TECHNICAL ASPECT OF *IN VITRO* EMBRYO PRODUCTION

Danilda H. Duran
Embryo Biotechnology Laboratory
Philippine Carabao Center, National Headquarters and Gene Pool
Muñoz, Nueva Ecija 3120 Philippines

ABSTRACT

This Bulletin discusses in vitro embryo production of water buffalo. Ovaries are collected from slaughtered females of genetically superior breeds. The Bulletin gives details about the procedures for oocyte recovery, in vitro maturation and fertilization. Finally, the techniques for embryo culture are described.

INTRODUCTION

Conventional embryo transfer technology is based on the superovulation of high-quality donor animal and the subsequent recovery of embryos by flushing the uterus a week after breeding to the bull of choice. In cattle, this technology is well established, but in water buffalo, considering the poor ovulatory response of this animal, the application of the technology is difficult. Further understanding of the physiological behavior is required, and improvements of treatment regimes to improve the ovulatory response. However, the need for genetically superior embryos is inevitable, given the need for genetic improvement in most Southeast Asian countries. Hence, we need to improve the system to make genetically superior embryo available for transfer.

An alternative to conventional superovulation procedures is *in vitro* embryo production. This technology allows the predictable supply of embryos from ovaries of slaughtered females or from live selected animals, via repeated recovery of primary oocytes. To date, this technique has given considerable success in both cattle and buffalo, achieving a success rate ranging from 30 - 50% development of preimplantation stage embryo *in vitro*. This technology does not only offer optimization of high-quality dams, but also allows the preservation and rapid multiplication of genetically superior characters by making embryos available for cloning, sexing and nuclear transfer.

This paper discusses the technical aspects of the procedures involved in the production of embryos *in vitro,* with special reference to the techniques used at the Embryo Biotechnology Laboratory of the Philippine Carabao Center.

PREPARATION OF MEDIA AND STOCK SOLUTIONS

Preparation of media and stock solutions for *in vitro* embryo production requires a sterile technique and accurate and careful weighing of components. The use of a Laminar flow cabinet is indispensable to avoid any contamination that may alter and spoil the prepared media. An analytical balance with readability of at least 10 µg and accuracy of ±0.1 µg is very important. All components of the medium must be of the highest grade.

The following are important factors for the preparation of culture media:

Water Source

A Millipore-Q system or its equivalent is used to produce high quality pyrogen-free water (Milli-Q) for the preparation of media. In our system, we used distilled water to pass through the Milli-Q system. Water for media preparation should have a resistance of >18 megahms-cm.

Glassware

Glassware for media preparation should be designated solely for this purpose. Glassware to be

Keywords: embryo, embryo transfer, *in vitro* fertilization, *in vitro* maturation, IVEP, IVM

used must be sterile and pass the standard tissue culture cleaning standards.

Use of Disposable Supplies

In many occasions, the use of disposable supplies such as Petri dishes, acrodisc filters, centrifuge tubes for media storage and pipette tips are indispensable for media preparation. This makes the *in vitro* embryo production (IVEP) work rather expensive. However, if a large number of oocytes are attended and development of pre-implantation embryo is increased, the cost is still minimal. The use of disposable materials is more beneficial and convenient in terms of eliminating contamination and ensuring purity and safety of the prepared media.

Filtration

Filtration of the prepared media is a pre-requisite before using or before storage. The use of a 0.22µm filter is recommended, considering that the size of the smallest bacteria is 0.3µm. It is necessary, however, to discard the first few drops of the filtrating medium to remove any contaminants from the filter and the container before the succeeding volume is finally filtered and stored.

Sterility within the Culture and Manipulation Area

The culture media used for the culture of oocytes and embryos are very prone to contamination. The humidity and temperature inside the incubator are factors that enhance the growth of many foreign organisms encountered by the culture media. Contamination could come from the air, from surfaces of the working area and from any materials used in the preparation of the culture media. Therefore, it is necessary to implement the following measures to avoid contamination of the culture medium for oocytes and embryos.

Workers should cover their mouth and nose, especially when talking during the preparation of *in vitro* culture media.
Disinfectant such as 70% alcohol should be applied to the working surfaces before the preparation of the culture media.
Hands must be washed and sterilized before handling the materials for media preparation and manipulation of oocytes and embryos.

