Chapter 3

3.0 MATERIALS AND METHODS

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3.1 GENERAL INTRODUCTION

There were 2 main laboratories involved in carrying out the experiments in the present study. They were Nuclear Transfer and Reprogramming Laboratory (NaTuRe), Institute of Research, Management and Consultancy (IPPP) and Embryo Micromanipulation Laboratory (EMiL), Institute of Biological Sciences (ISB) at the University of Malaya, Kuala Lumpur, Malaysia. The experimental goats were sourced from the ISB Mini Farm (University of Malaya) and the Department of Veterinary Services and Abattoir Complex, Shah Alam while the experimental bovine samples were collected from the Department of Veterinary Services and Abattoir Complex, Shah Alam and Senawang. Surgery for oocyte retrieval (laparoscopic oocyte pick-up, LOPU) was conducted once a week using one donor goat per surgery session at the NaTuRe Laboratory. After the oocyte-retrieval surgery, immatured oocytes were transported using portable temperature control container to the EMiL Laboratory for *in vitro* maturation and subsequent experiments. The duration of the research was from November 2009 to May 2011.

3.2 FACILITIES

The facilities used in the present study included NaTuRe Laboratory (for caprine oocyte-retrieval surgery), EMiL Laboratory (for subsequent *in vitro* maturation, fertilisation and culture and pathenogenetic activation as well as vitrification experiments), ISB Mini Farm (source of live experimental goats for oocyte samples), the Department of Veterinary Services and Abattoir Complex, Shah Alam and Senawang (source of experimental caprine and bovine ovaries samples).

3.3 EXPERIMENTAL ANIMALS

In the present study, does comprising of Boer crossbred, Jamnapari breed and local mixed-breed, age ranging from 6 to 42 months old were used. For laparoscopic oocyte pick-up, a total of 32 experiments were performed on 32 does; for embryo flushing, a total of 4 surgeries were performed on 5 does. Goats were underwent oestrus synchronisation and hyperstimulation. In each oocyte-retrieval surgery, 1 hyperstimulated-donor doe was used and the immatured caprine oocytes were retrieved from 2 ovaries while the frozen Boer buck semen was obtained from sperm preservation laboratory, ISB Mini Livestock Farm, University of Malaya. The semen was supplied in French straws (0.5 ml) and stored in the liquid nitrogen tank (-196ºC) in the laboratory where *in vitro* fertilisation was done. On the other hand, the experimental bovine ovaries were obtained from the Department of Veterinary Services and Abattoir Complex, Shah Alam and Senawang, in which ovaries were transported back to EMiL laboratory for ovary slicing and subsequent experiments and frozen bull semen was obtained from National Animal Biotechnology Institute, Jerantut, Pahang.

3.4 MATERIALS

Materials used in the present study included various equipment and instruments, glass-wares, lab-wares and disposables, chemicals, reagents and media as well as study samples.

3.4.1 Equipment and Instruments

Equipment and instruments used in the present study are listed in Appendix Table 1.1.

3.4.2 Glass-wares, Lab-wares and Disposable Items

Glass-wares, lab-wares and disposables items used in the present study are listed in Appendix Table 1.2.

3.4.3 Chemicals, Reagents and Media

All chemicals, reagents and media were purchased from Sigma Chemical Co., St. Louis, USA and plastics from Nunclon, Roskilde, Denmark unless otherwise stated in Appendix 1.3.

3.5 METHODS

3.5.1 Maintenance of Research Laboratory

For successful *in vitro* production experiments, it is crucial that the laboratory activities and facilities adhere to strict cleanliness and sterilisation regimes throughout all experimental procedures. The laboratory is ensured to be in a distraction-free and accident-free environment to optimise the outcome of *in vitro* production works. It is also of utmost importance to minimise the potential introduction of contaminants which bring hazardous to the oocytes and embryos.

The glass-wares and non-disposable items used in the present study included glass bottles, beakers, volumetric flasks, measuring cylinders, conical flasks, magnetic stirrer as well as conical tubes. After they were being used, the glass-wares were washed with diluted cleaning solution (7x®-PF) using a brush or sponge and immediately rinsed 5 times with water followed by 5 times with reverse osmosis (RO) water. After rinsing was completed, the cap of glass-ware was placed loosely and covered snugly with a layer of aluminium foil. A piece of autoclave tape was placed on the foil. Alternatively, non-disposable items were placed in an autoclaved bag and sealed (for magnetic stirrer, it was wrapped with aluminium foil before putting into an autoclave bag); a piece of

autoclave tape was placed on the seal-bag. All the glass-wares and non-disposable items were allowed to be autoclaved for a minimum of 25 minutes and dry cycled for 30 minutes. After autoclaving was done, all the items were transferred to oven for drying. The glass-ware cap was tightened a little bit to prevent any contaminants from entering. The cap was not tightened completely until the glass-ware had cooled to prevent a vacuum forming in the glass-ware. After being dried, all items were put at their particular storage cabinets.

For all *in vitro* production (IVP) experiments, this study used carbon dioxide (CO_2) (5%) in humidified air to maintain the correct physiological pH (pH 7.3 to 7.4) and a temperature of 38.5ºC. Monthly cleaning regime of the CO_2 incubator involves wiping the inside wall, doors and racks with sterile RO water and sterile towels or gauzes. The RO water in the tray which provides humidity should be changed with every cleaning regime. The CO_2 incubator must be monitored regularly and the LED display of temperature checked with independent thermometer readings. The opening and closing of the CO_2 incubator should be kept to minimum as it affects the stability of the oocyte or embryo culture environment.

Before and after any experiment was carried out, the inner surface of laminar flow work station, microscope stage as well as micropipettes was wiped with ethanol (70%). Water bath was cleaned with diluted cleaning solution (7x®-PF) using a sponge and rinsed 5 times with water followed by 5 times with RO water, subsequently fill the water bath with RO water and the water was changed frequently.

3.5.2 Preparation of Stocks and Media

Instead of purchasing commercially, all culture media used throughout the present study were prepared ‘in-house’. The water used to prepare all culture media was sourced from a ultrapure water purification system; with treatments of particulate filtration, activated

carbon filtration, reverse osmosis and electrodeionisation (EDI), ultraviolet oxidation system, followed by a Milli-Q PF Plus purification (18.2 MΩ-cm) and finally filtered through a membrane filter (0.22 µm) to eliminate trace particles as well as to prevent bacterial contamination from the environment. Typically, fresh culture media were prepared weekly. It was more convenient to prepare working solutions from a series of stock solutions as the preparation of culture media required accurate but time-consuming measurements. All stock solutions were sterile prepared by filtering through a syringe filter (0.22 µm), aliquot in microcentrifuge tubes (0.5 or 1.5 ml), completely sealed and stored in the refrigerator (4ºC) or freezer (-20ºC) as appropriate. For all the media, the pH was adjusted to 7.2-7.4 and osmolarity to 280-300 mOsm/kg.

3.5.2.1 Preparation of oestrus goat serum (OGS)

Oestrus goat serum was supplemented in the oocyte maturation and embryo culture media to provide additional growth factors, hormones and peptides.

3.5.2.1 (a) Blood collection

To prepare the oestrus goat serum, blood samples (200 to 300 ml) were collected aseptically via the jugular vein from goats synchronised for oestrus using vacutainer® tubes (without heparin) with a needle (21 gauge) (Figure 3.1). Freshly collected blood was distributed into sterile conical tubes (15 ml) and the tubes were kept in laminar flow at room temperature for approximately 3 hours to obtain the serum from clotted blood.

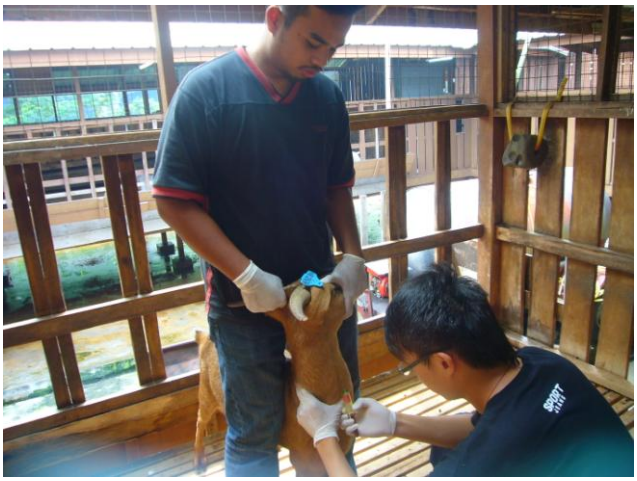

Figure 3.1: Blood collection was being carried out.

3.5.2.1 (b) Heat-inactivation

The tubes containing clotted blood were centrifuged (500 x *g*, 10 minutes, 25ºC). The supernatant (serum) was aspirated and placed in a sterile conical flask and heat-inactivated in water bath (56ºC, 30 minutes) to destroy components that might lead to cell lysis by antibody binding. After 30 minutes of treatment at 56ºC, serum was removed from the water bath. Prolonged heat treatment could cause deterioration of some components of the serum. After cooling to room temperature (25ºC), serum was centrifuged again (500 x *g*, 10 minutes, 25ºC) to sediment residual erythrocytes. The oestrus goat serum was then aliquot in microcentrifuge tubes (1.5 ml) and stored in the freezer (-20ºC) with a shelf life of 6 months. OGS was thawed (room temperature) immediately prior to use.

3.5.2.2 Preparation of media for laparoscopic oocyte pick-up

Heparinised saline and flushing medium were prepared for laparoscopic oocyte pick-up.

3.5.2.2 (a) Heparinised saline

The saline solution consisted of NaCl (4.5 g) and heparin (0.025 g) dissolved in autoclaved Milli-Q water (500 ml) (Table 3.1). After preparation, the saline was kept for 3 months in the refrigerator (4ºC) for future use.

Table 3.1: Composition of heparinised saline solution

Chemical (catalogue no.)	Concentration	Quantity/ 500 ml
NaCl (S5886)	0.9 (w/v)	4.5 g
Heparin (H0777)	0.05 mg/ml	0.025 g

3.5.2.2 (b) Flushing medium

The flushing medium served as the collection medium for laparoscopic oocyte pick-up was connected to an aspiration system (a vacuum pump). Micro-volumes of flushing medium was flushed into the follicles on the surface of the ovary; follicular fluids (containing oocytes suspended in flushing medium) from the follicles were subsequently aspirated and collected in a sterile round-bottom test tube (14 ml) which was pre-warmed by a test tube heating system. Flushing medium (300 ml) was prepared within 24 hours before oocyte retrieval. The flushing medium consisted of phosphate buffer saline (PBS) supplemented with gentamycin sulphate (50 µg/ml) and heparin (50 µg/ml) as shown in Table 3.2. The flushing medium was then filter-sterilised using syringe filter (0.22 µm pore size), aliquot into Terumo® luer slip syringe (50 ml) and kept warmed (38.5ºC) prior to oocyte retrieval.

Table 3.2: Composition of flushing medium

Chemical (catalogue no.)	Concentration	Quantity/300 ml
Phosphate-buffered saline (PBS) tablets (P4417)	1 tablet/100 ml	PBS (3 tablets) were dissolved in Milli-Q water (300 ml), sterilised by autoclaving.
Gentamycin sulfate salt (G3632)	30 µg/ml	Gentamycin sulphate (9 mg) was dissolved in PBS solution (300 ml) prior to use.
Heparin (H0777)	52 IU/100 ml	Heparin (1 mg*) was dissolved in PBS solution (300 ml) prior to use.

*1 mg of heparin contains 156 IU.

3.5.2.3 Preparation of media for ovary slicing

Saline supplemented with streptomycin and penicillin G as well as TL-Hepes was prepared for ovary slicing.

3.5.2.3 (a) Saline

Saline supplemented with streptomycin and penicillin G was used for ovary collection. NaCl (9 g), streptomycin (0.05 g) and penicillin G (0.06 g) were added and dissolved in Milli-Q water (1 litre) (Table 3.3). Saline was warmed at 37ºC before being put into thermos.

Table 3.3: Composition of saline supplemented with streptomycin and penicillin

Chemical (catalogue no.)	Concentration	Quantity/litre
NaCl (S5886)	0.9 (w/v)	9 g
Streptomycin (S1277)	0.06 mg/ml	0.05 g
Penicillin G (PEN-NA)	0.05 mg/ml	0.06 g

3.5.2.3 (b) TL-Hepes

TL-Hepes was used for oocytes searching and oocytes washing before being transferred to *in vitro* maturation medium. To prepare TL-Hepes stock solution, NaCl (6.66 g), KCl

(0.24 g), $NaHCO_3$ (0.168 g), $NaH_2PO_4.H_2O$ (0.056 g), Na L-lactate (60% syrup) (1.86 ml), $CaCl_2.2H_2O$ (0.3 g), $MgCl_2.6H_2O$ (0.1 g), Hepes $C_8H_{17}N_2O_4SNa$ (1.2 g), Hepes $C_8H_{18}N_2O_4S$ (1.2 g), penicillin G (0.065 g) and phenol red (0.005 g) were added to Milli-Q water (approximately 800 ml). The volume of solution was then topped up to 1 litre (Table 3.4). Finally, the solution was gently stirred (to ensure a homogenous mixture), filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile glass bottle (1 litre) with a shelf life of 2 weeks.

Table 3.4: Composition of TL-Hepes stock with a shelf life of 2 weeks (stored at 4ºC)

Chemical (catalogue no.)	Concentration (10X)	Quantity/litre
NaCl (S5886)	114.0 mM	6.66 g
KCl (P5405)	3.2 mM	0.24 g
$NaHCO_3$ (S5761)	2.0 mM	0.168 g
$NaH_2PO_4.H_2O$ (S9638)	0.4 mM	0.056 g
Na L-lactate (60% syrup) (L7900)	10.0 mM	1.86 ml
$CaCl_2.2H_2O$ (C3881)	2.0 mM	0.3 g
$MgCl_2.6H_2O$ (M2393)	0.5 mM	0.1 g
Hepes $C_8H_{17}N_2O_4SNa$ (H0763 or H3784)	10.0 mM	1.2 g
Hepes $C_8H_{18}N_2O_4S$ (H3375 or H6147)	10.0 mM	1.2 g
Penicillin G (PEN-NA)	100 IU/ml	0.065 g
Phenol red (P3532)		0.005 g

To prepare TL-Hepes working solution, gentamycin stock (50 µl), sodium pyruvate (0.0022 g) and BSA-FV (0.1 g) were added to TL-Hepes stock solution (100 ml) (Table 3.5). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile glass bottle (100 ml) with a shelf life of 1 week.

Table 3.5: Composition of TL-Hepes working solution with a shelf life of 1 week (stored at 4ºC)

Chemical (catalogue no.)	Quantity/100 ml
TL-Hepes stock solution	100 ml
Gentamycin stock (G3632)	50 µl
Sodium pyruvate (P4562)	0.0022 g
BSA-FV (A7030)	0.1 g

3.5.2.4 Preparation of *in vitro* maturation (IVM) medium

In vitro maturation medium was prepared a day before and equilibrated overnight in the CO_2 incubator (5%) prior to oocyte retrieval. The maturation medium used was TCM-199 supplemented with cystein (0.0009 g), TCM-pyruvate (100 µl), FSH (10 µl), gentamycin (5 µl), estradiol 17β (10 µl) and FBS (1 ml) (Table 3.6).

