CHAPTER II

MATERIALS AND METHODS

Experimental animals

Mature male (aged 8 to 15 weeks), mature female (aged 6 to 8 weeks), and immature female (aged 3 to 4 weeks) Swiss albino mice obtained from the National Experimental Animal Center , Mahidol University were used in this study. They were housed in a temperature controlled room at 70^0- 75^0F with a 14 hour light : 10 hour dark cycle at the Experimental Animal Center , Faculty of Medicine , Chulalongkorn University. They were fed with standard chow and tap water ad libitum.

Media preparation

1. Krebs Ringer Bicarbonate (KRB) medium preparation ; for 100 ml

Chemicals / Solutions	**KRB with Hepes**	**KRB without Hepes**
Stock A solution	10 ml	10 ml
Stock B solution	10 ml	10 ml

Penicillin G and		
streptomycin solution	1 ml	1 ml
Na lactate	0.45 ml	0.45 ml
Na pyruvate	0.0055 g	0.0055 g
$NaHCO_3$	0.0330 g	0.2100 g
Glucose	0.1008 g	0.1008 g
BSA	0.40 g	0.40 g
Hepes	0.548 g	------
Pnenol red	0.001 g	0.001 g

bring up volume to 100 ml with Milli-Q water

osmolarity 280 - 285 mOsm/l

pH 7.3 -7.5 ; adjusted by 0.1 N HCl or 0.1 N NaOH

sterilized by filtrating through a 0.22 micrometre filter

Stock A solution preparation ; for 100 ml

NaCl	5.82 g
KCl	0.358 g
KH_2PO_4	0.1633 g
$MgSO_4$	0.144 g

Stock B solution preparation ; for 100 ml

$CaCl_2 . 2H_2O$	0.2499 g

Penicillin G and streptomycin solution ; for 100 ml

Penicillin G	300 mg
Streptomycin	500 mg

2. Percoll (90%) in KRB with Hepes solution preparation ; for 100 ml

Chemicals / Solutions

Percoll	90 ml
NaCl	0.5534 g
KCl	0.0358 g
KH_2PO_4	0.0163 g
$MgSO_4$	0.0144 g
$CaCl_2 . 2H_2O$	0.0251 g
Penicillin G and Streptomycin solution	1 ml
Na lactate	0.45 ml
Na pyruvate	0.0055 g
$NaHCO_3$	0.0336 g
Glucose	0.1010 g
Hepes	0.548 g

bring up volume to 100 ml with Milli-Q water

pH 7.3 -7.5 ; adjusted by 0.1 N HCl or 0.1 N NaOH

sterilized by filtrating through a 0.22 micrometre filter

3. Percoll (45%) in KRB with Hepes solution preparation

Five- ml aliquots of Percoll (90%) were diluted with equal parts of KRB with Hepes medium to make Percoll (45%) in KRB with Hepes medium on the day of experiment.

4. T6 medium preparation ; for 100 ml

Chemicals / Solutions ; mM (g)

NaCl	99.23 (5.80)
KCl	2.68 (0.105)
$NaH_2PO_4 . 2H_2O$	0.36 (0.051)
$MgCl_2 . 6H_20$	0.47 (0.096)
$CaCl_2 . 2H_2O$	1.80 (0.261)
Na lactate	25.00 (3.63 ml)
Na pyruvate	0.25 (0.052)
$NaHCO_3$	25.00 (2.10)
Glucose	5.56 (1.0)
BSA	3 mg/ml
Penicillin G	100 μg/ml

