Studies for the Improvement of *in vitro* Culture Systems of Oocytes and Embryos in Water Buffalo

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BCB	brilliant cresyl blue
BO	Brackett and Oliphant
BSA	bovine serum albumin
COCs	cumulus-oocyte complexes
CR1aa	Charles Rosenkran's 1 amino acid medium
E_2	estradiol-17_β
EDTA	ethylenediamine tetraacetic acid
EFS40	ethylene glycol, ficoll and sucrose 40
EGF	epidermal growth factor
ET	embryo transfer
FBS	fetal bovine serum
FCS	fetal calf serum
FSH	follicle stimulating hormone
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
LH	luteinizing hormone
MII	metaphase II
mPBS	modified phosphate-buffered saline
mSOF	modified form of synthetic oviductal fluid
OPU	ovum-pick-up
PBS	phosphate-buffered saline
$PGF_{2\alpha}$	prostaglandin F_2 alpha
RPMI	Roswell Park Memorial Institute
TALP	Tyrode's albumin lactate pyruvate
TCM	tissue culture medium
TEM	transmission electron microscopy

CHAPTER I. General Introduction

1.1. The Water Buffalo

Water buffaloes (*Bubalus bubalis*) are of two types. These are the river type (2n=50) and the swamp type (2n=48). The river buffaloes are grown for dairy and meat and are found mostly in the Indian continent like Pakistan and Sri Lanka and in the Middle East and Southern Europe. Of lesser number are in South Africa and South America.

The swamp buffaloes are primarily used for draft and only secondary for meat. They are found mostly in South-East Asia such as Brunei, Cambodia, China, Indonesia, Laos, Malaysia, Philippines, and Vietnam.

Water buffaloes are important livestock for rural farmers in South-East Asia providing milk, meat and draft power. In countries where swamp buffaloes are the dominant buffalo breed, importation of river buffaloes has been initiated to improve milk and meat production.

The first generation crossbreds (2n=49) produced from crossing river and swamp buffaloes can grow faster to as much as 70 to 100 percent with its huge body conformation providing 60 to 80 percent more meat (Abanto *et al.*, 2000) and 200 to 300 percent more milk than its swamp buffalo counterparts (Castillo, 1975).

However, the risks and high costs of live animal importation for breeding purposes plus inherent reproductive problems such as delayed age of puberty, less pronounced signs of estrus, seasonal anoestrus, long period of post-partum anoestrus and low conception rates have limited the propagation of upgraded animals.

To solve this problem, assisted reproductive technologies such as artificial insemination have been introduced to overcome reproductive inefficiencies and to increase genetic gain by producing up-graded animals. However, crossbreeding to improve the blood line for milk and meat efficiencies requires years of continuous backcrossing to reach the desired animal genetics; hence, developing alternative tools is needed to facilitate production of purebred river buffaloes.

1.2. The Water Buffalo Industry in the Philippines

The Philippines is dominated by a swamp type buffalo called carabao or Philippine carabao. This is valuable for hauling and cultivation, and has potential for meat production but not for milk.

Buffaloes constitute a significant part of the domestic livestock in the Philippines. This is why genetic improvement for milk and meat has been a major concern for several decades. The genetic potential for growth and milk production was improved through the introduction of dairy buffalo germplasm from the 1980s. In the past until 1994, carabao's population declined significantly at an average rate of 1.9 percent per year. However, an increase in carabao population of 3.6 percent was achieved in 1995. This increase was attributed to reduced extraction rate because of the importation of buffalo meat from India and a massive upgrading program performed by the Philippine government through artificial insemination and bull loan.

At present, the water buffalo industry in the Philippines is constantly progressing with research and development programs in place. As efforts to produce more dairy/meat carabaos are intensified, a corresponding increase has occurred in carabao-based enterprises. This increased the demand for development of alternative tools for the production of purebred river buffalo considering the high cost and high risk of disease transmission associated with imported live animals for breeding purposes.

