CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 GENERAL INTRODUCTION

The present study was conducted at the Nuclear Transfer and Reprogramming (NaTuRe) Laboratory, Institute of Research, Management and Consultancy, IPPP, the University of Malaya, Kuala Lumpur, Malaysia from January 2007 to June 2009. The experimental goats were sourced from ISB Mini Farm (the University of Malaya) located approximately 1 km from the laboratory. Surgeries for embryo recovery and embryo transfer were conducted once or twice a week, using one donor with two recipient does per session. All media and sterilisation were prepared in the same laboratory.

3.2 EXPERIMENTAL ANIMALS

The donor and recipient does that were used in this study comprising of mixed parous Boer, Jamnapari crossbreed and local mixed breed goats. The age and body weight of the experimental goats ranging from 15 to 96 months old and 20-45 kg. Fed with Napier grass and commercial pellets twice daily, with *ad labium* access of water. All experimental goats were maintained under good conditions and with animal welfare guidelines. These goats were subjected to oestrus synchronisation, superovulation and embryo recovery or embryo transfer. A total of 36 surgery sessions were performed on 43 goats at the NaTuRe laboratory.

3.3 MATERIALS

Various equipment, chemicals, media, labwares and disposables used in this study are listed in Appendix Table 1.

3.4 METHODOLOGY

The methodology section has been divided into 3 main subsections, that is preparation for the successful ET environment, preparation of media and microtools and experimental procedures. Each of these subsections is further subdivided for ease of description.

3.4.1 Preparation for a Successful ET Environment

It is vital to ensure that laboratory activities, facilities and environment adhere to strict hygienic and cleanliness regimes throughout all experimental procedures. It is the responsibility of the laboratory user to maintain the laboratory in a distraction-free and accident-free environment to optimise the outcome of ET works. It is utmost importance to minimise the potential introduction of contaminants to the ET work area, particularly for embryo handling system.

3.4.1.1 Air Quality

Air quality is great importance in the laboratory that deals with embryos since incubators obtained air directly from the laboratory. This embryo handling area is separated from the embryo collection area to ensure no exposure to aerosols arising from animals and people. Smoking, eating and perfume are strictly prohibited in embryo handling and surgery rooms.

3.4.1.2 Water Quality

It is necessary to have a reliable source of ultrapure water system in the laboratory since water forms the basic and common component in all embryo handling media. *In vivo* embryos have virtually no defense mechanism once they were flushed out from doe's

reproduction tract, since their active and passive absorption mechanisms are mostly indiscriminate. The water used to prepare flushing media and saline was sourced from ultrapure water purification system; with treatments of particulate filtration, activated carbon filtration, reverse osmosis (RO) and electro-deionisation (EDI), ultraviolet oxidation system, followed by Milli-Q PF Plus purification (18.2 MΩ cm) and finally filtered through a membrane filter (90.22 µm) to eliminate trace particles as well as to prevent bacterial contamination from the environment.

3.4.1.3 Glassware Cleaning and Sterilisation

Washable glasswares used in the present study include glass bottles, beakers, volumetric flask, measuring cylinders, conical flasks, stir bars and conical tubes. Cleaning solution (7X*-PF™ , Australia) was used as a detergent for glassware washing was kept in squeze bottle until use. Used glassware was rinsed in water to wash away contaminant traces, and the label was immediately removed and soaked in 7X*-PF. The glassware was washed with diluted cleaning solution (7X*-PF) using brush or sponge and immediately rinsed five times with distilled water followed by five times reverse osmosis (RO) water. After completion, the cap was placed loosely and covered snugly with a layer of aluminium foil. A piece of autoclave tape was placed on the foil. Alternatively, conical tubes were placed in an autoclave bag and be autoclaved for a minimum 25 minutes and subsequently dry-cycled for 30 minutes. After autoclaving, the glassware cap was tightened a little bit to prevent any contaminants from entering and were air dried in the oven (Memmert Gmbh, Germany) at 60^{o}C. The cap was not tightened completely until the glassware had cooled to prevent a vacuum forming.

3.4.1.4 Plastic and Other Heat-Labile Equipment

Needles, catheters, transferring syringes and other glasswares or instruments that were not resistant to heat were sterilised by the application of ultraviolet (UV) light for 30 minutes inside the laminar flow work station. Flushing media and other solutions and microdrops for embryo handling and storing were prepared inside the laminar flow work station (Gelman Sciences, Australia) under fluorescent light.

3.4.1.5 Embryo Handling Area

The inside surface of laminar flow work station, microscope stages and other equipment were wiped with ethanol (70%; HmbG Chemicals, Germany) before initiating any experiment or procedure. The residual traces of ethanol were allowed to evaporate for at least 20 minutes before commencing the experimental work. Any spillage was wiped immediately with dry tissue, and if it was necessary, with ethanol (70%). The inside surfaces of laminar flow and microscope stages were wiped with ethanol (70%) after finishing experimental work. The water bath (Memmert GmbH, Germany) was cleaned and water was changed frequently.

3.4.1.6 Carbon dioxide (CO_2) Incubator

In the current study, a 5% CO_2 incubator in humidified air was used to culture embryos obtained from flushing the donor does before being transferred to recipient does. Carefully calibrated and accurately controlled CO_2 incubators are crucial for survival of subsequent transferred *in vivo* embryos. The CO_2 incubator (HERAcell 240; Kendro Laboratory Products, Germany) was monitored regularly and the LED display of temperature checked with independence readings. Throughout the experiments a temperature was maintained at 38.5^{o}C.

