CHAPTER 3

MATERIALS AND METHODS

Experiment 1. Effect of FSH treatment on retrieved bovine oocytes and in vitro maturation

Oocyte donors

Ten Holstein-Fresian cows aged averagely 6 years (range 2 to 11 years), with live weight of 420 kg and body condition score of 3 units (scale of 1 to 5, where 1= very thin and 5= very fat). All of the cows had of normal reproductive tracts with normal reproductive cycle. During the experimental periods, all cows had been fed with 2 kg of concentrate diet (18% proteins) and 6 kg of silage twice daily.

OPU equipments

Oocytes were collected using transvaginal follicle aspiration with Hill Aspirator (Maple Hill Embryo INC. Canada). The aspiration apparatus consists of a set of stainless steel tubes. An inner tube (needle holder; 0.7 cm-diameter, 40 cm-long) is threaded to accept a Luer lock with a nipple (Figure 8). The outer tube (needle holder; 1.5 cm-diameter, 35 cm-long) allows easy movement of the inner needle holder. A 55 cm-piece with 2 mm-ID silicone tubing (Baxter, T5715-90, UK) extends from the threaded Luer lock through the needle holder to a second Luer lock with attaches to a 15 g needle inserted in a silicone stopper. A second needle inserted in the stopper is attached to a hand held vacuum pump (Nalgene 6130-010) and the

stopper is placed in the opening of a 50 ml collection tube (Figure 9). The system is checked for potency and leaks; while the tubing is rinsed with aspiration fluid (phosphate buffer saline; PBS), prior to attaching a 19 g, 2.5 cm, thin walled, short bevelled needle (Becton-Dickinson, 305188) to the end of the needle holder.

FIGURE 8. An inner tube or needle rod of Hill Aspirater was threaded with Luer lock.

