CHAPTER 3

GENERAL PROTOCOLS FOR IN VITRO MATURATION, FERTILIZATION AND CULTURE (IVMFC) IN GOATS

3.1. OBJECTIVE, PERIOD AND PLACE OF STUDY

The objective of this study was to develop in vitro immature oocytes obtained from slaughtered goat, as a preparation for their subsequent fertilization and also culture of embryos in vitro. The study which was conducted between the year 1989 to 1994, was mostly held in the Animal Biotechnology - Embryo Laboratory (ABEL), at the Department of Physiology, Faculty of Medicine, University Kebangsaan Malaysia. Some work was also carried out in the ABEL of the Department of Zoology, University of Malaya, and the Institute of Advanced Studies, University of Malaya, Kuala Lumpur, Malaysia.

3.2. ANIMALS

Female local goats, the majority of which were between 1.5 to 2.5 years of age, were obtained from various farms in the state of Selangor, D. E. They were slaughtered in the Abattoir in Shah Alam, Selangor, D. E.

3.3. SOURCE OF OOCYTES

3.3.1. Collection of Ovaries

The ovaries were immediately removed after the goats were slaughtered. They were rinsed and kept in collecting medium (phosphate-buffered saline, PBS) at 39.0°C and transported within 45 min to the laboratory.

3.3.2. Collection and Classification of Oocytes

Within the laminar flow (Email Westinghouse Pty Ltd., Australia), the surfaces of the ovaries were sliced using a scalpel blade to obtain the oocytes. The ovaries were then flushed thoroughly with PBS using a 10 ml syringe . The collected oocytes were washed three times with PBS, followed by three times washed with washing medium (hepes buffered H-TCM 199,) in a sterile petri dish (60 x 15 mm)(Falcon 3002, Becton Dickinson & Co., USA).

Before transferring into in vitro maturation (IVM) medium, the oocytes were classified into two groups: group 1 oocytes were those surrounded with minimum 2 layers of cumulus cells and called cumulus - oocyte complexes (COCs) and group 2 were those without cumulus cell and called cumulus-free oocytes (CFOs) (Figure 3.1). Care was taken to pick up only oocytes with normal, uncracked zona pellucida,

having cytoplasm with granulated appearance and which filled the entire perivitelline space. This is important because some CFOs are from atretic follicles which certainly would not show development characteristics in ultrastructures. Therefore, the oocytes with cracked zona pellucida, partly filled perivitelline space with smooth appearance of cytoplasm, degenerated cytoplasm and those with vesicles were not chosen (Figure 4.3). For the sake of standardization, oocytes with patches of cumulus cells and oocytes covered with jelly-like substance were not taken.

After being collected and classified, the oocytes were washed several times with hepes-buffered medium and finally rinsed once with the IVM medium. The oocytes were transferred into microdroplets of IVM medium, incubated and examined at specified intervals.

3.4. PREPARATION OF MEDIA

3.4.1. Oocytes Collecting Medium

The medium used for washing the ovaries and the oocytes was phosphate buffered saline, PBS (Sigma Chem. Co., USA, Cat. No. 1000-3), pH 7.6 at 37.0°C. The sliced ovaries were washed three times before the oocytes were picked up and classified. Then the collected oocytes were washed in the washing medium described below (3.4.2).

3.4.2. Washing Medium

The washing medium used was hepes buffered H-TCM 199 (Sigma Chemical Co., St. Louis, MO, USA, Cat. No. M 2520), with Earle's salt and L-glutamine, and was modified by addition of the following (Table 3.1):

Table 3.1: Reagents used to modify the washing medium

Composition	µg/ml
Sodium pyruvate [1]	112
Streptomycine sulfate [2]	50

[1]Sigma Chem. Co., USA, Cat. No. P 3662; [2]Sigma Chem. Co., USA, Cat.No. S 9137

After preparation, the medium was sterilized by filtration using disposable filters of 0.22 µm pore size (Sterile Millex-GS, Millipore Product Division, Bedford, United Kingdom). The pH of the medium (7.4) was checked using pH meter (Orion model 420A). Its osmolality (280-290 mOsm/L) was checked using an automatic osmometer (Osmette A; Precision System, Inc., Massachusetts, 01776, USA). Unless used

immediately, the medium was kept in a refrigerator for up to two weeks.

