CHAPTER 2

RESEARCH DESIGNS AND METHODS

A. Research designs

1. Experiment 1 Effect of White Kwao on *in vitro* development

This experiment was conducted to determine if White Kwao could improve the developmental potency of mouse embryos. The two-cell stage embryos were collected from the oviducts of superovulated female mice. The embryos were washed in three-changes of PBI medium and finally washed in M16 medium supplemented with 0.4% bovine serum albumin (BSA). Only morphological normal embryos were randomly divided into three treatment groups, and cultured at 37°C in 5% CO_2 in air with 100 humidity. Cleavage was assessed every 24 hours under inverted microscope and the number of embryos developing to the hatched blastocyst stage was recorded on day 7 after hCG injection. Ten replicate were carried out in each treatment groups.

1.1 Differential cell counting to evaluate the effect of White Kwao on the number of cell allocate into inner cell mass (ICM) and trophectoderm (TE) of individual blastocyst

The number of ICM and TE cells of individual blastocysts were counted by differentially labeling the nuclei with two polynucleotide-specific fluorochromes, the propidium iodide (PI) and the bisbenzimide. All stained blastocysts were veiwed under a Olympus fluorescence microscope and individual nuclei were counted.

2. Experiment 2 Effect of White K̆wao on implantation rate

This experiment was undertaken to evaluate if White Kwao could improve subsequent implantation of mouse blastocyst. The superovulated mature female mice were mated with the vasectomized mice 2.5-day previously. A minimum of 10 embryos total must be transferred. After completion of the transfer, the foster mice were caged and maintained in the controlled room. After 15 days of maintenance, the foster mice were killed by cervical dislocation, opened the abdominal wall and the uterine wall for counting the implantation site. This experiment was repeated 10 animals for each groups.

