Selection of Oocytes for *in Vitro* Maturation

Once the COCSs are located within the Petri dish during searching under a microscope at X10 objective, the COCs were picked using micropipettes with a bore diameter of about 200 µm and transferred into a dish of fresh pre-warmed TALP- Hepes washing medium. The plastic tip used in picking the COCs must have a bore diameter wide enough to avoid disruption of the cumulus cells surrounding the oocytes. The presence of cumulus cells surrounding the immature oocytes is a prerequisite both for successful maturation of the oocyte and for embryo competence.

Only oocytes with compact multilayered cumulus investment and evenly granulated cytoplasm should be selected for *in-vitro* maturation. It should be noted, however, that the aspirated COCs may have a differences in their appearances [11]. Some will be denuded (with no cumulus cells attached to the zona pelucida), others being partly denuded (with one sided swollen cumulus cells, and others with an already expanded or spider-like cumulus cells. Others will also have uneven cytoplasm. Not all oocytes should be used for IVEP because they lack the potential to undergo normal maturation and will eventually end up as degenerated COCs after *in-vitro* fertilization. Further details on IVEP of Boran cows were described by Muasa in 2010, which contains a chapter providing guidelines on COC grading for IVEP [11].

***In Vitro* Maturation of COCs**

Prior to transfer to an *in vitro* maturation (IVM) medium, selected COCs must be washed 2-4 times in fresh pre-warmed TALP -Hepes medium and finally washed in *in-vitro* maturation medium. The medium used for *in-vitro* maturation (IVM) of oocytes varies among laboratories. It should be considered that the culture medium employed in IVM may affect the proportion of oocytes that reach metaphase II and become capable of undergoing fertilization. It also influences subsequent embryo development
[12]. The culture media employed in maturation of oocytes can be broadly divided into simple and complex.

Simple media are usually bicarbonate-buffered containing basic physiological saline with pyruvate, lactate and glucose. The main difference between various types of simple media is in their ion concentration and energy sources. The media are generally supplemented with serum or albumin, with trace amounts of antibiotics added (penicillin, streptomycin, gentamicin).

Complex media, on the other hand, contain, in addition to the components of the simple media, amino acids, vitamins, purines and other substances, mainly at levels they are found in serum. Fixed nitrogen is present as free amino acids.

This study used a widely-used complex media for IVM of cattle oocytes: Tissue Culture Medium 199 (TAC-199) with Earle's salt, L-Glutamine and 25 mM HEPES supplemented with 10% heat inactivated fetal calf serum (this can also be calf serum, steer serum or serum from cow in estrus), and supplemented with 1ug/ml of follicle stimulating hormone (FSH), Luteinizing Hormone (LH) and estradiol.

Other IVM media can be used such as Synthetic Bovine Oviductal Fluid medium and others. All others, however, require further supplements in order to promote maturation.

During IVM, extensive redistribution of intracellular organelles occurs. In addition to extensive cumulus expansion that occurs during maturation of oocytes during IVM, the mitochondria migrate to occupy a perinuclear location, and the cortical granules migrate outward to lie just beneath the vitelline membrane of the oocyte. At all times, the critical requirement of successful oocyte maturation is the capability to undergo normal embryonic development after sperm penetration and fertilization. An extensive description on how to evaluate and detect matured oocytes after IVM has

been described in depth by Muasa [11]. However, the following are the important considerations one must take into account during IVM procedure:

a) Water quality

Water is the major constituent of any IVM medium and due to the presence of several basic contaminants such as ionized and non-ionized solids and gases, particulate matter, microbials and pyrogens, the use of ultrapure water is highly recommended.