3.4.3. In Vitro Maturation (IVM) Medium

The in vitro maturation (IVM) medium used in this study was a modified TCM 199 medium described by Younis *et al.* (1991). This medium consists of buffered TCM 199 with Earle's salt, L-glutamine (b-TCM 199, Sigma Chem. Co., Cat. No. M 4530, St. Louis, MO, USA) and was modified by addition of the following (Table 3.2):

Table 3.2: Reagents used to modify the maturation (IVM) medium

Composition	/ml
Sodium pyruvate [1]	50 µg
Glucose [2]	5.5 mg
Streptomycine sulfate[3]	50 µg
Bovine serum albumin (BSA)[4]	4 mg
Heat-inactivated (56°C, 30 min) oestrus goat serum	20% v/v

[1]Sigma Chem. Co., USA, Cat. No. P 3662; [2]Sigma Chem. Co., USA, Cat. No. G 7021; [3]Sigma Chem. Co., USA, Cat. No. S 9137; [4]Sigma Chem. Co., USA, Cat. No. A 9647

After preparation, the medium was sterilized as explained before (3.4.2). The IVM medium was prepared immediately before use.

3.4.4. Medium for Washing of Sperm

Medium used for washing and capacitation of sperm was modified Krebs-Ringer bicarbonate (m-Kreb) solution described by Toyoda and Chang (1974). The contents of the medium were as in Table 3.3.

After being prepared, this medium was filtered as explained before (3.4.2) and equilibrated in 5% CO_2 incubator at 39.0°C for at least 1 hr before use.

3.4.5. In Vitro Fertilization (IVF) Medium

The in vitro fertilization (IVF) medium was modified Krebs-Ringer bicarbonate (m-Kreb) solution described by Toyoda and Chang (1974). It consisted of the reagents shown in Table 3.4.

Table 3.3: Composition of medium for washing of sperm

Composition	/100 ml
Sodium chloride ($NaCl$)[1]	6.91 g
Potassium chloride (KCl)[2]	3.56 g
Sodium bicarbonate ($NaHCO_3$)[3]	2.1 g
Potassium dihydrogen orthophosphate (KH_2PO_4)[4]	0.162 g
Sodium lactate[5]	21.58 mM/L (or 4.83 ml/L)
Calcium chloride ($CaCl_2$)[6]	0.189 g
Magnesium sulfate ($MgSO_4 7H_2O$)[7]	0.29 g
Sodium pyruvate[8]	0.028 g
Bovine serum albumin (BSA)[9]	600.0 mg
Streptomycin sulfate[10]	5 mg
Penicillin G[11]	7.5 mg

[1]Sigma Chem. Co., USA, Cat. No. S 5886; [2]Sigma Chem. Co., USA, Cat. No. P 5405; [3]Sigma, Chem. Co., USA, Cat. No. S 5761; [4]BDH AnalaR, Prod. Cat.No.10203; [5]Sigma Chem. Co., USA, Cat. No.L 7022; [6]Sigma Chem. Co., USA, Cat. No. L 2536; [7]BDH AnalaR, Prod. Cat.No. M 7774; [8]Sigma Chem. Co., USA, Cat. No. S 5280; [9]Sigma Chem. Co., USA, Cat.No. S 9647; [10]Sigma Chem. Co., USA, Cat. No. S 9137; [11]Sigma Chem. Co., USA, Cat. No. P 3032.

Table 3.4: Composition of fertilization (IVF) medium

Composition	/100 ml
Sodium chloride ($NaCl$)[1]	6.91 g
Potassium chloride (KCl)[2]	0.356 g
Sodium bicarbonate ($NaHCO_3$)[3]	2.1 g
Potassium dihydrogen orthophosphate (KH_2PO_4)[4]	0.162 g
Sodium lactate[5]	21.58 mM/L (or 4.83 ml/L)
Calcium chloride ($CaCl_2$)[6]	0.189 g
Magnesium sulfate ($MgSO_4\ 7H_2O$)[7]	0.29 g
Sodium pyruvate[8]	0.028 g
Glucose[9]	1.0 g
Bovine serum albumin (BSA)[10]	400.0 mg
Streptomycin sulfate[11]	5 mg
Penicillin G[12]	7.5 mg

[1]Sigma Chem. Co., USA, Cat. No. S 5886; [2]Sigma Chem. Co., USA, Cat.No. P 5405; [3]Sigma Chem. Co., USA, Cat. No. S 5761; [4]BDH AnalaR, Prod 10203; [5]Sigma Chem. Co., USA, Cat. No. L 7022; [6]Sigma Chem. Co., USA, Cat.No. C 2536; [7]BDH AnalaR, Prod. 7774; [8]Sigma Chem. Co., USA, Cat. No. P 5280; [9]Sigma Chem. Co., USA, Cat. No. G 7021; [10]Sigma Chem. Co., USA,Cat. No. A 9647; [11]Sigma Chem. Co., USA, Cat. No. S 9137; [12]Sigma Chem. Co., USA, Cat. No. P 3032

This medium was prepared immediately before use. It was filtered using millipore, and 200 μL each of microdroplets was prepared in the 4-well culture dish as shown before. The microdroplets were then equilibrated in the CO_2 incubator for minimum of 1 hr before use.

