Chapter II

Materials and Methods

1. Animals

1.1 Rats

Three-month old virgin female Wistar rats obtained from the breeding colony of Biology Department, Chulalongkorn University were used in this study. The animals were housed in an air conditioned room (22-25°C) with daily light schedule during 0600-2000h. Pellets food (Pokhand Animal Feed Co., Ltd., Bangkok) and tap water were available ad libitum. Only animals which exhibited a minimum of two normal 4 day cycles, determined by daily vaginal smears, were used. In the cyclic stage, the nucleated cells, the cornified cells, the first day leukocytes and the second day leukocytes found in the vaginal smears was designated as proestrus (O), estrus (Co or E), diestrus-day 1 (Di-1) and diestrus-days (Di-2) respectively (Long and Evan, 1922). Pseudopregnancy was induced by electrical stimulation of the cervix on the morning of proestrus and estrus described by Shelesnyak (1932). Day 1 of pseudopregnancy (L_1) was designated by the appearance of leukocytes in the vaginal smear. Pregnancy was induced by placing 2-3 females with a fertile male during the evening of proestrus. The presence of sperm plug of spermatozoa in the vaginal smear in the morning of the expected day of estrus was designated as Day 1 of pregnancy (L_0) The delivery day was designed as the first day of lactation (L_o). With no exception, all lactating mothers were

adjusted to nurse 10±1 infants throughout the period of experimentation.

1.2 Monkeys

Three adult female cynomolgus monkeys with regular menstrual cycles from the primate breeding colony of the Biology Department, Chulalongkorn University were used. Two monkeys (#75 and #101 were 5 years old and exhibited normal menstrual cycle (# 75 = 35.14±5.71 days, # 101 = 33.91±4.11 days) while other monkey (# 24) was over 13 years old and had shorter menstrual cycle length of 24 days with an evidence of infertile and galactorrhea (Cholvanich, 1986). They were housed individually under environmental temperature. The colony exposed to natural light and additional fluorescent light were also provided daily (0600-1800h). They were fed daily in the morning with monkey chow (Pokphand Animal Feed Co., Ltd., Bangkok) and in the afternoon with fresh fruits and vegetables. Daily vaginal swabbings were carried out for detection of the menstrual bleeding. The approximate date of ovulation was anticipated by using Dukelow et al's formula (1979) as follow:

$$\frac{\text{day of ovulation}}{\text{cycle length}} = 0.48$$

This would be used as the parameter for collection of luteal cells during various luteal phase.

2. Chemicals

2.1 Medium for luteal cell culture

- Dulbecco's modified Eagle medium (DMEM)
- nutrient mixture-F10 (Ham) (HAM-F10)

2.2 Agents for testing responsiveness of CL

- ovine-prolactin (o-PRL), biological activity 31 IU/mg,

- human Chorionic gonadotropin (hCG), biological activity 2500 iu/vial, and

- prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$, synthetic) 8.1 mg/vial.

2.3 Hormones and antisera for RIA.

2,4,6,7,16,17,^{3}H,-E_2 (specific activity 152 curies/mmol) and 1,2,6,7,^{3}H-P (specific activity 80-110 curies/mmol) purchased from Amersham International Ltd.

Standard hormones of E_2, P and antisera to E_2 and P obtained from WHO.

Note : All other chemicals and instruments which were used in this study are shown in appendix pages 162-166.

3. Collection of rat CL.

The CL were dissected from different reproductive stages of rats. Each group consisted of 4-6 rats.

reproductive stages	stages of CL collection
estrous cycle	E, Di-1, early Di-2 and late Di-2
pseudopregnancy	L_2, L_6, L_{12}
pregnancy	L_2, L_6, L_{12}, L_{20}
Lactation	L_2, L_{12}, L_{20}

Aseptic technique was used for all operation. The animals were killed by decapitation between 0830-0900 h, except rats in late Di-2 which were killed between 1500-1530 h. The ovaries were removed immediately and transfered to washing medium at room temperature. The CLs were carfully dissected from adhering tissue by free hand dissection. Newly formed CLs were different from old CLs by noting the big size, pink color and heavy degree of capillary invasion. There were two sets of CLs in lactating rat, the large yellow ones with a low degree of capillary invasion were pregnant CLs; the small, pink ones with a high degree of invasion were lactating CLs. The CLs which were obtained from the same physiological stages were pooled and stored in washing medium for luteal cell dispersion.