Table 3.6: Preparation of stock solutions for *in vitro* maturation medium

Chemical (catalogue no.)	Method of preparation
TCM-pyruvate (M4530; P4562)	Na pyruvate (0.0022 g) was added to TCM-199 (1 ml) in a microcentrifuge tube (1.5 ml), stored at 4ºC for 2-3 days.
FSH (Ovagen ™)	FSH powder (0.005 g) was dissolved to solution provided (1 ml), aliquot and stored (-20ºC).
Gentamycin stock (G3632)	Gentamycin (1 g) was added to DPBS (20 ml), syringe filtered (0.22 µm pore size), aliquot, wrapped with aluminium foil and stored (4ºC).
Estradiol 17β (E8875)	Estradiol (0.001 g) was added to ethanol (1 ml) in a cryotube, wrapped with aluminium foil and stored (4ºC).

To prepare the *in vitro* maturation medium, TCM-199 (8.9 ml) was measured using a disposable serological pipette (10 ml) and dispensed into a sterile conical tube (15 ml). Cystein (0.0009 g), TCM-pyruvate (100 µl), FSH (10 µl), gentamycin (5 µl) and FBS (1 ml) were added to the TCM-199. The medium was filter-sterilised using syringe filter (0.22 µm pore size). Estradiol 17β (10 µl) was added to the syringe filtered *in vitro* maturation medium (Table 3.7). The maturation medium was then equilibrated overnight in CO_2 incubator (5%) with the cap of the tube loosened.

Table 3.7: Composition of *in vitro* maturation medium

Chemical (catalogue no.)	Quantity/10 ml
TCM-199 (M4530)	8.9 ml
Cystein (C2529)	0.0009 g
TCM-pyruvate (M4530; P4562)	100 µl
FSH (Ovagen ^{TM})	10 µl
Gentamycin stock (G3632)	5 µl
FBS (GIBCO 10270)	1.0 ml
*Oestradiol 17β (E8875)	10 µl

*Oestradiol 17β was added after the *in vitro* maturation medium was syringe filtered.

3.5.2.5 Preparation of *in vitro* fertilisation (IVF) medium for caprine

In the present study, synthetic oviductal fluid (SOF) medium was used for caprine *in vitro* fertilisation.

3.5.2.5 (a) Preparation of synthetic oviductal fluid (SOF) medium

Synthetic oviductal fluid medium was prepared from a series of stock solutions (Stocks A, B, C and D). Components of stock solutions for SOF medium are shown in Tables 3.8 to 3.13 and 3.22. To prepare SOF-Stock-A solution (25 ml), a sterile conical flask (50 ml) was filled with Milli-Q water (approximately 20 ml). NaCl (1.5735 g), KCl (0.1334 g), KH_2PO_4 (0.0405 g), penicillin G (0.0150 g), streptomycin (0.0125 g), glucose (0.0676 g), Na L-lactate (60% syrup; 13.7 µl) and $MgCl_2.6H_2O$ (0.0249 g) were weighed by using a digital balance, added stepwise and dissolved in Milli-Q water (approximately 20 ml). The solution was gently stirred when Na L-lactate (60% syrup) was added. $MgCl_2.6H_2O$ was dissolved first in a separate container with Milli-Q water, then added last after all other chemicals had dissolved. The volume of solution was topped up to 25 ml (Table 3.8). Finally, the solution was gently stirred (to ensure a homogenous mixture), filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile conical tube (50 ml) with a shelf life of 3 months.

Table 3.8: Composition of Stock-A for SOF medium (10x) with a shelf life of 3 months (stored at 4ºC)

Chemical (catalogue no.)	Concentration (10x)	Quantity/25 ml
NaCl (S5886)	1077.0 mM	1.5735 g
KCl (P5405)	71.6 mM	0.1334 g
KH_2PO_4 (P5655)	11.9 mM	0.0405 g
Penicillin (sodium salt) (P7794)	600 µg/ml	0.0150 g
Streptomycin (S9137)	500 µg/ml	0.0125 g
Glucose (G7520)	15.0 mM	0.0676 g
Na L-lactate (60% syrup) (L4263) (w/w)	33.0 mM	13.7 µl
$MgCl_2.6H_2O$ (M2393)	4.9 mM	0.0249 g

SOF-Stock-B solution (10 ml) was prepared by weighing $NaHCO_3$ (0.2106 g) with a digital balance and dissolving in Milli-Q water (approximately 7 ml) in a sterile conical tube (15 ml). Then the volume of solution was topped up to 10 ml (Table 3.9). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile conical tube (15 ml) with a shelf life of 1 week.

Table 3.9: Composition of Stock-B for SOF medium (10x) with a shelf life of 1 week (stored at 4ºC)

Chemical (catalogue no.)	Concentration (10x)	Quantity/10 ml
$NaHCO_3$ (S5761)	250.7 mM	0.2106 g

SOF-Stock-C solution (5 ml) was prepared by weighing Na pyruvate (0.0182 g) with a digital balance and dissolving in Milli-Q water (approximately 4 ml) in a sterile conical tube (15 ml). Then the volume of solution was topped up to 5 ml (Table 3.10). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size), aliquot to microcentrifuge tubes (1.5 ml), wrapped with aluminium foil and stored (-20ºC, sealed) with a shelf life of 1 week.

Table 3.10: Composition of Stock-C for SOF medium (100x) with a shelf life of 1 week (stored at -20ºC)

Chemical (catalogue no.)	Concentration (100x)	Quantity/5 ml
Na pyruvate (P3662)	3.3 mM	0.0182 g

SOF-Stock-D solution (5 ml) was prepared by weighing $CaCl_2.2H_2O$ (0.1257 g) with a digital balance and dissolving in Milli-Q water (approximately 4 ml) in a sterile conical tube (15 ml). Then the volume of solution was topped up to 5 ml (Table 3.11). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size), aliquot to microcentrifuge tubes (1.5 ml) and stored (4ºC, sealed) with a shelf life of 3 months.

Table 3.11: Composition of Stock-D for SOF medium (100x) with a shelf life of 3 months (stored at 4ºC)

Chemical (catalogue no.)	Concentration (100x)	Quantity/5 ml
$CaCl_2.2H_2O$ (C7902)	17.1 mM	0.1257 g

3.5.2.5 (b) Medium for sperm preparation (sperm-SOF medium)

Sperm-SOF medium used for frozen-thawed goat semen preparation (sperm swim-up and sperm washing) was SOF medium supplemented with heat-inactivated OGS (20%) as shown in Table 3.12. The medium was prepared a day before the *in vitro* fertilisation experiment, filter-sterilised using syringe filter (0.22 µm pore size) and equilibrated overnight in CO_2 incubator (5%) prior to use.

Table 3.12: Composition of sperm-SOF medium (10 ml)

Component	Concentration (%)	Quantity/10 ml
SOF-Stock-A	10%	1000 µl
SOF-Stock-B	10%	1000 µl
SOF-Stock-C	1%	100 µl
SOF-Stock-D	1%	100 µl
OGS	20%	2000 µl
Milli-Q water	58%	5800 µl

3.5.2.5 (c) Medium for *in vitro* fertilisation (IVF-SOF medium)

IVF-SOF medium used for oocyte washing, sperm capacitation and insemination was SOF medium supplemented with heparin and heat-inactivated OGS (10%) as shown in Table 3.13. In the present study, heparin stock solution (2 mg/ml) was prepared by dissolving heparin (H3393; 12 mg) in a sterile saline (6 ml), sterilised by filtration using

a disposable filter (0.22 µm pore size), aliquot to microcentrifuge tubes (1.5 ml) and stored (-20ºC, sealed) until use. The IVF-SOF medium was prepared a day before the *in vitro* fertilisation experiment and filter-sterilised using syringe filter (0.22 µm pore size) and equilibrated overnight in CO_2 incubator (5%) prior to use.

Table 3.13: Composition of IVF-SOF medium (10 ml)

Component	Concentration (%)	Quantity/10 ml
SOF-Stock-A	10%	1000 µl
SOF-Stock-B	10%	1000 µl
SOF-Stock-C	1%	100 µl
SOF-Stock-D	1%	100 µl
OGS	10%	1000 µl
Heparin	2.5%	250 µl
Milli-Q water	65.5%	6550 µl

3.5.2.6 Preparation of *in vitro* fertilisation medium for bovine

In the present study, Brackett-Oliphant (BO) medium was used for bovine *in vitro* fertilisation.

3.5.2.6 (a) Preparation of Brackett-Oliphant (BO) medium

To prepare Brackett-Oliphant (BO) stock solution (100 ml), a sterile conical tube (100 ml) was filled with Milli-Q water (approximately 75 ml). All the chemicals were weighed using a digital balance. NaCl (6.5453 g), KCl (0.2997 g), $CaCl_2.2H_2O$ (0.3307 g), $NaH_2PO_4.2H_2O$ (0.1295 g), $MgCl_2.6H_2O$ (0.1057 g) and Phenol red (100 µl) were added stepwise and dissolved in Milli-Q water. The volume of solution was topped up to 100 ml (Table 3.14). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size and stored (4ºC, sealed) in a sterile glass bottle with a shelf life of 3 months.

Table 3.14: Composition of BO stock solution with a shelf life of 3 months (stored at 4ºC)

Chemical (catalogue no.)	Concentration	Quantity/100 ml
NaCl (S5886)	112.00 mM	6.5453 g
KCl (P5405)	4.02 mM	0.2997 g
$CaCl_2.2H_2O$ (C3881)	2.25 mM	0.3307 g
$NaH_2PO_4.2H_2O$ (04269)	0.83 mM	0.1295 g
$MgCl_2.6H_2O$ (M2393)	0.52 mM	0.1057 g
Phenol red (15100-043)	2 µg/ml	100 µl

To prepare Brackett-Oliphant (BO) working solution (100 ml), BO stock solution (10 ml), $NaCO_3$ (0.3108 g), glucose (0.25 g), Na pyruvate (0.0137 g) and gentamycin stock (100 µl) were added stepwise and dissolved in Milli-Q water (approximately 80 ml). The volume of solution was topped up to 100 ml (Table 3.15). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile glass bottle with a shelf life of 2 weeks.

Table 3.15: Composition of BO working solution with a shelf life of 2 weeks (stored at 4ºC)

Chemical (catalogue no.)	Concentration	Quantity/100 ml
BO stock solution	10%	10 ml
$NaHCO_3$ (S5761)	3.70 mM	0.3108 g
Glucose (G6152)	1.39 mM	0.2500 g
Na pyruvate (P3662)	0.12 mM	0.0137 g
Gentamycin stock (G3632)	1%	100 µl

3.5.2.6 (b) Medium for sperm preparation (BO-sperm preparation medium)

To prepare BO-sperm preparation medium which was used for sperm washing, BO working solution (4875 µl) was added with heparin stock (125 µl) (Table 3.16). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile conical tube (15 ml) with a shelf life of 2 weeks. The medium was equilibrated overnight in CO_2 incubator (5%) prior to use.

Table 3.16: Composition of BO-sperm preparation medium with a shelf life of 2 weeks (stored at 4ºC)

Chemical (catalogue no.)	Concentration	Quantity/5 ml
BO working solution	97.50%	4875 µl
Heparin (H3393)	2 mg/ml	125 µl

3.5.2.6 (c) Medium for *in vitro* fertilisation (BO-IVF medium)

To prepare BO-IVF medium which was used for oocytes washing, sperm capacitation and insemination in bovine, BO working solution (9750 µl) was supplemented with theophylline (0.009 g), heparin stock (250 µl) and BSA (0.060 g) (Table 3.17). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile conical tube (15 ml) with a shelf life of 1 week. The medium was equilibrated overnight in CO_2 incubator (5%) prior to use.

Table 3.17: Composition of BO-IVF medium with a shelf life of 1 week (stored at 4ºC)

Chemical (catalogue no.)	Concentration	Quantity/10 ml
BO working solution	97.50%	9750 µl
Theophylline (T1633)	5 mM	0.009 g
Heparin (H3393)	2 mg/ml	250 µl
BSA (A6003)	6 mg/ml	0.060 g

3.5.2.7 Preparation of medium for parthenogenetic activation

In parthenogenesis, calcium ionophore (0.0005 M) and 6-dimethylaminopyridine (0.2000 M) were used in the experiment.

3.5.2.7 (a) Preparation of calcium ionophore (CI)

To prepare the stock of calcium ionophore with 500 µM concentration, calcium ionophore (10 mg) was diluted with dimethyl sulfoxide (DMSO, D2650; 38.2 ml) (Table 3.18). The prepared calcium ionophore stock was then aliquot (10 µl) in microcentrifuge tube (100 µl) and stored in freezer (-20ºC). Working solution (100 µl)

was prepared by adding KSOM working solution (as described in Section 3.5.2.8 (b); 990 µl) to the calcium ionophore stock solution (10 µl) (Table 3.19).

Table 3.18: Composition of calcium ionophore stock solution

Chemical (catalogue no.)	Quantity
CI (C7522)	10 mg
DMSO (D2650)	38.2 ml

Table 3.19: Composition of calcium ionophore working solution

Chemical	Quantity (100 µl)
CI stock solution	10 µl
KSOM working solution	990 µl

3.5.2.7 (b) Preparation of 6-dimethylaminopyridine (6-DMAP)

To prepare the stock of 6-dimethylaminopyridine with 0.2 M concentration, 6-dimethylaminopyridine (250 mg) was diluted with Milli-Q water (7.7 ml) (Table 3.20). The prepared 6-dimethylaminopyridine stock was then aliquot (10 µl) in microcentrifuge tube (100 µl) and stored in freezer (-20ºC). Working solution (100 µl) was prepared by adding KSOM working solution (as described in Section 3.5.2.7; 990 µl) to the 6-dimethylaminopyridine stock solution (10 µl) (Table 3.21).

Table 3.20: Composition of 6-dimethylaminopyridine stock solution

Chemical (catalogue no.)	Quantity
6-DMAP (D2629)	250 mg
Milli-Q water	7.7 ml

Table 3.21: Composition of 6-dimethylaminopyridine working solution

Chemical	Quantity (1000 µl)
6-DMAP stock solution	10 µl
KSOM working solution	990 µl

3.5.2.8 Preparation of *in vitro* culture (IVC) medium

3.5.2.8 (a) IVC-SOF medium

The medium used for *in vitro* culture of caprine *in vitro* fertilised-embryo was IVC-SOF (Table 3.22). The medium was prepared a day before *in vitro* culture and filter-sterilised using syringe filter (0.22 µm pore size) and equilibrated overnight in CO_2 incubator (5%) prior to use.

Table 3.22: Composition of IVC-SOF medium (10 ml)

Component	Concentration (%)	Quantity/10 ml
SOF-Stock-A	10%	1000 µl
SOF-Stock-B	10%	1000 µl
SOF-Stock-C	1%	100 µl
SOF-Stock-D	1%	100 µl
OGS	10%	1000 µl
Milli-Q water	68%	6800 µl

3.5.2.8 (b) Potassium Simplex Optimisation (KSOM) medium

The medium used for *in vitro* culture of embryo for bovine *in vitro* fertilisation and parthenogenesis as well as vitrification of embryos was KSOM medium. KSOM stock solution was prepared by adding NaCl (0.5553 g), KCl (0.0186 g), KH_2PO_4 (0.0048 g), $MgSO_4$ (0.0024 g), Na L-lactate (60% syrup) (186 µl), Na pyruvate (0.0022 g), D-glucose (0.0036 g), $NaHCO_3$ (0.2101 g), $CaCl_2$ (0.0190 g), L-glutamine (0.0146 g) and EDTA (0.0004 g) to dissolve in Milli-Q water (approximately 80 ml). The volume of solution was topped up to 100 ml (Table 3.23). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile glass bottle (100 ml) with a shelf life of 1 month.