Streptomycin sulphate	50 μg/ml
Phenol red	0.001% (w/v)

bring up volume to 100 ml with Milli-Q water

osmolarity 280 - 285 mOsm/l

pH 7.3 -7.5 ; adjusted by 0.1 N HCl or 0.1 N NaOH

sterilized by filtrating through a 0.22 micrometre filter

Immature oocyte preparation for IVM

Immature female mice were intraperitoneally (i.p.) injected with PMSG (Sigma) (PMSG-primed mice) or with hCG (Serono) (hCG-primed mice) and killed by cervical dislocation (CD) 48 hours later. Ovaries were removed and placed in a petri dish with 2 ml Kreb Ringer Bicarbonate (KRB) medium containing Hepes (0.548 g/100 ml culture medium) and 0.4% bovine serum albumin (BSA ; Sigma). The antral follicles of each group were punctured with sterile needles (26G x ½") to release oocytes at various stages. Immature oocytes were separated and washed two times in KRB- Hepes- 0.4% BSA medium and once in maturation medium (M199 medium containing 0.29 mM pyruvate, 0.5 IU/ml hCG, 0.075 IU/ml human menopausal gonadotropin (hMG ; Serono), 10% fetal bovine serum (FBS ; Sigma), 0.05 mg/ml penicillin and 0.075 mg/ml streptomycin) (Barnes et al., 1995). Immature oocytes were

cultured in 1 ml maturation medium in each well of a 4-well tissue culture plate (Downs et al., 1988) under an atmosphere of 5% CO_2 in air at 37^0C. The number of matured oocytes showing first polar body (MII stage ; *in vitro* matured oocytes) was recorded at the end of the incubation period.

Percoll- gradient centrifuged (PGC) sperm preparation

Mature male mice were killed by CD, followed by removal of the caudae epididymis into 1 ml KRB-Hepes-0.4% BSA medium. The caudae epididymis were cut with scissors and incubated in a water bath at 37^0C for 15 minutes to release the sperm. The sperm was centrifuged at room temperature (RT) through a two- step discontinuous Percoll gradient (45 and 90%, Pharmacia) at 1500 rpm for 30 minutes. Sperm was separated on the basis of differential densities into a pellet fraction and resuspened in 1 ml KRB-Hepes-0.4% BSA medium and centrifuged at RT at 1500 rpm for 10 minutes. The PGC sperm concentration and motility were assessed microscopically using a hemocytometer before being inseminated to the *in vitro* matured oocytes (Tanphaichitr et al., 1993).

IVF procedure and embryo development

PGC sperm was capacitated in 1 ml KRB-3% BSA medium at 37^0C under 5% CO_2 in air for 1 hour and centrifuged at RT at 1500 rpm for 10 minutes (Tanphaichitr et al., 1993). Capacitated sperm (5 x 10^5/ ml) was coincubated with 20 *in vitro* matured oocytes in 50 µl KRB- 0.4% BSA medium at 37^0C under 5% CO_2 in air for 6 hours. Sperm-oocyte complexes were then washed two times in KRB-0.4% BSA medium in order to wash out the excess sperm (for prevention of polyspermy). Sperm-oocyte complexes were incubated again at 37^0C under 5% CO_2 in air overnight. The success rate of *in vitro* fertilization was determined by the number of 2-cell embryos (Odawara and Lopata, 1989 ; Tanphaichitr et al., 1993). In addition, two-cell embryos were washed two times in T6 medium and cultured in T6 medium at 37^0C under 5% CO_2 in air, and the percentage of blastocyst development was determined 120 hours after incubation (Odawara and Lopata, 1989).

Cyclic AMP enzymeimmunoassay system

This assay is based upon the competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP specific antibody. With fixed amounts of antibody and peroxidase-labelled cAMP, the amount of peroxidase-labelled ligand bound to

the antibody will be inversely proportional to the concentration of added unlabelled ligand.

The peroxidase ligand bound to the antibody is immobilized on to polystyrene microlitre wells precoated with a second antibody. Thus, any unbound ligand can be removed from the well by a simple washing procedure. The amount of peroxidase-labelled cAMP bound to the antibody is determined by addition of a tetramethylbenzidine. The reaction is stopped by addition of an acid solution, and the resultant colour read at 450 nm in a microlitre plate spectrophotometer.

Intraoocyte cAMP extraction procedure

The extraction of intraoocyte cAMP used in this study was prepared by ion exchange chromatography using a disposable Amprep SAX minicolumn (Amersham ; code RPN 1918).

