CHAPTER II

MATERIALS AND METHODS

2.1 Animals

Female golden hamsters aged 10-20 weeks old were maintained in a well-ventilated room at 25 ± 1°C with daily lighting of 14 hrs (between 0600 - 2000). Rodent standard chow (Chareonpokaphan, Thailand) and tap water were given *ad libitum*. Female animals exhibited at least two consecutive regular 4-day estrous cycles were selected for the experiments.

2.2 Natural ovulation and superovulation

The estrous cycle was determined by daily checking for post-estrous vaginal discharge according to the method of Deanesly (1938). The day when a sticky and viscous exudate was observed in the vagina, when it was gently squeezed, was designated as day 1 of the cycle. Female animals usually came into heat between 1800-2000 hr on day 4, and ovulation was estimated to be around 0100 hr of the following morning (Ward, 1946; Harvey et al., 1961). On the estrous day of their natural reproductive cycle, animals were paired overnight with male animals, and successful matings were confirmed by the presence of spermatozoa in the vaginal smears performed on the next morning. This was designated as day 1 of pregnancy (D_1).

Superovulation in adult hamsters (Greewald, 1962; Sato, 1962; Yanagimachi & Chang, 1964) was induced by intraperitoneal injections of 25 i.u. pregnant mare serum gonadotropin (PMSG, Sigma Co.) at 0900 - 1000 hr on day 1 of the cycle, followed at 72 hrs later by 25 i.u.

human chorionic gonadotropin (hCG, Sigma Co.). Gonadotropins-treated hamsters usually ovulated 12 to 16 hrs after the injection of hCG, a situation similar to those reported by Sato (1962) and Yanagimachi & Chang (1964). Following the hCG administration each female was paired overnight with a fertile male and treated in the same way as the naturally cycling hamsters.

2.3 Recovery of embryos

Sucessfully mated females were killed by cervical dislocation at various time intervals : 0900 - 1000 hr on D_1, D_2 and D_3, 1700 - 1800 hr on D_3, and 0700 - 0900 hr on D_4. Oviducts were removed by cutting through the ovarian bursa and utero-tubal junction, and uteri were separately removed with two cuts near the cervical end. Embryos from oviducts or uteri were flushed into a watchglass, using a 1 ml plastic syringe fitted with a blunt 30-gauge needle. The medium used for flushing was a modified Dulbecco's phosphate-buffer medium (PB_1, Whittingham, 1971). Zygotes were freed from surrounding cumulus cells by treating briefly with hyaluronidase (Bovine testes type I-S, Sigma Co.) 150 i.u./ml PB_1. A criteria for assessing fertilization was morphological change : the presence of second polar body is indicative of fertilization; these oocytes were then called zygotes. All embryos were washed in two successive changes of PB_1 before being divided for further treatments.

2.4 Procedure for studying the characteristics of surface structure of embryos by SEM

To study the surface structure of embryos, zona pellucida (ZP) was removed by immersing the embryos in 0.1% trypsin (Sigma Co.) in PB_1 for 1 minute. After trypsin treatment, the embryos were washed

in two successive changes of 0.1 M sodium cacodylate buffer pH 7.4, and immediately transferred to 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4°C for 30 minutes. The embryos were then washed two times in cacodylate buffer and transferred to 1% OsO_4 in 0.1 M cacodylate buffer pH 7.4 at 4°C for 1 hr. The embryos were then washed with the same buffer, and dehydrated through graded concentrations of ethanol : 50, 70, 80, 90, 95% for 10 minutes at each step and twice in 100% ethanol. The embryos were then dried by the critical-point drying apparatus, Hitachi HCP-2, using liquid carbon dioxide as a transitional medium. The dried embryos were mounted on a double-sided tape on the stub, and shadowed with gold in Polaron E-500 ion sputtering apparatus to increase the contrast and reduce the charging effect. Observations were made and photographs were taken from the Hitachi S-430 scanning electron microscope set at the accelarating voltage of 15-20 KV.