3.4.6. In Vitro Culture (IVC) Medium

The culture medium consists of buffered TCM 199 with Earle's salt, L-glutamine (b-TCM 199, Sigma Chem. Co., Cat. No. M 4530, St. Louis, MO, USA) and was modified by addition of the reagents shown in Table 3.5.

3.5. REPARATION OF MICRODROPLETS

Microdroplets were prepared in a laminar flow cabinet. An ultraviolet (uv) lamp of the cabinet (Email Westinghouse Pty Ltd., Australia) was switched on for 0.5 hr before the preparation. The preparation of the microdroplets was carried out under fluorescent light. Using a 1.0 ml syringe (Japan Medical Supply Co. Ltd), incorporating a millipore filter, 500 μL IVM medium were each deposited into sterile 4 - well culture dish (Cat. No. 176740, Nunclon). For fertilization (IVF) and IVC media, droplets of 200 μL of each medium were used. The microdroplets were then equilibrated for a minimum of 1 hr in a CO_2 incubator (Jouan IG150) before being used for culturing the oocytes. The incubator contained a humidified 5% CO_2 in air at 39.0°C.

Table 3.5: Composition of culture (IVC) medium

Composition	/100 ml
Heat-treated (56°C, 30 min)	
goat oestrus serum	10% v/v
Bovine serum albumin (BSA)[1]	500 mg
Sodium pyruvate[2]	25 mg
Taurine (2-aminoethanesulfinic acid)[3]	12.51 mg
L-glutamine (l-2- aminoglutaramic acid)[4]	14.61 mg
Penicillin G[5]	7.5 mg
Streptomycine sulfate[6]	50 mg/L

[1]Sigma Chem. Co., USA, Cat. No. A 9647; Chem. Co., USA, Cat.No. P 3662; [3]Sigma Chem. Co., USA, Cat No. H 1384; [4]Sigma Chem. Co., USA, Cat. No. G 1517; [5]Sigma Chem. Co., USA, Cat. No. P 3032); [6]Sigma Chem. Co., USA, Cat. No. S 9137.

The culture medium was prepared immediately before use. It was filtered using millipore before 200 μL each of microdroplets was prepared in the 4-well culture dish. The microdroplets were then equilibrated in the CO_2 incubator at 39.0°C for minimum 1 hr before use.

3.6. PREPARATION OF SPERM

The frozen semen for IVF procedure in this study was obtained from the Reproductive Physiology Laboratory, Institute of Advanced Study, University of Malaya. The standard procedures of handling and freezing of semen in this laboratory are given in Appendix 1.

3.6.1. Thawing of the Frozen Semen

For thawing, the straws were taken out of liquid nitrogen, thawed at 37.0°C in a water bath for 2 min. The semen was put in the sterile graduated test tube by cutting both ends of the straws and washed as shown in 3.6.2.

3.6.2. Washing of Semen for IVF

In the laboratory, a small aliquote of semen was observed under light microscope (Olympus CH-2, Japan). Ejacu-

lates showing motility of the spermatozoa less than 2 in a 0 to 5 scale were discarded. The criteria in grading the motility were as follows: 0 - no movement, all cells dead; 1 - very slow, non-progressive movement; 2 - slow but progressive movement; 3 - moderate velocity and linear movement; 4 - fast movement; 5 - very fast and linear movement.

Semen of 0.5 - 1.0 ml was mixed thoroughly in 3 ml sperm medium. The mixture was centrifuged at 600xg for 10 min at 39.0°C (Sorval RT 6000 refrigerated centrifuge, Ou Pont). After centrifugation, the supernatant was aspirated. This procedure was repeated twice. These sperm were then subjected to swim-up procedure (3.6.3).

3.6.3. Swim-up Procedure

After washing twice with sperm medium, the sperm pellet was subjected to swim-up procedure, again using sperm medium. In this procedure, by using a pipette, the medium was allowed to run slowly along the tube. The preparation was equilibrated for 1 hr at 39.0°C under 5% CO_2 in air. This technique allows the good sperm to swim-up to the top layer of the medium, leaving the dead or non-viable sperm at the bottom.

3.6.4. Capacitation of Sperm

Fertilization medium was added with 0.5 mg/10 ml heparin (sodium salt, Sigma Chem. Co., USA, Cat. No. H 5765). The microdroplets of heparin-treated fertilization medium was prepared and equilibrated in the CO_2 incubator at 39.0°C.