Table 3.23: Composition of KSOM stock solution with a shelf life of 1 month (stored at 4ºC)

Chemical (catalogue no.)	Concentration	Quantity/100 ml
NaCl (S5886)	95 mmol/litre	0.5553 g
KCl (P5405)	2.5 mmol/litre	0.0186 g
KH_2PO_4 (P5655)	0.35 mmol/litre	0.0048 g
$MgSO_4$ (M7506)	0.2 mmol/litre	0.0024 g
Na L-lactate (60% syrup) (L-7900)	10 mmol/litre	186 µl
Na pyruvate (P4562)	0.2 mmol/litre	0.0022 g
D-glucose (G6152)	0.2 mmol/litre	0.0036 g
$NaHCO_3$ (S5761)	25 mmol/litre	0.2101 g
$CaCl_2$ (C5670)	1.71 mmol/litre	0.0190 g
L-glutamine (G3126)	1 mmol/litre	0.0146 g
EDTA (E9884)	0.01 mmol/litre	0.0004 g

To prepare KSOM working solution, KSOM stock solution (9.85 ml) was added with BSA (0.04 g), MEM (50 µl) and BME (100 µl) (Table 3.24). Finally, the solution was filter-sterilised using syringe filter (0.22 µm pore size) and stored (4ºC, sealed) in a sterile conical tube (15 ml) with a shelf life of 1 week. The medium was equilibrated overnight in CO_2 incubator (5%) prior to use. Additional D-glucose (0.004 g) was added to the KSOM working solution for late stage embryo development (P.J. Kwong, research postgraduate scientist of ABEL laboratory, personal communication, 2011).

Table 3.24: Composition of KSOM working solution with a shelf life of 1 week (stored at 4ºC)

Chemical (catalogue no.)	Quantity/10 ml
KSOM stock solution	9.85 ml
BSA (A6003)	0.04 g
MEM (M7145)	50 µl
BME (B6766)	100 µl

3.5.3 Preparation of Hand-controlled Pipette and Mouthpiece

A hand-controlled pipette was used for handling the oocytes and embryos. It was constructed as shown in Figure 3.2, consisting of a pulled-glass Pasteur pipette attached to a mouthpiece.

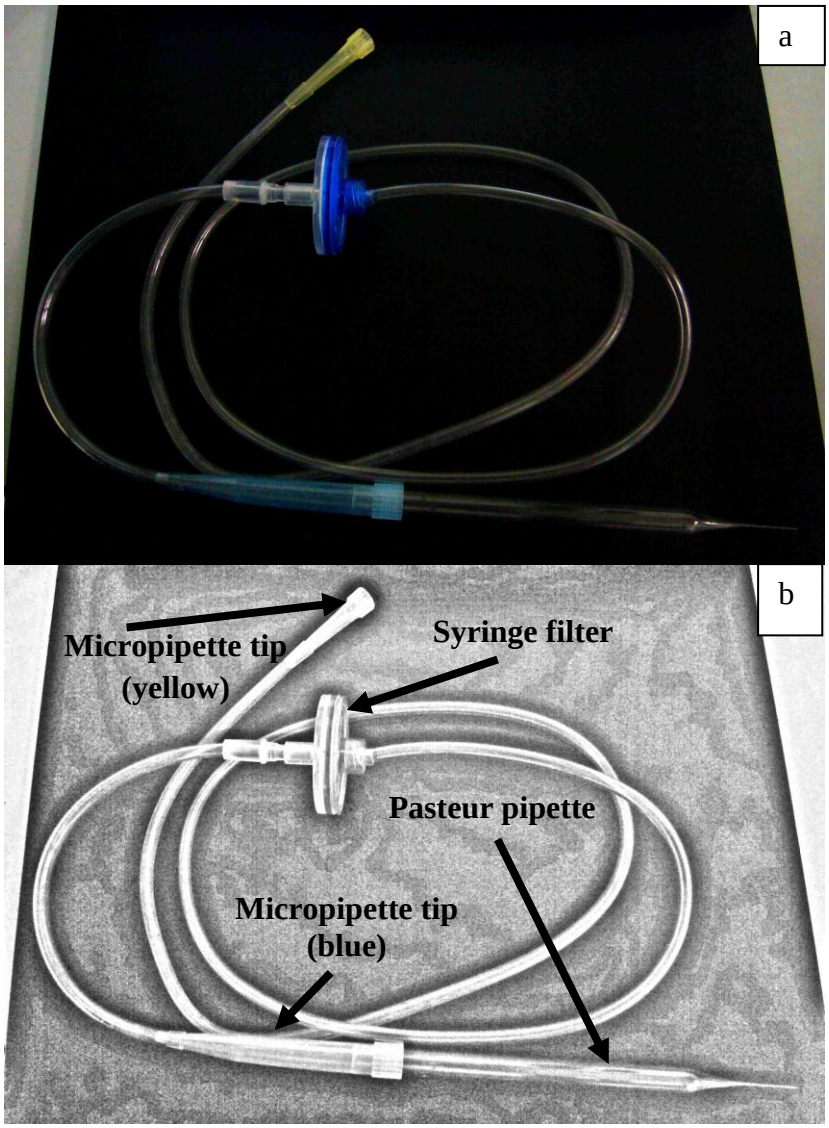

Figure 3.2: Hand-controlled pipette with mouthpiece. (a) Original photograph; (b) Labelled photograph.

3.5.3.1 Cleaning and sterilisation of pipette

The glass Pasteur pipettes were cleaned and sterilised by autoclaving and thereafter, dried thoroughly in the oven (56ºC) overnight.

3.5.3.2 Preparation of hand-controlled pipette

The glass capillary was softened by rotating it in a fine flame until the glass became soft. The glass was then immediately withdrawn from the heat and both ends were quickly pulled smoothly to produce a tube with an internal diameter of approximately 200 µm and 400 µm for embryo and cumulus oocyte complex, respectively. For a neat break,

the pulled portion of the capillary was scribed with a diamond stone and snapped at the scribed portion. It was important to achieve an evenly snapped straight tip as a jagged capillary end may potentially cause damage to the zona pellucida during manipulation of the oocyte/embryo. Moreover, sharp edges of the pipette were easily caught by the plastic surface of the culture dish, causing the pipette to break and tend to collect more debris or lose oocytes/embryos during manipulation. The tip of the pipette was fire polished by touching the flame quickly to achieve a smooth edge of the pipette tip.

3.5.4 Preparation of Silicone Oil

Silicone oil was used throughout the *in vitro* production experiments to layer droplets of culture medium containing oocytes/embryos. It was necessary to overlay the droplets of culture medium with equilibrated oil as the oil serves as a physical barrier that separates droplets from the atmosphere and airborne particles or pathogens. It was also preventing evaporation and delaying gas diffusion, thus stabilising the pH, temperature and osmolarity of the microenvironment surrounding the oocyte/embryo during handling outside the CO_2 incubator (5%).

It was crucial to wash silicone oil (1x) prior use for *in vitro* production to remove possible water-soluble contaminants. This was done by gently shaking the oil with ultrapure water (Milli-Q) in a sterile glass bottle or disposable tissue culture flask in the ratio of 3:2 (oil to aqueous solution). The mixture in the bottle was then left to be settled at room temperature shielded from direct sunlight to avoid possible embryonic toxic overlay. After 3 days, the upper layer of oil was aspirated slowly and stored in a sterile glass bottle.

3.5.5 Oocyte Retrieval and Embryo Flushing

In the present study, there were different sources of oocytes (laparoscopic oocyte pick-up, ovariectomy and abattoir ovary) for *in vitro* produced embryos. To obtain *in vivo* produced embryo, embryo flushing was carried out in our laboratory.

3.5.5.1 Preparation of donor goat for laparoscopic oocyte pick-up

The goat oocyte samples obtained from laparoscopic oocyte pick-up were selected to undergo oestrus synchronisation and hyperstimulation prior surgery. A schematic representation of the processes involved in donor goat is shown in Figure 3.3.

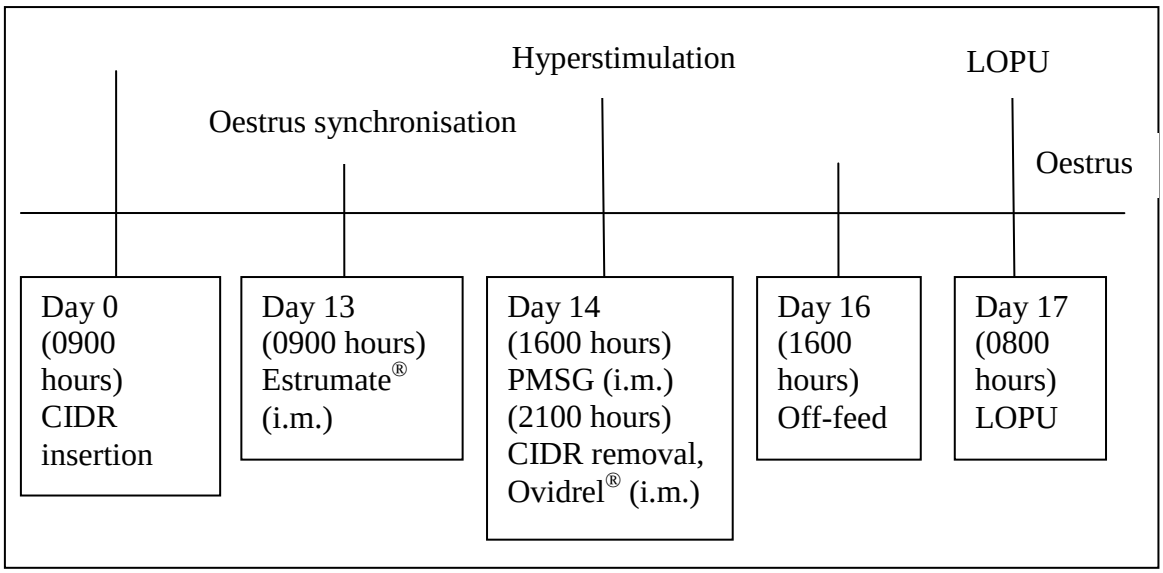

Figure 3.3: A schematic representation of the treatments performed on a donor goat for laparoscopic oocyte pick-up. Dosage of hormone treatments was described in the text.

3.5.5.1 (a) Oestrus synchronisation of donor goat for laparoscopic oocyte pick-up

Oestrus synchronisation is a standard procedure for manipulating the oestrous cycle of donor goats such that it is possible to carry out the oocyte retrieval during laparoscopic oocyte pick-up on a selected date and time (Figure 3.3). In the present study, oestrous cycle of a donor goat was synchronised using insertion of a Controlled Intravaginal

Drug Release device (CIDR, 0.3 g progesterone) into the vagina for 14 days (at 0900 hours on day 0) using a sterile controlled intravaginal drug release applicator with water-based lubricant (K-Y Jelly) (Figure 3.4). The controlled intravaginal drug release is made of an inert silicone elastomer that is non-porous and does not readily absorb bodily fluids; once properly inserted deep into the donor's vagina, the controlled intravaginal drug release unfolds into a 'T' like formation that aids in retention. Daily monitoring of the device was performed to confirm that it had not been inadvertently removed. At 36 hours prior to controlled intravaginal drug release removal, a luteolytic treatment of Estrumate® (125 µg cloprostenol) was administered intramuscularly (i.m.; at 0900 hours on day 13) to regress corpus luteum that facilitated initiation of pro-oestrus and eventually resulted in oestrogen surge for the onset of heat (oestrus).

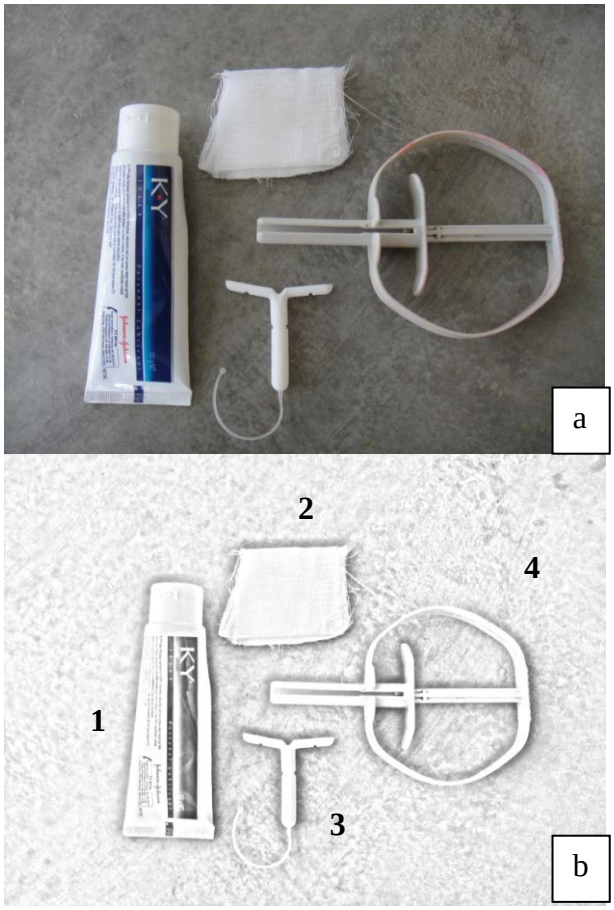

Figure 3.4: Devices used during CIDR insertion. (a) Original photograph; (b) Labelled photograph. The items are (1) K-Y Jelly; (2) gauze; (3) CIDR and (4) CIDR applicator.

3.5.5.1 (b) Hyperstimulation of donor goat for laparoscopic oocyte pick-up

Hyperstimulation is a procedure conducted following oestrus synchronisation to recruit multiple growths of follicles in the ovary. Pregnant mare's serum gonadotrophin (1500 IU) was administered (i.m.; at 1600 hours on day 14). Upon the removal of controlled intravaginal drug release (at 2100 hours on day 14), Ovidrel® (250 IU chorionic gonadotrophin) was administered (i.m.; at 2100 hours on day 14) to stimulate multiple follicular development prior to oocyte-retrieval surgery (at 0800 hours on day 17).

3.5.5.2 Preparation of donor goat for embryo flushing

The goat embryo samples obtained from embryo flushing were selected to undergo oestrus synchronisation, hyperstimulation and mating prior surgery. A schematic representation of the processes involved in donor goat is shown in Figure 3.5.

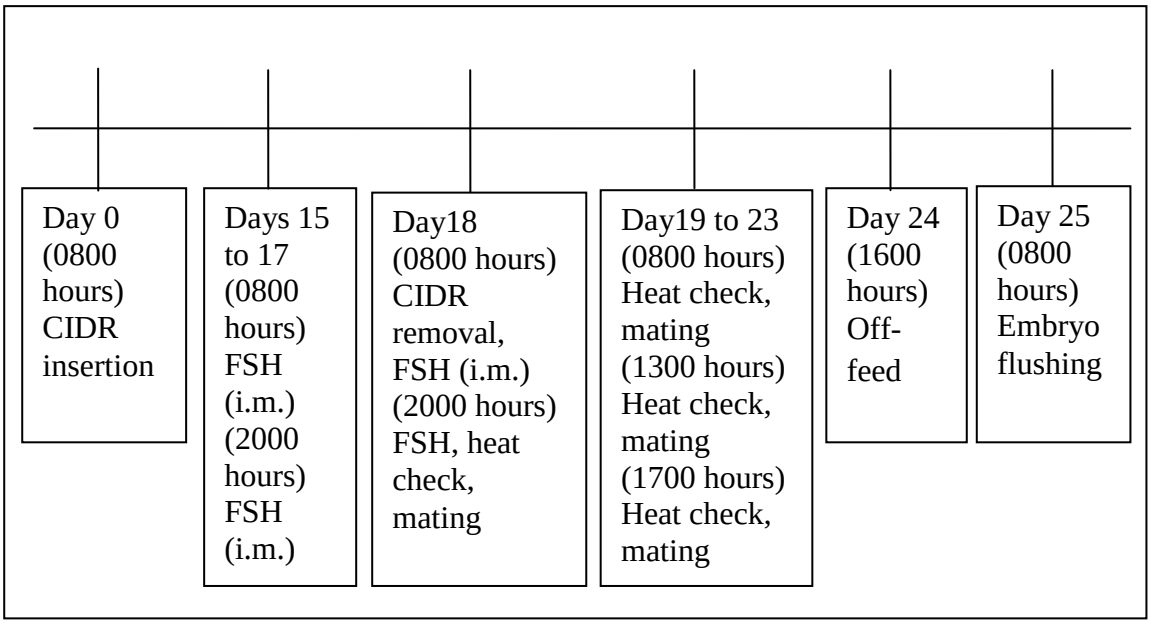

Figure 3.5: A schematic representation of the treatments performed on a donor goat for embryo flushing. Dosage of hormone treatments was described in the text.