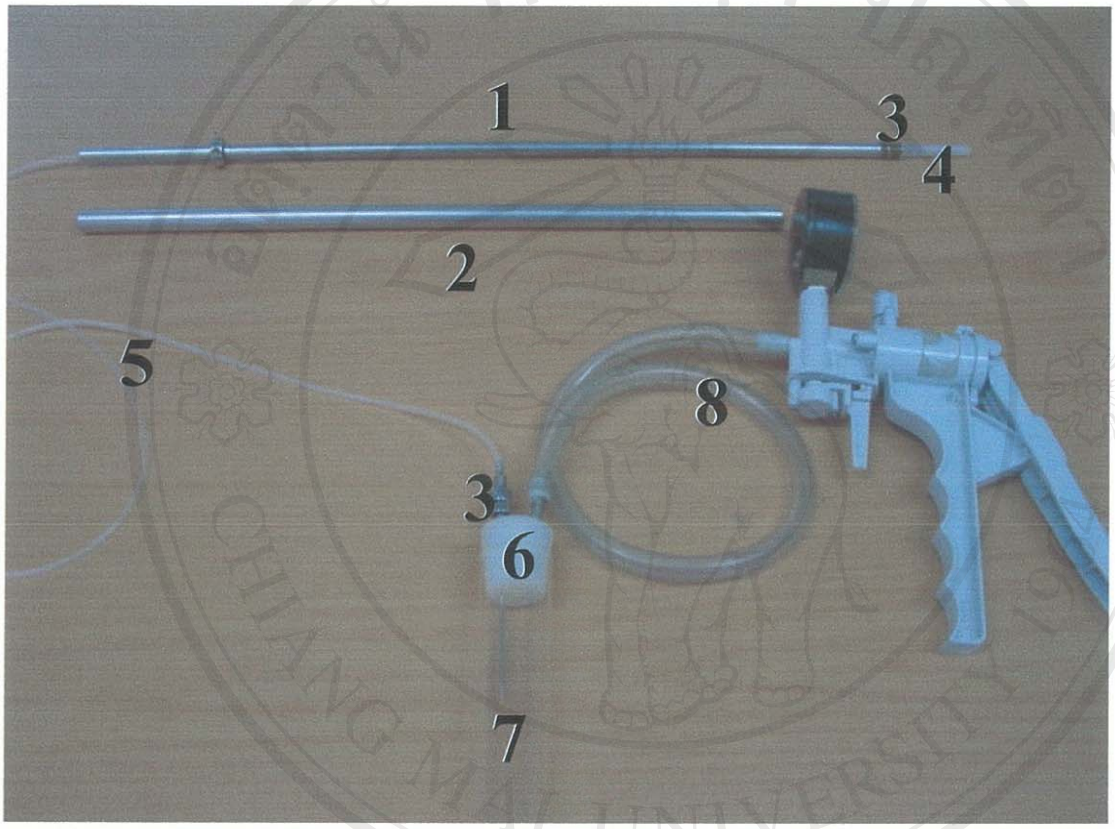

FIGURE 9. Hill Aspirator apparatus consists of inner tube (1), outer tube (2), two Luer lock (3), 19 g short bevelled needle (4), tubing system (5), silicone stopper (6), collecting tube (7) and vacuum pump (8).

Follicle stimulation and oocyte collection

Ten cows were randomly assigned into two treatment groups (n= 5/ group) that used 5 cows per oocyte collection session. Group I received no FSH treatment and n-OPU was performed twice weekly. Group II received FSH treatment starting 5 days prior to n-OPU, which was performed once every two weeks. The FSH was administered intramuscularly twice daily in 8 A.M. and 4 P.M. for 3 days in decreasing doses (50, 50 + 30, 30 + 20, 20 mg) to give a total dose of 200 mg FSH (Folltropin-V, Vetrepharm (A/Asia) Pty. Ltd., Australia) (modified from Sirard et al., 1999). The treatments were administered for 4 times in 8 weeks and the aspiration was performed on day 5 or 48 hours after the last FSH injection. Both groups were carried out for 8 weeks, after which crossover treatment between those two groups were continuing for another 8 weeks, with and 4 weeks resting period interposed. The aspiration session of FSH treatment group was performed 8 sessions and non-FSH treatment group was performed 32 sessions in aspiration period 5 months.

Before follicle aspiration, the system (silicone tubing and 19 gauge short bevelled needle) was rinsed with PBS supplemented with 2,500 IU/L heparin and 1% FCS, thereafter 3-4 ml PBS was retained in the collection tube. The oocyte donor was restrainted in stall and epidural anesthesia used 4 ml of 1% lidocaine hydrochloride. The vulva area was cleaned thoroughly by water and towel tissues. After preparation of donor, the needle guide and needle rod combined with the systems were inserted into the vagina. By rectal palpation, the ovary was placed on the end of the needle guide ensuring that there was no rectum between the wall of the vagina and the ovary. The ovary was punctured at one pole and the vacuum was commenced at 100 mmHg. After all of ovary areas were aspirated, changed the needle guide to the other side fo

ovary and continuing the procedure. When finish aspiration, the system were rinsed with PBS 10 ml and the collection fluid was incubated in 37°C waterbath until finished all 5 donor cows. The fluid was filtrated with embryo filter cup and the number of retrieved oocytes were counted and classified into 4 categories (Figure 10); A) compacted cumulus oocyte, B) partially denuded cumulus oocyte, C) completely denuded cumulus oocyte and D) expanded cumulus oocyte (Hasler, 1998).

