CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Animals

Nature albino rats of the Charles Foster strain weighing 250-300 g were used in these studies. The strain was obtained from the Physiology Department, Faculty of Medicine, Chulalongkorn University. The animals were maintained in our laboratory in an air conditioned room (25°C) exposed to 12 hours of light per day and they were fed ad libitum with excess amount of food (Zuellig) and water.

The rats were divided into three groups:

1. Donor rats were healthy female rats used for inserting the IUD and collecting the uterine fluid.

2. Recipient rats were fertile female rats at various stages of pregnancy used for testing the antifertility activity of inorgonic phosphate (P_i) and other testing materials.

3. Male rats were healthy male rats used for copulation.

2. Chemicals

Chemicals and solvents used in the present investigation were of reagent grade.

Phenol reagent (Folin-Ciocalteu reagent) was prepared in our laboratory by the method of Folin and Ciocalteu (12).

3. Chemicals for scintillation counting

Carrier-free ^{32}P-orthophosphate in dilute HCl solution is the product of the Radiochemical Center, Amersham.

Toluene and methanol were from J.T. Baker Chemical Co.

Naphthalene and p-dioxane were bought from E. Merck Ag., Darmstadt.

Ethylene glycol was obtained from BDH Chemicals Ltd.

2,5-Diphenyloxazole (PPO), 1,4-bis-[2,5-phenyloxazolyl] benzene (POPOP) and Triton X-100 were products of Packard Instrument Inc.

4. Chemicals for gel filtration

Sephadex G-25 and Blue dextran 2000 were obtained from Pharmacia Fine Chemical AB, Uppsala, Sweden.

Potassium chromate (analytical grade) was from E. Merck Ag., Darmstadt.

5. Chemicals for Gel-electrophoresis

Acrylamide, N,N'-bis-methylene acrylamide, tris (hydroxymethyl) aminomethane and coomassie brilliant blue were bought from the Sigma Chemical Company.

Ammonium persulphate, glycine, sodium lauryl sulphate, glycerol, N,N,N',N'-tetramethylethylenediamine (TMED) and bromophenol blue were obtained from BDH Chemicals Ltd.

Trichloroacetic acid was the product of May & Baker Ltd.

METHODS

1. Mating procedure

Pregnancy was induced by pairing one female rat (recipient) with two male rats. The female was examined for the sperm plug in the next morning, and the day of finding a plug was designated as day 1 or the first day of pregnancy.

2. Insertion of intra-uterine device (IUD) and collection of uterine fluid

All surgical procedures was carried out through a mid-ventral laparotomy under ether anaesthesia. Full aseptic precautions were used and the animals were rested for 10 to 14 days before being used in the next operation.

Vaginal cytological studies of the donor female rats were carried out daily during 8.00-9.00 AM by observing the cells from vaginal smear under microscope at 10 x 10 maghification. The rats which showed at least 2 normal oestrus cycle were used in each experiment. The normal oestrus cycle of the rat is completed in four to five days (13).

At oestrus stage, silk suture (number 4-0) IUD was inserted as described by Doyle and Margolis (14) as shown in Fig. 1. The uterine fluid collected from the left control uterus during oestrus stage was designated "control fluid", and the uterine fluid collected from the right IUD-bearing uterus was called "IUD fluid". Uterine fluid from each side was pooled and stored at -70°C not more than 8 weeks.