3.5.5.2 (a) Oestrus synchronisation and hyperstimulation of donor goat for embryo flushing

Oestrous cycle of a donor goat was synchronised using insertion of a Controlled Intravaginal Drug Release device (0.3 g progesterone) into the vagina for 17 days (at 0800 hours on day 0) using a sterile controlled intravaginal drug release applicator with water-based lubricant (K-Y Jelly). Daily monitoring of the device was performed to confirm that it had not been inadvertently removed. Follicle stimulating hormone (20 mg) was administered intramuscularly (i.m.; at 0800 hours and 2000 hours from day 15 to 18). Controlled intravaginal drug release was removed on day 18 at 0800 hours.

3.5.5.2 (b) Detection of oestrus and mating

The does were visually observed and also by the use of a fertile buck for oestrus signs (started at 2000 hours on day 18). The does were recorded as being in oestrus if they flag their tails and stood to be mounted. All does in oestrus were allowed to be served by the buck and the first day of service was considered as day 0 of the oestrous cycle. The donors were then allowed to be with the buck as a pair in the same pen during the night and an additional mating was allowed at every 4 to 5 hours with different buck during the day until the doe no longer in oestrus and refused to be mated.

3.5.5.3 Setting up of surgical instruments, CO_2 insufflator system, light system, imaging device and aspiration-flushing system for laparoscopic oocyte pick-up

On the day prior to surgery, non-autoclavable surgical instruments (atraumatic grasper, trocar and cannula, fibre optic cable, light probe for endoscope and flushing system tubing) were disinfected by immersing completely in Gigasept® solution (10%) for 10 minutes followed by rinsing twice in sterile autoclaved distilled water and subsequently

drained dry before placing on a sterile surgical table-cum-trolley. After being autoclaved and dried completely in the oven (56ºC), autoclavable surgical instruments were UV sterilised overnight prior the day of surgery. The outer wrapping of the sterilised surgical pack (containing surgical sets, scalpel blade, catgut suture, sterile gauze, sterile towels, sterile gloves, sterile drapes, round bottom tubes and aspiration needle) was opened and carefully unfolded without touching the sterilised instruments inside. All the surgical instruments were assembled and arranged in a consistent order on a sterile surgical table-cum-trolley which was covered with a sterile table cloth.

The laparoscopy equipment used consisted of a laparoscope, light cable, light source, trocar (7 mm) for the laparoscope, atraumatic grasping forceps and smaller trocar (5 mm). The light probe was connected to a light system via fibre optic cable. The light system, in turn, was connected to a charge coupled devices (CCD) camera and computer monitor. The lens of the light probe and the entire length of the fibre optic cable were disinfected with ethanol (70%) and white balance was performed using a sterile gauze. The follicle aspiration set consisted of a puncture pipette, tubing, pre-warmed collection tubes (38.5ºC) and an electronic vacuum pump. The collection medium for oocyte retrieval (flushing medium) was pre-warmed (38.5ºC) and filled in a sterile luer slip syringe (50 ml) and placed horizontally in the electronic vacuum pump (aspiration system). The other sterile luer slip syringes filled with flushing medium were kept in a polystyrene box with a glass bottle with luke-warm water in it to keep the temperature of the flushing medium during laparoscopic oocyte pick-up. The collection tubes (disposable round-bottom test tubes, 14 ml) with inlet and outlet ports in the stopper were placed in a test tube heating system to maintain constant temperature (38.5ºC) during laparoscopic oocyte pick-up. The inlet and outlet ports were connected to the aspiration pipette and to a vacuum pump, respectively, with plastic tubing. The vacuum pressure was regulated with a flow valve (-100 mm Hg) and measured as drops

of collection medium per minute entering the collection tube, routinely it was adjusted to 50 to 70 drops per minute. The complete aspiration set was gas sterilised and rinsed with collection medium prior to use (Figure 3.6).

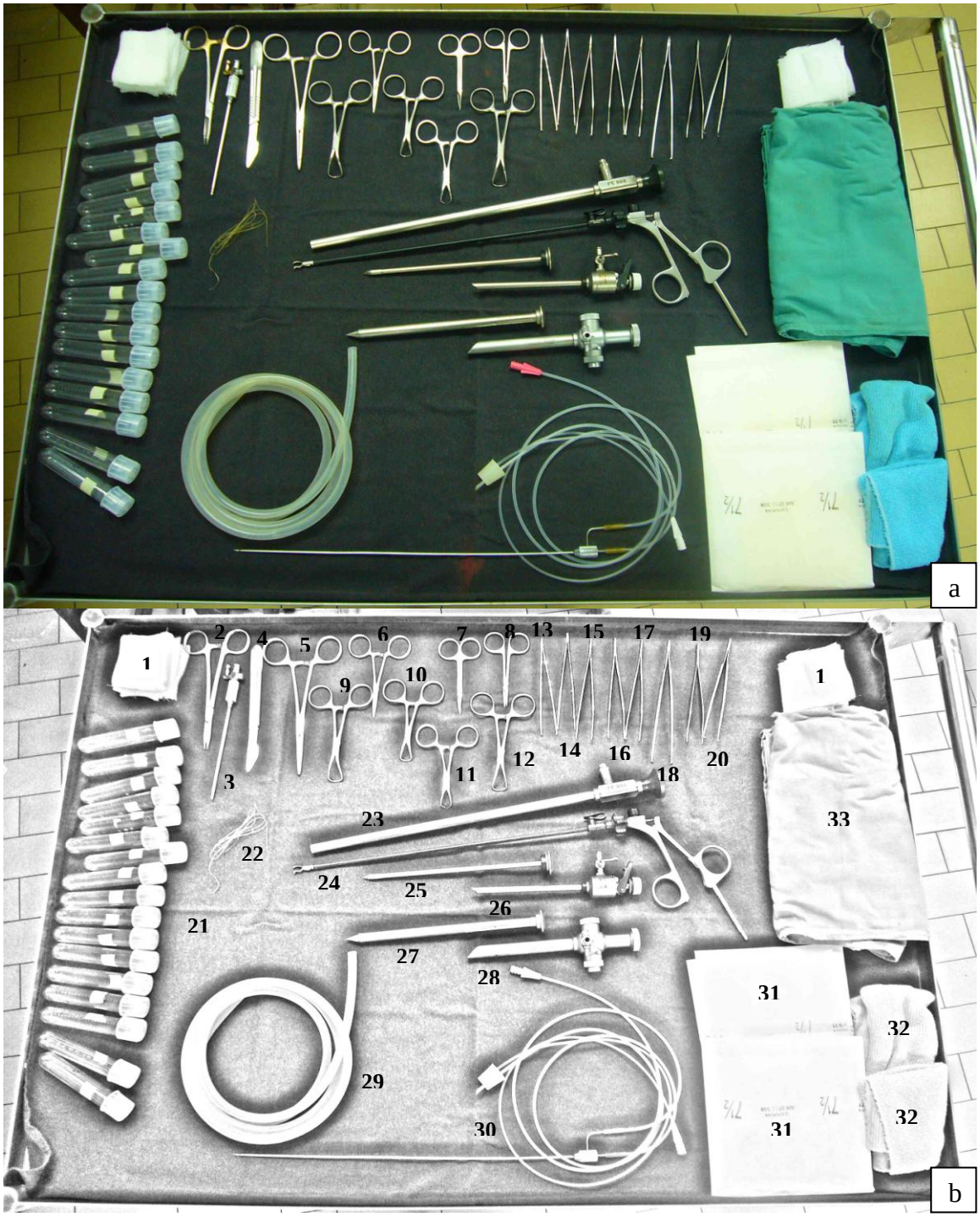

Figure 3.6: Setting up of surgical instruments on surgical trolley for laparoscopic oocyte pick-up. (a) Original photograph; (b) Labelled photograph. The items included (1) sterile gauze, (2, 7, 8) different types of surgical scissors, (3) Veress needle, (4) scalpel with surgical blade, (5, 6, 9-12) different types of haemostatic forceps, (13-20) different types of forceps, (21) collecting tubes, (22) suture, (23) light probe for endoscope, (24) atraumatic grasper, (25, 27) trocar, (26, 28) cannula, (29) silicone tubing for flushing system, (30) aspiration needle, (31) sterile gloves, (32) sterile hand towel and (33) drape for animal.

3.5.5.4 Setting up of surgical instruments, CO_2 insufflator system, light system and imaging device for embryo flushing

The setting was generally similar as described in Section 3.5.5.3, excluding the follicle aspiration set which consisted of a puncture pipette, tubing, pre-warmed collection tubes (38.5ºC) and an electronic vacuum pump (Figure 3.7).

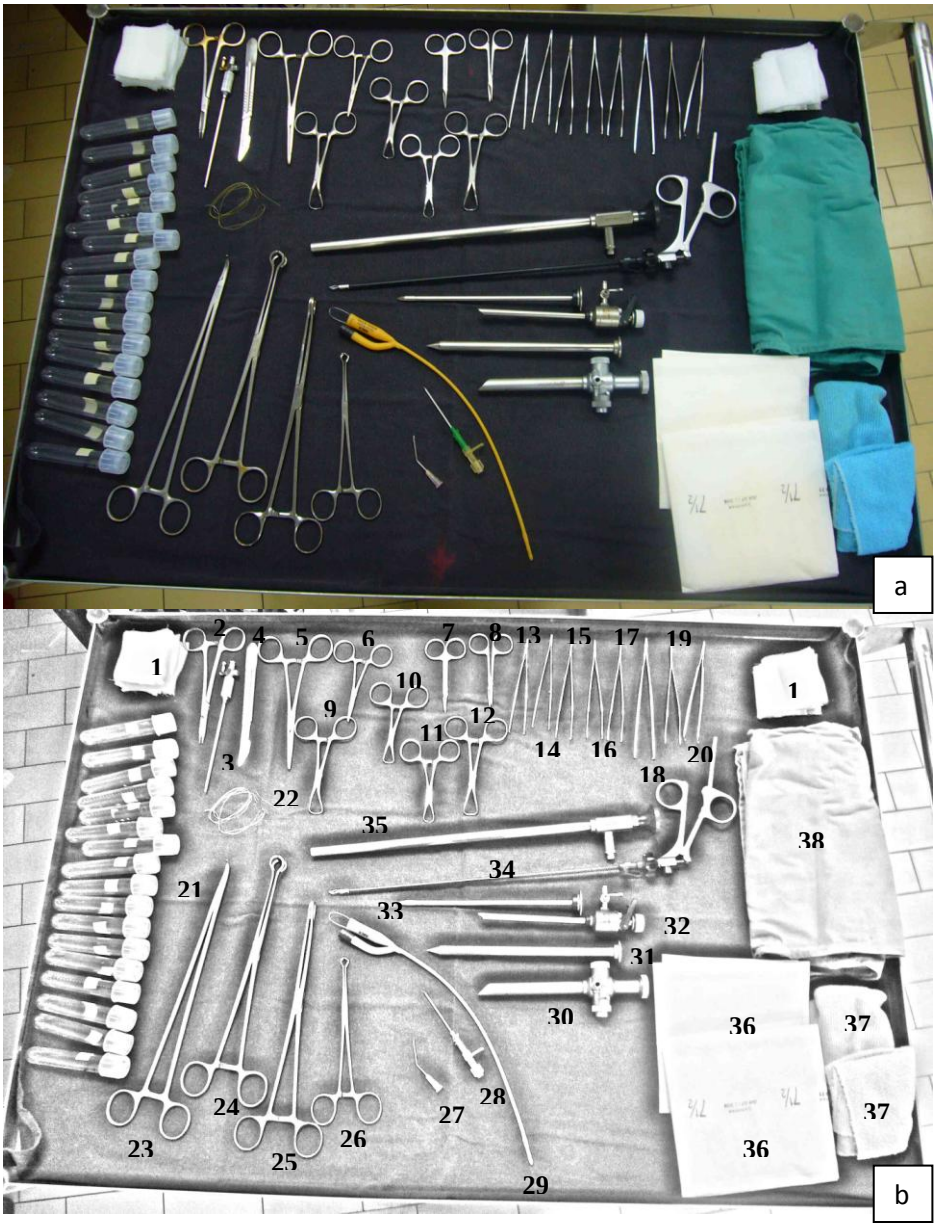

Figure 3.7: Setting up of surgical instruments on surgical trolley for embryo flushing. (a) Original photograph; (b) Labeled photograph. The items included (1) sterile gauze, (2, 7, 8) different types of surgical scissors, (3) Veress needle, (4) scalpel with surgical blade, (5, 6, 9-12, 23-26) different types of haemostatic forceps, (13-20) different types of forceps, (21) collecting tubes, (22) suture, (27) needle, (28) Teflon I.V. Catheter, (29) Foley catheter, (30, 32) cannula, (31, 33) trocar, (34) atraumatic grasper, (35) light probe for endoscope, (36) sterile gloves, (37) sterile hand towel and (38) drape for animal.

3.5.5.5 Sedation and anaesthetisation of donor goat

Identification tag of the goat was determined before the sedation and anaesthetisation. Preparation for surgery was initially facilitated by administration of anaesthetic to a donor goat using a mixture of xylazil and ketamil (1.1 ml xylazil mixed with 50 ml ketamil; 1 ml) via intramuscular (i.m.) injection (11 mg/kg body weight). The anaesthetised donor goat was placed on a clean restraining cradle. The restraining cradle was set at a 45º angle with the head of the animal lowered to facilitate the laparoscopy procedure. Using clean gauzes, the abdominal area of the donor goat was disinfected with diluted Hibiscrub (10%) and the hair was shaved. After shaving, the bare skin was disinfected again with absolute Hibiscrub (100%) and subsequently with iodine solution (10%). The donor goat was then covered with a sterile drape with an opening that revealed the disinfected bare skin and was ready for oocyte-retrieval surgery. The reason of using drape was to isolate the surgical site and minimise wound contamination. The drape was positioned without the fabric dragging across a non-sterile surface and secured in place with towel clamps at the 4 corners of the surgical site. The donor goat was maintained under anaesthesia with administration (intramuscularly) of the mixture of xylazil and ketamil (0.1 mg/kg body weight) at regular intervals as required while laparoscopy surgery was in progress.

3.5.5.6 Laparoscopic oocyte pick-up (LOPU)

Laparoscopic oocyte pick-up is a microsurgical procedure to retrieve oocytes from matured follicles seen as ‘pimple-like’ protrusions on the surface of the ovary via 4 incisions on the abdomen of the donor goat. Oocytes were retrieved by aspiration of follicular contents under laparoscopic observation. Before the actual oocyte retrieval process began, the donor goat was sedated and anaesthetised, surgical instruments and accessories were disinfected. During oocyte retrieval, test tubes (each containing 3 ml of

follicular fluid) were passed to embryologists for oocytes searching under stereomicroscopes. After oocyte retrieval, the donor goat underwent post-surgical treatment.