3.4.1.7 Silicone Oil

It is necessary to covered medium droplet with thin layer of silicone oil if the *in vivo* embryos need to be stored in uncover container for more than 20 minutes. Silicone oil serves as physical barrier to prevent evaporation and contamination and to regulate the rate of gas exchange between the medium and surrounding atmosphere for stabilising the pH, temperature and osmolarity of the media. Silicone oil needs to be washed before using to remove any possible water-soluble contaminants. This can be done by gently shaking the oil with ultrapure water (Milli-Q) or sterile saline solution (0.9% NaCl) in a sterile glass bottle or disposable tissue culture flask in the ratio of 3:2 (oil to aqueous solution). This 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 ($4^{o}C$). The oil was then equilibrated with holding medium by incubating overnight in CO_2 (5%) incubator using tissue culture flask (50 ml) with the cap loosened.

3.4.2 Preparation of Microtool

The preparation of hand-controlled hand-control micropipette is important as a tool used for handling of embryos. It consists of a pulled-haematocrit capillary attached to a silicone tubing at one end. The other end of the silicone tubing was completely sealed with silicone glue. Micropipette was made from Pyrex glass tubing with a 4-mm outside diameter. The glass in 10 cm length, was heated in the centre with Bunsen burner and pulled to make outside diameter less than 1mm straight tube. It was important to achieve an evenly snapped straight tip because a jagged capillary end may potentially cause damage to the zona pellucida during manipulation of embryos. The pulled portion was scored with a diamond pencil and subsequently was broken to make two pipettes. All ends were fire polished because sharp edges of the pipette were easily hooked to

plastic surface of the searching dish, causing the pipette to break and tend to collect more debris during manipulation. For sterilisation, they were rinsed 10 times with RO water and autoclaved in clean glass test tube covered with aluminum foil. When using it, the pipette was connected to a silicone tubing at one end.

3.4.3 Preparing Media for ET Experiment

In vivo embryos depend on the ambient fluid to maintain their physiological integrity. Media used in the present study were prepared either 'in-house' or bought direct from suppliers. These media are adequate to maintain embryos for routine short-term interval between collection and transfer and not suitable for longer period of *in vitro* embryo handling.

3.4.3.1 Preparation of Normal Physiological Saline (0.9% NaCl)

Normal physiological saline (0.9% NaCl) was used throughout the study. It is needed during the donor post-operative treatments to fill up the peritoneum cavity. Exteriorisation of reproductive tract may cause bleeding and, therefore, such blood was washed or diluted with saline to prevent or to minimise the formation of post-operative adhesions to the reproductive tract especially the uteri and ovaries. The saline also was conveniently used for rinsing the surgical and other related instruments and facilities, if necessary. Normal physiological saline (0.9% NaCl) was prepared in a one litre bottle (Durran, Germany) by adding 9 g sodium chloride (0.9% w/v; Sigma-Aldrich Co., USA) in one litre of Milli-Q water (Reference, Millipore Asia limited, Malaysia). After preparation, the saline was sterilised by autoclaving and kept for 3 months in refrigerator (4^{o}C) for future use.

3.4.3.2 Preparation of Embryo Flushing Medium

The embryo flushing medium was used to fill the uterine horn or oviduct of the superovulated donor during embryo collection procedure. During flushing, the outflow of the medium was carried out by flushing the embryos into the collecting container. The flushing medium (300 ml) was prepared within 24 hours prior to embryo retrieval. The flushing medium consisted of PBS (phosphate-buffered saline; Oxford, UK) supplemented with 9 mg of antibiotic (gentamycin sulphate, G1264; Sigma-Aldrich Co., USA), heparin (9 mg) (G 0777; Sigma-Aldrich Co., USA) and 0.3 g bovine serum albumin (BSA, A6003; Sigma-Aldrich Co., USA) as shown in Table 3.1. Macromolecular BSA was added to the medium to prevent embryos from float or stick to the plastic containers. The flushing medium was filter-sterilised using syringe filter (0.22 µm pore size), aliquot into Terumo® luer slip syringe (50 ml) and kept warmed (38.5^{o} C) prior embryo retrieval.

Table 3.1: Composition of flushing medium (300 ml).

Chemical component	Final Concentration	Quantity/300 ml
Phosphate-buffered saline (PBS)	1 tablet/100 ml	3 PBS tablets dissolved in 300 ml Milli-Q water, sterilised by autoclaving
Gentamycin sulphate	30 µg/ml	9 mg dissolved in 300 ml PBS solution prior to use
Heparin	30 µg/ml	9 mg dissolved in 300 ml PBS solution prior to use
Bovine Serum Albumin (BSA)	0.1%	0.3g dissolved in 300 ml PBS solution prior to use

3.4.3.3 Preparation of Embryo Handling Medium

Supplementing handling media with high concentration of albumin will decrease surface tension, which reduces the tendency of the embryo to float and adhere to the

culture dishes used. A volume of 5 ml of handling medium (Emcare; ICPbio Ltd., NZ) was supplemented with 0.02 g of BSA (0.4%). The medium was prepared a day earlier before embryo recovery and filter-sterilized using syringe filter (0.22 µm pore size) and equilibrated overnight in CO_2 incubator (5%) prior to use.

3.4.4 Experimental Procedure

3.4.4.1 Management of Donor and Recipient Animals

Donor and recipient does were selected based on ISB farms record to ensure only cyclic, good body condition and diseases free animals were chosen for this study. Fertile bucks with good health and libido were identified and used for natural mating. Selected animals were kept in separate group or individual pen at least for one month before oestrus synchronisation to prevent any unnecessary stress resulted from new animals mixing. Different coloured-paint spray marker was used for identification of treated animals in addition to ear tags. One dedicated pen was used as natural mating and fasting pen.