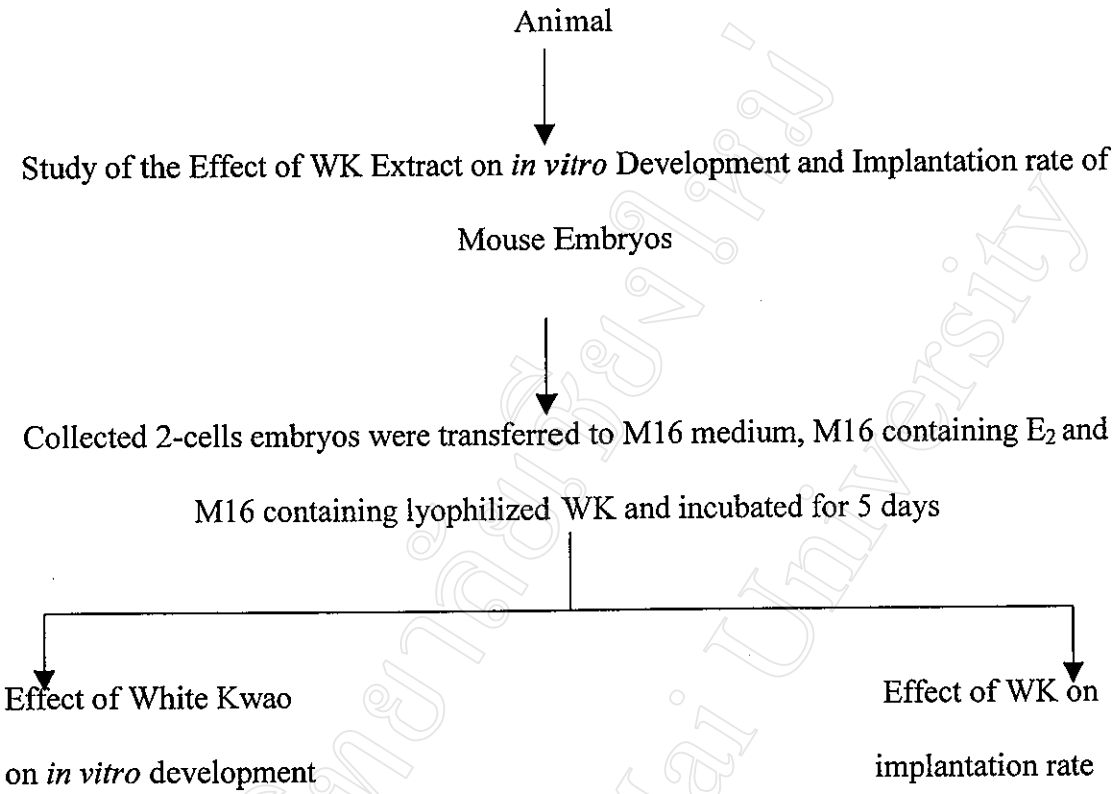

Figure 7 Summary diagram of the procedure in this research

B. Experimental animal

Male and female (random bred) ICR-strain mice were purchased from the National Laboratory Center, Nakornpratom and raised at the Animal Center, Faculty of Medicine, Chiang Mai University. The female mice used in this study were approximately 6-8 weeks old, weighing about 20-30 g. Male mice were approximately 12-16 weeks old, weighing about 40-60 g. and have been mated within 3-7 days to prove its fertility. Males were caged in individual cages and females in-group of five to ten were detained in a controlled room at 24-25 °c for 14 hours light cycle and 10 hours dark cycle. The animals were fed with a standard mice chow and tap water *ad libitum.*

C. Preparation of Lyophilized White Kwao

The tuberous root of White Kwao were collected during May 2001 from Mae Tang District, Chiang Mai Province, Thailand. After being dug up, the root was pealed, sliced into thin pieces. They were exposed to sunlight for 8 hours, and dried again in an oven at 60 °c for 48 hours or until completely dried. The roots were then mashed with a grinder. The White Kwao became a light brown powder. The fine powder of White Kwao was extracted with distilled water at a ratio of 1:5 (w/v) for 1 day. After filtered through filter paper, the filtrate was heat-evaporated at 60 °c and then completely dried with a lyophilizer. The lyophilized White Kwao was weighed and kept at -20 °c until being used. (พรรณิภา ชุมศรี, 2523)

D. Preparation of Media

All media used in this study were in-house prepared. The constituents of each media are shown in appendix I. All the prepared media were sterile filtered through 0.22 μm membrane filter into sterile containers, pre-incubated at 37 °c in 5% co_2 in air for at least 12 hours before used, to maintain a pH of 7.34-7.4. The media can be kept at 4 °c and routinely used within 1 month.

D.1 Embryo collecting and washing medium.

Phosphate buffer saline (PBI) supplement with 0.4% bovine serum albumin (BSA fraction V) was used for collecting the embryos from the oviducts and washing the embryos before placing them into the culture medium.

D.2 Embryo culture medium

All 2-cells stage embryos were cultured for 5 days in the modified M16 medium was supplemented with 0.4% bovine serum albumin (BSA fraction V) (Whittingham, 1971).

D.2.1 Preparation of lyophilized White Kwao into M16 medium

Rodkaew (2001) reported that the concentration of Lyophilized White Kwao (10 mg/ml) significantly ($p<0.05$) increased the maturation of oocyte from GV-stage to metaphase II. So that this study used Lyophilized White Kwao at the concentration of 10 mg/ml added into 10 ml M16 medium supplemented 0.4% bovine serum

albumin (BSA fraction V), and then sterilizing by filtering through 0.22 μm membrane filter unit. The medium was checked for estradiol levels by the electro-chemiluminescent immunoassay. This analysis was repeated three times.

D.2.2 Preparation of M16 medium containing ethinylestradiol 17-β

Ethinylestradiol 0.52 μg/ml were added into M16 medium supplemented with 0.4% bovine serum albumin (BSA fraction V), and then sterilized by filtering through 0.22 μm membrane filter unit. The medium was checked for estradiol levels by the electro-chemiluminescent immunoassay. This analysis was repeated three times.

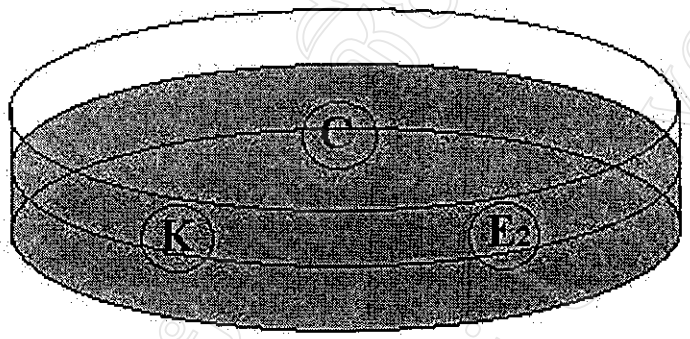

Figure 9 The plastic petri-dish containing microdrop(50 µl) of M16 (C: control), M16 containing ethinylestradiol (E_2: estrogen), and M16 containing WK (K: White Kwao)

D.3 Setting Up Microdrop Cultures.