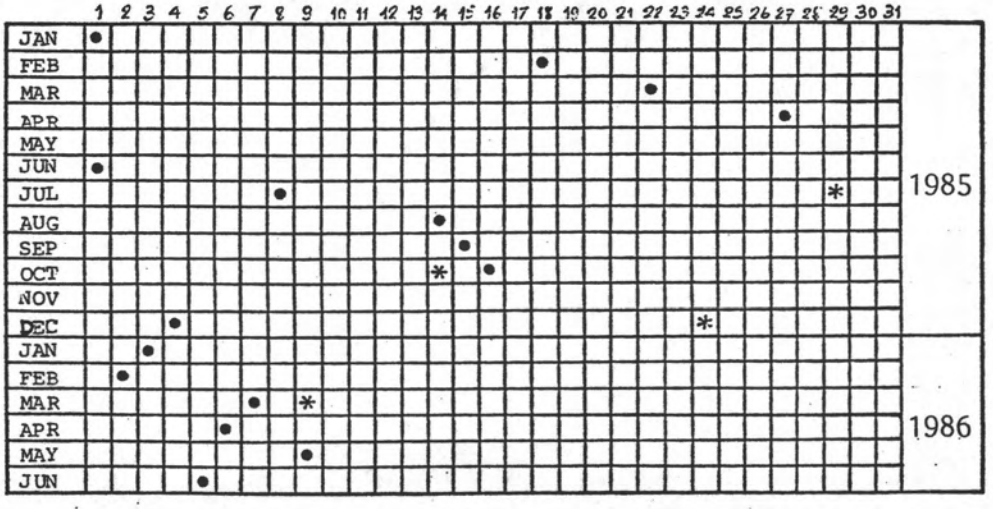
4. Collection of rat plasma samples.

Prior to corpus luteum dissection on the anticipated date, blood samples were collected from decapitated rat in heparinized conical tube, centrifuged immediately at 3,200 rpm., 4°C, 30 minutes. Plasma was collected and kept in deep freezer (-20°C) for P and E_2 radioimmunoassays.

5. Collection of monkey corpus luteum

Monkey CLs were obtained by partial lutectomy from various stages of the CL life during menstrual cycle. After the anticipation date of ovulation, the stages of monkey CLs were classified into 4 groups as follows:

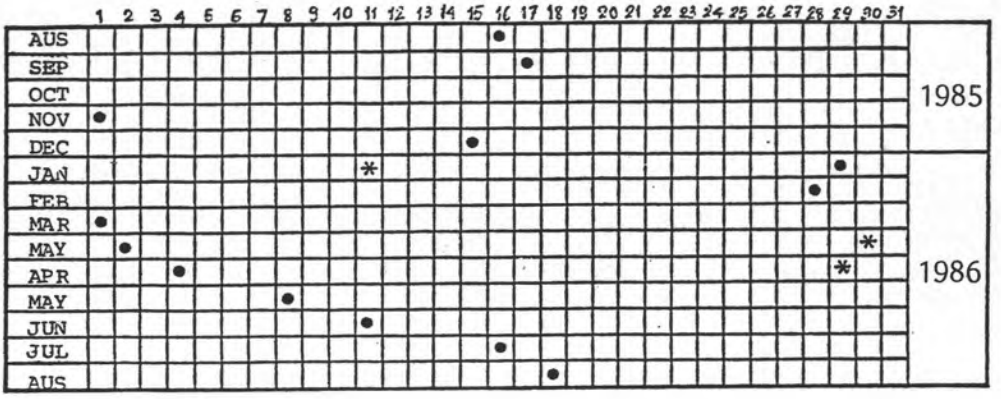
- 15-18 days prior to menses as early luteal phase.
- 8-9 days prior to menses as mid luteal phase.
- 3-5 days prior to menses as late luteal phase.
- during menstrual bleeding peroid as luteolytic phase (see Fig. 1,2,3)



● first day of menstrual cycle

* the day of lutectomy

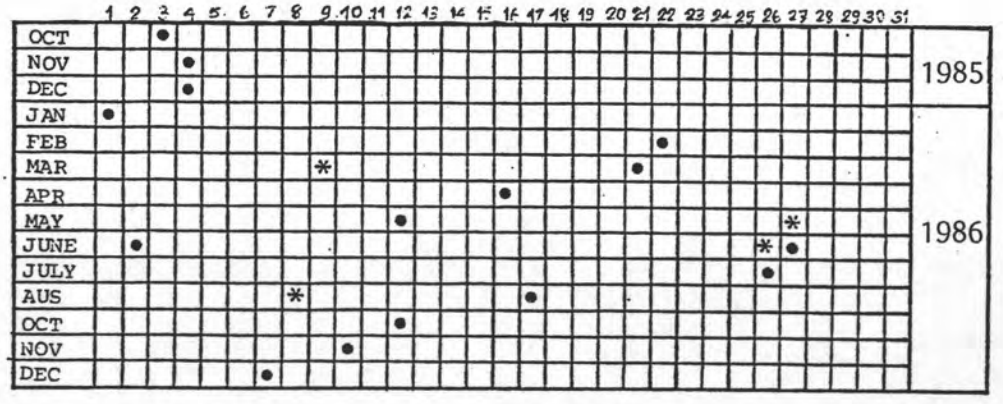
Fig. 1 Menstrual records of cynomolgus monkey # 101



• first day of menstrual cycle

* the day of lutectomy

Fig. 2 Menstrual records of cynomolgus monkey # 75



• first day of menstrual cycle

* the day of lutectomy

Fig. 3 Menstrual records of cynomolgus monkey # 24

When the animals were in the anticipated stages, they were starved 12 hours before operation. The animals were anesthesized by ketalar injection (Parke-Devis Detroit, 15-20 mg/kg, intramuscular) and supplemented with atropine sulfate (0.5 ml/individual, intramuscular) in order to inhibit saliva secretion. Prior to performing the operation, each animal had to be checked laporascoptically with the aid of cystoscope (in order to ascertain that the monkey had CL in the ovary). When the CL was detected, it was dissected out approximately ½-¾ of the whole CL by laporatomy and was then transfered to HAM-F10 nutrient for dispersion, simultaneously. Aseptic technique was necessary during operation and luteal cell dispersion. The lutectomized monkeys were housed in individual cage. They were observed for the menstrual bleeding by daily vaginal swabbings. After partial lutectomy, lutectomized animals were found to have reestablishment of regular menstrual cycle. The subsequent lutectomy was performed after at least one cycle.