Storage and Maintenance of Media and Reagents for IVEP

It is important not to compromise the success of IVEP by using expired media and reagents, or by improper handling and storage. It should be noted that media used for IVEP usually have a short shelf life, so the expiry date must be observed, and the media must be kept in a proper storage environment. To maintain the viability and efficacy of media and reagents, preparation of aliquots is recommended to avoid frequent opening and possible contamination. Storage containers should be sealed thoroughly to avoid any moisture contamination that may alter the pH or osmolarity of the culture medium.

COLLECTION OF OVARIES

For purposes of genetic improvement, ovaries must be collected from genetically superior breeds. Ovaries are collected within 10 minutes after the slaughter of the animals, and kept in a sealed container containing physiological saline (0.9% NaC1 with 100 µm/ml Streptomycin and 100 IU/ml Penicillin), at a temperature ranging from 28-33°C. In other reports, storage at 20°C is preferable, but in our experience, such low storage temperatures can reduce the development potential of the oocytes. Careful observation of the temperature of the saline is important to maintain the viability of the ovaries during the period of collection.

The question of the time interval between animal slaughter and oocyte recovery from the ovaries and the temperature of the holding medium is an important one. In general, if the time spent from ovary collection to oocyte recovery is short, 30 - 33°C temperature of the physiological saline is better. However, in cases where the slaughterhouse is distant from the laboratory, a decrease in the temperature is recommended, but the temperature should still be kept above 28°C to maintain the development potential of the oocytes. Prolonging the period of ovary collection may significantly affect the viability of the oocytes for *in vitro* maturation. It is recommended that oocytes should be collected from the ovaries within a 6-hour period. Beyond six hours, development potential is greatly compromised.

OOCYTE RECOVERY

Before oocyte recovery, it is necessary to wash the ovaries in fresh, sterile physiological saline (without antibiotics) to further remove any contaminants. Briefly rinsing the ovary in 70% ethyl alcohol is recommended, to eliminate surface organisms before processing begins. The ovaries are dried lightly with sterile paper towels before primary oocytes are recovered from 2-8 mm vesicular follicles to avoid contaminating the aspirates during oocyte retrieval. It is important to remove the big follicles (beyond 9 mm) because they contain secretions that cause jelly formation in the aspirates. This may affect the retrieval of oocytes during searching.

Recovery of the oocytes from the ovaries may be done by:

- Oocyte aspiration,
- Slicing the ovaries, *or*
- Follicle dissection from slaughtered animals, *or*
- Ovum pick-up from live animals.

For the ovaries collected from slaughtered animals, the aspiration method is commonly employed because of the convenience associated with its application. Aspiration of oocytes is done using a needle attached to a 10-ml syringe. To avoid disruption of the surrounding cumulus cells, an 18-gauge needle is used. Possible toxicity associated with syringes containing rubber plungers and siloxane lubricants is avoided by washing and sterilizing glass syringes under the stringent conditions used for tissue culture glassware. For livestock, the use of plastic disposable syringes is acceptable.

One of the difficulties associated with the aspiration approach lies in the fact that oocytes may only be retrieved from some 30-60% of the punctured follicles. To minimize this effect, it is necessary to prime the needle and syringe with approximately 0.25-0.5 ml of aspiration medium. This is to provide the initial fluid that helps ensure the retrieval of the follicular contents, especially in collecting the first few oocytes.

The common aspiration medium used is modified phosphate-buffered saline with 3% (v/v) inactivated calf serum. Aspiration medium can be prepared once, stored in a refrigerator and made available for one week's activity. However, it is very important to pre-warm the aspiration medium up to 38°C before use. Alternatively, Medium-199 with Earle's salts, L-glutamine, 25 mM HEPES, supplemented with 3% (v/v) inactivated calf serum (heat-treated at 56°C for 30 min) and trace amounts of antibiotics, can also be used.

After aspiration, the contents of the syringe are slowly dispelled into a sterile centrifuge or test tube with minimum disruption of the cumulus-oocyte complex (COC). Once the last ovary of a particular batch is processed, the oocytes are allowed to settle to the bottom of the tube for at least 5 minutes. The precipitate is taken using a sterilized Pasteur pipette and poured into sterile Petri dishes for subsequent searching of COCs. As much as possible, recovery work must be done in a sterile environment with a room temperature of 25°C.

