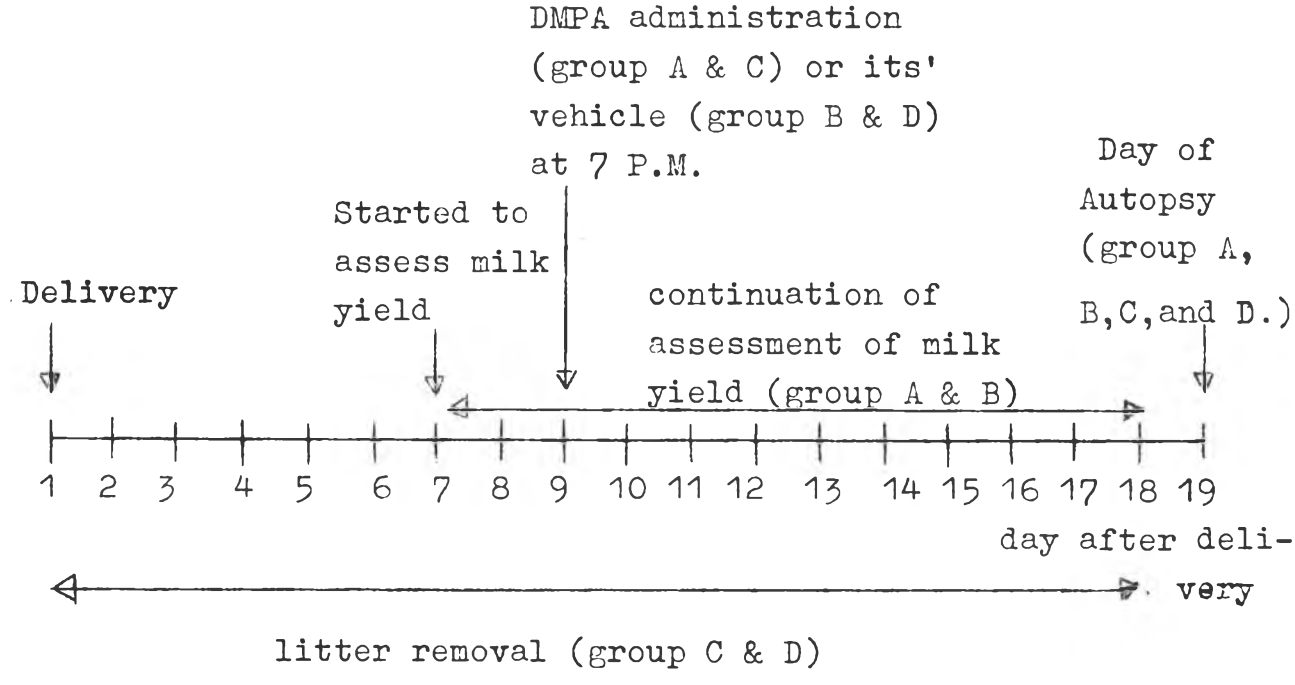
The purpose of the first two groups (A and B) was to see the effect of DMPA on milk secretion in lactating rats, while the last two groups. (C and D) was to see the effect of DMPA on histology of pituitary and mammary gland.

Biological parameter for milk secretion was assessed by body weight increment of the suckling infants. With no exception, the number of litters were reduced to five, three females and two males, to ensure that the suckling stimulus would be similar for all lactating mothers. Milk secretion was assessed twice daily starting from day 7 to day 18 of lactation. Before the first nursing of the day, litters were separated 6 hours from their mothers from 6.00-12.00 A.M. For the purpose of ejection of the rest of milk remained in mammary alveoli, 1.0 mU Syntocinon (oxytocin) was injected intraperitoneally into each mother prior to each suckling. The litters were allowed to suck for 90 min. Body weight of the litters were recorded before and after each suckling. For the second nursing of the day, the litters in both groups again were separated

from their mothers,this time from 2.00-6.00 P.M., after which litters were permitted to suck again for 60 min. Weighed the litters before and after the suckling. The purpose of the second nursing was to see the refilling rate of milk in the mammary gland. When the weight gain of the litters after suckling became constant or no more fluctuate than 30% for three consecutive days, DMPA (5 µg/gm. body weight) were injected intramusculary to the lactating mother. For control group,vehicle of DMPA were injected instead. In litters removal group (group C and D),litters were removed on the day of delivery. DMPA or vehicle was injected intramusculary on the same day as group A and B. The schedule of this experiment was summarized in Fig II

In the morning of day 19 of lactation, all of the mothers in every group were secreficed by an overdose of ether. The pituitary gland was promptly removed and placed in sublimate formal (9:1) fixative. At the same time the right abdominal mammary glands were removed with approximately 3 mm. of tissues surrounded and fixed in Bouin's fluid.

After fixing for 2 hours, pituitary glands were carefully dissected free of surrounding tissue and washed in running tap water for 2 hours. They were placed briefly on the filter paper to remove excess water and



Figrue II Schedule of experiment on the effect of DMPA on milk secretion in lactating rats.

weighed with electrobalance and were kept for histological study.

2. Effect of DMPA on growth and sexual development of the litters

Twenty lactating rats were used and were randomly devided into 2 groups of ten rats each as follows :

group A : 5 µg/gm. body weight DMPA was injected intramuscularly to mother on day 3 of lactation.

group B : DMPA's vehicle was injected on the same day of animals in group A.

As in other groups previously mentioned, on day 3 of lactation the number of litter of each mother was reduced to 5, three females and 2 males, to ensure that the suckling stimulus would be similar for all the lactating mothers and there would be adequate milk available for all the litters. Growth rate and various parameters of sexual developments were followed in thirty-two females and nineteen males whose mothers recieved DMPA (group 2 A), comparing with twenty-eight females and nineteen males whose mother received vehicle of DMPA (group 2 B) until 60 days of age. Detail of the studies were as follows :

2 a. Effect on body growth and sexual maturation of the suckling female litters

The following factors studied for the females ;

(a) daily weight gain

(b) day of vaginal opening

(c) day of the first regular estrous cycle

(d) ability to become pregnant after exhibiting 2 or more regular 4-5 days estrous cycle and had about 3 months of age

(e) maintenance of pregnancy and labour

(f) number of the litters borned

(g) abnormalities of the litters (if any)

(h) lactational performance as assessed by weight curves of their litters

2 b. Effect on body growth and sexual maturity of the male litters

The following factors studied for the 19 males whose mother were injected DMPA :

(a) daily weight gain

(b) fertility approach when mated with normal female

(c) ability to sire normal healthy pups