Because of this, reproductive biotechnologies became an attractive alternative tool. One of the very promising reproductive technologies for propagating purebred river buffaloes is the combination of embryo *in vitro* production (IVP), cryopreservation and embryo transfer (ET) techniques developed in cattle and found to be applicable in buffaloes.

1.3. Reproductive Potential of Water Buffaloes

Reproductive efficiency in buffaloes is affected by a number of variables. Among these are late maturity in both sex and reduced fertility after natural mating

(Bodhipaksha *et al*., 1978; Chantarakhana *et al*., 1981; Fischer and Bodhipaksha, 1992; Na-Chiengmai, 2002).

A review of the management data on buffalo research station reports showed the following: (i) an average age at first calving of 4.2±0.8 years ranging from 3.6 to 4.9 years; and (ii) an average calving interval of 573.3±117 days ranging from 490 to 700 days (Na-Chiengmai, 2002).

The weaning age of buffalo is around 8 months after calving. A long period of calving interval is an important factor affecting the reproductive efficiency. The inherent long gestation period, long service period, and late period of reproductive maturity make the production of highly upgraded buffaloes through continuous backcrossing a relatively long process. In essence, it takes about 15 to 20 years or 4 to 5 generations of backcrossing to produce an animal that can express milk production of similar volumes as that of purebred dairy buffalo.

The combination of recently developed reproductive technologies such as ovum-pick up (OPU) and embryo IVP can accelerates genetic improvement for production traits and reproductive efficiency of females. The feasibility of OPU has already been reported in pre-pubertal buffaloes, heifers and cows (Boni, 1994; Kitiyanant *et al*., 1995; Pavasuthipaisit *et al*., 1995; Boni *et al*., 1997; Promdireg *et al*., 2000; Techakumphu *et al*., 2000; Presicce *et al*., 2002; Techakumphu *et al*., 2004a, b). These reports suggest that reproductive potential of females can be maximized if OPU and embryo IVP are applied. Hence, it is imperative to study the factors necessary to improve the success rate of the application for reproductive biotechnologies in this species.

1.4. Application of Reproductive Biotechnologies in Water Buffaloes

Artificial insemination through natural or synchronized estrus is the earliest reproductive biotechnique commonly used for up-grading water buffaloes in the Philippines. Using this concept, Cruz *et al*. (1991) attempted to enhance genetic improvement in water buffalo, more particularly for dairy type buffaloes, using superovulation and ET and capitalizing on the production potentials of superior animals. However, the response showed a relatively low yield of *in vivo*-derived embryos. This

confirmed previous findings of poor response of buffaloes to superovulation treatments (Vlakhov *et al*., 1985; Karaivanov, 1986; Madan, 1990, Karaivanov *et al*., 1990; Misra, 1993). This resulted in a shift of interest to embryo IVP technology as a potential alternative method for propagating superior animals.

Efforts to establish embryo IVP techniques in buffaloes in the Philippines started in 1992. However, limited technical resources and laboratory facilities made developments very slow. In 1996, an *in vitro*-produced crossbred (male river x female swamp) buffalo embryo "Malakas" was delivered to term by a swamp recipient (Ocampo *et al*., 1996). This calf was a result of *in vitro*-produced embryos freshly transferred to the recipient animal. Yet the limited number of embryos made it difficult to assess the success rate of IVP and ET in those trials.

This is why it is imperative to establish a system for embryo IVP, cryopreservation and ET technology in the Philippines in order to hasten the propagation of river buffaloes for milk and meat. Through this, alternative tools for water buffalo genetic improvement can be developed. This could provide the rural farming families an elite animal that could be a source of additional income and improve their economic and overall well being, generate employment, overcome malnutrition problems, and address food security in the country. In essence, the embryo IVP system and efficiencies need to be improved. Factors affecting the development of the oocytes have to be determined to identify ways of improvement.

Similarly, to carry out ET of *in vitro*-produced embryos, the embryos must be in a frozen state in order to allow storage and transport. Thus, a cryopreservation method with high efficiency of preserving the viability of the embryos needs to be developed.