While anaesthetised goat was restrained in a cradle in the standard position for laparoscopic surgery, a small incision was made on the disinfected abdominal area and a trocar connected to a CO_2 tank via the CO_2 insufflator was inserted into the incision to insufflate the abdominal cavity with CO_2. The peritoneum cavity was filled with filtered CO_2 in order to facilitate visualisation of the reproductive tract.

Three small incisions (3 to 5 mm) were made once the peritoneum cavity was expanded. One of the incisions was made near the umbilicus to facilitate insertion of trocar for passing the light probe, one on the right side of lower-ventral abdomen to insert the trocar for passing the grasping forceps and one on the left side of lower-ventral abdomen for passing oocyte retrieval needle.

Using the grasper, the uterine horns were gently manipulated to allow visualisation of each stimulated ovary. Once an ovary was identified, the ovarian ligament was grasped, without damaging the oviduct, to effectively stabilise the ovary for follicular puncture. The ovarian characteristics (size of ovary, stimulation response and estimated number of follicles) were recorded for each experimental donor animal prior to the follicle puncture procedure. During the surgery, the ovaries were exposed by pulling the fimbria in different directions using the grasping forceps and the follicular contents were aspirated from all follicles (±3 mm in diameter) visible on the surface of the ovaries by puncturing follicles with the aspiration needle (Figure 3.8).

The collection tube containing aspirated fluid (3 to 5 ml) was dispensed into a sterile Petri dish (90 mm) for oocyte searching under a stereomicroscope (magnification 20x to 40x). Oocytes were then washed in 3 droplets of flushing medium followed by 3

droplets of IVM medium and subsequently cultured according to oocyte grading for subsequent experiment.

After aspiration, the ovary was rinsed repeatedly with warm heparinised (38.5ºC) physiological saline using a sterile syringe (50 ml) filled with heparinised saline introduced through one of the trocars to aid in reducing adhesions following oocyte aspiration. The ovary was then released and the contralateral ovary was similarly aspirated. The incisions were sutured and the incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The donor goat was administered with oxytetracycline (20 mg/kg body weight) via intramuscular injection once in 4 days within the duration of 2 weeks to prevent possible post-surgical infection.

Figure 3.8: Laparoscopic oocyte pick-up was being carried out.

3.5.5.7 Ovariectomy

In cases where the donor goat failed to be superovulated due to repeated oocyte retrieval surgeries or when the ovaries were physically abnormal such as having adhesion, these ovaries were surgically removed (ovariectomised) from the donor goat.

At the abdominal part of the donor goat, a midline skin incision (vertical abdominal incision) was made (4 to 6 cm). Using blunt dissection to tunnel subcutaneously, lateral to the skin incision, the muscles of the posterior abdominal wall

were separated in order to enter the abdominal cavity. The ovaries were located in a fat pad just beneath the muscles. Using forceps, the periovarian fat was gently grasped to lift and exteriorise the ovary. Mosquito forceps (a small and curved haemostatic forceps) were used to crush the fallopian tube and cranial-most part of the uterine horn distal to the ovary, being careful not to crush or contact the ovary. The ovary was removed by cutting above the clamped area. The uterine horn was placed back carefully into the abdomen. The incisions were sutured and the incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The donor goat was administered with oxytetracycline (20 mg/kg body weight) via intramuscular injection once in 4 days within the duration of 2 weeks to prevent possible post-surgical infection.

A Petri dish (35 mm) containing warm (38.5ºC) flushing medium was prepared to collect the ovaries that were surgically removed from the anaesthetised-ovariectomised goat. The ovaries were then recorded as left or right ovary. Under the laminar flow, each ovary was freed from the surrounding tissues and overlying bursa using surgical scissors and forceps. The ovary was placed in a Petri dish (90 mm) containing TL-Hepes working solution (5 ml) on a heating stage (38.5ºC). The ovary was held with the help of forceps and each ovary was sliced individually (Figure 3.9). Checker-board incisions were made along the whole ovarian surface using a quarter sections of a stainless steel razor blade held with a sterile haemostat. While retrieving the oocytes by slicing method, the ovary was kept completely dipped in the TL-Hepes working solution. The Petri dish was then examined under a stereomicroscope (magnification 20x to 40x). Oocytes were then washed with TL-Hepes working solution followed by *in vitro* maturation medium and subsequently cultured according to oocyte grading for subsequent experiment.

3.5.5.8 Abattoir

For bovine samples and some of the caprine samples, ovaries were obtained from the Department of Veterinary Services and Abattoir Complex, Shah Alam (Selangor) and Senawang (Negeri Sembilan). Two thermoses (one for rinsing the ovaries and another one for collecting the ovaries) were filled with warmed (38.5ºC) saline supplemented with streptomycin and penicillin G. A pair of surgical scissors was used to cut the ovaries from the donor animals. The excess tissues of the ovaries were trimmed and rinsed with the warmed saline before transferred to the thermos.

After reaching laboratory, the ovaries were poured into a beaker with warmed saline. Under the laminar flow, the ovary was placed in a Petri dish (90 mm) containing TL-Hepes working solution (5 ml) on a heating stage (38.5ºC). The ovary was held with the help of forceps and each ovary was sliced individually (Figure 3.9). Checker-board incisions were made along the whole ovarian surface using a quarter sections of a stainless steel razor blade held with a sterile haemostat. While retrieving the oocytes by slicing method, the ovary was kept completely dipped in the TL-Hepes working solution. After slicing, the ovaries were rinsed with TL-Hepes working solution in a small beaker to ensure that all oocytes were retrieved. The Petri dish was then examined under a stereomicroscope (magnification 20x to 40x). TL-Hepes working solution in the small beaker was poured into the Petri dish for searching as well. Oocytes were then washed with TL-Hepes working solution followed by *in vitro* maturation medium prior cultured according to oocyte grading (if sufficient number of oocytes available) for subsequent experiment.

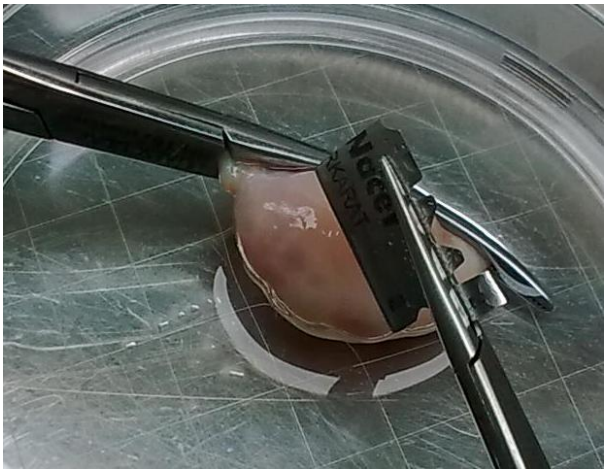

Figure 3.9: The slicing of ovary.

3.5.5.9 Embryo flushing

The ovarian response for the hormonal treatment was initially examined by laparoscopy. If 1 or more corpora lutea were observed, laparotomy was performed. The number of corpora lutea from the 2 ovaries for each donor animal was counted and recorded (Figure 3.10).

Using clean gauzes, the abdominal area of the donor goat was disinfected with diluted Hibiscrub (10%) and the hair was shaved. After shaving, the bare skin was disinfected again with absolute Hibiscrub (100%) and subsequently with iodine solution (10%). A distance of 2 cm away from the udder, a 4 cm long incision was made on the skin along the mid-abdominal wall using a scalpel blade. The incision was continued further on the underlying tissues and peritoneum layer along the linea alba.

The uterine horns along with the ovaries were exteriorised and the number of corpora lutea in each ovary was recorded. Using the modified technique of flushing of embryos described by Tervit and Havik (1976) in sheep, a puncture wound was made on each uterine horn near the bifurcation of the uterus using a small pair of haemostatic forceps. A 2-way size 8 Foley Catheter was then inserted through the puncture to a depth of 3 cm (Figure 3.11). The balloon of the catheter was sufficiently inflated to

completely obstruct the lumen at the caudal end of each uterine horn. Closer to the anterior end of the uterine horn, a Teflon intravenous (I.V.) catheter placement unit fixed with a needle (20 gauge) was introduced (Figure 3.12). This was followed by the removal of intravenous catheter needle and the introduction of flushing medium (20 ml) into the uterine horn using a sterile disposable syringe (20 ml) fixed to the end of the Teflon catheter. The uterine horn was gently massaged and the fluid contents of the uterine horn were collected through the free end of the Foley catheter. This was repeated with a further flushing medium (20 ml). Subsequently the same procedure was used for the flushing of the other uterine horn if at least 1 corpus luteum was present in the respective ovary.

After flushing, the abdominal cavity was filled with heparinised saline (250 ml) to prevent adhesion. The incisions were sutured and the incision area was sprayed with antiseptic and insecticide containing cyphenothrin. The donor goat was administered with oxytetracycline (20 mg/kg body weight) via intramuscular injection once in 4 days within the duration of 2 weeks to prevent possible post-surgical infection.

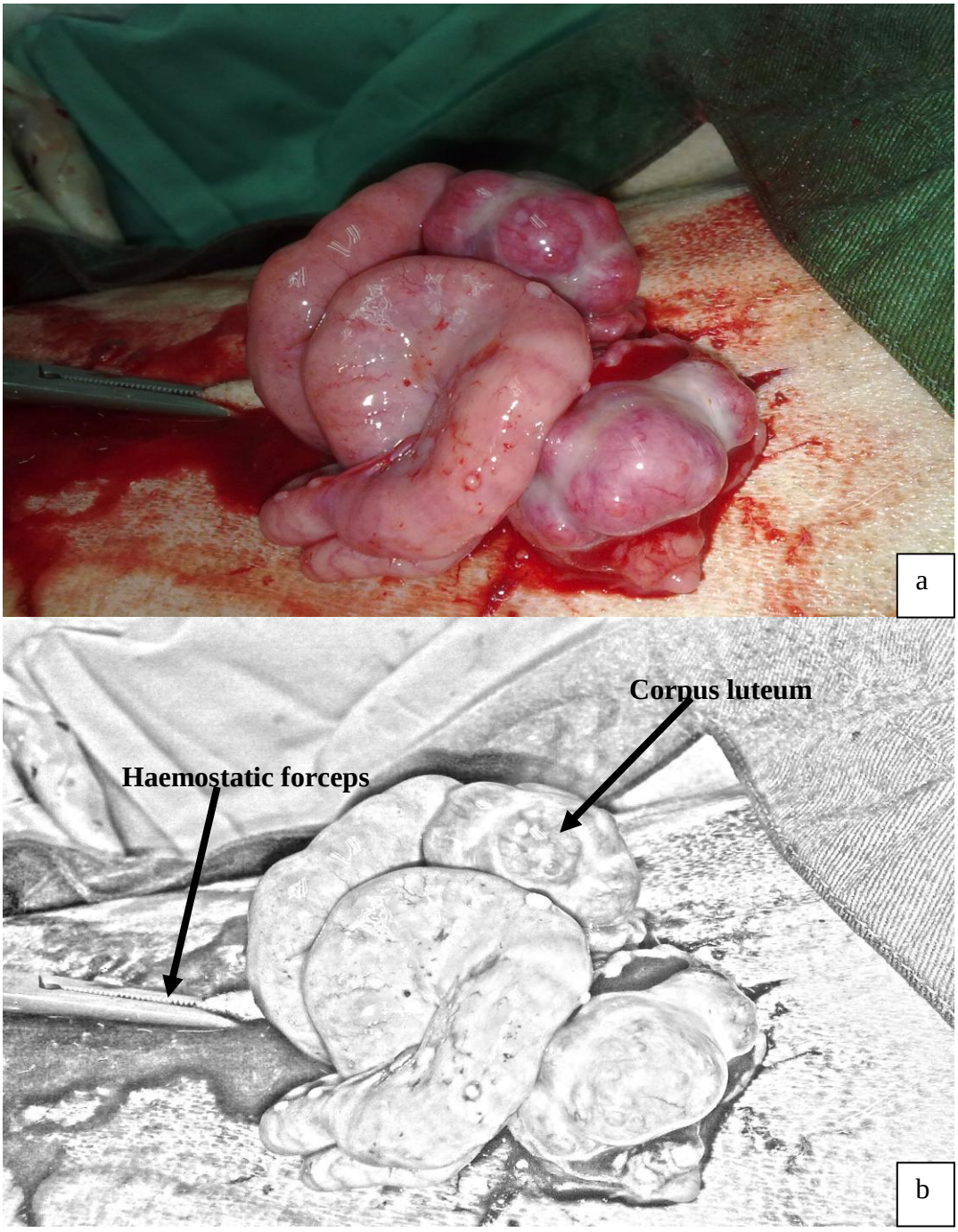

Figure 3.10: The uterine horn and ovaries were exteriorised for observation of the ovarian morphology.

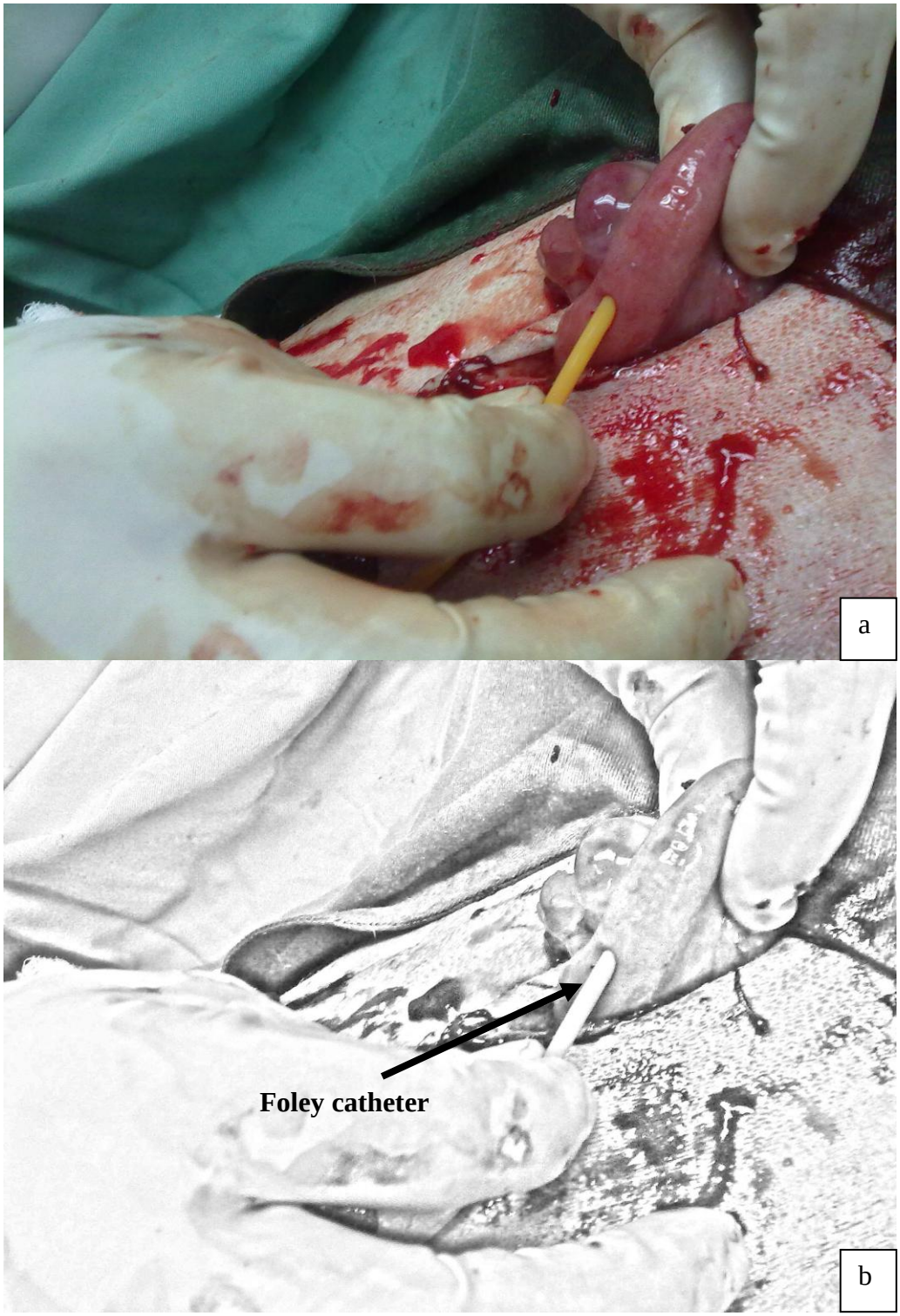

Figure 3.11: A 2-way Foley catheter was inserted through the puncture towards the utero-tubal junction and the catheter balloon was sufficiently inflated to completely obstruct the lumen at the caudal end of each uterine horn.