3.4.4.2 Oestrus Synchronisation of the Donor and Recipient

Requirement for synchronisation of oestrous cycles of the donor and recipients have been recognised as one of factors influencing the success of the embryo collection and embryo transfer. In this study, oestrous cycle of the donor and recipient does was synchronised by inserting the Controlled Intravaginal Drug Release Device (CIDR; 0.33 g natural hormone progesterone; EAZI-BREED CIDR, Pharmacia & Upjohn Limited, NZ) into the vagina of the does for a period of 13 days. The CIDR releases progesterone at a controlled rate into blood stream of the does. In other words, the progesterone is released by diffusion from silicone rubber elastomer moulded over a nylon spine which is shape to retain the device in the vagina. Daily monitoring of the device was

performed to ensured that it had not accidentally dropped from the vagina of the does. At 48 hours prior to CIDR removal, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha)}$, a luteolytic synthetic analogue of tromethamine salt (75µg Estrumate, Schering-Plough, Australia) was administrated (at 0700 hours) intramuscularly to regress the existing corpus luteum. Once luteolysis has occurred, the ovulatory follicle produced high levels of oestradiol which resulted the does to be in oestrus with subsequent ovulation. The oestradiol peak is responsible for triggering overt oestrus behavior in the does. Plasma oestradiol concentration returned to basal level at time of ovulation.

Ovulation in recipient does had been induced by the administration of 200-500 IU PMSG (FOLLIGON; Intervet International, Netherlands) at 48 hours prior to CIDR removal. The PMSG is a gonadotrophin which is high in FSH as well as LH activities. Due to its FSH-activities, serum gonadotrophin stimulated the growth of the interstitial cells of the ovary as well as the growth and maturation of the follicles. Consequence to LH-activities, PMSG induces ovulation in the recipients. In this study, PMSG was used to induced ovulation in recipient only because it is convenient and cheaper than FSH.

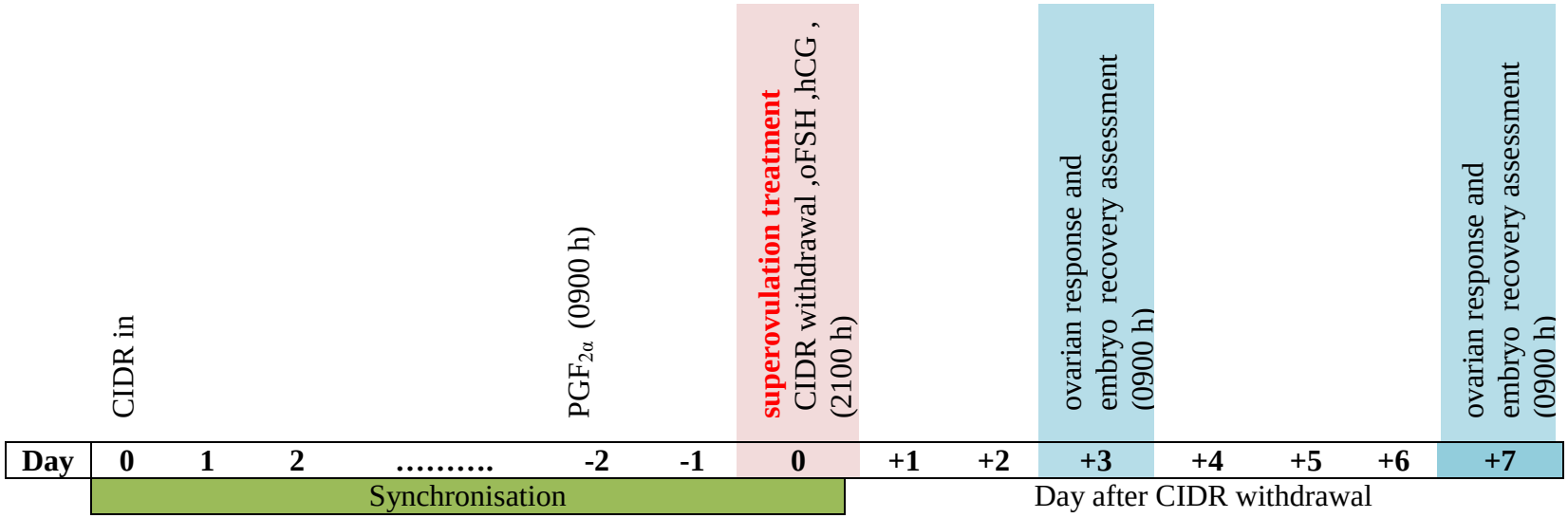

Figure 3.1. A schematic representation of the superovulation protocol performed on S group donor doe.
*Dosage of hormone treatments was described in the text.