1. rinse an Amprep SAX minicolumn with 2 ml methanol

2. rinse the minicolumn with 2 ml distilled water

3. apply the fresh 150 mature (MII stage) denuded oocytes to the minicolumn

4. pass 3 ml of acidified methanol through the minicolumn and collect the eluate for the determination of the intraoocyte cAMP

Measurement of intraoocyte cAMP procedure

1. prepare assay buffer and standards ranging from 2 - 128 fmol/50 µl

2. equilibrate all reagents to room temperature and mix before use

3. label polypropylene or glass tubes (12 x 75 mm) for standards and test samples

4. set up the microtitre plate (coated with donkey anti-rabbit IgG) with sufficient wells to enable the running of all blanks, standards and samples as required. Recommended positioning of blank, non-specific binding, standard (0-128 fmol) and sample wells.

5. prepare the acetylation reagent by mixing 1 volume acetic anhydride with 2 volumes of triethylamine in a glass vessel. Mix well. (Sufficient reagent for 50 acetylations may be attained by mixing 0.5 ml acetic anhydride with 1.0 ml triethylamine).

6. pipette 1 ml assay buffer into the standard acetylation tube.

7. pipette 1 ml of each sample into the appropriately labelled acetylation tubes.

8. carefully add 25 ml of the acetylation reagent to all acetylation tubes containing standards and samples, ensuring a clean pipette tip is used each time.

Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid. Each tube should be vortexed immediately following addition of the acetylating reagents.

9. pipette 100 ml of antiserum (rabbit anti-cAMP) to all wells except the blank and the non-specific binding wells.

10. pipette duplicate 50 ml aliquots from all acetylation tubes including the standard into the appropriate wells.

11. pipette 150 ml assay buffer into the non-specific binding wells.

12. cover the plate with the lid provided, gently mix and incubate at 3-5 ^{0}C for exactly 2 hours.

13. pipette 100 ml cAMP peroxidase conjugate into all wells except the blank.

14. cover the plate, gently mix and incubate at 3-5^{0}C for exactly 60 minutes.

15. aspirate and wash all wells four times with 400 ml wash buffer. Blot the plate on tissue paper ensuring any residual volume is removed during the blotting procedure.

16. immediately dispense 150 ml enzyme substrate into all wells, cover the plate and mix on a microplate reader for exactly 60 minutes at room temperature (15-30^{0}C).

17. pipette 100 ml 1.0 M sulphuric acid into each well, mix the contents of the plate and determine the optical density in a microplate reader at 450 nm within 30 minutes.

Experiment I : Effects of PMSG and hCG concentrations for the stimulation of immature female mice on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development.

This experiment was designed to investigate the most suitable concentration of PMSG and hCG for the stimulation of immature female mice to obtained immature oocytes for the IVM/IVF studies, and to compare the effects of PMSG and hCG on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development. Denuded and cumulus cell - enclosed immature oocytes were obtained from the either 5, 7.5, or 10 IU gonadotropins stimulated immature female mice. Immature oocytes of each group were cultured in maturation medium (10 oocytes per ml) under a humidified mixture of 5% CO_2 in air at 37^0C for 24 hours (conventional procedure, Downs et al. 1988 ; Das et al. 1992). At the end of culture, maturation was assessed by the percentage of oocytes that reached the MII stage. *In vitro* matured oocytes of each group were then inseminated with PGC sperm. The success rate of *in vitro* fertilization was determined by the percentage of 2-cell embryos and the percentage of *in vitro* fertilization among these groups were compared. In

addition, two-cell embryos from each group were washed two times in T6 medium and cultured in T6 medium at 37^0C under 5% CO_2 in air, and the percentage of embryos developed to blastocyst stage were determined 120 hours after incubation (Trounson and Gardner, 1993) (see in conceptual framework I) .

Experiment II : Effects of oocyte maturation duration on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in PMSG- primed and hCG-primed mice.