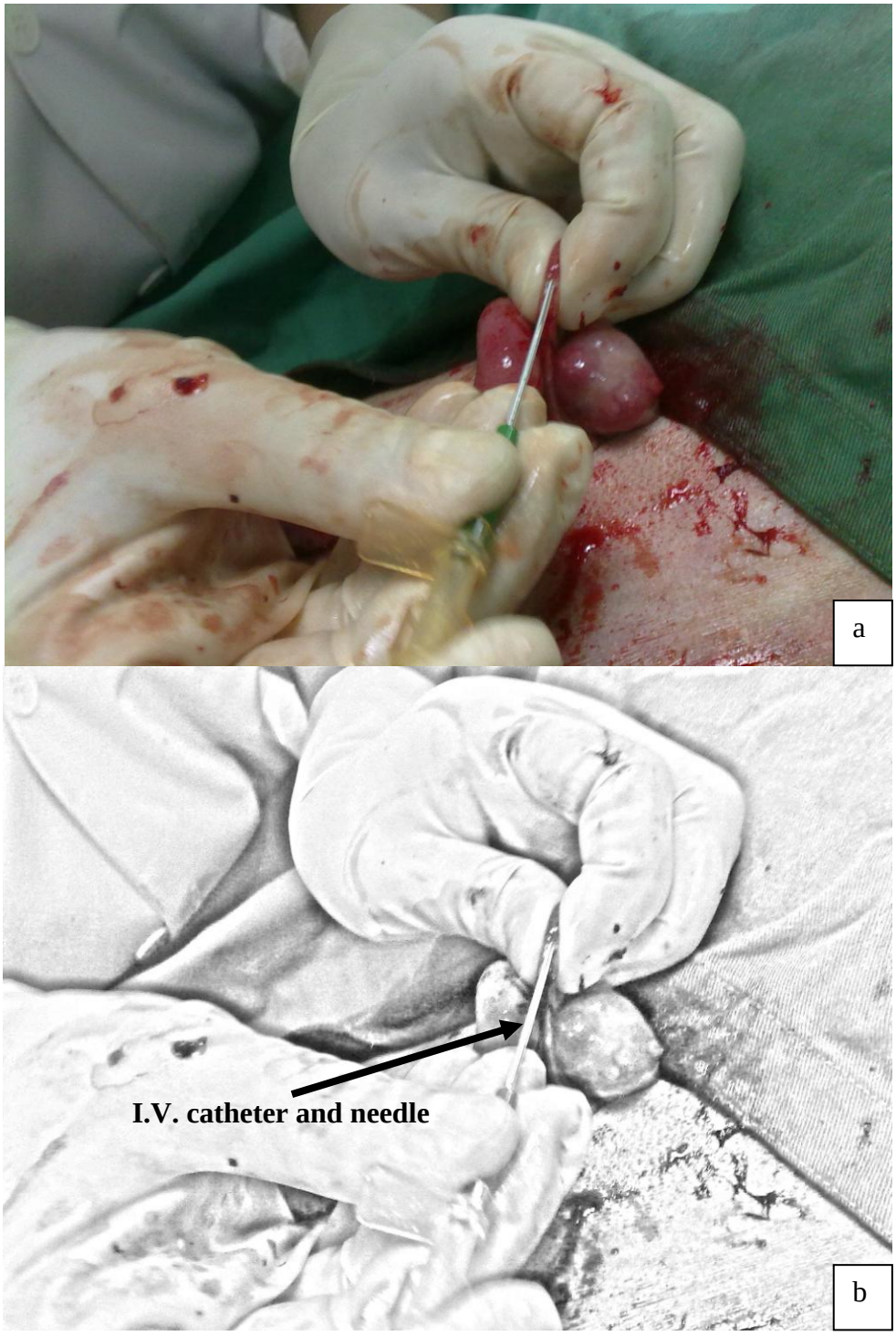

Figure 3.12: Teflon intravenous catheter placement unit was inserted at the anterior end of the uterine horn.

3.5.5.10 Post-surgery management

When oocytes-retrieval surgery was completed, all the surgical instruments were cleaned with diluted 7x®-PF solutions, using a sponge and immediately rinsed 5 times with running tap-water followed by 2 times with RO water. Each instrument was inspected to ensure that all debris (especially blood clot) had been removed. After physical cleaning, instruments were drained dry. The outer surface of the aspiration needle was disinfected with ethanol (70%) and the openings were flushed with ethanol (70%) using a syringe (20 ml) attached to a needle (18 gauge), then drained dry and packed in a surgical bag. Autoclavable surgical instruments were packed in the autoclave bag and all the autoclavable lab-wares were autoclaved by autoclave machine. An indicator test strip with the date of sterilisation was placed on the surgical pack and the aluminium foil which covered the opening of the lab-wares. Non-autoclavable surgical instruments were packed individually in a clean transparent bag and dried in the oven (56ºC) before proceeding to UV sterilisation (30 minutes).

3.5.6 *In Vitro* Production (IVP) of Embryos

The *in vitro* production of embryos involved the retrieval of immatured oocytes from the sources of laparoscopic oocyte pick-up, ovariectomy or abattoir to undergo the *in vitro* maturation process, followed by *in vitro* fertilisation or parthenogenetic activation and subsequent *in vitro* culture of embryos. The entire *in vitro* experiments took approximately 10 days.

3.5.6.1 *In vitro* maturation (IVM)

A Petri dish (35 mm) with *in vitro* maturation droplets (100 µl) which were overlaid with equilibrated silicone oil was prepared overnight. The dish was kept in CO_2

incubator (5%). Under the stereomicroscope (magnification 20x to 40x), the cumulus oocyte complex (COC) was located and picked up, using a hand-controlled pipette. Oocytes from laparoscopic oocyte pick-up were then washed in 3 droplets of flushing medium followed by 3 droplets of *in vitro* maturation medium, while oocytes from ovariectomy and ovary slicing were washed with TL-Hepes working solution followed by 3 droplets of *in vitro* maturation medium. The collected cumulus oocyte complexes were then matured and incubated overnight in CO_2 (5%) incubator at 38.5°C for 18 to 21 hours (caprine laparoscopic oocyte pick-up source), 24 to 27 hours (caprine ovariectomy or abattoir source) or 22 to 25 hours (bovine) according to oocyte grading (if sufficient number of oocytes available). The cumulus oocyte complexes were graded based on the criteria stated in Table 3.25 (Figure 3.13).

Table 3.25: Oocyte grading based on the cumulus cellular vestments and cytoplasm uniformity

Oocyte grade	Cellular vestments and cytoplasm uniformity
Grade A	COC with complete 5 layers of cumulus cells with evenly granulated cytoplasm
Grade B	COC with partially 5 layers or complete 3-4 layers of cumulus cells with evenly granulated cytoplasm
Grade C	COC with partially 3-4 layers or complete 1-2 layers of cumulus cells with evenly granulated cytoplasm
Grade D	COC with partially 1-2 layers of cumulus cells with evenly granulated cytoplasm
Grade E	Naked oocytes

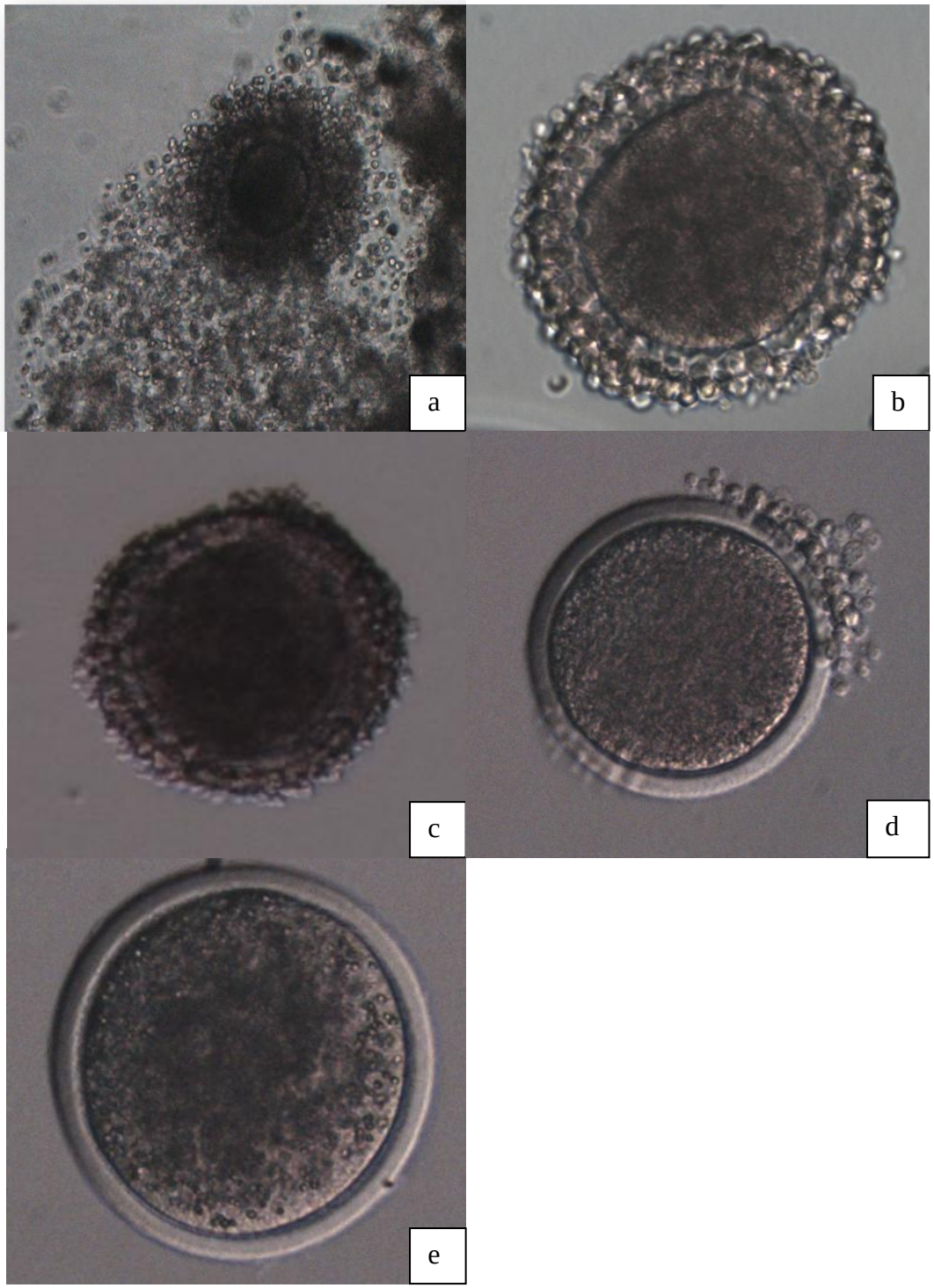

Figure 3.13: Photomicrographs of immature oocyte at different grades. (a) Grade A, (b) Grade B, (c) Grade C, (d) Grade D and (e) Grade E oocytes (adapted from Rahman, 2008).

3.5.6.2 *In vitro* fertilisation (IVF)

At the end of *in vitro* maturation, the cumulus cells of oocytes were partially removed by repeated pipetting to leave few corona cell layers surrounding the oocytes. The

medium used for *in vitro* fertilisation was a SOF-based medium (caprine) and BO medium (bovine).

3.5.6.2 (a) Sperm swim-up

A frozen straw of semen was removed aseptically from the liquid nitrogen (LN_2) tank using a pair of long forceps and pre-thawed at room temperature (25ºC, 1 minute) followed by thawing in a water bath (37ºC, 3 minutes). The straw was removed from the water bath and outer surface of the straw was disinfected with ethanol (70%) and allowed to dry. Equilibrated overnight sperm-SOF medium (caprine) or BO-sperm preparation medium (bovine) (3 ml) was placed in a conical tube (15 ml) held at approximately 60º to the horizontal surface for sperm swim-up (designated 'swim-up tube'). Using a pair of sterile scissors, the straw was cut at both ends (first was the end with cotton plug, then the seal end) and the semen was slowly released at the bottom of the swim-up tube aided by a syringe (1 ml) with a yellow micropipette tip in it (pointed end of tip was inserted into the syringe). Without agitating the mixture of semen and medium, the swim-up tube was carefully held at 60º to the horizontal surface in the CO_2 incubator (5%) and incubated (38.5º) in humidified air (45 minutes) to allow sperm swim-up.

3.5.6.2 (b) Sperm washing

At the end of the swim-up duration, the swim-up tube was carefully taken out from the CO_2 incubator (5%) without agitating the tube. The upper layer of the medium containing highly motile sperm (sperm suspension) was aspirated (1 ml) from the swim-up tube and placed in a sterile conical tube (15 ml) by using a disposable pipette. Using a sterile pipette, sperm-SOF medium (caprine) or BO-sperm preparation medium (bovine) (2 ml) was added to the sperm suspension to make a total volume of 3 ml.

With the tube tightly capped, the tube was centrifuged for 5 minutes (200 x *g*) and the supernatant was discarded. Fresh sperm-SOF medium (caprine) or BO-sperm preparation medium (bovine) was added to the pellet to a total volume of 3 ml. The tube was flicked to mix the pellet with fresh sperm-SOF medium (caprine) or BO-sperm preparation medium (bovine). With the tube tightly capped, the tube was again centrifuged for 5 minutes (200 x *g*) and the supernatant was discarded carefully without agitating the sperm pellet.

3.5.6.2 (c) Assessment of sperm concentration

Haemacytometer can be used to count sperm (Table 3.26). A haemacytometer has 2 chambers and each chamber has a microscopic grid etched on the glass surface. The chambers are overlaid with a glass cover-slip that rests on pillars exactly 0.1 mm above the chamber floor (Figure 3.14).

The sperm pellet in the conical tube was resuspended in IVF-SOF (caprine) or BO-IVF medium (bovine) (100 µl). For sperm suspension (5 µl) was then diluted (1:10) with IVF-SOF medium (caprine) or BO-IVF medium (bovine) (50 µl) and mixed well by pipetting the suspension up and down in the microcentrifuge tube (0.5 ml) 5 to 7 times using an adjustable micropipette to obtain a uniform suspension.