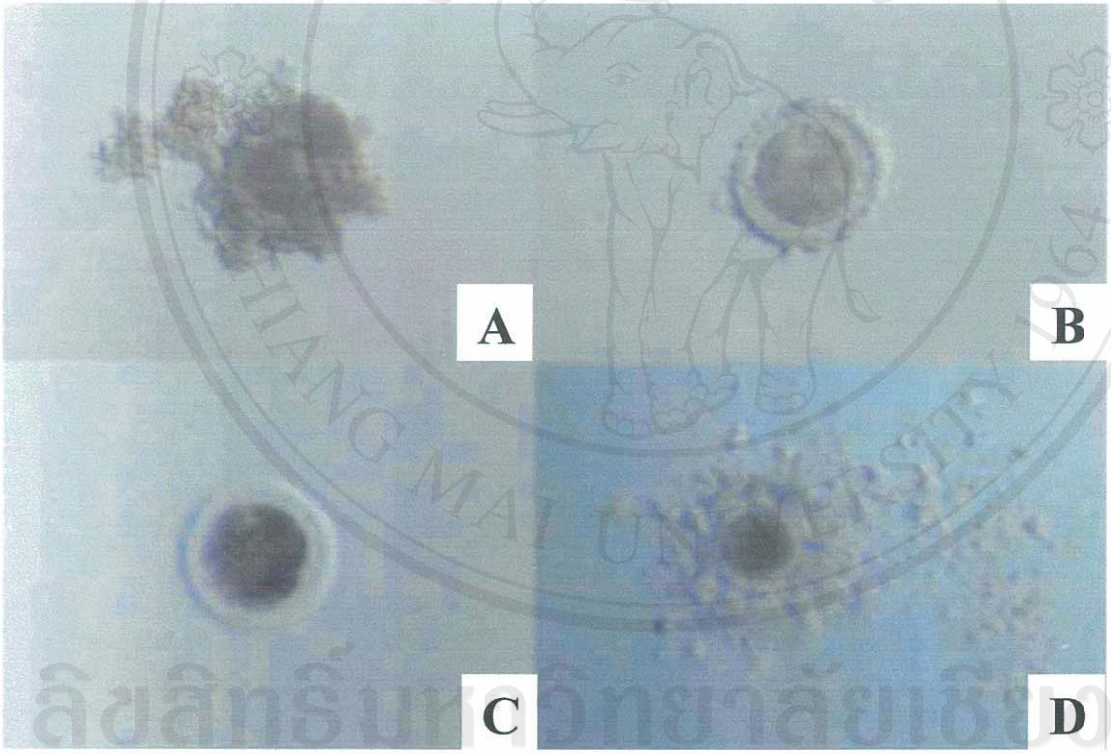

FIGURE 10. Classification of oocyte; A= compacted cumulus oocytes or cumulus oocyte complexes, B= partially denuded cumulus oocytes, C= completely denuded cumulus oocytes and D= expanded cumulus oocytes.

In vitro maturation

Maturation was performed in TCM-199 (Earle's salt, Gibco) medium supplemented with sodium pyruvate (28 mg/L), L-glutamine (146 mg/L), penicillin (50,000 IU/ml), streptomycin (50 mg/ml), epidermal growth factor (EGF) (10 μg/L), FSH (1 mg/L), hCG (500 IU/L), Estradiol (1 mg/L) and 10% FCS. Usable oocytes (category A and B) were placed in 500 μl of IVM medium in 4-well dish and incubated at 39 °C under 5% CO_2 in humidified air for 22- 24 hours.