b) Buffering Systems and Osmolality

The pH of the IVM media changes drastically whenever exposed to non-CO_2 controlled atmosphere. Hepes or phosphate-buffered media for short-term work with oocytes and embryos does not require a carbon-dioxide controlled gas phase to maintain a relatively constant pH. For this reason, these media should be commonly used for washing, and for storage of oocytes and embryos outside the incubator. The optimum range of osmolality of the IVM medium should range between 275 and 285 mOsmol. If the osmolality of the IVM medium is not within this range, it should be discarded and a new one prepared. In some instances, the measured osmolality of a medium is less than the calculated value. This can be attributed to the incomplete dissociation of ions once they reach mM concentrations. In our system we utilized TALP-Hepes as washing media and osmolality of IVM made was 280 mOsmol.

c) Flux Culture

This involves the regular, gentle agitation of the culture dishes to prevent the attachment and subsequent differentiation of cumulus cells within maturation tubes. Micro droplets of IVM media covered by oil offer advantages including preventing or reducing the evaporation of water, protection from microbial contamination, attenuation of temperature and gas fluctuations, and ease of examination during culture period. This practice is advisable.

d) Maturation Time

Some discrepancies exist in the length of time needed for IVM of cattle oocytes. The culture medium and the supplements used, as well as the quality of oocytes, influence the length of time required before oocytes attain the maturation stage (Metaphase II). This study showed that while there was no significant difference between maturation period in terms of maturation and cleavage rates, there was a significant difference in final blastocyst yield in favor of the 24 h maturation period. Thus, the 24 hr maturation period for IVM of oocytes is recommended.

e) Other considerations

i) Antibiotic cover

This provides cover against growth and proliferation of microorganisms during the period of IVM culture. The concentration of antibiotics must be non-toxic and their inclusion in the medium needs to be combined with rigorous standards of hygiene in the IVM laboratory.

ii) Light environment

As part of routine maturation, fertilization and culture, the cattle embryos are inevitably exposed to varying amounts of light. The lesions caused by prolonged exposure of high light intensity under the microscope may contribute to embryo development failure. In principle, oocytes and embryos should not be over exposed to light longer than is necessary. Maturation and other events normally occur under conditions of darkness in the animal's reproductive organ and in our case, the laboratory windows were covered with opaque curtains.

iii) Temperature and Gas phase

The success of IVEP is temperature-dependent. Changes in temperature exposure to oocytes can lead to temperature shocks that can induce chromosomal abnormalities. The temperature range of 38 - 39°C is found to be ideal for cattle oocytes during IVEP. The incubation temperature was set at 38.5°C in all IVEP procedures. Gas phase was also observed to have a very big influence on the success of Boran IVEP. Development to the morula and blastocyst

stage of the embryo was found to be highly dependent on the gaseous environment of the incubator during IVEP. The best is an environment of 5% CO_2, 5% O_2 and 90-100% humidity.

iv) Culture supplements

The supplements usually added to the culture medium are the following:

v) Proteins

The most commonly used protein source is serum added in a concentration of 5 to 10%. Proteins in serum have macromolecules attached such as hormones, vitamins and fatty acids, as well as chelated metal ions and pyrogenes. Bovine serum albumin was used for protein supplement. The role of protein in the culture media may not only be a fixed nitrogen source, but also as a chelator of toxic metal ion. In this system we used 10% fetal calf serum.

vi) Hormones and Growth factors

Some of the hormones used as supplements include FSH, LH, oestradiol and prolactin. The direct action of these hormones on oocyte maturation and early development of embryo are not well established, but their addition to the culture media improves development of pre-implantation stage embryos. Growth factors added include IGF (insulin-like growth factor) and EGF (epidermal growth factor). These growth factors have mitogenic effects and also stimulate RNA and protein synthesis. In our? case we only used 1µg/ml of FSH, LH and estradiol.

vii) Culture Apparatus

In thsese experiments, it was observed that the type of culture apparatus used for *in vitro* culture of oocytes and embryos affected the outputs of *in vitro* maturation and also development of pre-implantation stages of the embryos. The use of ordinary Petri dishes was not as beneficial as the use of the Nunc 500um deep holed petri dishes, which was found to greatly enhance embryo development.