1.5. Milestone in Embryo *in vitro* Production and Cryopreservation in Water Buffaloes

Some milestones in embryo IVP in buffalo have been attained. Yet, embryo IVP in buffalo is not yet an established technology despite tremendous potential in genetic improvement (Gasparrini, 2002). A number of problems need to be solved before this technology can be applied routinely in buffalo breeding (Nandi *et al*., 2002).

Oocytes for embryo IVP are either collected from live animals by OPU or from ovaries of slaughtered animals. OPU provides the most reliable source of genetic material to enhance female contribution to genetic progress while abattoir-derived ovaries have very little impact on genetic improvement although these provide a cheap and abundant source of oocytes for research and propagation of desired breed of animal. Neglia *et al*. (2003) found out that retrieval of good quality oocytes was lower in OPU than aspirating from slaughter house-derived ovaries but of a higher blastocyst yield (29.7% versus 19.9%; $P<0.05$). Also, embryos arrested at tight morula stage (11.1% versus 22.3%; $P<0.05$) was of a lower proportion in live animals than abattoir ovaries.

In ovary collection and handling, physiological saline (0.9% sodium chloride with 100 µg streptomycin/mL and 100 IU penicillin/mL) at 28 to 33ºC is the most common medium used for storage of ovaries. Ravindranatha *et al*. (2003) found that storage of ovaries at 4ºC for 12 or 24 h significantly ($P<0.05$) reduced the developmental potential of oocytes. However, in cattle, Lucci *et al*. (2004) found that ovarian pieces stored at 4°C for up to 18 h kept the percentage of normal follicles similar to that of the control while storage of ovarian pieces at 20°C for 12 or 18 h significantly reduced the percentage of morphologically normal follicles. However, within 6 h storage at 20°C, normal follicles were preserved.

On the other hand, Schernthaner *et al*. (1977) indicated that bovine ovaries stored for 24 h between 15 and 21ºC did not influence the IVP of blastocysts. This was confirmed by other authors indicating that 20°C is optimum for ovary storage for blastocyst development (Osaki *et al*., 2004).

These reports have demonstrated conflicting results. Nonetheless, they suggest that ovary storage affects the developmental competence of the oocytes. The very limited reports about buffalo suggest a need to consider this aspect of *in vitro* embryo production to improve the success rate of embryo production.

On the other hand, the average number of visible surface follicles in water buffalo ovaries is 5.20±0.97 (Kumar *et al*., 1997). Aspirations of follicles, slicing of ovaries, and puncturing or dissecting of follicles (Boni, 1994; Boni *et al*., 1994; Das *et al.*, 1996) were the methods used to recover oocytes. Oocyte recovery rate was greatest using slicing (1.2, Kumar *et al*., 1997; 3.9, Gasparrini, 2002) but is time

consuming with a highly heterogeneous population of oocytes.

Follicle dissection is not practical in water buffaloes. The follicles are deeply embedded in the stromal region of the ovary (Gasparrini, 2002) so aspiration is the most widely accepted technique due to the speed of operation (Nandi *et al*., 2002). Aspiration method in the presence of fluid medium Dulbecco's phosphate buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA) enhanced oocyte retrieval and provided energy support. This method rescued the oocytes upon removal from the follicles resulting in higher development potentials among oocytes (Hufana-Duran *et al*., 1997).

In selecting oocytes for *in vitro* maturation (IVM), irrespective of the species, oocytes are selected by compaction of cumulus-corona investment and homogeneity of the ooplasm. In water buffalo, several studies have revealed that oocytes with compact and evenly granulated cumulus cells give higher maturation rates (Suzuki *et al*., 1991; Chauhan *et al*., 1998a; Abdoon *et al*., 2001). This finding confirmed the observations in cattle (De Loos *et al*., 1989; Smith *et al*., 1996). It was shown that 57.2 percent of the oocytes collected from bovine ovaries at abattoirs have compact thick cumulus cells, 9.6 percent have thin cumulus layers, and 7.5 percent are naked (Shioya *et al*., 1988).