The haemacytometer was first rinsed with RO water followed by the disinfection with ethanol (70%) using a lens tissue and allowed to dry in the laminar flow before use. A special rectangular cover-slip (provided by the manufacturer) was placed over the counting chambers on the haemacytometer. Diluted sperm suspension (10 µl) was loaded to both counting chambers by placing the micropipette tip at the edge of the cover-slip. Capillary action will draw the sperm suspension into the chamber. The entire volume of the chamber was filled, but was not overfilled as doing so will give an inaccurate count. The haemacytometer was viewed under a microscope at 100x

magnification. The sperm were visible above the grid of the counting chamber (Figure 3.15).

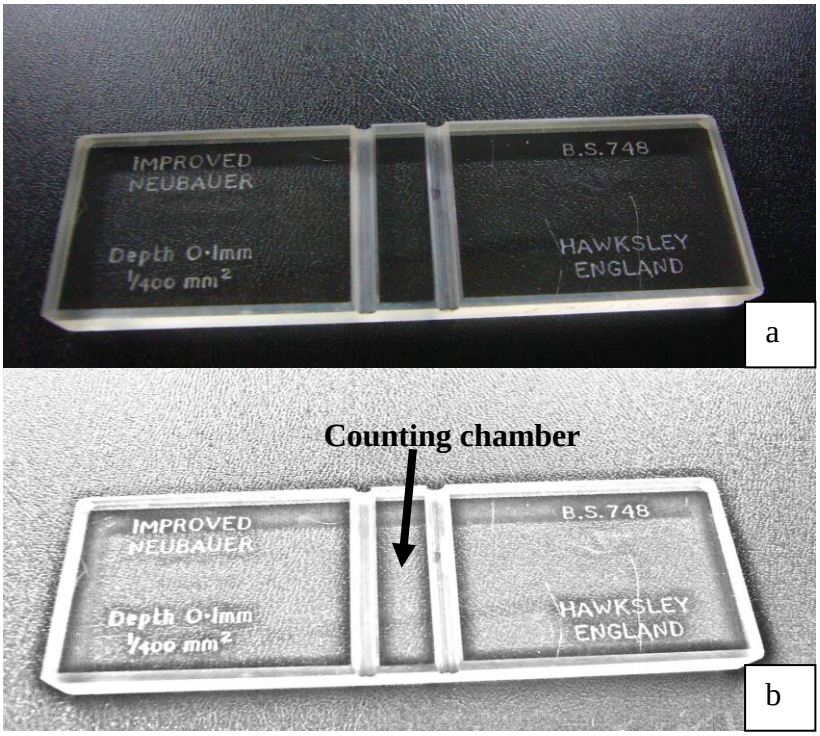

Figure 3.14: The 'Neubauer' type chamber of haemacytometer. (a) Original photograph; (b) Labelled photograph.

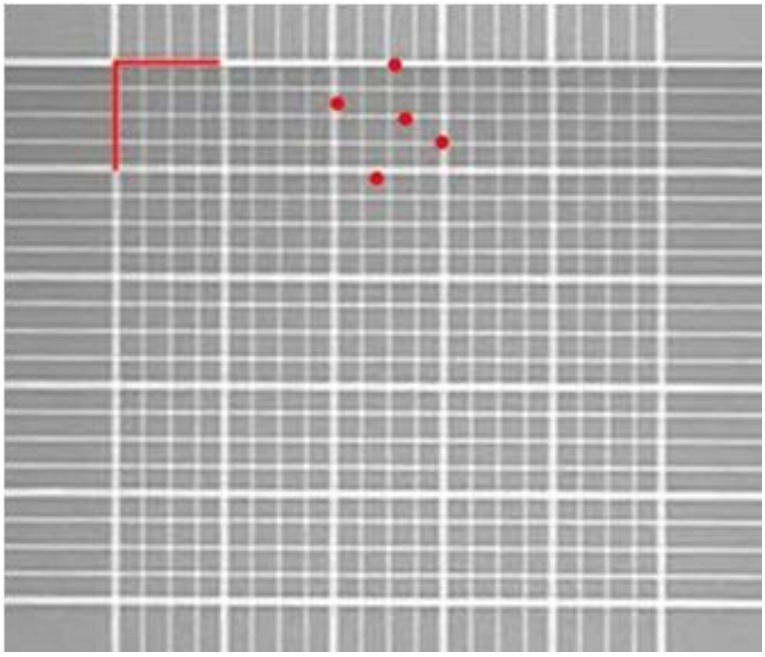

Figure 3.15: Assessment of sperm concentration using haemacytometer. The full grid on a haemacytometer contains 9 squares, each of which is 1 mm square. The central counting area of the haemacytometer contains 25 large squares and each large square has 16 smaller squares. Only sperm that lied on the lines of 2 sides of the large square were counted to avoid counting cells twice. The example above shows red lines where cells on the line would be counted. If red dots represent sperms, one would count 3 sperm in the top middle large square. Adapted from Collection And Evaluation of Semen (2002). http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/hemacytometer.html

Table 3.26: Calculating sperm concentration

No. of sperm in 5 of the 25 large squares within the central counting area of two chambers	Mean no. of sperm	Mean no. of sperm per chamber	No.of sperm per ml of diluted sample	Dilution factor	No. of sperm per ml of original sperm sample
100 sperm and 128 sperm	114	114 x 5= 570	5,700,000	10	5,700,000 x 10= 57,000,000

3.5.6.2 (d) Preparation of oocytes for *in vitro* fertilisation

Using a sterile hand-controlled pipette, oocytes were transferred from the *in vitro* maturation medium culture dish to the droplets of *in vitro* fertilisation medium (100 µl). Oocytes were partially denuded by sucking in and out from the pipette. The oocytes left

with 1 or 2 layers of cumulus cells were washed thrice with droplets of *in vitro* fertilisation medium (100 µl) before being transferred to the insemination culture dish and kept in the CO_2 incubator (5%) to maintain its temperature and gas equilibration until required for insemination.

3.5.6.2 (e) Sperm capacitation and insemination

While conducting the assessment of sperm concentration, the capacitation-treated sperm were incubated in CO_2 incubator (5%). After the sperm concentration had been determined, sufficient volume of sperm suspension was added to the fertilisation droplets (30 µl) containing 5 to 10 matured oocytes to yield final concentration $1.0x10^6$ sperm/ml.

3.5.6.3 *In vitro* culture of *in vitro* fertilised zygotes

The dish containing *in vitro* culture droplets under silicone oil were equilibrated overnight at 38.5ºC in CO_2 incubator (5%). The culture medium used for *in vitro* embryo culture was as described in Section 3.5.2.8. After 8 to 14 hours or 18 to 24 hours sperm insemination duration, all the presumptive zygotes were removed from the insemination droplets and transferred to *in vitro* culture droplets (100 µl). The presumptive zygotes were lightly denuded to dislodge residual sperm and cumulus cells. All zygotes were subsequently transferred to new *in vitro* culture droplets (100 µl) for washing. After washing thrice, zygotes were transferred to the *in vitro* culture droplets for culture (38.5ºC, 5% CO_2). Each culture droplet (100 µl) contained 5 to 10 presumptive zygotes.

Under the inverted microscope, all presumptive zygotes were observed for the presence of 2 polar bodies. The culture dish containing zygotes or embryos were then

cultured at 38.5ºC in CO_2 (5%) and humidified air for 7 days (8 days post-insemination). The embryos were observed and evaluated daily for embryonic development.

3.5.6.4 Parthenogenesis

Petri dish with droplets of calcium ionophore working solution and 6-dimethylaminopyridine working solution overlaid by silicone oil were pre-incubated (38.5ºC, 5% CO_2) 3 hours prior the experiment. Oocytes which were cultured in *in vitro* maturation droplets were completely denuded and looked for oocytes with polar body (matured oocytes). The matured oocytes were washed with 3 droplets of calcium ionophore and incubated in it for 5 minutes. After 5 minutes, the oocytes were washed with 3 droplets of 6-dimethylaminopyridine and incubated in it for 5 hours. The oocytes were washed with 3 droplets of preincubated *in vitro* culture droplets and cultured after 5 hours. The cleavage rates were recorded daily.

3.5.7 Vitrification of Embryos

Embryo vitrification technique involved preparation of vitrification and warming solutions, construction of cryoleaf and a vitrification protocol proper.

3.5.7.1 Preparation of vitrification and warming solutions

The vitrification and warming medium composed of the solutions stated as below (Table 3.27).

Table 3.27: Solutions for vitrification and warming procedures

Solutions (catalogue no.)	Concentration	Method of preparation (storage duration)
HM (Holding medium) (P4417)	1x	PBS supplemented with OGS (20%), filter-sterilised and stored (4ºC). (3 months, without addition of OGS)
SM (Sucrose medium) (S9378)	1M	Sucrose (17.115 g) was added to PBS (40 ml), solution was warmed (40-45ºC), mixed with stirrer, topped up to final volume (50 ml), filter-sterilised and stored (4ºC); supplemented with OGS (20%) prior to use. (3 months, without addition of OGS)
EG (Ethylene glycol) (E9129)	1x	EG was aliquot to a sterile tube (3 ml), tight-closed and stored (room temperature). (2 weeks)
DMSO (Dimethyl sulfoxide) (D2650)	1x	DMSO was aliquot to a small sterile glass bottle (5 ml), wrapped in aluminium foil, tight-closed and stored (room temperature). (2 weeks)

3.5.7.2 Construction of cryoleaf

A French straw (0.25 ml) was cut into 4. One end of each cut straw was pressed with sealer to create a small flat area for loading embryo. The other end of each short straw was dyed with a xylene-free marker pen to aid in the identification inside liquid nitrogen (Figure 3.16).

Figure 3.16: Hand-made cryoleaf.

3.5.7.3 Vitrification

Before starting the vitrification procedure, all the vitrification solutions were warmed (37ºC) in a water bath for 30 minutes. All the manipulations were performed on a stage warmer (37ºC). The cryoleaf were dyed using a xylene-free marker pen in order to be visible in the liquid nitrogen. A foam box (15x17x15 cm) was disinfected with ethanol (70%) before filling with liquid nitrogen. The filling was aided by first marking the level (80% of the foam box) before filling the liquid nitrogen. The foam box was then covered to allow horizontal cooling.

A sterile 4-well dish was used as the vitrification dish and filled with vitrification solutions (HM in well-1 and well-2; VS1 in well-3 and VS2 in well-4, respectively) as shown in Table 3.28. The dish was covered and warmed (5 to 10 minutes) on a stage warmer (38.5ºC). Meanwhile, the polystyrene box was filled again with liquid nitrogen to compensate evaporation.

Table 3.28: Experimental outline of vitrification

Vitrification dish	Medium	Duration
Well-1	800 µl HM	1 minute
Well-2	800 µl HM	3-5 minutes
Well-3	(VS1): 850 µl HM + 75 µl EG + 75 µl DMSO	3 minutes
Well-4	(VS2): 670 µl SM + 165 µ EG + 165 µl DMSO	45 seconds

By using a hand-controlled pipette, embryos were placed into well-1 and left for 1 minute subsequently into well-2 for storage of embryos during stepwise vitrification. At 1 cycle of vitrification, 1 to 2 embryos were transferred into well-3 (VS1) with minimal possible volume of medium. The timer was started (3 minutes) once this was done. Towards the last 30 seconds, a droplet (20 µl) was made of medium from well-4 (VS2) on a new Petri dish (35 mm). When 3 minutes times up, embryos were transferred in the minimal volume into the droplet (20 µl) of VS2. Under the microscope, the cryoleaf was held with the left hand and placed within the focus of the microscope at low magnification (10x). Using a transfer pipette on the right hand, the embryos in the droplet were taken up in a minimum volume (<3 µl) and placed onto the cryoleaf. All the steps were done within 45 seconds when embryos were exposed to the VS2. The cryoleaf was immediately held with long forceps and plunged quickly into liquid nitrogen (Figure 3.17).

Vitrification was repeated with subsequent embryos. Only 1 to 2 embryos were loaded for each cryoleaf in each cycle of vitrification. VS2 medium in the droplet (20 µl) was made in each new cycle of vitrification.

3.5.7.4 Warming

All warming solutions were warmed (37ºC) in a water bath (30 minutes) prior the experiment. A sterile 4-well dish was used as the warming dish and filled with warming solutions (TS in well-1 and well-2; DS in well-3 and HM in well-4, respectively) as shown in Table 3.29. The dish was covered and warmed (5 to 10 minutes) on a stage warmer (37ºC).

Table 3.29: Experimental outline of warming

Warming dish	Medium	Duration
Well-1	(TS): 800 µl HM + 400 µl SM*	5-10 minutes
Well-2	(TS): 800 µl HM + 400 µl SM*	5 minutes
Well-3	(DS): 800 µl HM + 200 µl SM*	5 minutes
Well-4	800 µl HM	5 minutes

*The solution was mixed by aspirating in and out (after finish filling all well)

The well-1 of the warming dish containing thawing solution (TS) was placed within the viewing field of the microscope focus. By using long forceps, cryoleaf was removed from liquid nitrogen then the cryoleaf was held with the thumb and index. In 3 seconds, the cryoleaf containing vitrified embryos (in VS2) was immersed into well-1 of the warming dish and gently agitated under the focus of microscope by totally submerging the vitrified liquid droplet. The embryos were released once the VS2 droplet melted. The released embryos were then transferred into well-2 of the warming dish. After 5 minutes each, embryos were subsequently transferred into well-3, then well-4 before washing thrice in equilibrated *in vitro* culture medium and cultured in *in vitro* culture droplets (Figure 3.18).

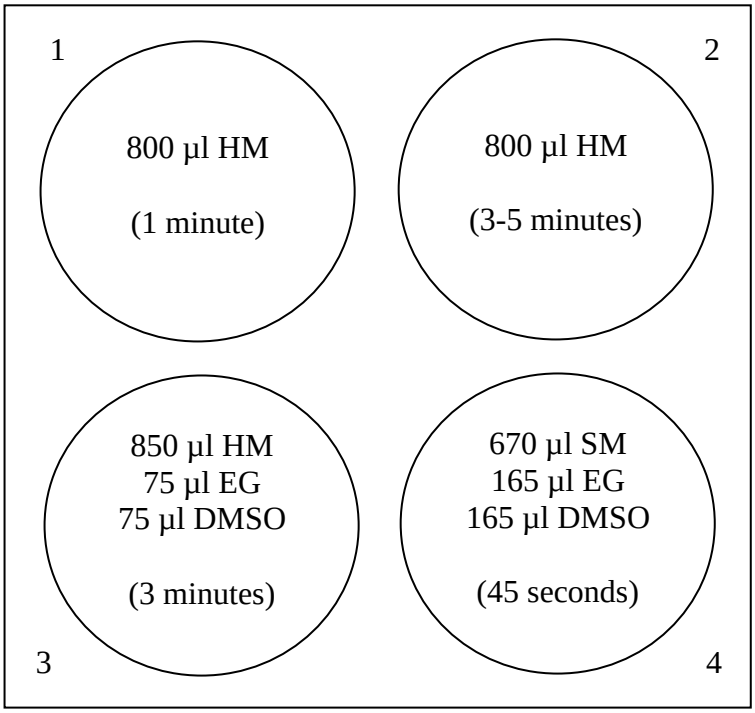

Figure 3.17: A schematic representation of the steps involved in vitrification procedure. Exposure duration was indicated in the bracket.