Sperm Treatment and Capacitation

To ensure a successful fertilization of the oocytes during IVEP, the sperm cells that are used must be viable, motile, capacitated and capable of expressing acrosome reaction. Thus, the cells must bind to the zona pellucida of the oocyte and pass through its vitelline membrane. This is only possible if the cells are able to fuse with the oolemma and be incorporated into the oocyte. This ability is normally acquired by the sperm cells as they pass through the epididymis through maturation process and within the female genitalia through the process of capacitation.

The process of capacitation is critical for fertilization. Only capacitated cells can undergo acrosomal reaction, a vital step that aids the cell to penetrate the ova to achieve fertilization. Capacitation is a process that involves complex biochemical and physiological changes within the sperm cell. During this process, gradual removal or alteration of the acrosomal protective coat from the sperm surface occurs. This then permits exposure of acrosomal enzymes needed for effective penetration of the ova and also exposure of sperm cell receptors that aid in binding onto the zona pellucida receptor sites.

In the laboratory, capacitation procedures are aimed at stimulating the sequence of events that normally occur in the female reproductive tract. The seminal proteins and any dead cells within the ejaculated and/ frozen semen are removed thorough either a process of swim-up through capacitation medium or by centrifuge process through elevated ionic strength. The following are the components of an effective capacitation medium.

a) Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) plays a key role in removing cholesterol and/or zinc ion from the sperm cell. Bovine serum albumin protein has considerable binding capacity for both cholesterol and zinc molecules. Cholesterol and zinc usually stabilize sperm cell membrane and must be removed during capacitation to enable acrosomal reaction and penetration of the ova.

b) Heparin

Heparin is a glucoseaminoglycan molecule added to the medium to mimic *in-vivo* heparin-binding proteins present usually present in female genitalia during capacitation. Heparin proteins play a role in fertilization by inducing the process of capacitation.

c) Caffeine

Caffeine is a cyclic nucleotide phosphodiesterase inhibitor used as a motility-stimulating agent in bull sperm. When caffeine inhibits phospodiesterase within the sperm cell the intracellular accumulation of cyclic adenosine monophosphate (cAMP) occurs resulting into activated sperm motility.

d) Calcium Ionophore

Calcium is added to increase calcium ion (Ca^{2+}) content of the medium to facilitate sperm motility and ova penetration during fertilization.

Swim-up technique of sperm capacitation

In-vivo sperm capacitation usually takes about 6 h within the female reproductive system. In the laboratory, we conducted a swim-up technique for this process.

Prior to swim up, the semen is evaluated by placing a drop on a pre-warmed glass slide and checked under a microscope for vigour and motility. Only semen that attained over 50% motility was used. For swim-up, 0.25 ml of the semen was then pipetted and gently released at an angle of 45° at the bottom of the 10ml sterile centrifuge tube containing 1.0 ml of swim up -TALP medium as shown in figure 2 above.

The swim up was done in a 5% CO_2 buffered incubator for 60 minutes at 38.5°C to allow the motile sperm to swim up through the medium as they also at the same time undergo capacitation [13]. After one hour, about 0.8 ml of the upper layer of the medium (supernatant) with the vital sperm, was pipetted into a 15ml-apirogenic tube and centrifuged at 10,000 rotations per minute (RPM) for 5 minutes at room temperature. The supernatant was then discarded and the sperm cell pellet located at the bottom of the tube was re-suspended with 100ul of fertilization (Fert-TALP) medium.

***In Vitro* Fertilization (IVF)**

Successful IVF can only take place with appropriate preparation of sperm and oocyte, and also in culturing conditions that are favorable to the metabolic activity of the male and female gametes. In cattle, IVF of *in vitro* matured oocytes is usually carried out 17-24 hours within an incubator set at 38.5^{o}C, at 95-100% humidity and 5%CO_2 [14]. *In vitro* fertilization (IVF) is carried out in a 100 µl micro droplets of IVF medium containing sperm cells at 1 x 10^6 sperm/ml concentrations, to fertilize 10-15 oocytes. In order to avoid going into the laboratory at odd hours, it is recommended, as we practiced, to conduct IVF for 24hrs [15]. The oocytes were processed by washing twice in pre-warmed IVF medium.