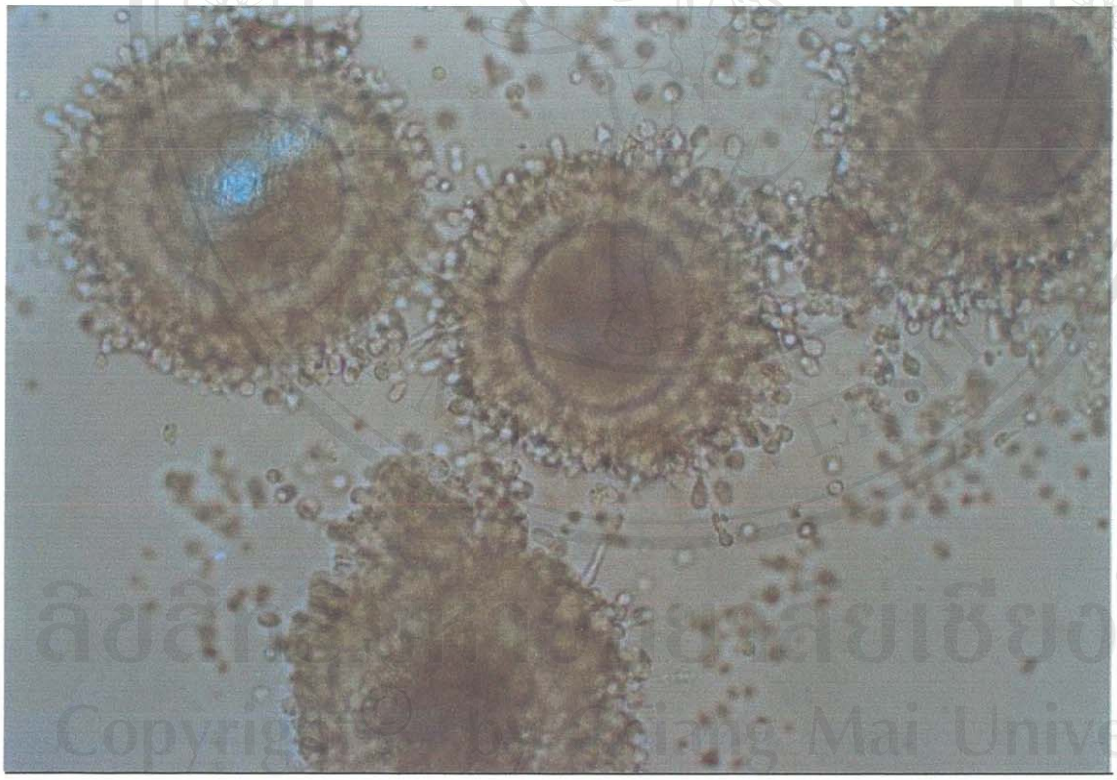

Figure 11. Bovine oocytes after in vitro maturation, the cumulus cells were expanded and loose arraged of cells entrapped in a sticky matrix (400X).

Morphological evaluation

At the end of IVM (incubated 24 hours in CO_2 incubator), expanded cumulus oocytes (Figure 11) were denuded by pepetting them in 3% (w/v) sodium citrate solution. They were then fixed in a freshly prepared 1:3 acetic acid: ethanol solution for 48-72 hours before being stained with aceto-orcein [1% (w/v) orcein and 45% (v/v) acetic acid] followed by aceto-glycerol (1:1:3 glycerol: acetice acid: distilled water) and then evaluated under a light microscope at 400X magnification. Oocytes were categoried ad being at one of the following stages: metaphase I (included germinal vesicle, germinal vesicle breakdown, anaphase I and telophase I), metaphse II (Figure 12) and degenerated oocytes.