2.5 Procedure for studying the ultrastructure of embryos by TEM

Embryos were fixed for 30 minutes in 2.5% glutaraldehyde in 0.1 M cacodylated buffer, pH 7.4 at 4°C. After fixation, the embryos were washed twice in cacodylate buffer and transferred to 1% OsO_4 in cacodylate buffer at 4°C for 1 hr. Then embryos were washed in distilled water and stained *en bloc* with 1% uranyl acetate in 0.1 M sodium acetate buffer, pH 5.1 at room temperature (RT) for 1 hr. Thereafter, embryos were embedded in 2% agar. After agar solidification, samll blocks, each containing 1-2 embryos, were cut and dehydrated in graded ethanol. Embryos were transferred through two changes of propylene oxide(P.O.)for 20 minutes each, then a mixture of P.O. and Araldite at the ratio 2:1 (V/V) and 1:2 for 1 hr and overnight, respectively. Then the embryos were

embedded in Araldite and polymerized at 30°, 45° and 60°C for 1, 2 and 3 days, respectively.

Three to four embryos of each stage were sectioned with MT-II Porter-Plum ultramicrotome fitted with glass knives. Thin sections of 300-500 Å were cut and picked up with formvar-film-coated single hole (400-600 μm) and/or Athene Robertson grids. The sections were stained with saturated uranyl acetate in 70% ethanol for 15 minutes and counter-stained with Reynold's lead citrate for 15 minutes (Reynold, 1963). They were finally examined under a Hitachi H-300 transmission electron microscope.

2.6 Preparation of cytoskeletons of embryos

To investigate the organization of the cytoskeletal components in preimplantation embryos, ZP of embryos from D_1 to D_4 were removed as previously described. The embryos were then fixed briefly in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C for 2-5 minutes. They were washed twice in cytoskeleton buffer as described by Small et al. (1978). The buffer contains : 137 mM NaCl; 5 mM KCl; 1.1 mM Na_2HPO_4; 0.4 mM KH_2PO_4; 4 mM $NaHCO_3$; 5.5 mM glucose; 2 mM $MgCl_2$; 2 mM EGTA and 5 mM PIPES at pH 6.0-6.1. The specimens were then extracted to get rid of soluble components, by treating with 0.5% glutaraldehyde and 0.5% Triton X-100 in cytoskeleton buffer with constant shaking for 1 hr at RT. After washings in cytoskeleton buffer and cacodylate buffer, they were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 20 minutes and post-fixed in 0.2% osmium tetroxide in the same buffer at 4°C for 1 hr. Specimens were stained *en bloc* with 1% uranyl acetate in 0.1 M acetate buffer pH 5.1 in the dark at RT for one hr and then prepared for TEM as previously described.

Summaries of the procedures for studying the ultrastructure of preimplantation embryos

female hamsters
↓
primed with 25 i.u. PMSG (IP)
↓
primed with 25 i.u. hCG (IP) - mated with fertile males
↓
collected embryos on D_1 - D_4

SEM
↓
dissolved ZP with 0.1% trypsin
↓
fixed in 2.5% glutaraldehyde
↓
postfixed in 1% OsO_4
↓
dehydrated through increasing concentrations of ethanol
↓
critical-point-dried
↓
coated with gold
↓
SEM

TEM
↓
fixed in 2.5% glutaraldehyde
↓
postfixed in 1% OsO_4
↓
stained *en bloc* with 1% uranyl acetate
↓
dehydrated through increasing concentrations of ethanol
↓
embedded in Araldite
↓
sectioned and stained with lead citrate and uranyl acetate
↓
TEM

cytoskeletal components
↓
dissolved ZP with 0.1% trypsin
↓
fixed in 2.5% glutaraldehyde for 2-5 minutes
↓
extracted with 0.5% glutaraldehyde and 0.5% Triton X-100 for 1 hr.
↓
fixed in 2.5% glutaraldehyde
↓
post fixed in 0.2% OsO_4
↓
stained *en bloc* with 1% uranyl acetate
↓
dehydrated through increasing concentrations of ethanol
↓
embedded in Araldite
↓
sectioned and stained with lead citrate and uranyl acetate
↓
TEM

2.7 Characterization of cell surface carbohydrate residues of preimplantation embryos and uteri

2.7.1 Collection of preimplantation embryos and uteri

Embryos and uteri were collected as described previously at 0900-1000 hr on D_1, D_2, D_3 and D_4.