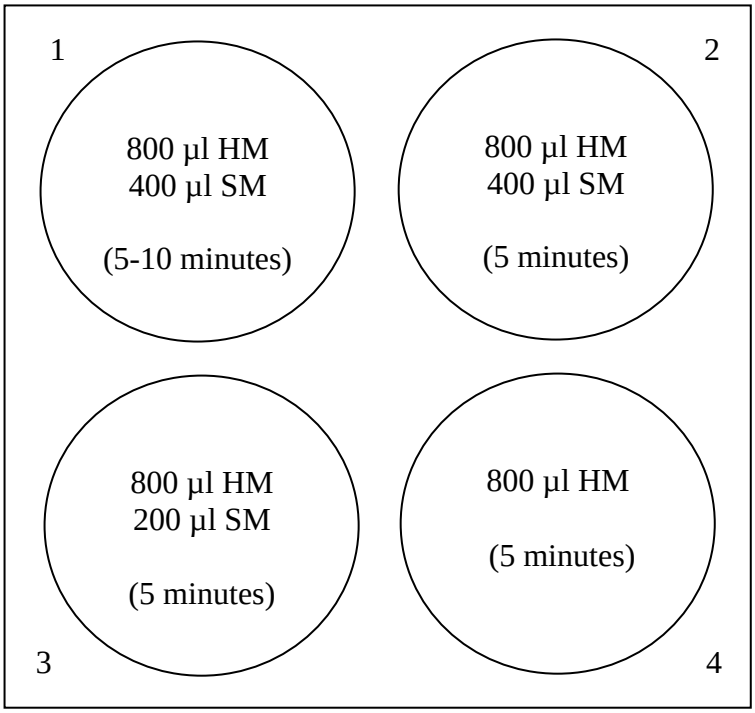

Figure 3.18: A schematic representation of the steps involved in warming procedures. Exposure duration was indicated in the bracket.

3.6 EXPERIMENTAL DESIGN

The present study was carried out to investigate the effect of caprine oocytes obtained from different sources (laparoscopic oocyte pick-up, ovariectomy and abattoir) and bovine oocytes (abattoir) on the quality of oocytes as well as the embryo developmental competence via different sperm insemination duration in *in vitro* fertilisation and through parthenogenetic activation. An attempt to cryopreserve embryos using vitrification was carried out in this study as well (Figure 3.19).

3.6.1 Effect of Oocyte Grading and Insemination Duration on Cleavage Rate of Embryos Obtained from *In Vitro* Fertilisation in Bovine (Experiment 1)

In this experiment, the effects of oocyte quality and insemination duration on the developmental competence of *in vitro* matured-fertilised embryos were studied. Immatured abattoir-derived bovine oocytes were cultured in *in vitro* maturation droplets according to oocyte grades for duration of 22 to 25 hours. The cumulus oocyte complexes were classified into 5 grades namely Grades A, B, C, D and E based on the cumulus cellular vestments and cytoplasm uniformity (Table 3.25). However, in this experiment, only oocytes from Grades A, B and C as well as mixed grades (Grades A + B + C) were included in the results as Grades D and E oocytes were developmentally incompetent.

At the end of *in vitro* maturation culture of oocytes, the maturation rate was determined by the expansion of cumulus cells. Matured oocyte was recorded as 'matured' if it was with cumulus expansion (sun-burst); alternatively, oocyte with unexpanded cumulus was recorded as 'immatured'. Matured oocyte could also be determined with the presence of the first polar body. The maturation rate was calculated as a percentage of the number of oocytes matured per total oocytes used for the respective oocyte grades.

The matured oocytes were subsequently used for the *in vitro* fertilisation to be inseminated *in vitro* with frozen-thawed semen. Frozen-thawed semen was treated with the swim-up procedure for 45 minutes (38.5ºC, 5% CO_2 in humidified air) using the BO-sperm preparation medium [Section 3.5.6.2 (a)]. Meanwhile, matured oocytes were partially denuded to remove the cumulus cells and subsequently placed in the insemination droplets (BO-IVF medium) for the respective oocyte grades [Section 3.5.6.2 (d)]. After swim-up, sperm washing was performed by centrifuging twice (200 x *g*; 5 minutes) [Section3.5.6.2 (b)]. By using a haemacytometer, sperm concentration was assessed [Section 3.5.6.2 (c)]. A sufficient volume of sperm was added to the insemination droplets containing partially denuded oocytes to yield a final concentration of $1.0x10^6$ sperm/ml [Section 3.5.6.2 (e)].

After 8 to 14 hours or 18 to 24 hours of insemination duration, all presumptive zygotes were removed from the insemination droplets and lightly denuded to dislodged residue sperm and cumulus cells [Section 3.5.6.3]. Zygotes/oocytes were recorded as 'fertilised' in the presence of a second polar body or recorded as 'unfertilised' in the absence of a second polar body. The fertilisation rate was recorded as percentage of oocytes fertilised per total matured oocytes used for the respective oocyte grades.

The embryos were cultured in *in vitro* culture droplets according to oocyte grades (38ºC, 5% CO_2 in humidified air). The embryo development was observed daily. The cleavage rate was recorded as percentage calculated from the number of presumptive zygotes cultured in *in vitro* culture, in the respective oocyte grades.

The rates of maturation, fertilisation and cleavage were recorded in percentages form for the respective oocyte grades. The maturation rate was calculated as number of matured oocytes per total number of oocytes used; fertilisation rate was calculated as number of fertilised oocytes per number of matured oocytes; and cleavage rate was calculated as number of embryos obtained per number of fertilised oocytes. Effect of

oocyte grades and insemination duration on maturation rate, fertilisation rate and cleavage rate were determined.

3.6.2 Effect of Oocyte Grading and Insemination Duration on Cleavage Rate of Embryos Obtained from *In Vitro* Fertilisation in Caprine (Experiment 2)

In this experiment, the effects of oocyte quality and insemination duration on the developmental competence of *in vitro* matured-fertilised embryos were studied. Immatured caprine oocytes derived from laparoscopic oocyte pick-up, ovariectomy and abattoir were cultured in *in vitro* maturation droplets according to oocyte grades for a duration of 18 to 21 hours (caprine laparoscopic oocyte pick-up source) and 24 to 27 hours (caprine ovariectomy or abattoir source). The cumulus oocyte complexes were classified into 5 grades namely Grades A, B, C, D and E based on the cumulus cellular vestments and cytoplasm uniformity (Table 3.25). However, in this experiment, only oocytes from Grades A, B and C as well as mixed grades (Grades A + B + C) were included in the results as Grades D and E oocytes were developmentally incompetent.

At the end of *in vitro* maturation culture of oocytes, the maturation rate was determined by the expansion of cumulus cells. Matured oocyte was recorded as 'matured' if it was with cumulus expansion (sun-burst); alternatively, oocyte with unexpanded cumulus was recorded as 'immatured'. Matured oocyte could also be determined with the presence of the first polar body. The maturation rate was calculated as a percentage of the number of oocytes matured per total oocytes used for the respective oocyte grades.

The matured oocytes were subsequently used for the *in vitro* fertilisation to be inseminated *in vitro* with frozen-thawed semen. Frozen-thawed semen was treated with the swim-up procedure for 45 minutes (38.5ºC, 5% CO_2 in humidified air) using the sperm-SOF medium [Section 3.5.6.2 (a)]. Meanwhile, matured oocytes were partially

denuded to remove the cumulus cells and subsequently placed in the insemination droplets (IVF-SOF medium) for the respective oocyte grades [Section 3.5.6.2 (d)]. After swim-up, sperm washing was performed by centrifuging twice (200 x *g*; 5 minutes) [Section 3.5.6.2 (b)]. By using a haemacytometer, sperm concentration was assessed [Section 3.5.6.2 (c)]. A sufficient volume of sperm was added to the insemination droplets containing partially denuded oocytes to yield a final concentration of $1.0x10^6$ sperm/ml [Section 3.5.6.2 (e)].

After 8 to 14 hours or 18 to 24 hours of insemination duration, all presumptive zygotes were removed from the insemination droplets and lightly denuded to dislodged residue sperm and cumulus cells [Section 3.5.6.3]. Zygotes/oocytes were recorded as 'fertilised' in the presence of a second polar body or recorded as 'unfertilised' in the absence of a second polar body. The fertilisation rate was recorded as percentage of oocytes fertilised per total matured oocytes used for the respective oocyte grades.

The embryos were cultured in *in vitro* culture droplets according to oocyte grades (38ºC, 5% CO_2 in humidified air). The embryo development was observed daily. The cleavage rate was recorded as percentage calculated from the number of presumptive zygotes cultured in *in vitro* culture, in the respective oocyte grades.

The rates of maturation, fertilisation and cleavage were recorded in percentages form for the respective oocyte grades. The maturation rate was calculated as number of matured oocytes per total number of oocytes used; fertilisation rate was calculated as number of fertilised oocytes per number of matured oocytes; and cleavage rate was calculated as number of embryos obtained per number of fertilised oocytes. Effect of oocyte grades and insemination duration on maturation rate, fertilisation rate and cleavage rate were determined.

3.6.3 Effect of Oocyte Grading in Bovine and Caprine on Production of Parthenogenetic Embryos (Experiment 3)

The objective of this experiment was to produce parthenogenetic embryos using chemical activation for different oocyte grades in bovine and caprine. For the bovine, oocytes were from abattoir-derived while for the caprine, the oocytes were from laparoscopic oocyte pick-up, ovariectomy and abattoir-derived.

Oocytes which were cultured in *in vitro* maturation droplets were completely denuded and oocytes with polar body were identified. The matured oocytes were washed with 3 droplets of CI and incubated in CI droplets for 5 minutes after which the oocytes were washed with 3 droplets of 6-DMAP and incubated in 6-DMAP droplets for 5 hours. The oocytes were washed with 3 droplets of pre-incubated *in vitro* culture droplets and were cultured in *in vitro* culture droplets (38ºC, 5% CO_2 in humidified air) and the embryo development was observed daily until day 7 of *in vitro* culture. The maturation rate and cleavage rate were calculated as described in Sections 3.6.1 and 3.6.2. Effect of oocyte grades and species on maturation rate and cleavage rate were determined.

3.6.4 An Attempt to Cryopreserve Caprine and Bovine Embryos Using Vitrification Technique (Experiment 4)

The objective of this experiment was an attempt to develop a protocol for vitrification of caprine and bovine embryos in our laboratory. Embryos were derived from *in vivo*-(embryo flushing) and *in vitro*-fertilisation of oocytes from laparoscopic oocyte pick-up, ovariectomy and abattoir sources. The embryos were divided into 2 groups: a) for toxicity screening after exposure to vitrification solution and b) for evaluation of survival rate after vitrification. For the former, the embryos were exposed to vitrification solution (combination of dimethyl sulfoxide, ethylene glycol and sucrose)

for 3 to 4 minutes and then the survival rate was evaluated after exposed to warming solution. For the latter, the embryos were vitrified and then devitrified to evaluate the survival rate by culturing *in vitro* in 5% CO_2 incubator at 38.5ºC in humidified air.

3.7 STATISTICAL ANALYSIS

Data from Experiments 1, 2, 3 and 4 were analysed in percentages form. Calculation of percentages was described in the respective experimental design. Effects of oocyte grading and insemination duration on cleavage rate of embryos obtained from *in vitro* fertilisation in bovine (Experiment 1); effects of oocyte grading and insemination duration on cleavage rate of embryos obtained from *in vitro* fertilisation in caprine (Experiment 2); effects of oocyte grading in bovine and caprine on production of parthenogenetic embryos (Experiment 3); as well as an attempt to cryopreserve caprine and bovine embryos using vitrification technique (Experiment 4), were all analysed by analysis of variance (ANOVA) and Duncan's multiple range tests (D-MRT), using the SPSS statistical software package version 17 excluding Experiment 4. A probability of $P<0.05$ was considered significant for all statistical tests. Values were presented as mean±SEM.

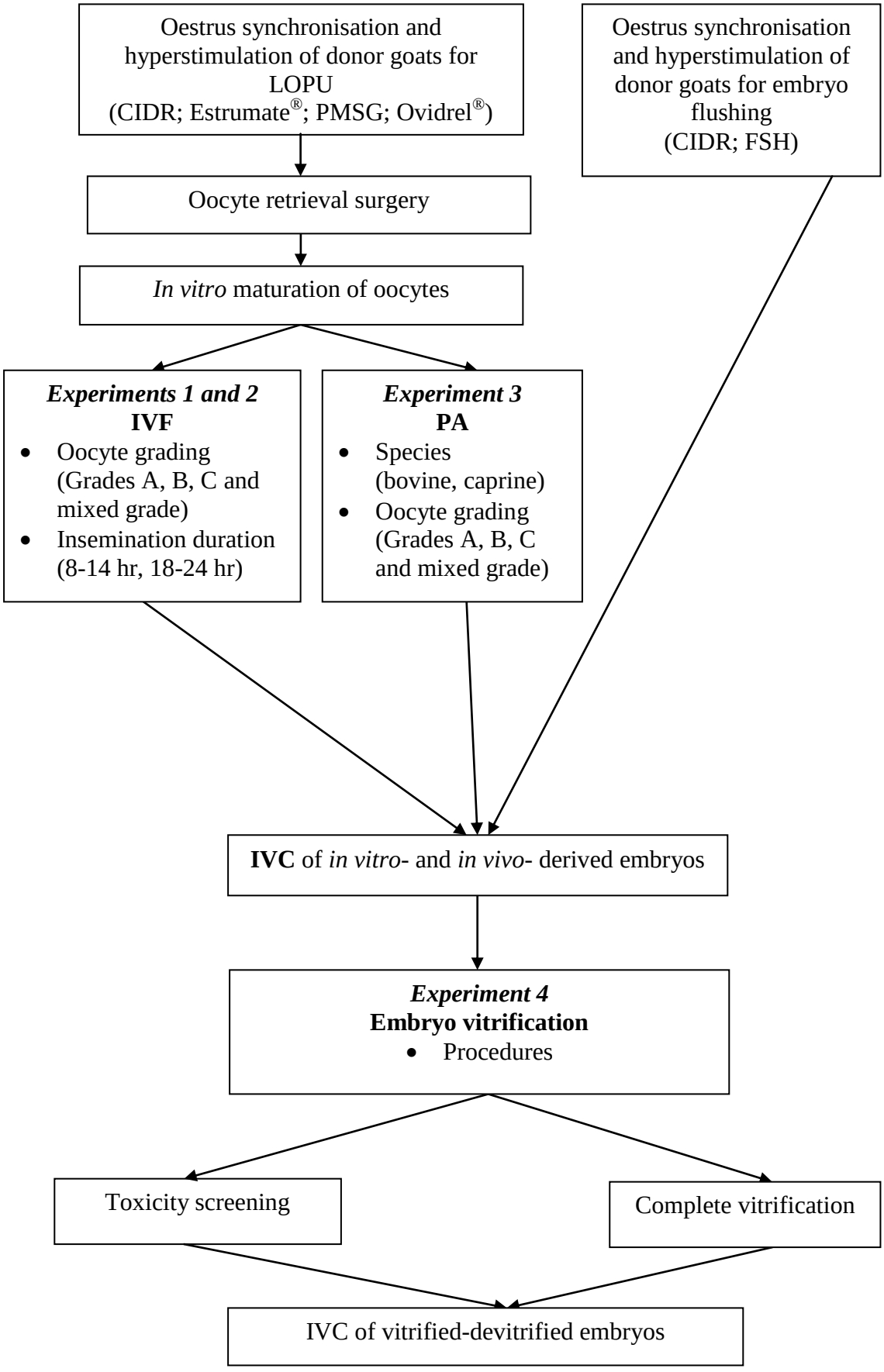

Figure 3.19: A flow chart for schematic overview of the experimental design.