Sometimes the ovaries are sliced in order to retrieve oocytes. In fact, this method enhances the retrieval of a larger number of oocytes. However, it requires a longer period of retrieval and is not advisable when manipulating large number of ovaries because the extended period of oocyte retrieval would lower the *in vitro* maturation potential of oocytes.

The third method used for slaughtered animals is follicle dissection. This method is also effective in retrieving good-quality oocytes. However, it may also increase the time needed for the retrieval process.

On the other hand, *in vivo* retrieval of oocytes involves superovulation and recovery either by 1) an endoscope inserted through the skin of the right paralumbar fossa, 2) by laparoscopy or 3) by ultrasound guided follicular aspiration or ovum pick-up. These techniques require treating the donor animal with local anaesthesia, and collection of oocytes near the time of expected ovulation.

SELECTION OF OOCYTES FOR *IN VITRO* MATURATION

After the ovaries have been obtained, they are searched for the oocytes. Once located in the Petri dish, oocytes are lifted in a sterile glass pipette with a bore diameter of about 400 µm and transferred into a dish of fresh pre-warmed washing medium (this may be the same as the aspiration medium). The glass pipette used in holding oocytes 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 requisite for successful maturation *in vitro,* so they must be protected during oocyte retrieval.

Oocytes with compact multilayered cumulus investment and evenly granulated cytoplasm are selected for *in vitro* maturation. It should be noted, however, that the collected oocytes may have a different appearance. Some are denuded (no cumulus cells attached), some are partly denuded, others have swollen, expanded or spider-like cumulus cells, while others have deformed ooplasm. These oocytes should not be used, because they lack the potential to undergo normal maturation and will eventually end as degenerating oocytes after *in vitro* fertilization.

IN VITRO MATURATION OF OOCYTES

Prior to transfer to an *in vitro* maturation medium, selected oocytes must be washed four times in fresh pre-warmed aspiration medium and subjected to a final wash in *in vitro* maturation medium before the *in vitro* maturation droplets are pooled. The oocytes must be free from any contaminants if successful *in vitro* maturation is to take place.

The medium used for *in vitro* maturation 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 embryonic development. The culture media employed in maturation of oocytes *in vitro* can be broadly divided into simple and complex.

Simple media are usually bicarbonate-buffered systems containing basic physiological saline with pyruvate, lactate and glucose. The main differences between various types of simple media lie in differences in their ion concentration, and in the concentration of the 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 at which they are found in serum. Fixed nitrogen is present as free amino acids.

The most widely used complex media for IVM of cattle and buffalo oocytes is Tissue Culture Medium 199 (TAC-199) with Earle's salt, L-Glutamine and 25 mM HEPES supplemented with 10-20% heat inactivated serum (be it a fetal calf serum, calf serum, steer serum or an estrus cow serum). Other culture media used are Ham's F-10, MEM, IVMD 101, RPMI and Synthetic Bovine Oviductal Fluid medium and others. All these however, require further supplements in order to promote maturation.

During IVM, extensive redistribution of intracellular organelles occurs. Mitochondria migrate to occupy a perinuclear location, and the cortical granules migrate outward to lie just beneath the vitelline membrane. At all times, there is a need to realize that the crucial test of successful maturation is the ability of the oocytes to undergo normal embryonic development after sperm penetration and fertilization.

Important Considerations for *in vitro* Maturation

Water quality

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

Buffering Systems and Osmolarity

These will vary, depending on whether the medium is exposed to air or to a carbon dioxide-enriched 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 are commonly used for washing, and for storage of oocytes and embryos outside the incubator. The optimum range of osmolarity of the medium used for culturing oocytes and embryos is generally between 275 and 285 mOsmol. If the osmolarity of the medium is not within this range, it should be discarded and a new batch prepared. In some instances, the measured osmolarity of a medium is less than the calculated value. This can be attributed to the incomplete dissociation of ions once they reach m*M* concentrations.

Flux Culture

This involves the regular, gentle agitation of the culture dishes to prevent the attachment and subsequent differentiation of cumulus cells. For the most part, the use of microdroplets under oil has certain advantages. These include preventing or reducing the evaporation of water, protection from microbial contamination, attenuation of temperature and gas fluctuations, and ease of examination during culture.