This experiment was designed to test the hypothesis that hCG, rather than PMSG, increases the percentage of *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in designated durations of oocyte maturation process. As has been shown in previous experiment that 7.5 IU of gonadotropins was the most suitable concentration for the stimulation of immature female mice to obtain immature oocytes for the IVM/IVF study in our laboratory and the percentage of *in vitro* matured oocytes, fertilization, and preimplantation embryo development of the cumulus cell - enclosed oocytes was higher than that of the denuded oocytes. Therefore, cumulus cell - enclosed oocytes from 7.5 IU PMSG- primed and 7.5 IU hCG-primed mice were used in this experiment.

Oocytes were cultured in maturation medium (10 oocytes per ml) under a humidified mixture of 5% CO_2 in air at 37^0C for 3, 6, 9, 12, 15, 18, 21, and 24 hours. At the end of each culture, maturation was assessed by the percentage of oocytes that reached the MII stage. *In vitro* matured oocytes of each group were then inseminated with PGC sperm. The success rate of *in vitro* fertilization was determined by the number of 2-cell embryos. Subsequently, two-cell embryos were washed two times in T6 medium and cultured in T6 medium at 37^0C under 5% CO_2 in air, and the percentage of embryos developed to blastocyst stage were determined 120 hours after incubation (see in conceptual framework II).

Experiment III : Effects of oocyte maturation duration on the level of intraoocyte cAMP, zona pellucida thickness and zona pellucida hardness of oocytes obtained from PMSG- primed and hCG-primed mice.

Many investigators have suggested that the lower *in vitro* fertilization rate of *in vitro* matured oocytes than that of *in vivo* matured oocytes probably caused by a change in the zona pellucida (ZP) to zona hardness (Downs et al., 1988 ; Ducibella et al., 1990 ; DeMeestere et al., 1997 ; Hinsch et al., 1997). In addition, many investigators have showed that intraoocyte cAMP affected oocyte maturation but whether that affected fertilization and

embryo development have not been shown (Eppig and Downs, 1984 ; Cork et al. 1990 ; Mehlmann et al. 1996). This experiment was designed to test that oocytes from hCG-primed mice, at the designated incubation durations, rather than PMSG-primed mice, effectively induced oocyte maturation indicated by the decrease of intraoocyte cAMP level, zona thickness and zona hardness.

Cumulus cell-enclosed immature oocytes were obtained from 7.5 IU PMSG- primed and hCG-primed mice and cultured in maturation medium (10 oocytes per ml) under a humidified mixture of 5% CO_2 in air at 37^0C for 3, 6, 9, 12, 15, 18, 21, and 24 hours. At the end of each culture period, mature oocytes were collected for the determination of the level of intraoocyte cAMP, the ZP thickness and the ZP hardness. Mature oocytes were examined for their intraoocyte cAMP by enzymeimmunoassay (Biotrak RPN 225 ; Amersham life science, using a commercially available test kit according to the manufacturer's specifications). The ZP thickness and the ZP hardness of mature oocytes were checked by ocular micrometer, and the time required for digestion of ZP by 1 mg/ml α- chymotrypsin, respectively. For assessing ZP hardness, 20 mature oocytes were quickly washed in M199 medium + 3 mg/ml BSA, and transferred into 100 μl Dulbecco's phosphate-buffered saline containing 0.1% α-chymotrypsin at pH 7.2 under paraffin oil. Dishes were warmed on a slide-warming tray set at 37^0C and observed at one- minute intervals for ZP lysis.The ZP digestion time is defined as the time required for 50% of the ova to become