6. Collection of monkey plasma samples

Blood samples (3 ml each) were collected by femeral puncture (under ketalar anesthesia) on the day of lutectomy and on days 1 and 5 of post operation. They were drained into heparinized conical tubes and centrifuged immediately at 3200 rpm, 4°C for 30 minutes. Plasma was collected and kept in deep freezer (-20°C) until P and E_2 radioimmunoassays were performed.

7. Luteal cell culture

7.1 Medium for rat luteal cell culture : Dulbecco's Modified Eagle Medium (DMEM) 500 ml.

5 grams of DMEM powder, 25 mM (2.9878 gm) HEPES (n-2-hydroxyethyl piperazine-N-2-thanesulfonic acid), 4.4 mM (1.85 gm) $NaHCO_3$, 50 iu/ml penicillin, 0.1 mg/ml streptomycin and 50 iu/ml fungizone were dissolved completely in 447.4 ml of tridistilled water, adjusted pH of medium to 7.2-7.3 by using 1 N NaOH or 1 N HCL. Sterilizaiton of medim was done by filtering through 0.22 μm millipore filter. After 50 ml of fetal bovine serum (GiBCo) was added, the medium was aliquoted into 10 ml per bottle and kept at $4^{o}C$ until used. The medim prepared each time was used within 1 month.

7.2 Medium for monkey luteal cells culture : Nutrient mixture F-10 (Ham) (or HAM-F10, GIBCo) 500 ml.

5 grams of HAM-F10 medium powder, 2.4 mM (0.6 gm) $NaHCO_3$ (Merck, Ltd.), 25 mM (2.97875 gm) HEPES (BDH Chemicals Ltd.), 50 iu/ml penicillin, 0.1 mg/ml streptomycin and 50 iu/ml fungizone were dissolved completely in 447.4 ml tridistilled water, pH of the medium was adjusted to 7.2-7.3 by using 1N NaOH or 1N HCL. The medim was steriled by filtering through 0.22 μm millipore filter; added 50 ml of fetal bovine serum and aliquoted into 100 ml per bottle, and kept at $4^{o}C$ until used. The media prepared each time was used within 1 month.

7.3 Washing medium : culture medium without fetal bovine serum.

7.4 Luteal cell disperion

Corpora lutea of rats or monkeys were dispersed by using the method of Sala et al., (1979) with minor modifications as follows.

The corpora lutea were washed twice in washing medium to remove excess blood cells and then minced into fragments by clean, sharp surgical microscissor. The luteal tissue fragments were dispersed with 0.1% of collagenase in 10 ml of medium in a flask with constant slow stirring was carried out in water bath at 37^{o}C, for 30 minutes. Then, it was further dispersed by gentle aspiration (mechanical dispersion) with a flame-polished tip and large pore pastuer pipette for 15-20 times. Tissue fragments were allowed to settle for about 30 seconds, the turbid supernatant containing isolated cells was collected and centrifuged at 1000 rpm for 10 minutes, at room temperature. The pellet was then washed with washing medium twice. Ultimately, the pellet was resuspended in 3 ml of culture medium, the viability, concentration and responsiveness to exogenous agents of the luteal cells were determined. These isolated luteal cell suspensions were preincubated for 60 minutes before used.

7.5 Determination of cell viability and cell concentration

Preparation of Hank's Balanced salt solution (HBSS) 500 ml:

5 grams of HBSS powder and 0.45 mM (0.175 gm) $NaHCO_3$ were disolved completely in 500 ml of tridistilled water, pH of the solution was adjusted to 7.1-7.2 yb 1.0 N HCL or 1.0 N NaOH and kept at 4^{o}C until used.

Cell viability was judged by "The trypan blue stain exclusion method" (Tennants, 1964). An equal volume of 0.2% trypan blue in HBSS and isolated luteal cell suspension were mixed. The dye was allowed to penetrate into cells for about 5-10 minutes at room temperature. The viable cells were examined under light microscope and the unstained cells were scored for viable cells. The viable cell concentration was determined by hemocytometer, counting viable cells in five areas as shown in Fig. 4 and calculating the mean of viable cells per area (width, length = 1 mm (0.1 cm), depth = 0.1 mm (0.01 cm), volume = $10^{-4}cm^3$) was calculated. Cell concentration is the amount of viable cells per area x 10^4 cells/ml of isolated luteal cell suspension.