Effect of Maturation Time

Some discrepancies exist in the length of time needed for *in vitro* maturation of cattle and buffalo 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). Evidence indicates that while there is no significant difference between treatments (maturation period) in terms of maturation and cleavage rates, there is a significant difference in final blastocyst yield in favor of the 24 h maturation period. It is therefore important to assess the effect of the culture medium employed for IVM of oocytes before identifying the time for *in vitro* fertilization.

Other considerations

Antibiotic Cover

This provides cover against growth and proliferation of microorganisms during the period of culture. The concentration of antibiotics generally recommended is known to be non-toxic, but their inclusion in the medium must obviously be combined with rigorous standards of hygiene in the IVF laboratory.

Light Environment

As part of routine maturation, fertilization and culture, the cattle and buffalo embryos are inevitably exposed to varying amounts of light. The lesions caused by prolonged exposure, especially at high light intensity under the microscope, contributes to embryo development failure. In principle, oocytes and embryos should not be exposed to light any longer than is necessary. Maturation and other events normally occur under conditions of darkness in the animal's reproductive organ.

Temperature and Gas Phase

The success of IVEP is temperature-dependent. Changes in temperature expose oocytes to temperature shock, which induces chromosome abnormalities. The temperature range of 38 - 39°C is found to be beneficial for both cattle and buffalo oocytes. At the embryonic stage, however, tolerance to lower temperatures was observed. Gas phase was also observed to have a very big influence on the success of IVEP. Development to the morula and blastocyst was found to be dependent on the gas phase environment during *in vitro* culture. The best gas phase observed was 5% CO_2, 5% O_2 and 90% N_2.

Culture Supplements

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

Protein

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 is also used for protein supplement. The role of protein in the culture media may not only be as a fixed nitrogen source, but also as a chelator of toxic metal ion.

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 preimplantation stage embryos. Growth factors include IGF (insulin-like growth factor), EGF (epidermal growth factor) and TGF-L. These growth factors were found to have mitogenic effects, and to stimulate RNA and protein synthesis.

Culture Vessel

In our experiments, we observed that the type of culture vessel used for *in vitro* culture of oocytes and embryos affects the occurrence of *in vitro* maturation and development into pre-implantation stages in buffalo oocytes. The use of ordinary Petri dishes was not beneficial. Dishes that are classified as tissue culture dishes, and that have undergone in-house treatment (culture surface is coated with albumin or other growth promoting substances), were found to enhance embryo development.

SPERM TREATMENT AND CAPACITATION

To enhance successful fertilization of the oocytes, sperm cells must be motile, have the ability to undergo capacitation and express the acrosome reaction. Sperm must possess the capacity to bind to the zona pellucida and viteline membrane by acquiring the correct binding proteins during maturation, and exposing these binding sites to the oocyte at the appropriate time. They must be able to fuse with the oolemma and be incorporated into the oocyte. The sperm acquire many of these capabilities as they pass through the epididymis.

Capacitation of the sperm involves a complex series of biochemical and physiological reactions. It is believed that an important part of the capacitation process is the gradual removal or alteration of the protective coat from the sperm surface, especially in the region of the acrosome. The removal or alteration of this coat permits exposure of receptor sites, allowing sperm to interact with oocyte receptors.

In vitro capacitation procedures are aimed at stimulating the sequence of events that normally occur in the cow's reproductive tract. This involves the removal of seminal proteins and other substances that coat the sperm membrane of the ejaculated semen. To do this, sperm cells are subjected to thorough washing and exposure in media with elevated ionic strength. The following are some of the components of capacitation medium:

Bovine Serum Albumin

This plays a key role in removing cholesterol and/or zinc from sperm because this protein has considerable binding capacity for both molecules. These molecules are known to stabilize cell membrane. To enhance capacitation and acrosome reaction, cell membrane in both oocytes and sperm must be destabilized.

Heparin

This is a glucoseaminoglycans added to sperm treatment medium. The use of heparin reflects the *in vivo* mechanism that heparin-binding proteins present in seminal plasma. Evidence suggests that heparin-binding proteins play a role in fertilization by attaching to the sperm surface, enabling heparin-like CAGs in the female tract to induce capacitation, with eventually, an improved fertilization rate.