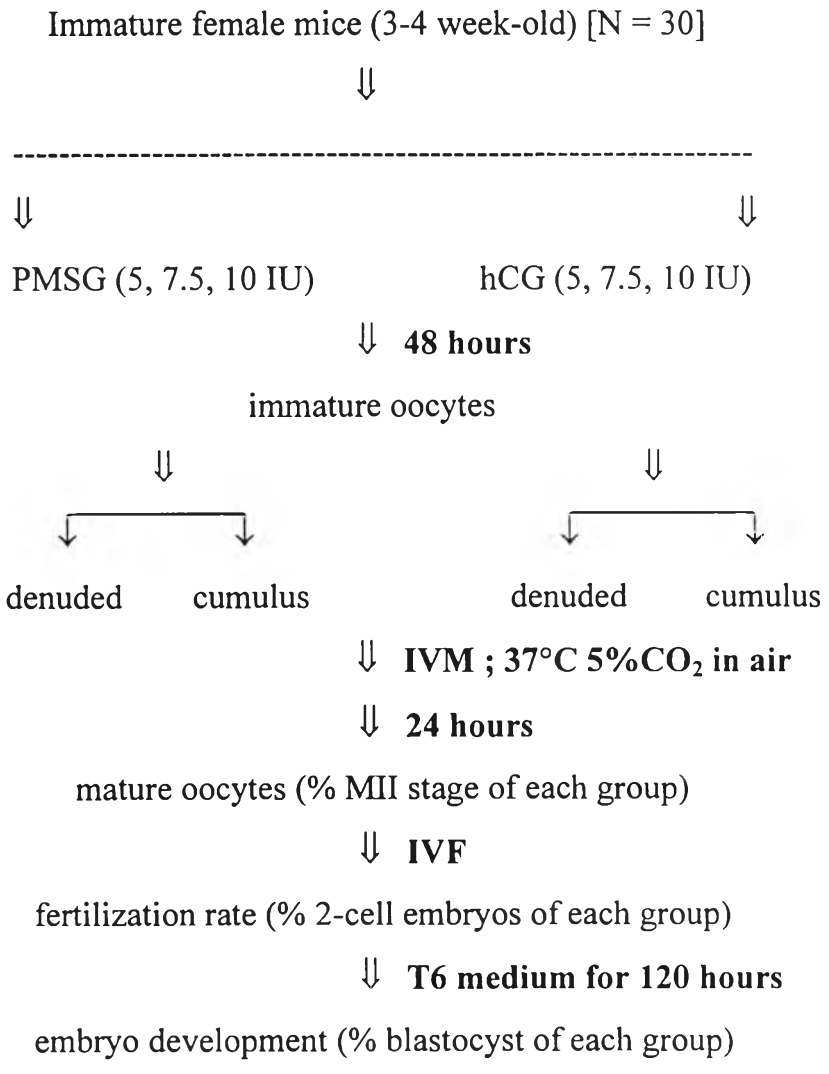
completely ZP- free as observed microscopically (Downs et al., 1986) (see in conceptual framework III) .

Data collection and analysis

Data were presented as means ± SD with at least three independent replicates. For evaluating the differences between groups with categorical data, analysis of variance (ANOVA) was applied. When a significant F-ratio was defined by ANOVA, the means were compared using Z test. Differences were considered significant at $P< 0.05$.

Conceptual framework [N = 270]

I. Effects of PMSG and hCG concentrations for the stimulation of immature female mice on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development.



note : N is number of mice

II. Effects of oocyte maturation duration on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in PMSG- primed and hCG-primed mice.

Immature female mice (3-4 week-old) [N = 80]

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⇓ ⇓

PMSG (7.5 IU) hCG (7.5 IU)

⇓ **48 hours**

immature oocytes *{cumulus-enclosed}*

⇓ **IVM ; 37°C 5%CO_2 in air**

3, 6, 9, 12, 15, 18, 21, and 24 hours

⇓

mature oocytes (% MII stage of each incubation duration)

⇓ **IVF**

fertilization rate (% 2-cell embryos of each incubation duration)

⇓ **T6 medium for 120 hours**

embryo development (% blastocyst of each incubation duration)

III. Effects of oocyte maturation duration on the level of intraoocyte cAMP, zona pellucida thickness and zona pellucida hardness of oocytes obtained from PMSG- primed and hCG-primed mice.

Immature female mice (3-4 week-old) [N = 160]

⇓

⇓ ⇓

PMSG (7.5 IU) hCG (7.5 IU)

⇓ **48 hours**

immature oocytes *{cumulus-enclosed}*

⇓ **IVM ; 37°C 5%CO_2 in air**

3, 6, 9, 12, 15, 18, 21, and 24 hours

⇓

mature oocytes (% MII stage of each incubation duration)

⇓

intraoocyte cAMP concentration zona thickness zona hardness