Sperm preparation for IVF

The right concentration of the sperm was determined by use of a Neubauer chamber using a 1:20 dilution factor as previously described [16, 17, 18]. Five micro litre of the capacitated semen after swim up was mixed with 95ul of water in order to kill the sperm and also achieve a 1:20 dilution factor. Then this was mixed thoroughly and loaded into both sides of Neubauer chamber and the numbers of sperm cells in both fields of the Neubauer chambers counted under a microscope. The total number of sperm cells counted was used to determine the spermatozoa concentration (sperm cells/ml). Experience indicated that whenever using a dilution factor of 1:20 in Neubauer chamber to calculate sperm concentration for IVF, a final concentration of 1 X 10^6 sperm/ml can be obtained by using semen volume equivalent to the volume of IVF droplet divided by the number of counted cells after taking into account the percentage of the post-thaw motility [16, 17]. Thus Volume of semen to use = volume of IVF droplet / (no. of counted cells /10 x % post thaw semen motility).

The calculated final volume of semen was then drawn and added to a droplet of IVF to be part of the 100ul of the final IVF droplet. Then the oocytes are added in the subsequent volume of IVF remaining to make up the required final 100ul IVF droplet.

In-Vitro Culture (IVC) of Embryos

In this system the zygotes were co-cultured with a monolayer of their cumulus cells. The presence of a somatic cell monolayer in the culture medium during in -vitro culture of the developing embryo enhances its developmental potential. The layer supports and provides the developing zygotes with a comfortable environment by secreting some growth factors to enhance their development. It is important to use culture medium that has been pre-conditioned in the incubator at least 1-2hrs prior to *in vitro* culture (IVC).

In vitro culture (IVC) is usually carried out for seven days. At the third day, in order to remove any metabolic waste resultant during cumulus-oocyte complex maturation and provide the embryos with a fresh medium that could sustain their metabolic requirements, half of the medium is drawn out and another fresh one added [17,18,19].

Before IVC, oocytes are washed 2-4 times in a washing medium (culture medium) to remove the excess sperm cells attached to the zona pellucida. It is important to remove excess sperm cells to avoid the presence of dead and denaturing cells that may contaminate the IVC medium [20]. In vitro embryo culture, like other procedures of IVEP is carried out inside a water-jacketed incubator with 5% CO_2 at 38.5°C.

It is advisable to check for cleavage rate three days (72 hrs) after the initiation of IVC whenever changing the IVC medium. At that time, most of the zygotes will be over 5-cell stage. Blastocysts used during embryo transfer are expected to be formed at day seven of IVC. During the evaluation times, gentle shaking of the culture dish is advised to allow for a uniform environment among embryos, and to allow for unified distribution of any autocrine growth factors that the embryo may have secreted. Also recommend that oocytes that have not cleaved are removed from the culture droplets at those times.

Other Important Considerations for Boran IVEP

a) Cleaning of Glassware

Cleanliness of the glassware is of utmost importance. Used glassware is rinsed thoroughly with tap water and soaked in detergent overnight. The following day, glassware is rinsed thoroughly at least 20 - 40 times in tap water, and then submerged in a 3-5% hydrochloric acid (HCl) for a minimum of 2 hours. It is then rinsed in running water for about 15 minutes, and rinsed again 20 - 40 times in tap water. Thereafter, glassware is rinsed with distilled water and placed upside-down to dry by heating it at 120°C for 2 - 4 hours. Glassware is then packed separately in aluminum foil and sterilized by heating at 120°C for 4 hours.

An ultrasonic cleaner is also used for heavily soiled and difficult to wash glassware such as volumetric flash, test tube and volumetric pipettes. Cleaning by sonication takes around 15 minutes in hot water. After sonication, glassware is rinsed 20 - 40 times with

tap water and finally rinsed with distilled water. Thereafter, the drying and sterilization protocol described above is practiced.