Caffeine

This is a cyclic nucleotide phosphodiesterase inhibitor that has been employed as a motility-stimulating agent in bull sperm. It achieves its effect by inhibiting phospodiesterase, which results in an intracellular accumulation of cAMP that activates respiration and sperm motility in bull sperm.

Calcium Ionophore (A23187)

This is employed to bypass early stages of capacitation of bull sperm by increasing Ca^{2+} content of the cell and inducing AR.

IN VITRO FERTILIZATION

Successful IVF requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. In cattle and buffalo, IVF of *in vitro* matured oocytes is usually carried out after 23-24 hours. This is because the peak of oocytes at metaphase II stage is at this period. A 100 µl microdroplets of BO medium containing caffeine, heparin and BSA with 10 x 10^6 sperm/ml is prepared to accommodate 10 oocytes. This is because 1-oocyte/10 µl is an optimum proportion for a successful *in vitro* fertilization of oocytes. Co-culture of sperm-oocyte for *in vitro* fertilization was done in a humidified environment with 5% CO_2 and 38.9°C temperature for 6 to 7 hours. The incubation period was determined by the fact that sperm capacitation inside the female reproductive tract occurred for about 6 hours.

Before *in vitro* matured oocytes are subjected to IVF microdroplets, they are first partially denuded of the surrounding cumulus cells to allow easy penetration of the sperm cells. They are washed twice in pre-warmed IVF medium to maintain the defined component of the IVF medium.

EMBRYO CULTURE

The presence of a somatic cell monolayer in the culture medium during *in vitro* culture of the developing embryo was found to be very important in enhancing its development potential. The provision of cell basement support provides the developing zygotes with a comfortable environment, and secretes some growth factors supportive to further development *in vitro*. In our system, the cumulus cells that were detached from the cultured oocytes were kept, and the same culture droplets were used for the culture of embryo after *in vitro* fertilization. However, the culture medium was replaced with a fresh one (pre-incubated for at least 2 hours inside the incubator) prior to the transfer of the embryo. This was 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.

After the sperm-oocyte co-culture and prior to transfer to the *in vitro* culture droplets, oocytes are washed four times in a washing medium (culture medium) to remove the excess sperm cells attached to or surrounding the zona pellucida. It is important to remove excess sperm cells, to avoid the presence of denaturating cells that may contaminate the components and eventually the efficiency of the culture medium. Therefore, oocytes are transferred to the *in vitro* culture droplets for further development.

Embryo culture is done inside a water-jacketed incubator with 5% CO_2 at 38°C humidified air. Observation of the cleavage rate is done approximately 24 hours after the initiation of *in vitro* culture. Observation on the development of pre-implantation embryo is done up to 8 to 10 days of *in vitro* culture. To make observation easier, oocytes that have not cleaved are removed from the culture droplets, leaving only those that have undergone cleavage. During the observation period, gentle shaking of the culture dish is advised to allow a uniform environment among embryos, and to allow distribution of any autocrine factors that the embryo may have secreted during *in vitro* culture.

Other Important Considerations for IVEP

Cleaning of Glassware

Cleanliness of the glassware is of utmost importance. Used glassware is rinsed thoroughly with tap water and soaked in biological detergent (Reasol) overnight. The following day, glassware is rinsed thoroughly at least 20 - 40 times in tap water, and then submerged in a 3-5% HC1 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.

All ultrasonic cleaner is also used for heavily soiled and difficult to wash glassware such as volumetric flash, test tube, Pasteur 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.

Availability of Back-up Equipment and Equipment Repair Kit

In some cases, equipment needs repair when it malfunctions or becomes defective. Back-up equipment must be available, to avoid compromising the ongoing activity. Staff working at the laboratory must be familiar with any repair measures that may need to be implemented.

REFERENCES

Aman, R.R. and J.E. Parks. 1994. Effects of cooling and rewarming on the meiotic spindle and chromosome of *in vitro* matured bovine oocytes. *Biol. Reprod.* 50: 103-110.

Bracket, B.G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod.* 12: 260-274.

Chuangsoongneon U. and M. Kamonpatana. 1991. Oocyte maturation, *in vitro* fertilization and culture system for developing preimplantation swamp buffalo embryos using frozen thawed semen. *Buffalo Journal* 2: 189-198.