Consequently, about 50 percent of the naked oocytes undergo maturation division and half are fertilized even though 20 percent of the naked oocytes were abnormal or degenerated when examined microscopically. These results suggest that the nature of the cumulus cells and the ooplasm of the oocytes are indicators of developmental competence. However, further studies are needed to define the concrete selection parameters of developmentally competent oocytes to improve the *in vitro* culture (IVC) system.

On the aspect of IVM of the oocytes, efforts were made to examine the best culture medium for buffalo oocytes IVM. Abdoon *et al*. (2001) assessed the efficiency on oocyte maturation of Charles Rosenkran's 1 amino acid (CR1aa), Charles Rosenkran's 2 amino acid (CR2aa), Tissue Culture Medium (TCM) 199, Minimum Essential Medium and RPMI (Roswell Park Memorial Institute) media containing 5% estrous cow serum, 10 µg follicle stimulating hormone (FSH)/mL, 50 µg gentamycin/mL and 5 mM taurine as supplements. They found that CR1aa was the best

with 21 percent blastocyst development.

Meanwhile, they also found out that TCM 199 was found best as it enhanced 81.7±14.5 percent nuclear maturation when supplemented with 20% buffalo estrus serum, FSH (0.5 µg/mL) and estradiol-17$_\beta$ (E_2, 1 µg/mL) but not when luteinizing hormone (LH) was added (Totey *et al*., 1992). On the other hand, Ham's F-10 was found best when supplemented with 20% buffalo estrus serum and 5 µg LH/mL with an IVM rate of 76.8±18.3 percent but not when FSH was added. This indicated failure of LH to synergistically act with FSH and E_2.

Madan *et al*. (1994a) demonstrated a 78.1 percent fertilization rate in oocytes cultured for 20 to 24 h in TCM 199 with 10% buffalo estrus serum alone. On the other hand, the use of defined and semi-defined media in buffalo IVM (Abdoon *et al.*, 2001; Gupta *et al*., 2002; Raghu *et al.*, 2002b) were also reported to have a positive effect. Buffalo oocytes can be developed up to the blastocyst stage in serum-free, semi-defined media containing BSA, FSH, epidermal growth factor (EGF) and insulin-transferrin-selenium (Raghu *et al.*, 2002b). These results indicate that efficiency on the production of embryos varies with media and supplements.

The use of antioxidants as supplement in the IVC medium was also examined. Reports showed that cysteamine supplementation (50 µM/mL) during IVM did not improve IVM and *in vitro* fertilization (IVF) rates but improved embryo development (22.6%) and quality (19.3%) (Gasparrini *et al*., 2000). On the other hand, the use of beta-mercaptoethanol was found to significantly improve the proportion of oocytes that exhibited synchronous pronuclei formation (31.8±5.1% versus 17.9±3.3%, $P<0.05$). Blastocysts developed faster with cavitation occurring at 156 h post insemination than those from oocytes matured in the absence of beta-mercaptoethanol (Songsasen and Apimeteetumrong, 2002). These suggest that antioxidants are needed in the culture medium to maintain a favorable environment for the development of oocytes and embryos.

On the other hand, growth factors were added to IVM medium because preimplantation embryos produced *in vitro* are at a developmental disadvantage when compared to their *in vivo* counterparts. Heyner *et al*. (1993) demonstrated that this disadvantage can be overcome to some extent by adding growth factors or washings

from the reproductive tract.

Positive effects were observed in various growth factors. These are EGF (Chauhan *et al.*, 1999; Raghu *et al*., 2002b), EGF plus fibroblast growth factor (Gupta *et al*., 2002), insulin-like growth factor-I (Pawshe *et al*., 1998), and insulin-like growth factor-II (Chauhan *et al*., 1998c) in oocyte culture media. These have been shown to increase cumulus expansion, nuclear maturation and post-fertilization cleavage rates in water buffalo. Insulin-like growth factor-I showed positive interaction with FSH in the presence or absence of granulosa cells on meiotic maturation and synergistically enhanced DNA synthesis, protein synthesis and steroidogenesis in the presence of granulosa cells. However, LH was found to suppress insulin-like growth factor-I.