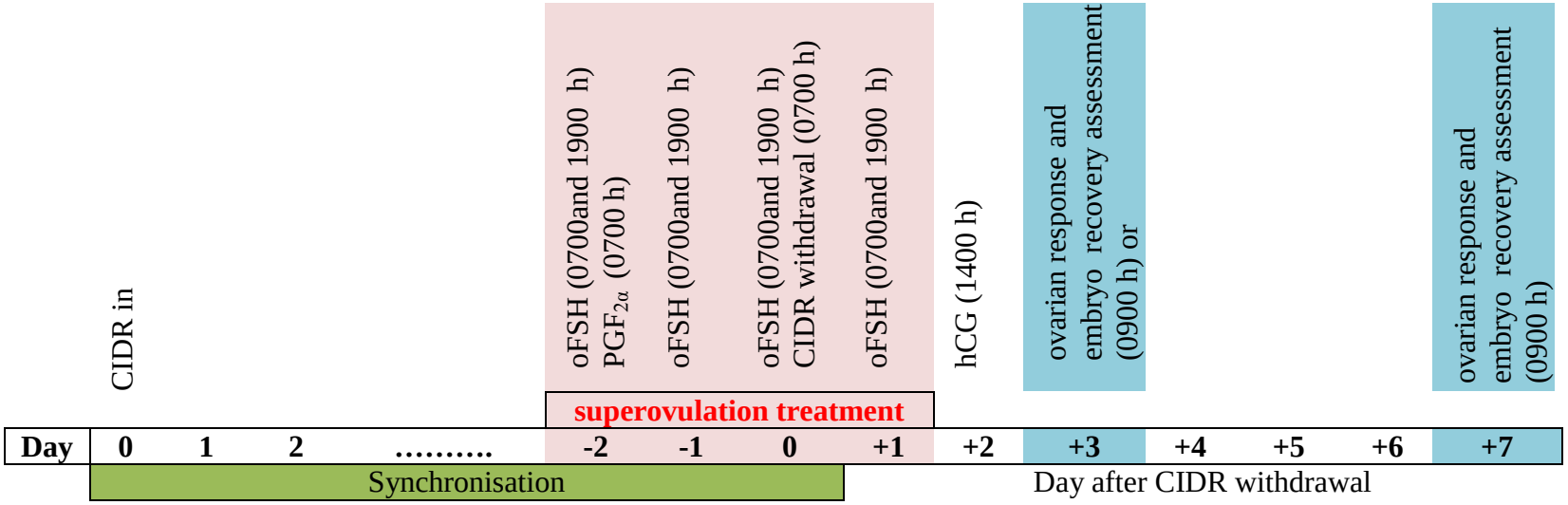

Figure 3.2. A schematic representation of the superovulation protocol performed on M_1 and M_2 groups donor doe.
*Dosage of hormone treatments was described in the text.

3.4.4.3 Superovulation Protocol of Donor

Superovulation protocol is a procedure to promote the growth and maturation of multiple follicles in the donors ovaries which leads to multiple-ovulation. In this present study, superovulation was obtained with gonadotrophin treatment consisting of NIADDK-oFSH-17, ovine pituitary Follicle Stimulating Hormone (oFSH; OVAGEN^{TM} , ICPbio Limited, New Zealand). A total of 35 synchronised-donor does were assigned randomly into 3 groups according to their superovulation protocol, S (n=13), M_1 (n=13) and M_2 (n=9). Donor goats in S group were subjected to the single administration protocol. This protocol was develop based on the existing protocol of our laboratory for LOPU (Rahman, 2008). M_1 and M_2 superovulation protocols were modification of protocol S, using multiple injection of two different total dosages of oFSH (8.8 and 14.1 mg). Superovulation treatment for goats in M_1 and M_2 groups starting 2 days before and finishing a day after CIDR withdrawal for 4 consecutive days at 12 hours intervals (at 0700 and 1900 hours). CIDR removal was concurrent with the fifth oFSH injection (at 0700 hours). For the purpose of synchronising the ovulation time, goats in group M_1 and M_2 received a i.m. injection of 250 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet, Netherland), 36 hours after CIDR withdrawal (0700 hours).

M_2 (n=9) does received highest total dosage of 14.1 mg oFSH distributed in 8 i.m. injections (twice daily, 12 h intervals) in decreasing dosages: 2.64, 2.64; 1.76, 1.76; 1.76, 1.76 and 0.88, 0.88 mg respectively. M_1 (n=13) does received a total dosage of 8.8 mg oFSH distributed in 8 i.m. injections in equal dosages (1.1 mg x 8), twice daily at 12 h intervals. The superovulation treatment protocol for donor goats in S (n=13) group starting upon withdrawal of CIDR (at 1900 hours), a single injection of gonadotrophin treatment (8.8 mg oFSH) and 250 IU hCG (Ovidrel®, PreFilled Syringe, Industria Farmaceutica Serono, S.P.A., Bari, Italy) were administered intramuscularly upon

CIDR removal (2100 hour). The protocols of oestrus synchronisation and superovulation are show in Figures 3.1 and 3.2**.**

3.4.4.4 Oestrus Detection and Breeding of Donor

The does were visually observed for signs of oestrus. Oestrus detection and matings were performed with fertile adult bucks at 24, 36 and 48 hours after CIDR withdrawal. Each doe was tested for oestrus using an individual buck bringing to her. Doe with standing heat was allowed to be mated with a fertile buck and remained together for 3 additional days in mating

3.4.4.5 Preparation of the Animals for Surgery

3.4.4.5.1 General Preparation

Surgical instruments and accessories used for surgery were classified into two categories based on the method of cleaning and sterilisation, i.e autoclavable instruments and non-autoclavable instruments. The autoclavable instruments included surgical sets e.g. forceps, scissors, curve haemostatic forceps, haemostatic forceps, scalpel, surgical gauze, hand towels, drapes for animal and surgical trolley, whereas non-autoclavable instruments included silicone tube for oviduct flushing, Foley catheter, syringe and needle. Other disposables used in the surgery included catgut suture, scalpel blade, sterile gloves, round-bottom test tube and petri dishes.

3.4.4.5.2 Preparation of Surgical Instruments on Surgical Trolley

On the day of surgery, autoclavable surgical instruments in the autoclave bag were ready to use after being autoclaved and dried completely in the oven (56^{o}C). Before opening a sterilised surgical pack, the sterilisation date, adhesive indicator tape (appropriate colour change for autoclaved instruments) and pack description should be

checked carefully. The upper wrapping of the surgical pack 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 drape. The air system of the Foley catheter balloon was checked to ensure the balloon will hold air. The Foley catheter was rinsed with sterile saline, and a plastic stylet was inserted into the catheter lumen. Surgical instruments were routinely placed in a sequential order so that items used first placed nearer to the surgeon.

3.4.4.5.3 Responsibility of the Surgical Team

In our laboratory setting, with limited surgical facilities, only certain sophisticated sterile procedures were performed, such as the requirements of all members to wear an appropriate clean laboratory coat (dedicated for surgery purpose only), face musk and sterile glove. However, all members involved in the surgery paid strict attention to aseptic techniques.