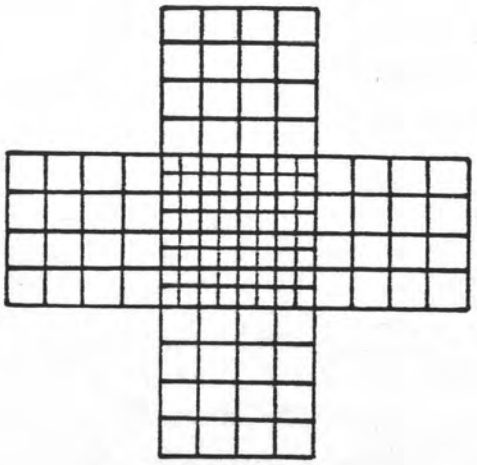


Fig. 4 Showing 5 areas of hemocytometer.

7.6 Determination of isolated rat luteal cell responsiveness

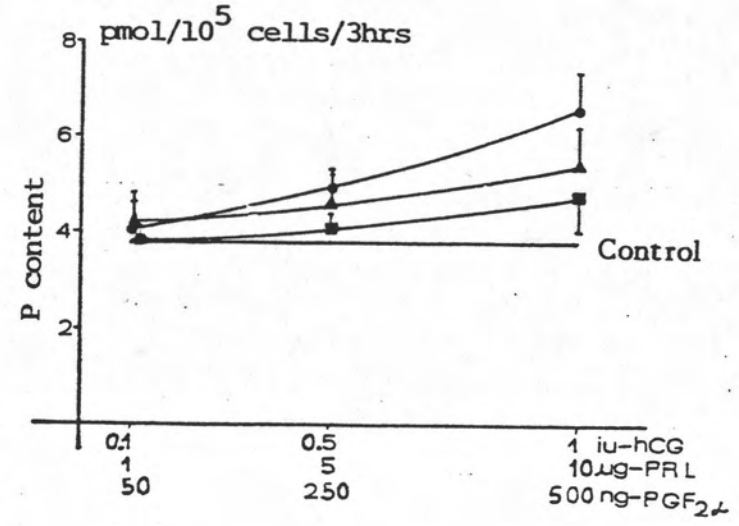
Isolated rat luteal cell suspension from cyclic rat at estrous stage, pseudopregnant stage L_2, pregnant stage L_2 and lactating stage L_2 were used. They were cultured individually with and without hormone added in doses of 1, 5 and 10 folds. Hence hCG 0.1, 0.5 and 1.0 iu/10^5 luteal cells/2 ml DMEAM, o-PRL 1, 5 and 10 µg/10^5 luteal cells/2 ml DMEM and $PGF_{2\alpha}$ 50, 250 and 500 µg/10^5 luteal cells/2 ml DMEM were designated as treated groups. The luteal cell culture without hormone was kept as control group. Each group was performed in triplicate. They were incubated in multiwells dish (24 wells/dish) at 37°C under 5% CO_2 and 95% humidified air for 3 hours. At the beginning of culture peroid, aliquoted 10^5 luteal cells mixed with 95% ethanol 500 µl for killing cells and kept in deep freezer for zerotime P determination. At the end of the incubation period, the medium and luteal cells were collected and kept at -20°C for P assay. P content was determined and expressed in production rate (pmol/10^5 cells/3 hrs).

production rate = P content during 3 hour incubation - P content at zerotime

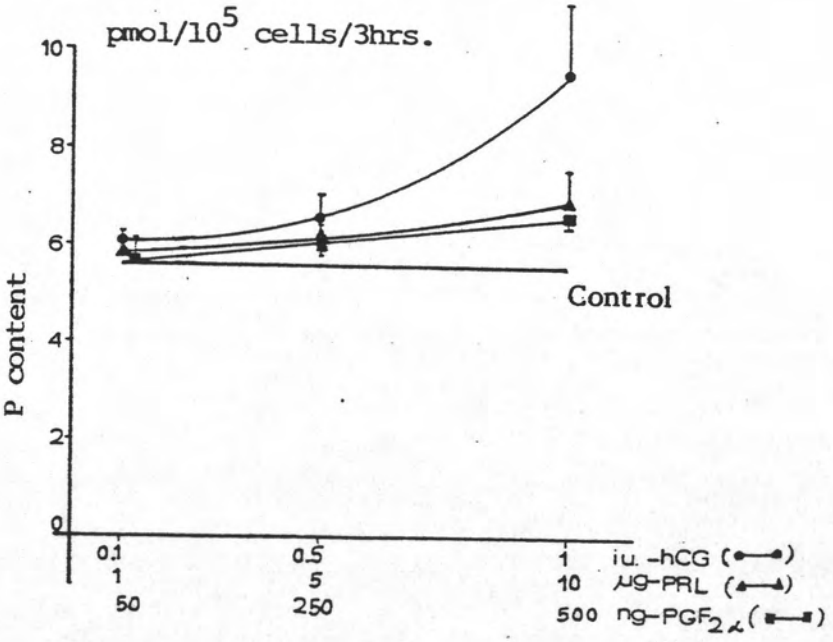
Figure 5, 6 show the results on linear semilog scale with production of P on y-axis and dose of hormones treatment on x-axis. From these results, hCG 0.5 iu/ml, PRL 5 µg/ml and PGF_2 250 ng/ml were chosen for further experiment.