2.7.2 Lectin labelling techniques

In order to study the changes of cell surface carbohydrate residues, three types of lectins were used : Con A; WGA and RCA_1.

2.7.2.1. Con A labelling technique

Two-steps labelling technique using horseradish peroxidase to react with Con A was employed. ZP of embryos were dissolved as previously described. Eight to ten embryos of each stage were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4°C for 20 minutes and washed twice in 0.1 M phosphate buffer saline (PBS) pH 7.2. The embryos were subsequently treated with Con A (500 µg/ml PBS) at RT for 30 minutes. They were washed twice in PBS, exposed to 1000 µg/ml of HRP in PBS for 30 minutes, and washed thoroughly again in PBS, and then incubated in 400 µg/ml of 3,3' diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer pH 7.6 in the dark (Graham & Karnovsky, 1966) for 15 minutes. Then, 0.05% H_2O_2 was added to the solution and the embryos were further incubated for another 20 minutes. After washing twice in Tris HCl buffer and 0.1 M cacodylate buffer, they were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 hr, and processed for TEM as previously described. Three embryos from each stage were sectioned and examined by TEM without staining.

Following the flushing of the uterine lumen with 0.1 M cacodylate buffer, the uteri were diced into small pieces, which were fixed in glutaraldehyde and further processed for TEM as described above for

the embryos.

Control specimens were treated with the same procedure but without Con A incubation.

2.7.2.2 WGA and RCA_1 labelling technique

HRP-conjugated lectins (WGA, RCA_1) purchased from Sigma Co. were used. Embryos and small pieces of uteri were obtained and fixed as described previously in Con A treatment. They were then incubated in 500 µg/ml of WGA-HRP or RCA_1-HRP in PBS at RT for 20 minutes. After washing in PBS, specimens were exposed to DAB and H_2O_2 in the same procedure as described previously and then processed in a similar fashion as Con A treatment. Control groups were treated similary but without exposure to the lectins.

2.8 Procedure for intrauterine injection of Con A and WGA to determine their effects on implantation

Female hamsters were mated with fertile males as described previously (Section 2.2). Pregnant animals were divided into eight groups of ten animals each as follows :

Group 1 : Animals were left untreated to serve as natural control

Group 2 : Animals received 0.1 ml normal saline (NS) to serve as sham control

Group 3, 4, 5 : Animals received 200, 400 and 800 µg Con A/ 0.1 ml NS, respectively

Group 6, 7, 8, 9 : Animals received 50, 100, 200 and 400 µg WGA/ 0.1 ml NS, respectively

All administrations were performed on both uterine horns on D_3 at 1700-1900 hr. In the morning of D_8, animals were laparotomized

Summaries of the procedures for studying sugars on the surface of preimplantation embryos and uteri

female hamsters

↓

primed with 25 i.u. PMSG (IP)

↓

primed with 25 i.u. hCG (IP) and mated with fertiled males

↓

collected embryos and uteri on D_1-D_4

Embryos	Uteri
↓	↓
dissolved ZP with 0.1% trypsin	
↓	
fixed in 2.5% glutaraldehyde	fixed in 2.5% glutaraldehyde
↓	↓
*incubated in a solution containing Con A 500 µg/ml PBS	*incubated in a solution containing Con A 500 µg/ml PBS
↓	↓
** incubated in a solution of HRP 1000 µg/ml PBS	**incubated in a solution of HRP 1000 µg/ml PBS
↓	↓
incubated in DAB 400 µg/ml Tris-HCl + H_2O_2	incubated in DAB 400 µg/ml Tris-HCl + H_2O_2
↓	↓
stained with 1% OsO_4	stained with 1% OsO_4
↓	↓
dehydrated through increased concentration of ethanol	dehydrated through increase concentration of ethanol
↓	↓
embedded in Araldite	embedded in Araldite
↓	↓
sectioned	sectioned
↓	↓
TEM	TEM