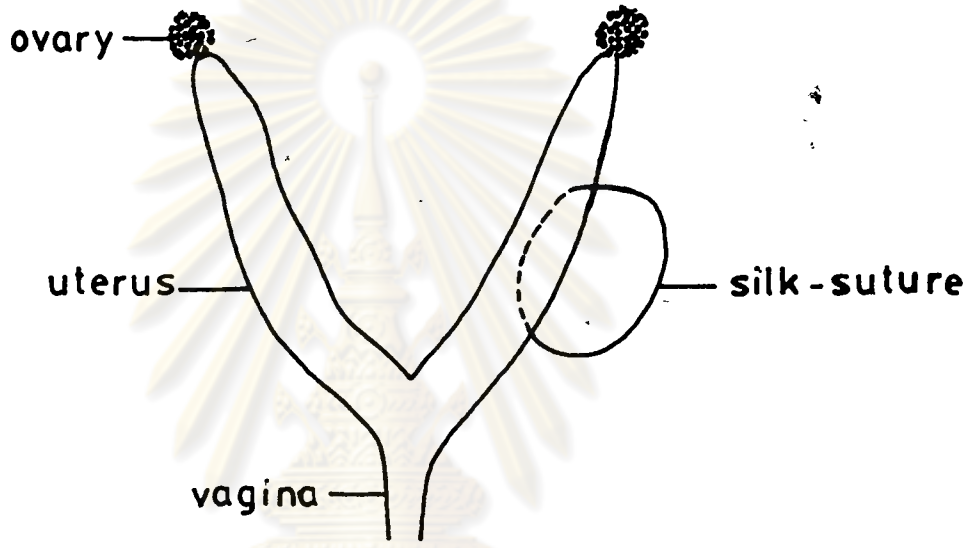

Figure 1. Insertion of an Intra-uterine device

The protein concentration of the pooled uterine fluid was determined according to Lowry, et al. (15) using crystalline bovine serum albumin (Sigma) as standard. Determination of inorganic phosphate concentration was carried out as described by Morin and Prox (16) using o-phenylenediamine as reductant.

3. Bioassay for contraceptive activity of inorganic phosphate (P_i)

Testing fluid (0.2 ml) was injected into the right uterus of the recipient rats on various days of preganancy, and the same amount of proper control fluid was injected into the left uterine of the same rat. The number and development of fetuses were observed on day 15 of pregnancy.

There are three kinds of fetus observed on day 15 of pregnancy (Fig. 2):

1. Normal fetus. The normal fetus has spherical shape and average diameter of 9-10 mm. The embryo inside the amniotic sac can be seen via uterine incision.

2. Abnormal fetus. The abnormally small fetus also has spherical shape, but only 5-6 mm in diameter and very dense.

3. Clotting material. This is observed as fused clotting blood of irregular size and can be counted in a number.

4. The Experiments with ^{32}P-orthophosphate (^{32}P-P_i)

^{32}P-P_i in dilute HCl solution (specific activity 37 MB_q/ml) was neutralized with 0.1 N NaOH and diluted in sterile deionized water, pH 7.0 to the final specific activity of 3.25 MB_q/ml.

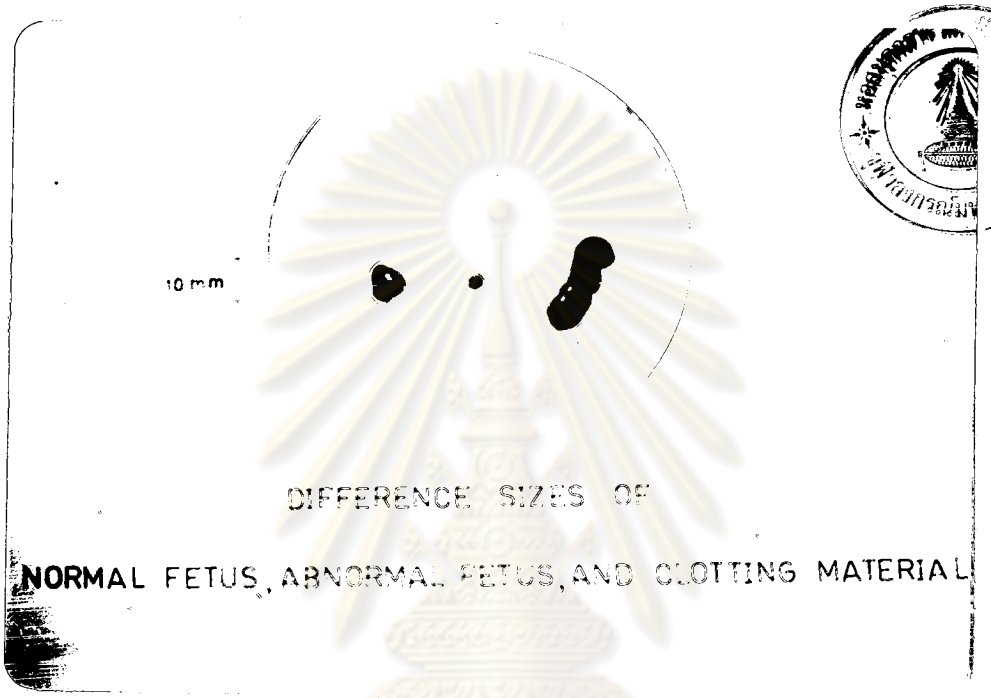

Figure 2. Difference sizes of normal fetus, abnormal fetus and clotting material

There are three kinds of fetuses observed on day 15 of pregnancy after bioassay for contraceptive activity of inorganic phosphate (P_i) :

Normal fetus,

Abnormal fetus and,

Clotting material .