Dispensed 50 µl drops of M16, M16 consisting of lyophilized White Kwao 10 mg/ml and M16 consisting of ethinylestradiol 0.52 µg/ ml in an array on the bottom of tissue culture dish and then flood the dish with 2.5 ml of mineral oil. The purpose of putting the oil on the top was to stabilize the drops of medium and also protect the evaporation of medium and CO_2. The petri dishes were placed in 5% CO_2 in air, at 37 ^{o}c overnight to maintain a pH of 7.34-7.40.

E. The study of the Effect of White Kwao Extract on *in vitro* Development of Mouse Preimplantation Embryos.

Pregnant Mare's Serum Gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were used to mimic Follicle-Stimulating Hormone (FSH) and Lutinizing Hormone (LH). Female mice (about 3-5 mice at a time) were injected intraperitoneally with 5 IU of PMSG to stimulate ovarian follicle development, followed by an intraperitoneal injection with 5 IU of hCG at 48 hours later induce ovulation. Immediately after hCG injection, the mice were placed with fertile proved male mice in individual cages, they were examined on the following morning for the presence of a sperm plug. Female mice with sperm plug were killed by cervical dislocation 40-44 hours after hCG injection.

The abdomen was cut open using the aseptic technique. The oviduct, ovary and fat pad was pulled out and cut between the oviduct and the ovary as shown in Figure 9. The oviduct and attached segment of uterus can now be transferred to 35

mm petri-dish containing approximately 50μl of warm PB I medium. The two-cells stage embryos were flushed from oviducts using 0.1 ml of PBI in a 1 ml-syringe attached to a 30-gauge needle. To reduce the risk of tearing the oviducts, the end of the needle was cut and ground to a blunt tip. The syringe should be free of bubble air and the medium flew smoothly before inserting the needle into the ovidcut. Hold the oviduct in place with fine forceps, insert the end of the needle into the opening of the oviduct or infundibulum (Figure 11). Two-cell stage embryos were collected and transferred to a petri dish containing 50μl of warm PB I medium by using a pasteur pipette. They were washed three times in PBI and M16 and transferred into a drop of medium. They were randomly cultured in three different treatment groups.

1. M16 medium as the control group
2. M16 medium containing 0.52 μg/ml ethinylestrdiol-17β as the positive control group
3. M16 medium containing 10 mg/ml lyophilized White Kwao

All of the cultures were placed into 5% CO_2 in air, at 37 °C for 5 days. The developments of the embryos were observed under inverted microscope every 24 hours for 5 days. Embryo developmental rate in each culture medium was recorded. This experiment was repeated 10 times.

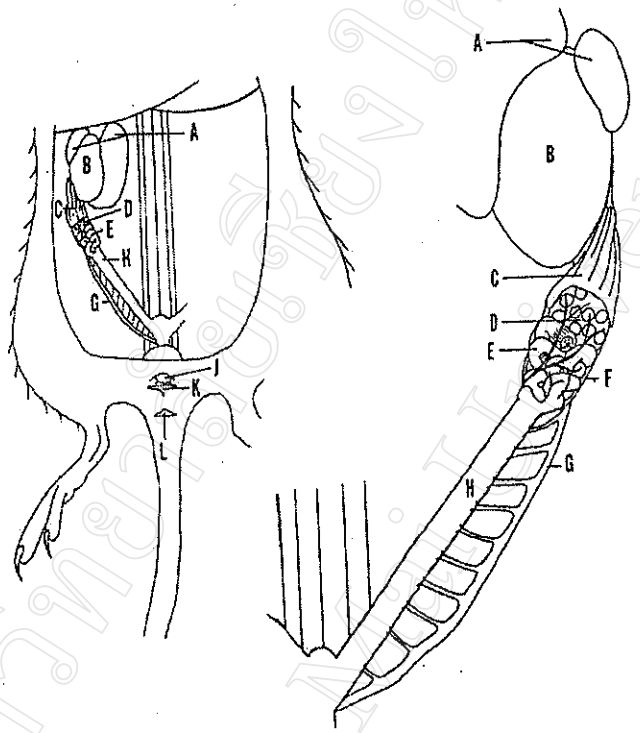

Figure 10 Female internal reproductive organs A. Peritoneal fat; B. Kidney; C.Periovarial fat body with peritoneal attachment at upper end; D. Ovary; E. Oviduct bursa; G. Uterine blood vessels; H. Uterus; (Keen and Rafferty, 1970)