3.4.4.5.4 Sedation and Anesthetisation of the Donor Does

Identity tag of the donor does were determined, taken to the fasting pen and deprived of food and water at least 12 - 16 hours prior to surgical. On the day of performing embryo recovery surgery, the donor goats were prepared in an area separated from where surgery was performed. Surgery preparation was facilitated by first inducing anaesthesia to a goat with xylazine hydrochloride (0.22 mg/kg body weight) and ketamine hydrochloride (1.1 mg/kg body weight) via intramuscular injection. The anaesthesia goat was placed on clean small ruminant restraining cradle. The restraining table was set at 45^{o} angle, with the head of the animal lowered, to facilitate the laparoscopy procedure. Using clean gauzes, the abdominal area of the 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 abdomen of the goat was then covered with a sterile drape with an opening that exposed the disinfected bare skin and ready for the surgery. 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. While laparotomy surgery was in progress, the donor goat was maintained under anaesthesia with administration (i.m.) of ketamine hydrochloride (0.1 mg/kg body weight) at regular intervals (approximately 30 minutes).

3.4.4.6 Embryo Recovery

In this study, *in vivo* embryos were recovered through laparotomy surgical procedure, exposing the reproductive tract by a midventral incision under general anesthesia. The side of the flushing was determined according to the age of the embryos. Two types of flushing procedures were used, uterine horn flushing for recovering the embryo of age more than day 5 and oviduct flushing for the embryo, age below day 5.

3.4.4.6.1 Laparotomy Surgery Procedure

The anaesthestised donor goat was surgically laparotomised by making 4 cm mid-ventral incision on the skin 2 cm away from the udder using a scalpel blade (Size 15, BB 515, Aesculap, Germany). The incision was continued further on the underlying tissues and peritoneum layer along the linea alba. The uterine horns along the ovaries were exteriorised. The ovaries were examined for the present of corpora lutea (CL) and anovulatory follicles to evaluate the ovarian response of the hormonal treatments.

3.4.4.6.1.1 Uterine Horn Flushing

The 7 days old embryos were recovered by flushing the uterine horn with PBS (PBS; Dulbecco's phosphate buffered tablet, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 0.2% BSA (bovine serum albumin, Fraction V, Sigma Chemical Co.) from the base of the uterine horn towards the utero-tubal junction. A puncture wound was made on uterine horn near to bifurcation of the uterus using a small pair of haemostatic forceps (BH 104, Aesculap, Germany). A two way Foley Catheter was inserted through the puncture hole to a depth of 3 cm. The balloon of the catheter was sufficiently inflated for completely obstruct the lumen at the caudal end of uterine horn. Closer to the anterior end of the uterine horn, a Teflon intravenous (I.V) catheter placement unit fixed with a 20 gauge needle (Vasoscan, B. Braun, Germany) was introduced. Later the I.V. catheter needle was removed and 20 ml of flushing medium was introduced into the uterine horn using a 20 ml sterile disposable syringe (Terumo, Japan). Flushing medium was slowly flushed into uterine horn, the pressure caused the uterine fluid to gush through the Foley catheter, with enough turbulence to flush the embryos into collection tube attached at free end of the Foley catheter. This flushing procedure was repeated with additional 20 ml medium.

3.4.4.6.1.2 Oviductal Flushing

Genital tract of oestrus-exhibiting donor was exposed by midventral laparotomy surgery. Embryo recoveries were performed by flushing both oviducts with 2 ml flushing medium, supplemented with 0.2% BSA using a 2 ml syringe fixed with a 26 gauge needle at the utero-tubal junction towards infundibulum opening. The end of the utero-tubal was blocked with a thumb and forefinger digit to avoid backflow of the flushing medium. A 10 cm long silicone tube catheter (cut from Wallace embryo transfer catheter unit) was inserted through fimbriae into the infundibulum opening to a

depth of 1-2 cm and kept fixed by a small forceps. Flushing media was collected into container at the end of silicone tube for embryos isolation and assessment.

3.4.4.6.2 Isolation of Embryos

The proper procedure for classifying embryos is to isolate them, remove the debris, and separate them into transferable (viable) and non-transferable (non-viable) embryo groups. Before embryo retrieval, the stereo-microscope was attached to a heating stage at 38.5^{o}C prior to embryo search. When the collection container about 3 ml filled with flushing fluid, the container was sent to embryos processing laboratory and put on the test tube rack in the water bath (36^{o}C) for 5-10 minutes to allow the embryos to settle at the bottom of the container. About 2 ml of the fluid was then removed using disposable plastic pipette, bottom 1 ml of the fluid was swirled and poured into flat-bottom of searching plastic petri dish. Each container then was rinsed at least twice with 1 ml of handling medium to dislodge any retained embryos for the embryos flushed from oviduct. The flushing fluid was directly put onto warmed stereo-microscope stage for the embryos evaluation. The embryos recovered were first visually assessed under stereomicroscope (SZH10; Olympus Optical Co., Ltd, Japan). Once identified, the embryos were transferred immediately to fresh handling medium and embryos were washed thoroughly using 3 changes of washing medium. Viable and non-viable embryos were separated into different dishes. The detailed ooplasmic morphology of the viable and non-viable recovered embryos was recheck for confirmation and photograph under an inverted microscope (IX71; Olympus Optical Co., Ltd, Japan) at 20 x magnification (eye piece) and they were graded into 4 categories as Grade 1, Grade 2, Grade 3 and Grade 4 (refer to Table 3.3). Transferable embryos (Grade 1,2 and 3) were kept in 5% CO_2 incubator at 38.5^{o} until use.