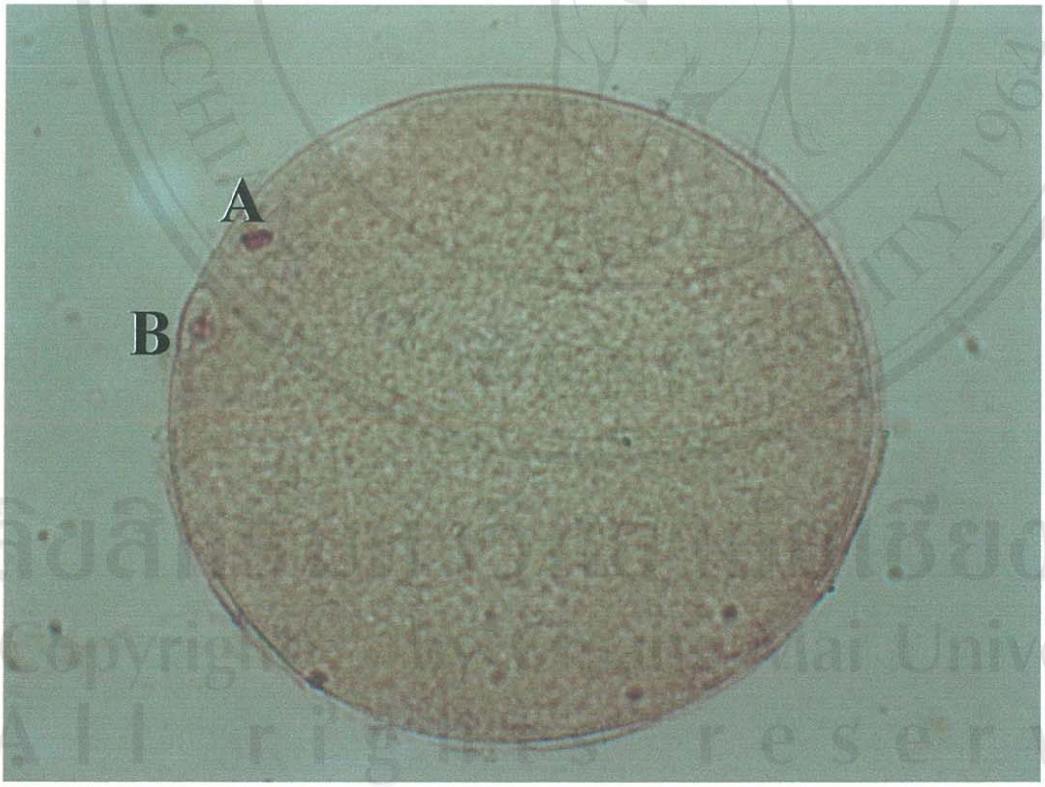

Figure 12. Bovine oocyte at metaphse II and to detect this stage from metaphse plate (A) and first polar body (B) (400X).

Experiment 2. Effect of FSH treatment on developmental competence of bovine oocytes

Source of oocytes

The retrieved oocytes were obtained by n-OPU technique from 3 donor cows per treatment. The data of non-FSH treatment group obtained from 10 aspiration sessions and FSH treatment group obtained from 6 sessions. The retrieved oocytes were performed under a stereoscope and placed in TCM-199 hepes medium, rinsed in the same media two times before IVM. Only usable oocytes (category A and B) were placed into IVM medium and incubated at 39 °C under 5% CO_2 in humidified air for 22- 24 hours.

Preparation of spermatozoa, in vitro fertilization and culture

Frozen/thawed semen from single bull was used for insemination. Spermatozoa were washed in capacitation media (TALP-hepes) by centrifugation at 300g for 5 min. After the supernatant was discard, motile and non-motile spermatozoa were separated by a swim-up method (Parrish et al., 1986b). Motile spermatozoa were counted with Makler counting chamber under light microscope at 100X magnification. For IVF, approximately one million motile spermatozoa in capacitation media were added to each of 500 µl fertilization medium [supplemented with pyruvate (22 mg/ml), penicillin (50,000 IU/ml), streptomycin (50 mg/ml), penicillamine (0.3 mg/ml), hypotaurine (0.11 mg/ml), epinephrine (0.46 mg/ml), heparin (10µg/ml) and non-fatty acid BSA (6 mg/ml) adjusted pH 7.6] in a 4-well dish. The inseminated oocytes were then incubated at 39 °C under 5% CO_2 in

humidified air for 18-20 hours. Thereafter, the cumulus cells were removed from presumptive zygotes by gentle pipetting and cultured in 50 μl microdrops of SOF medium covered with mineral oil and co-culture with vero cells at 39 °C under 5% CO_2 in humidified air. The culture media was changed 50% of volume (25 μl) every 3 days. The developmental competence of embryos were examined every day and number of cleaved embryos, morulae and blastocysts were recorded.

Statistical analysis

Retrieved oocytes were expressed as means ± SEM and the significance of differences between means was evaluated by Student's t-test. Statistically, oocyte quality and maturation rate were analyzed by Chi-square test using the SPSS, Version 10, for Windows software (SPSS, Inc.,USA). Differences were considered significance when $P< 0.05$.

The embryo evaluation data were analyzed by Chi-square test using the SPSS, Version 10, for Windows software (SPSS, Inc.,USA). Differences were considered significance when $P< 0.05$.