Figure 11 Procedure for flushing the oviducts (Keen and Rafferty, 1970)

F. Differentially labeling of inner cell mass and trophectoderm nuclei of mouse blastocysts.

The number of trophectodermal (TE) and inner cell mass (ICM) cells of individual hatched blastocysts were counted by differentially labeling the nuclei with two polynucleotide-specific fluorochromes, propidium iodide (PI) was specified TE and bisbenzimide was specified ICM. The hatched blastocysts were incubated in H6BSA for a minimum of 20 minutes, and then in TNBS (2,4,6-Trinitrobenzenesulfornic acid) on ice for 10 minutes, and washed in H6PVP. The embryos were then incubated in a rabbit anti-mouse lymphocyte anti-serum for 10 minutes at room temperature. After they were washed in H6 PVP, the embryos were incubated in 23μl of guinea pig complement (diluted 1:10 in H6BSA) supplemented with 2 μl of propidium iodide (1 mg/ml) for 10 minutes at 37 °C, 5% CO_2 in air and washed in H6PVP. Embryos were then fixed in 990μl of ice-cold absolute ethanol and stained with 10 μl of bisbenzimide (Hoechst) (2.5μg/ml). The embryos were left in bisbenzimide at 4°C for three days.

The embryos were mounted by being placed into absolute ethanol for 5-8 minutes. On a glass slide a tiny drop of ultra pure glycerol was placed and an embryo was transferred into the middle of a drop. A cover slip was placed over the drop, and firmly pressed. The embryos were viewed under a fluorescent microscope, and the numbers of nuclei were counted. Photographs were made using Kodak ASA 100 color print film.

G.The study of the Effect of the White Kwao Extract on implantation rate of Mouse blastocysts.

G.1. Vasectomy of male mice for preparation of pseudopregnancy female mice

The male mice, approximately 7 weeks old, were anesthetized with ether deeply enough for vasectomy. They were restrained in the supine position and the abdomen was wiped with 75% alcohol. Using fine forceps and scissors, a transverse incision of about one-centimeter was made on the mons pubis. (Opening abdominal wall that appeared plugged with fatty tissue, this was seized and pulled out, bringing the testis spermatic cord with it.) The vas deferens was identified and cut in two segments (with ligation). A single wound was sutured by silk, scrubbed with 75% alcohol. The vasectomized mice were placed in individual cage and maintained in a controlled room at 24-25 °C and 14:10 light: dark cycle. The animal should be allowed to recover for a week before being used for pseudopregnant mating.

G.2. Transfer of blastocysts into the uterine horn of pseudopregnant recipients

Approximately 6-8 weeks old female mice were superovulated and mated with vasectomiced mice. The blastocysts were taken from each culture medium and transferred into the uterine lumen of 2.5 days pseudopregnancy recipients. The experimental mice were operated from the level of the lowest rib and extending caudally for 3 centimeters or so, a single midline incision of the mid-dorsal skin was

made. The fat pad was seized and exteriorized through a sterile slitted gauze pad, bringing the oviduct and uterine horn with it. It was punctured with the bent needle on an empty syringe, and the needle tip introduced into the lumen. The needle was pulled out almost entirely, and the puncture hole was held open by retracting the needle laterally. The micropipette should be insert into the puncture on the uterine horn. A minimum of 10 embryos will be transferred.

Upon completion of the total 10 embryos transfer, the uterine horn, oviduct and ovary were replaced carefully within the abdominal cavity and the incision was repaired. Then, they were housed in stainless cages and maintained in a controlled room at 24-25 °C and 14:10 hours' light: dark cycle for 15 day. After 15 days of maintenance, the pseudopregnancy was killed by cervical dislocation, opened the abdominal wall and the number of implantation site in the uterine horn was examined.

H. Statistical Analysis

The effect of White Kwao extract on *in vitro* development of preimplantaion embryo was analyzed using χ^2 test. Statistical significance was accepted at $p<0.05$.