Culture condition of rat luteal cells.

number of luteal cells : 10^5 cells
culture medium : DMEM 2 ml
incubation peroid : 3 hours (short term incubation)
: 11 days (long term incubation)

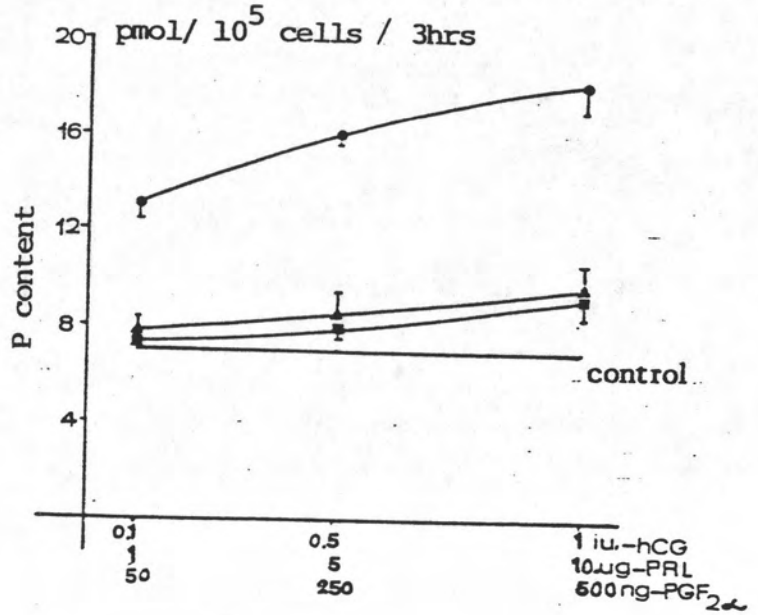


A.Estrus



B.Lactating stage L2.

Figure 5. Showed the responsiveness of luteal cells to hCG, PRL and $PGF_{2\alpha}$ on P production (mean±S.E., pmol/10^5 cells/3 hrs, n = 3). A. Estrus and B. Lactating stage L_2.



C.PSP stage L2

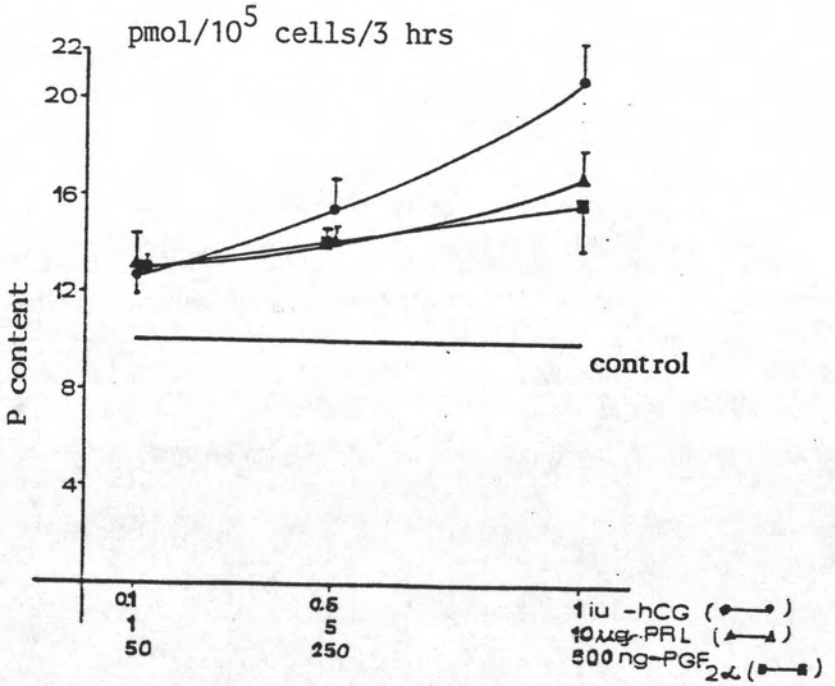


Figure 6. Showed the responsiveness of luteal cells to hCG PRL and $PGF_{2\alpha}$ on P production (mean±S.E., pmol/10^5 cells/3 hrs, n = 3). C. PSP stage L_2 D. Pregnancy stage L_2.

Day 0 - day 1 and day 1 - day 11 were designated as preincubation peroid (without hormone application) and incubation period (with hormone treatment) respectively. The rat luteal cells were cultured in multiwells under 5% CO_2 and 95% humidified air at 37°C.

The protocol of experiments were used as follows:

The viability of luteal cells and the hormone secreting ability of rat CL in vitro.