On the other hand, supplementation of EGF (20 ng/mL) to IVM medium was more beneficial than fibroblast growth factor (20 ng/mL) and vasoactive intestinal peptide (20 ng/mL). Along with pregnant mare serum gonadotrophin, EGF showed the highest improvement of buffalo oocyte maturation and embryo cleavage than fibroblast growth factor (Nandi *et al*., 2003). Addition of vasointestinal peptide to the oocyte maturation medium did not improve the results.

Raghu *et al*. (2002b) studied the *in vitro* development of buffalo oocytes up to the blastocyst stage in serum-free, semi-defined media containing BSA, FSH, insulin-transferrin-selenium and EGF. Results showed that supplementation with FSH and EGF significantly ($P<0.05$) increased the maturation rates of buffalo oocytes. Yield of blastocysts was higher ($P<0.05$) in media containing EGF and insulin-transferrin-selenium. However, no study yet has been done on the effects of combination of FSH, E_2 and EGF; hence, it needs to be investigated.

Co-culture method during IVM was also examined to assess the benefits of somatic cells in the maturation of oocytes especially in oocytes that lack surrounding cumulus cells after retrieval from the follicles. The developmental competence of denuded buffalo oocytes was found very low and could not be improved by the addition of cumulus cells to the culture medium during IVM (Das *et al*., 1997). However, a 6-day old monolayer of cumulus cells coved to bring higher maturation rates in artificially denuded oocytes (Pawshe and Totey, 1993).

The supplementation of sera from various sources on the culture medium has

been examined to determine the effect in the culture of oocytes and embryos. Sera have been known as rich source of biological residues for growth and development. Totey *et al*. (1993a) indicated that fetal calf serum (FCS) is more effective in achieving maturational competence than with buffalo estrus serum.

However, sources of sera seem to influence oocyte maturation as studies showed. These sources are buffalo estrus serum, superovulated buffalo serum, fetal bovine serum (FBS) (Chauhan *et al*., 1998a), FCS (Totey *et al*., 1993a), serum from buffalo at different stages of the estrous cycle (Samad *et al*., 1998), steer serum (Chauhan *et al*., 1998a; Nandi *et al.*, 2001b) and human serum (Chuangsoongneon and Kamonpatana, 1991). Results showed that the proportion of cleaved embryos developing to blastocyst stage was higher ($P<0.05$) with superovulated buffalo serum than with buffalo estrous serum, FBS and steer serum (Chauhan *et al*., 1998a). These suggest that the differences on the source of the serum supplement have varied effects on the maturation buffalo oocytes.

Attempts were made to partially or completely replace the basic medium (TCM 199) with cystic and normal follicular fluid containing regulatory proteins, growth factors, gonadotrophins, steroids, inhibin, meiosis-activating sterol and other nutrients (Gupta *et al.*, 2001); FBS with easily processed and inexpensive steer serum (Chauhan *et al*., 1998a; Nandi *et al*., 2001b); pure FSH with locally available pregnant mare serum gonadotrophin having both FSH and LH bioactivity (Gupta *et al*., 2001; Abdoon *et al*., 2001). Findings were encouraging but efficiency was still variable.

The efficacy of follicular fluid from different size follicles [small (<3 mm), medium (3 to 8 mm) and large (>8 mm)] to replace the medium for oocytes maturation was tested. Results suggested that follicular fluid was capable of developing buffalo oocytes to embryonic stage *in vitro* but efficacy was lower than that of the serum. This means regardless of high maturation rates after use of IVM in media containing follicular fluid or IVM in whole follicular fluid, low blastocyst rates were obtained after IVF (Nandi *et al*., 2004). Similar results were obtained in cattle (Avery *et al*., 2003) suggesting that follicular fluid lacks development activating factors necessary for the oocytes to achieve full development potential.

Studies on the effect of incubation temperature to determine the optimum

temperature for culturing oocytes and embryos and handling sperm cells showed that 38.5ºC during IVM for 20 to 24 h was optimum for embryo production *in vitro*. However, storing frozen-thawed sperm at 25ºC for 2 to 8 h significantly decreased its ability to cleave the oocytes (Ravindranatha *et al*., 2003).