3.7. PREPARATION OF OVIDUCTAL EPITHELIAL CELLS FOR CO-CULTURE

In order to culture and determine the developmental capacity of inseminated oocytes, oviductal epithelial cells were prepared as for cattle (Eyestone and First, 1989) with some modification. The epithelial cells used for co-culture system were those at the ampullary region of the oviduct. The stage for the selection of ampulla was based on the observation that the ovary was at the corpus hemorrhagicum stage i. e. the presence of trace of blood at the site whereby ovulation has just occurred. The right choice of this stage of oestrus cycle is important because this is a period whereby the epithelial cells were ready for the fertilization and the cleavage of embryos along the oviducts before these embryos undergone implantation in the uterine wall.

The ampulla was trimmed free of connective tissue and washed with PBS. Using a pair of forceps, the proximal part of the ampulla was gripped tightly while the ampulla was gently squeezed beginning from proximal to distal part of it using another pair of forceps. Extruded mucosal tissue was transferred to a conical tube containing 5 ml of hepes-bufferred TCM 199. The cells were washed in this medium three times. The cells were rinsed once with the IVC medium before incubation within the same medium overnight. These cells were then examined under light microscope at 40x magnification. The live epithelial cells showed worm-like movement of cells with actively moving cilia.

3.8. INSEMINATION OF OOCYTES

Only oocytes surrounded with cumulus cells, cumulus-oocyte complexes (COCs) were used for IVF study. This was based on the findings reported in Chapters 4 and 5 that these oocytes were presumably those which attained maturing stage in vitro and therefore more superior compared with CFOs. After incubation for maturation between 20 to 22 hr, the oocytes were washed thoroughly to remove the cumulus cells. This was achieved by transferring the oocytes into the washing medium followed by vortexing them. The cumulus-removed oocytes were then washed three times with the oocytes washing medium and followed by three times washing in

the fertilization medium. The oocytes were then transferred into microdroplets of the heparin-added fertilization medium. 0.2 ml of IVC medium containing epithelial cells were added into the microdroplets. Twenty μL each of sperm ($1x10^6$/ml) was added into each microdroplet which contained less than 10 oocytes.

3.8.1. Determination of sperm concentration

From time to time, the concentration of sperm in the microdroplet containing oocytes was determined using haemocytometer.

3.9. IN VITRO CULTURE OF EMBRYOS

At about 16 hr post-insemination, the embryos were washed thoroughly using washing medium and then transferred into IVC medium. Few drops of IVC containing epithelial cells was added. The embryos were further cultured and examined under the stereomicroscope for cleavage. Photographs were taken using a camera attached to the inverted microscope.

3.10. PROTOCOL FOR IVMFC PROCEDURES

Procedures for IVMFC involved the preparation of media and oocytes and sperm handling. The exact timing for the procedures were as shown in Table 3.6.

Table 3.6: Protocol for IVMFC procedure

Day	Events
0	Preparation of media: 1. PBS 2. hepes H-TCM 199
1	Preparation of media: 1. IVM medium 2. IVC medium Preparation of IVM microdroplets. Collection of ovaries and oocytes. Preparation of oviductal epithelial cells. IVM.
2	Preparation of IVF microdroplets. Treatment of sperm: 1. washing 2. swim up 3. capacitation Wash and transfer of oocytes into IVF medium. IVF.
3	Preparation of IVC microdroplets. Wash and transfer of 1-cell embryos into IVC medium. IVC.
4	Record for development
5	"
6	"
7	"

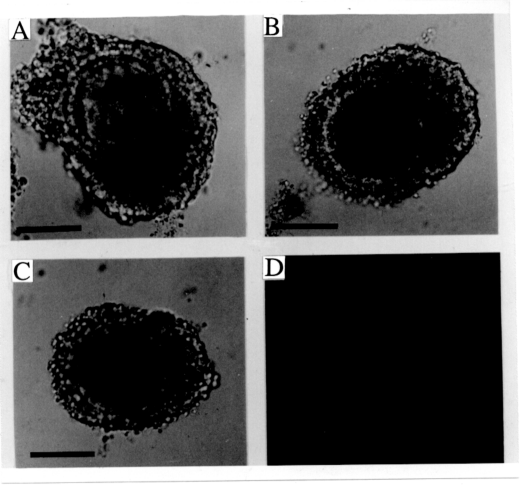

Figure 3.1. Classification of oocytes. Various types of oocytes, all of which were grouped as cumulus-oocyte complexes, COCs (A,B and C) and cumulus-free oocytes, CFOs (D). A, oocyte with 5 or more layers of cumulus cells; B, oocyte surrounded by 3-4 layers; C, oocyte with 2-3 layers. Scale bar: A,B,C: 60 μm; D : 50 μm