Two sets of isolated luteal cell suspensions obtained from the same pool of corpora lutea from each stage were used. The first set was cultured for determination of the viability of luteal cells which were attached to the culture dish during 11 days of incubation. In general the attached luteal cells were found within 18-24 hours incubation and were deattached by trypsinizaiton with 0.2% trypsin in HBSS at 37°C, for 10-20 minutes. (Corredor and Flickinger, 1983) The viability of luteal cells was determined by dye exclusion test (Tennants, 1964). The second one was performed for determination of the P secreting ability of luteal cell in culture. Each set was incubated 11 days in the same condition and peroid. Media were collected and replaced on day 1,3,5,7,9 and 11 of incubation. While the attached luteal cell viability in the first set was determined, media in the second set, were collected and centrifuged at 1000 rpm, for 10 minutes at room temperature and the supernatants were kept in deep freezer (-20°C) for P determination.

Effects of various agents on isolated rat luteal cells.

The isolated luteal cell suspension from each stage was used and assigned into 2 groups. Each group was designated into 7 treatments as follows:

treatment	exogenous hormone added		
	hCG (0.5/ml)	PRL 5 µg/ml	$PGF_{2\alpha}$ 250 ng/ml
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+

The first group was incubated 3 hours and designated as short term incubation. At the beginning of the incubation, aliquot of 10^5 isolated luteal cells was mixed with 95% ethanol for killing cells. This solution was used as zero time incubation. It was kept in deep freezer until P and E_2 assays. After 3 hours incubation, the medium and luteal cells were collected and kept in deep freezer until P and E_2 assays. P and E_2 secreting ability of luteal cells during short term incubation were determined and expressed as production rate in pmol/10^5 cells/3 hrs.

The second group was incubated for 11 days and designated as long term incubation. Media were collected and replaced with fresh 2 ml of culture medium and hormone application as in the previous experiment

on day 1,3,5,7,9 and day 11 of incubation. The collected media were centrifuged at 1000 rpm for 10 minutes at room temperature, and the supernatants were kept in deep freezer for P and E_2 determination. P and E_2 secreting ability of rat luteal cells during long term incubation were determined and expressed in pmol/10^5 cells. At the end of the experiment (11 days after incubation) viability of the remaining luteal cells in all groups was determined with 0.2% trypsin in HBSS for luteal cells deattachment (Correcdor and Flickinger, 1983) and stained with 0.2% trypan blue in HBSS (Tennants, 1964).

7.7 Determination of isolated monkey luteal cell responsiveness.

The isolated monkey luteal cell suspensions obtained from early, mid, late luteal phase and luteolytic periods were used and cultured. The protocol of the experiments were as follows:

number of cells : 5×10^4 cells

culture medium : HAM-F10 1 ml.

incubation condition : 37°C under 5% CO_2 and 95% humidified air.

incubation peroid : 11 days of incubation, day 0 - day 1 was designed as pretreatment period (without hormone application) and day 1 - day 11 as treatment peroid (with hormone application).

Secreting ability of these isolated monkey luteal cells was determined for the steroid. They were devided into 7 treatments and incubated for 11 days. Media were collected and replaced with fresh 1 ml of HAM-F10 nutrient and hormone application on day 1,3,5,7,9

and 11 of incubation as follows:

treatment	hCG 0.5 iu/ml	PRL 5 µg/ml	$PGF_{2\alpha}$ 250 ng/ml
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+

The collected media were centrifuged at 1000 rpm for 10 minutes at room temperature and the supernatants were kept in deep freezer for P and E_2 determination. P and E_2 secreting ability of luteal cells were determined and expressed in pmol/$5x10^4$ cells. The viability of isolated luteal cells of monkeys during day 1 - day 11 of incubation were not determined since there were not enough cells obtained from the fraction of ½-¾ of CL for each experiment, but was determined, at the end of the experiment (day 11 of incubation).

8. P and E_2 Radioimunoassay (RIA).

50 µl and 200 µl of plasma samples were used for P and E_2 assays respectively. The results were expressed in nmol/L.

25 µl of the cultured media from day 1 - day 5 and 50 µl from day 7 - day 11 of incubation were used for P assay, 100 µl of the culture media were used for E_2 assay. P and E_2 contents in plasma and medium were extracted with 5 ml of diethyl ether and using specific antibodies by using the method of WHO manual (1980).

8.1 Protocol.

Day 1 : Duplicate samples (medium and plasma), triplicate recovery of extraction (RCE, 50 µl of tracer), triplicate recovery of assay (RCA, 100 and 200 fmol of standard steroids), quardruplicate quanlity control (Q.C., 50 µl and 200 µl of pooled serum for P and E_2 assay). All were extracted by adding 5 ml fresh anhydrous diethyl ether and vortexing simultaneously for 1 minute; the aqueous phase was frozen in dry ice/ethanol bath, the organic phase decanted, evaporated to dryness at 40-50°C and all were redissolved in 0.5 ml BS. The RCE tubes were aliquoted 0.25 ml into a counting vial and added with 4.5 ml scintillant for counting and determined % of RCE. P standard was prepared within the range of 37.5-1200 fmol/0.5 ml (see appendix page 170) or E_2 standard within the range of 25-800 fmol/0.5 ml (see appendix page 170). Tracer (0.1 ml) was added to samples, standards, non specific binding tubes (NSB tubes, 0.6 ml BS), maximal binding tubes (Bo tubes, 0.5 ml BS), QC, RCA and total count tube (TC, 0.6 ml BS). Antisera (0.1 ml) was later added to each tube, except the NSB tubes, vortexed, covered and incubated for 18-48 hours at 4°C.