3.4.4.6.3 Embryo Washing

Prior to collection, the embryos were exposed only to sterile condition of the donor oviduct or uterine environment. The possibility of contamination of the recovered embryo during handling in the laboratory can be minimised by employing a sterile environment. In the present study, the recovered embryos were collected in the first well of a Nunc four-well dish. To ensure the removal of the pollutant and pathogen that may present before the transfer of viable embryos into the recipient, the collected embryos were then washed 3 times by passing through the subsequent well before transferred to the microdroplet for incubation. During this process, care has been taken to avoid contamination from the instruments and glassware involved. The glass pipettes used for washing and loading the embryos were always kept under stelised condition. Embryo washing process was carried out under the laminar flow.

The washing culture dish for embryo retrieval was prepared using a 4-well culture dish containing handling medium (Emcare; ICPbio Ltd., N.Z) with 0.4% BSA equilibrated overnight and kept in incubator (5% CO_2) until used. As soon as embryos were isolated from flushing fluid, they were pipetted using micropipette in as small volume of fluid as possible into the first well of washing dish. No more than 10 embryos should be placed in a single well, and embryos from different donors were placed in different washing culture dishes. The embryos were agitated gently by siphoning and emptying movement of the micropipette. The embryos were then transferred to the second well with as little handling fluid as possible. The second well was agigated gently before the embryos were transferred to the third well. After the third wash. The embryos were separated into viable (transferable) and non-viable (unfertilised and severely degenerated) embryos groups. They were then transferred into the 100 µl microdrop covered with silicone oil and kept in 5% CO_2 incubator at 38.5^{o}for future use.

3.4.4.7 Assessment of Ovulation and Embryo Recovery

The ovarian response in terms of number of corpora lutea was assessed by laparotomy on day 3 or 7 after the CIDR withdrawal. Embryos were obtained by surgical access to the genital tract. The ovaries of the donor does were visually examined. The number of corpus luteum (CL) and anovulatory follicles on the right and left ovary were recorded. Goats were considered responsive to superovulation treatment when at least one or more corpora luteum present. The total number of structures recovered (embryos and ova) per doe flushed was evaluated and classified using a stereomicroscope according to their morphological appearance.

3.4.4.8 Assessment of Embryo Viability

The structure recovered during flushing was examined microscopically, photographed and evaluated under a stereomicroscope for identification and classification. The structures of recovered embryos were categorised according to their stages of development and quality using morphological criteria. The embryos were classified as unfertilised ova (no cleavage), degenerated embryos (embryos that not develop into the normal stage of development on day of recovery, 2- cell or earlier for Day 3 recovery and 8-cell or earlier stage for Day 7 recovery) or as viable (transferable) embryos of Grades 1, 2 and 3. Grade 1 embryos were morphologically intact and had an even granulation and cell distribution, Grade 2 were embryos with small deviations, e.g. a few exuded blastomeres, while Grade 3 embryos demonstrated an uneven cell organisation, a loosened structure, with numerous free blastomeres. Only embryos that were competent in their developmental stage and in viable grades (1, 2 and 3) were then transferred to the recipient. Table 3.2 shown the developmental competence of the recovered embryo based on flushing site and Table 3.3 shows the embryo grading based

on the morphologically appearance of the blastomeres and degree of cytoplasmic fragments.

Table 3.2. The development competence of the recovered embryo based on flushing sites (Jainudeen, Wahid and Hafez., 2000).

Flushing site	Stage of embryonic development	Days after ovulation
Oviduct	1 – Cell	0-1
	2 –Cell	0-1
	4 –Cell	1-2
	8 –Cell	2-3
	Early morula	2-4
Uterus	Compacted morula	4-5
	Early blastocyst	5-6
	Blastocyst	6-7
	Expanded blastocyst	7-8
	Hatching blastocyst	8-9

Table 3.3. Embryo grading based on the morphologically appearance of the blastomeres and degree of cytoplasmic fragment.

Embryo grade	Blastomeres	Cytoplasmic fragments
1	equal size	no
2	equal size	less than 20%
3	distinctly unequal size	between 20-50%
4	equal or unequal size dan degenerated embryo/ova	above 50%

3.4.4.9 Indexes of the Superovulatory Response

In this experiment, the following parameters were observed and recorded for each donor doe: number of corpora lutea (CL), number of anovulatory follicles (AF), total number of recovered structures (RS), number of viable embryos (Grade 1,2 and 3) and number of non-viable embryos (Grade 4 and degenerated). The rate of recovery (RR) was obtained by dividing, in every donor doe, the total number of RS by the total number of

CL. The rate of non-viable embryos (NVR) was obtained by dividing the number of NV by the total number of RS. The rate of viability (VR) results from dividing the number of viable embryos by the total number of RS. All rates are expressed as percentages for clarity of the manuscript. The responded donors were categorised according to their numbers of their ovulation, doe with 0 CL were considered as not response, 1-2 CL as ovulated doe and 3-10, 11-18 and more than 18 CL as superovulated donor doe. Does also were categories according to their numbers of anovulatory follicles (AF), 0, 1-2, 3-10, 11-18 and more than 18 CL AF.

3.4.4.10 Surgical Embryo Transfer

Two surgical methods of embryo transfer were performed in present study, that is the uterine and oviduct transfers, depending on the developmental stage of the viable embryos. The oviduct transfer was performed to transfer viable embryos that were collected on day 3 or before (1, 2, 4, 8, 16 cells stage embryos) or uterine transfer for embryos that were collected on day 7 and after (morula and blastocyst). Recipient was sedated and prepared as donor doe before surgery. A mid-ventral incision was performed and the reproductive tract and ovary were exteriorised for CL examination. If at least one corpus luteum (CL) was present, the embryos were transferred to the side that ipsi-lateral with it. The embryos would be deposited into both sides (ipsi- and contra-lateral). Two to four embryos were transferred to each deposition site, depending on availability and quality of the recoverred embryos.