4.1 In vivo experiment

The fate of ^{32}P-P_i injected into the right uterus was studied by injecting ^{32}P-P_i (0.32 MBq) in 0.2 ml of 0.006 M cold phosphate buffer pH 7.0 intraluminally into the right uterus of the recipient rats via mid-ventral incision on day 5 of pregnancy. At various time intervals after injection, the rats were killed and both uteri were flushed with 1-2 ml of ice-cold normal saline. The flushing collected from each uterine horn was designated "fraction 1 (F_1)". The horn was then cut open and gently scrapped with a cover slit to remove the endometrial cells. The endometrial cells were suspended in 1-2 ml ice-cold normal saline, homogenized and the endometrial homogenate of each uterine was designated "fraction 2 (F_2)". The whole uterine leftover, consisted of muscular layers, was minced and homogenized in normal saline and called "fraction 3 (F_3)". Duplicate aliquots (100 µl) were dissolved in 0.5 ml of 10% hyamine 10-X hydroxide solution (scintillation grade) by incubating overnight at 37°C in a scintillation vial. The radioactivity was counted for 20 minutes in 4.4 ml of Triton X-100 scintillation fluid (17) in a Packard Liquid Scintillation Counter, Prias Model PL.

4.2 In vitro experiments

4.2.1 Binding of ^{32}P-P_i on Sephadex G-25 column

Binding of ^{32}P-P_i with some biomolecules in the control and IUD fluid was studied on a Sephadex G-25 column chromatography. By using Sephadex G-25 (Pharmacia), fractionation range (MW.) 1,000-5,000 dalton, packed in a column of 1 cm in diameter and 11 cm high with the

total bed volume of 3.64 ml. The void volume (3.5 ml) and the elution volume (7 ml) of the column were determined by using Blue dextran 2000 and potassium chromate as markers. The sample was eluted with deionized water containing 0.02% sodium azide at the flow rate of 1 ml/min (operating pressure 46 cm) and a volume of 0.5 ml was collected for each fraction (17 drops).

The elution profile of free P_i on Sephadex G-25 column was observed by layering 0.5 ml of ^{32}P-P_i (1.62 MBq) on the top of the column. The fractions collected were counted for radioactivity in the presence of 4.5 ml Bray's scintillation fluid (18).

The binding of ^{32}P-P_i with some biomolecules in the uterine fluid was carried out by preincubating each component (^{32}P-P_i) 1.62 MBq/0.5 ml, control or IUD fluid 1.25 mg protein/0.5 ml) separately at 37°C for 1 hr. At time 0, the components were mixed together well and were either layered immediately on the Sephadex G-25 column as described previously, or further incubated at the temperature as specified in each experiment before applying onto the column. The radioactivity of each fraction was determined by counting in 4.5 ml of Bray's Scintillation fluid with correction for decaying.

4.2.2 SDS-polyacrylamide gel electrophoresis

Pooled fractions 1-9 and 10-15 from column Sephadex G-25 were lyophilized in a Freeze-drier (Edwards) and dissolved in deionized water 600 µl. An aliquot of 50 µl was counted for the radioactivity in the presence of 4.95 ml Bray's scintillation fluid and showed the radioactivity in the range of 3,000-30,000 cpm. The same volume of solution

(50 µl) was mixed with 3 µl of tracking dye (0.05% Bromophenol blue in water), 1 drop of glycerol, 5 µl of mercaptoethanol and 2% SDS just before loading on the gel surface. Electrophoresis was performed at a constant current of 3 mA per gel

After 1 hr of electrophoresis the gels were removed, froze and longitudinally cut into two halves. One half was fixed in 12% TCA for at least 30 minutes to fix the protein bands and stained for 2 hrs. in 1% coomassie brilliant blue in 7% acetic acid, then destained in 7% acetic acid overnight to locate the protein bands. The stained gels were scanned in a Beckman spectrophotometer Model 25 at 650 nm with a scan rate of 10 cm/min, and a chart speed of 5 in/min.

The other half of the gel was cut into pieces of 0.25 mm long. Each piece was incubated at 37°C overnight in 0.5 ml of 30% hydrogen proxide in a scintillation vial. The radioactivity in the gel peice was then counted in the presence of 4.5 ml Triton X-100 scintillation fluid.