Day 2 : In an ice bath, 0.2 ml of cold charcoal-dextran suspension continually mixed by magnetic stirrer was quickly added to all tubes, except TC tubes. All tubes were vortexed. Fifteen minutes after the first tube received charcoal-dextran suspension, all tubes were centrifuged for 15 minutes at 2500 rpm (4°C). After centrifugation, the supernatant in each tube was quickly decanted into a counting vial, added with 4.5 ml scintillant, capped, labelled, shaken, left for at least 1 hour, and counted for 5 minutes each with Rack-beta liquid scintillantion counting.

8.2 Validation of the RIA.

The cross reaction of P antiserum with other steroids likely to be present in serum was tested in the following table by WHO Matched Reagent Program (1981). The cross reaction was calculated at B/Bo (B = standard counts, Bo = maximum binding counts) of 50% and was shown as the following.

cross reaction substance	% cross reaction
cortisol	< 0.01
Testosterone	< 0.3
17α-hydroxyprogesterone	< 3.0
20α-dihydroxyprogesterone	< 3.0

The cross reaction of estradiol -17β antiserum with other steroids likely to be present in serum was also tested. It was calculated at B/Bo of 50% and was shown as follows:

cross reaction substance	% cross reaction
Estrone	1.7
Testosterone	< 0.0002
cortisol	< 0.0001

Sensitivity : The sensitivity of assays was determined from every standard curves which were drawn on a semilog graph paper with an x-axis scale as percentage of radio-activity bound (% B/Bo, Bo = maximal binding counts, B = standard counts) and Y-axis scale as various doses of standard hormone. This sensitivity of assay was calculated at B/Bo of 90%.

Precision : The pooled sera of cynomolgus monkeys were used as quality control samples (typically 50 µl and 200 µl of pooled serum for P and E_2 assay respectively). The quality control samples were included in every assay and expressed the steroid levels in nmol/l. Calculated the intra and inter assay variability of steroid levels of the quality control samples. The precision of assay was expressed in percentage of coefficient variation

$$(\% \text{ CV} = \frac{\text{mean of steroid levels}}{\text{standard deviation}} \times 100)$$

Accuracy : The known concentrations of hormones were used (typically 100 and 200 fmol.). These triplicate known samples were included in every assay. This accuracy of assay was expressed in % of known sample concentration after assay/ known samples concentration before assay.

Table. 1 shows the validation of P and E_2 assay

validation of assay \ assay	P	E_2
sensitivity	10-40 fmol/tube	7-15 fmol/tube
precision :		
1. intra-assay serum hormone-level	15.15±0.52 pmol/ml	787.5±17.7 fmol/ml
% CV	3.42	2.52
2. inter assay serum hormone-level	14.43±0.75 pmol/ml	744.25±82.48 fmol/ml
% CV	5.16	11.04
3. % accuracy :		
- 100 fmol/0.1 ml/tube	85.5±3.75	89.75±5.44
- 200 fmol/0.2 ml/tube	76.18±4.35	77.13±4.65
% NSB	4.48±1.16	5.08±1.31
% RCE	92.83±9.22	90.57±5.42
% Bo	47.27±7.56	42.67±5.75

9. Statistical analysis

The results were presented as mean ± S.E., two-way analysis of variance and Turkey's test were used in the statistical evaluation of the results. A probability of $P < 0.01$ and $P < 0.05$ were considered to be statistically different.