3.4.4.10.1 Uterine Transfer

Surgical embryo transfer (ET) was done in the recipient does that were responsive to synchronisation and superovulation treatment. A 2 cm slit was made with a scalpel blade (size 15, BB 515, Aesculap, Germany) at the upper side of the does abdomen near

to the udder. The reproductive tract and ovaries were located and exteriorised over the surface of the abdomen. The ovaries of the recipient were evaluated in the presence of CL. The embryos were deposited near the tip of the same uterine horn, ipsi-lateral to the corpus luteum. One to four fresh *in vivo* embryos were loaded into a Wallace embryo transfer catheter unit attached to a 1 ml syringe along with a small amount of holding medium (about 0.1 ml). The tip of the embryo transfer catheter was inserted through a small puncture hole on the uterine horn, made by using a 18 gauge hypodermic needle (Terumo, Japan). The embryos were carefully deposited into the lumen of anterior part of the uterus horn (2–4 cm from the utero-tubal junction). The procedure was repeated to the other uterus horn if necessary. The incision was then closed, using three layers of sutures.

3.4.4.10.2 Oviductal Transfer

A 2 cm slit was made with a scalpel blade (size 15, BB 515, Aesculap, Germany) at the upper side of the does abdomen near to udder. The oviduct tract and ovaries were located and exteriorised over the surface of the abdomen. The ovaries of the recipient were observed for the presence of CL. The anterior end of oviduct tube was identified and the fimbria was raised with the small forceps to ease the insertion of the tip of embryo transfer catheter into infundibulum opening. Two embryos from the storage culture dish were aspirated using Wallace embryo transfer catheter. The tip of the catheter tube was then inserted through infundibulum opening. The embryos were carefully deposited into the oviduct through infundibulum with a depth of 2-3 cm. The incision was then closed using three layers of sutures.

3.4.4.11 Post-surgical Treatments of Does

The donor and recipient doeas were sutured using catgut (size 2, Aesculap, Germany) and Terramycin antibiotic power (Pfizer, New York) was spread on to the sutured surface. The surgical was sprayed with Gusanex aerosol (Dichlofenthione; Coopers, Pitman Moore, Malaysia) as a fly repellent. An antibiotic injection containing 200 mg Oxytetracycline/ml (Terramycin/LA, Pfitzer, New York) was given intramuscularly at a dosage rate of 1 ml per 10 kg body weight and continued every 4 days for 2 weeks to avoid post-surgical infection. To prevent multiple pregnancies in the donor, 2 ml $PGF_{2\alpha}$ was given i.m. after surgery or a day after.

3.4.4.12 Pregnancy Diagnosis

Pregnancy of the recipient does was diagnosed at 30 and 60 days after ET. A real time ultrasound scanner (Aloka SSD500V, Tokyo, Japan) equipped with a 5.0 mHz linear array transducer for the transabdominal approach was used. The coupling agent for ultrasound transmission used was a carboxymethylcellulose gel as contact fluid. The recipient doe to be scanned was restrained in standing position in a handling crush. The abdominal and inguinal hair at the right side of the doe approximately 5 cm in front of the rear leg and 2.5 cm above the udder were shaven properly to expose the skins. The contact gel was applied both to the shaven area and on the transducer. Then the transducer was aimed approximately 45^{o} upwards and forward for the uterus to be scanned across. The transducer was placed then moved carefully into the gel-skin area to eliminated the production of the air bubbles between transducer and skin. The doe was considered as pregnant when amniotic fluid and foetuses displaying heart-beat was observed.

3.5 EXPERIMENTAL DESIGN

The primary objective of this study is to compare the goat ovarian response of 3 different oFSH superovulation protocols. The study not only focuses on the aspect of ovarian response but also embryo recovery and transfer. Figure 3.3. illustrates the schematic overview of the experimental design used in this study.

3.5.1 Effect of Dosage Level and Administration Patterns of oFSH on Ovarian Response in Superovulated Donor Does

The objective of this experiment was to analyst the ovarian response of superovulated donor goats by comparing 3 different oFSH superovulation protocol. In this experiment, a total of 35 healthy does were randomly selected and subject to oestrus synchronisation. The oestrus synchronised does were divided into 3 groups, S (n=13), M_1 (n=13) and M_2 (n=9) for gonadotrophin (oFSH) superovulation treatment. Donor goats in S and M_1 received the same total dosages of low level oFSH (8.8 mg), given in single (S) and multiple equal dosages (M_1) injection. Meanwhile donor goats in M_2 group were received multiple injection of the high level of oFSH (14.08 mg) in decreasing dosages (see section 3.4.4.3 for detail of superovulation protocol). Ovarian response indicators such as ovulation (CL) and anovulatory follicles were observed during embryos recovery on day 3 or 7 after CIDR removal.

3.5.2 Effect of the Breed on Ovarian Response in Superovulated Donors Does

This experiment was conducted to compare the ovarian response and the quality of embryos recovered in 3 different breeds of local donor goats. In this experiment, all the donors in experiment 1 were identified and further divided according to their breed, i.e. local mixed-bred, Boer crossbred and Jamnapari. The ovarian response and embryos

produced, subsequent to superovulation treatment were analysed for the effect of the breeds.

3.5.3 Effect of the Body Weight on Ovarian Response in Superovulated Donors Does

This experiment was conducted to compare the ovarian response and the quality of embryos recovered in 2 different body sizes of local donor goats. In this experiment, all the donors in experiment 1 were identified and further divided according to their body weight, 20- 30 kg and 31-50 kg. The ovarian response and embryos produced, subsequent to superovulation treatment were analyzed for the effect of the body.