* This step was omitted in control groups

** In case of WGA and RCA_1, this step was omitted because HRP is linked to WGA or RCA_1 in the preceding step.

and the number of implantation sites were counted. Statistical significant difference among groups was analyzed by using Turkey's HSD.

2.9 Histological preparation of uterine tissues

To investigate effects of lectins on the uterus, additional three pregnant animals of each group in section 2.8 were prepared. These animals were killed in the morning of D_4 and D_6, uteri of each animal were collected and processed for light microscopy. Uterine tissues were cut into pieces of approximately 0.5 cm long and fixed in Bouin's overnight. The tissue were washed successively with 70% alcohol in order to remove the fixative thoroughly. After dehydration in the increasing concentrations of ethanol, the tissues were infiltrated with dioxane. Finally, they were embedded in paraffin and sections were cut at 5-7 μm thick, and stained with Hematoxylin and Eosin. The tissues were examined and photographed using an Olympus Vanox Microscope.

2.10 Embryo transfer

2.10.1 Preparation of embryos for transfer

Female hamsters were superovulated and mated as described in section 2.2. They were then killed by cervical dislocation between 1030-1900 hr on day 3 of pregnancy. Uteri were separately removed and blotted on a piece of sterile filter paper to remove excess blood. Contents from both uteri were flushed into an embryological watchglass with a 1 ml plastic syringe fitted with a blunt 30-gauge needle. The medium used for flushing was a PB_1. Embryos from 8-cell to blastocyst stages were picked up in a finely drawn sterile Pasteur pipette, and washed free of cell debris with two successive changes of fresh PB_1. Seven groups of these embryos were subjected to the following treatments.

Group 1 : Embryos were incubated in 0.85% NS at 37°C for 10 minutes before transfer to surrogate mothers.

Group 2, 3, 4 : Embryos were incubated in 200, 500 and 1000 µg Con A/ml NS, respectively at 37°C for 10 minutes before transfer

Group 5, 6, 7 : Embryos were incubated in 200, 500 and 1000 µg WGA/ml NS, respectively at 37°C for 10 minutes before transfer.

After 10 minutes, all embryos were washed twice in fresh PB_1 and transferred thereafter to the pseudopregnant recipients. Six embryos were transferred into each uterus.

2.10.2 Preparation of surrogate mothers

2.10.2.1 Preparation of vasectomized males

Ten male hamsters aged three to four months old were vasectomized by cutting both vas deferens, and left to recuperate for at least one month. Then they were mated with normal females on estrous day of the cycle for three times. The sterility of vasectomized males was proven by checking spermatozoa in vaginal smear as previously described in 2.2. Sterility was acclaimed when no spermatozoa was found in the vaginal smear.

2.10.2.2 Preparation of pseudopregnant recipients

Natural cyclic female hamsters were caged overnight in pair with vasectomized males of proven sterility in the afternoon on day 4 of the cycle. They were assumed to successfully mate with the males. The day following mating was designated as day 1 of pseudopregnancy.

2.10.3 Procedure for embryo transfer

Synchronized pseudopregnant recipients (at day 3 of pseudopregnancy) were anaesthetized with ether, oviducts and uteri were exposed by dorsolateral incisions of the skin and muscular layers.

Embryos were deposited into uterine lumen via a needle puncture in the uterine wall slightly below the utero-tubal junction. Following the transfer operation, organs were returned into the abdominal cavity and muscle and skin layers were sutured.

These recipients were killed on D_7, the number of implantations in uterine horns were counted and tested for significant difference, using Chi-Square Test.