Duran, D.H. 1996. Studies related to the effects of Dimethyl Sulphoxide (DMSO) on the culture systems of bovine oocytes *in vitro*. Unpub. M.Sc. Thesis, Miyazaki University, Japan. 164 pp.

Duran, D.H., Y. Tsuzuki, K. Ashizawa and N. Fujihara. 1996. Enhanced bovine embryo development from cultured cells and conditioned medium treated with Dimethyl Sulfoxide (DMSO). In: *Congress Programme of the 13th International Congress on Animal Reproduction* (Darling Harbour, Sydney, Australia) June 30 - July 4, 1996, Vol. 3, pp. 22-18.

Duran, D.H., F.P. Aquino, R.V. De Vera, P.B. Pedro and L.C. Cruz. 1998. Embryo development of *in vitro* matured swamp buffalo oocytes cross-fertilized in vitro with riverine buffalo semen. In: *Recent Developments in Animal Production 1997*. PSAS, College, Laguna, Philippines, pp. 251-255.

Duran, D.H., F.P. Aquino, R.V. de Vera, F.V. Mamuad and L. C. Cruz. 1998. Blastocyst formation in serum supplemented culture medium of *in vitro* matured swamp buffalo cross-fertilized *in vitro* with riverine buffalo semen. In: *Proceedings of the 5th World Buffalo Congress* (Royal Palace, Caserta, Italy)

Oct. 13-16, 1997, pp. 793-799.

Duran, D.H., F.P. Aquino, R.V. de Vera, P.B. Pedro and L.C. Cruz. 1998. Effects of some physical parameters on embryo development of buffalo oocytes matured and fertilized *in vitro*. *Phil. Jour. Vet. Anim. Sci.* 24, 1-2: 54-64.

Gardner, D.K. and M. Lane. 1993. Embryo Culture Systems. In: *Handbook of In Vitro Fertilization*, A. Trounson and D.K. Lane, (Eds.). CRC Press Inc., pp. 85-114.

Gordon, Ian. 1994. *Laboratory Production of Cattle Embryos*. CAB International, Wallingford, Oxon OX10 8DE, UK. 672 pp. ISBN 0 85198 928 4.

Gordon, Ian. 1996. *Controlled Reproduction in Cattle and Buffaloes*. Controlled Reproduction in Farm Animals Series No. 1, Vol. 1. CAB International, Wallingford, OX10 8DE, UK. 492 pp. ISBN 0 85199 1149.

Hunter, R.F.F. and C. Polge. 1966. Maturation of follicular oocytes in the pig after injection of human chorionic gonadotropin. *Jour. Reprod. Fertil.* 12: 525-531.

Jainudeen, M.R., Y. Takahashi, M. Nihayah and H. Kanagawa. 1993. *In vitro* maturation and fertilization of swamp buffalo (*Bubalus bubalis*) oocytes. *Animal Reproduction Science* 31: 205-212.

Madan, M.L., S.K. Singla, M.B. Chauhan and R.S. Manik. 1994. *In vitro* production and transfer of embryos in buffaloes. *Theriogenology* 41: 139-143.

Ocampo, M.B., L.C. Ocampo, N.D. Lorenzo, R.V. de Vera, F.P. Aquino and L.C. Cruz. 1996. Pregnancies resulting from swamp buffalo oocytes matured and fertilized *in vitro*. In: *Recent Developments in Buffalo production*, V.G. Momongan, R.T.J. Ducusin, C.P. Maala, O.L. Bondoc and A.N. del Barrio (Eds.). College, Laguna, Philippines. pp. 210-214.

Rogers, B.J., 1981. Factors affecting mammals *in vitro* fertilization. In: *Bioregulators of reproduction*. Academic Press, New York, pp. 459-486.

Totey, S.M., M. Taneja and G.P. Talwar. 1991. *In vitro* maturation and fertilization of follicular oocytes from water buffalo. *Theriogenology* 35: 284.

Totey, S.M., G. Singh, M. Taneja, C.H. Pawshe and G.P. Talwar. 1992. *In vitro* maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus bubalis*). *Jour. Reprod. Fert.* 95: 597-607.

Wildt, D.E. 1990. Potential applications of IVF technology for species conservation. In: *Fertilization in Mammals*, B.D. Bavister, E. Roldan, and J. Cummins, (Eds.). Serono Symposium, U.S.A., pp. 344-364.