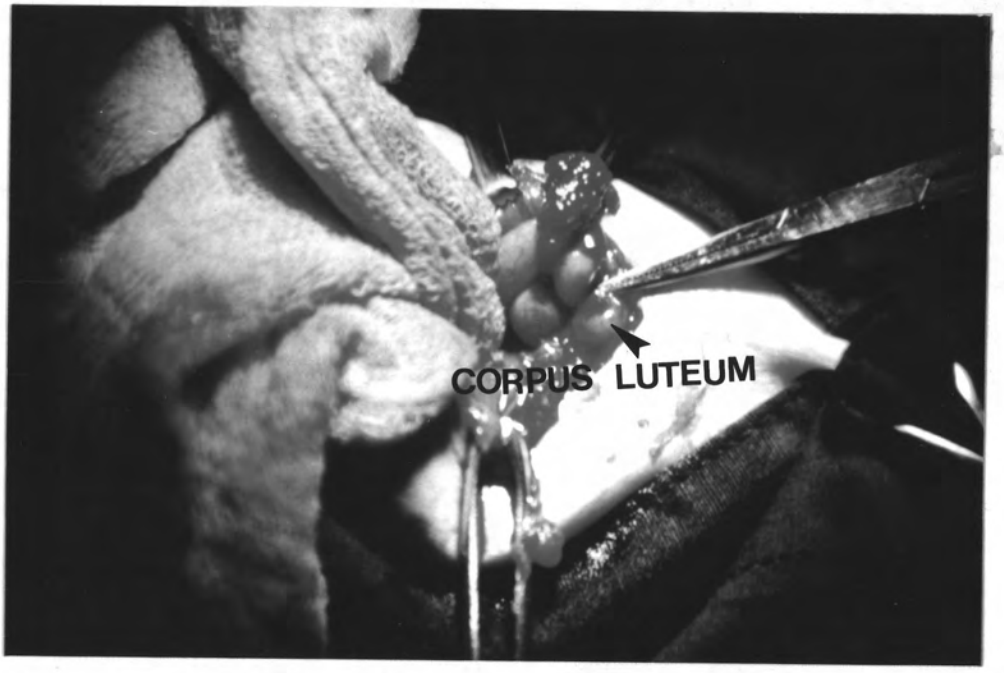


Figure 7. Surgical technique for partial lutectomy in monkey no. 101. Operation took place during mid ltueal phase (8 days prior to the next menstruation).

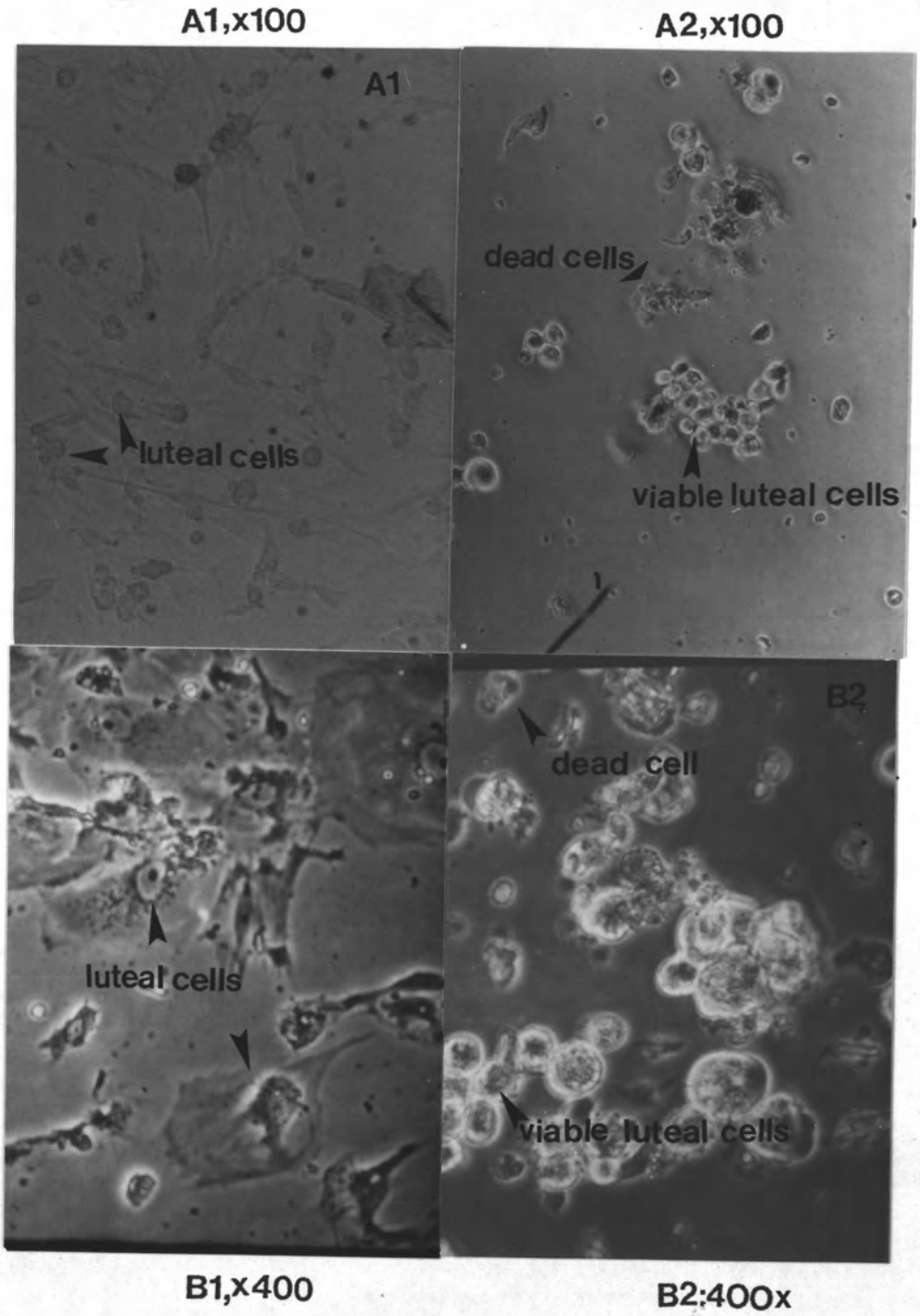


Figure 8. Illustration of the rat luteal cells (A1, A2) and monkey luteal cells (B1, B2); attached luteal cell in culture dish (A1, B1) and deattached luteal cells after 0.1% trypsin treatment for 10 minutes (A2, B2).