3.5.4 Effect of the Day of Embryo Retrieval on the Stages of Embryonic Development and the Quality of the Embryos

The objective of this experiment was to compare the stages of embryonic development of recovered embryos from 2 different day of recovery and to investigate the quality of the *in vivo* embryos produced. In this experiment, 12 superovulated does were successful flushed on Day 3 or Day 7 after CIDR withdrawal. The ovarian response and embryos recovery parameters such as the total stimulated follicles, ovulation, anovulatory follicles, structures (embryos and ova) recovered, stages of development, quality of embryos and degenerated ova/embryos were observed in this experiment.

3.5.5 Effect of Sources of the Embryos on Goats Embryo Transfer

This experiment was conducted to produce gestation and kid from *in vivo* and ICSI embryos transfer. Thirteen synchronised does with at least 1 CL present on either side of the ovaries were used as recipients in this experiment. Each recipient doe received not more that 4 embryos in each laparotomy transfer session. Out of 13 recipient does, 5

received a total of 8 ICSI embryos at morula stage of development that were produced in our laboratory by other co-researcher (Rahman, 2008) and 8 recipient goats received 32 *in vivo* embryos at different stages of development. For gestation status, all recipients subjected to ultrasound pregnancy diagnosis at 60 days after embryo transferred. Litter size was observed during kidding.

3.6 STATISTICAL ANALYSIS

The data presented in various experiments in the present study were mean±standard error of means (mean±SEM) and were analysed using one-way analysis of variance (ANOVA). The effects of different factors on number of corpora lutea (CL), number of anovulatory follicles, total number of recovered structures (RS), number of viable embryos (NF) and non-viable embryos, were compared and the significant differences between the means were further analysed using Duncan Multiple Range Test (DMRT) to show the specific differences among the factors on the parameter measured and $P<0.05$ was considered significant. The analysis was carried out with SPSS (Statistical Packages for Social Sciences) for windows, version 17, IBM Inc, USA.

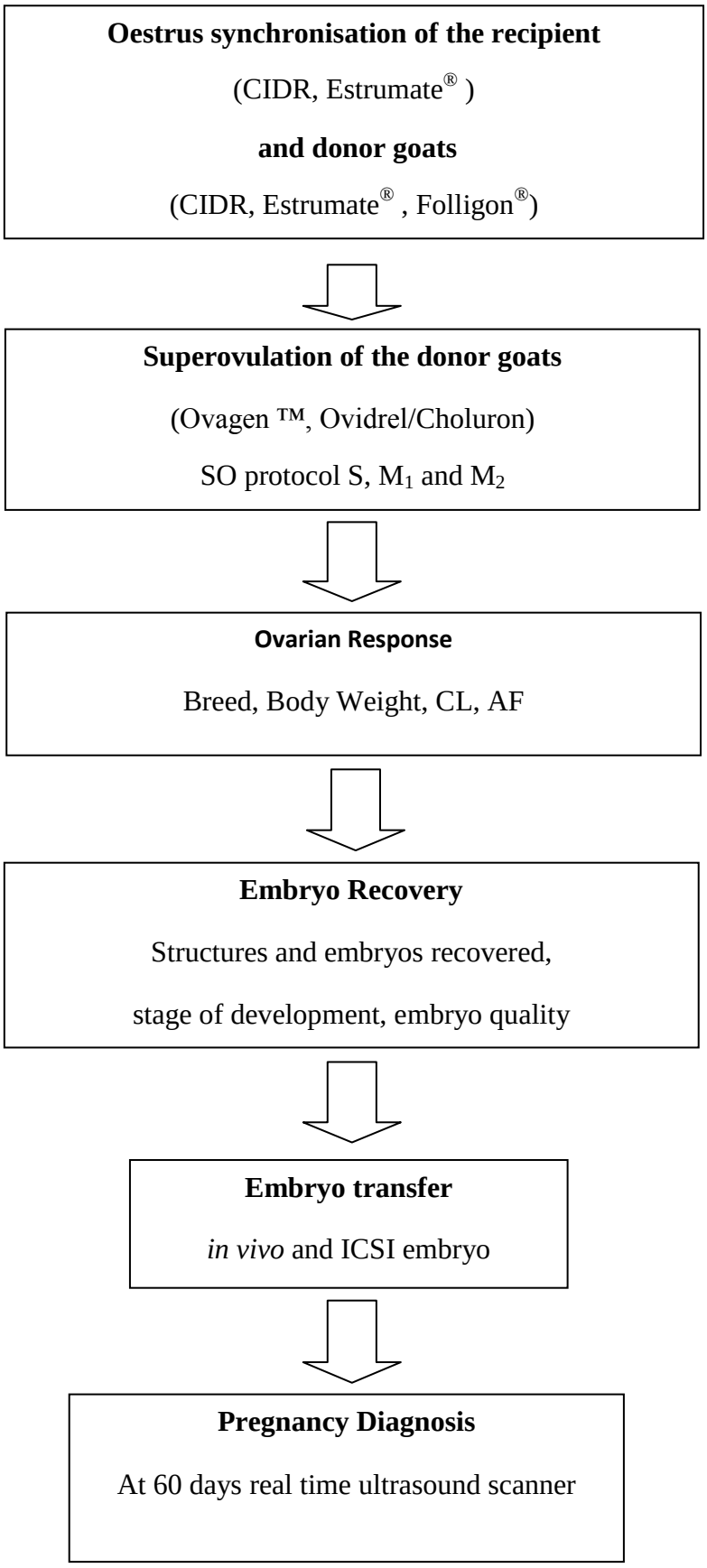

Figure 3.3. A schematic overview of the experimental design for the effect of superovulation on avarian response and subsequent embryo transfer in goats.