The optimum duration of IVM to determine the optimum time for IVF was examined by Neglia *et al*. (2001) and they found out that by confocal microscopy, 70 and 87 percent of *in vitro* cultured oocytes were at metaphase II (MII) stage at 15 to 16 h and 19 h, respectively. This suggested shorter IVM period for water buffalo oocytes. However, in the review made by Gasparrini (2002), 20 to 24 h was reported as the optimum time for IVM of buffalo oocytes.

The variations in the length of maturation suggested that oocytes used for IVM are in a heterogeneous population. This means that effective and efficient selection parameters for a homogeneous population of oocytes need to be determined to minimize variation in the length of culture requirements.

To produce an embryo, matured oocytes have to be fertilized. In IVF, the common method of sperm preparation is washing the frozen-thawed semen with a BO medium (Brackett and Oliphant, 1975) and collecting the washed spermatozoa by centrifugation. The use of modified TALP (Tyrode's albumin lactate pyruvate) or BO medium as basic medium to capacitate frozen-thawed semen was compared. Findings reported that frozen-thawed spermatozoa prepared in BO medium and treated with 5 mM caffeine plus 10 µg heparin/mL showed higher fertilization rate (29.8%) than those treated in TALP and treated with 10 µg heparin/mL (19.6%) (Totey *et al*., 1992). This was confirmed when sperm processed in BO medium had significantly ($P< 0.05$) higher ability to cleave the oocytes than the TALP medium (Ravindranatha *et al*., 2003). These results have shown that BO medium is superior to TALP for use in IVF.

On the other hand, Gasparrini (2002) indicated that IVF performed in TALP in the presence of heparin, hypotaurine and penicillamine under their system yielded 60 to 65 percent cleavage rate after sperm swim-up in modified version of Ham's medium suggesting that separating the motile sperm cells could improve the cleavage rate after IVF. This was supported by other experiments which indicated that fertilization rate, cleavage and blastocyst development were significantly improved when fresh ejaculated

spermatozoa treated with 5 mM caffeine plus 10 µg heparin/mL in BO medium was used (Totey *et al*., 1992).

Nonetheless, while fresh semen gives better fertilization rates than frozen-thawed semen, the practicality of using fresh buffalo semen in IVF is negligible due to drastic changes in buffalo semen quality with season. The low motility of frozen-thawed semen has been overcome by collecting motile buffalo spermatozoa by the swim up (Nandi *et al*., 1998), or percoll gradient technique (Totey *et al.*, 1996). However, the swim up technique is time consuming as it requires an additional 1.5 h to collect the motile spermatozoa.

Meanwhile, the use of percoll gradient was found efficient as it requires only a minute to separate the motile spermatozoa. The problem is the banning of percoll in 1996 due to high levels of endotoxins. This required identifying other materials that could replace percoll.

To enhance sperm-capacitation and enable the spermatozoa to penetrate the oocyte, sperm motility enhancer such as heparin and caffeine (Totey *et al*., 1993b; Nandi *et al*., 1998) was used. Other motility enhancers are theophylline (Chauhan *et al*., 1998e), pentoxifylline (Ramesha *et al*., 2000), taurine (Abdoon *et al*., 2001) and a mixture of penicillamine, hypotaurine and epinephrine (Totey *et al*., 1996). Efficiency of these motility enhancers to improve fertilization rate varies and must be tested considering the differences among bulls.

On the other hand, the concentration of sperm cells in the IVF medium has a significant effect in the success of IVF. Totey *et al.* (1993b) reported that $2x10^6$ sperm cells/mL with 20 h of sperm-oocyte incubation period is optimum for buffalo IVF. Higher sperm concentrations (3×10^6 or 4×10^6) resulted in higher oocyte penetration rates but gave rise to polyspermy. This was confirmed by Nandi *et al*. (1998) who found out that increasing the sperm concentration from 1 to 2 million through 9 to 10 million sperm/mL for 6 h incubation increased the cleavage rate significantly. Nonetheless, this had no effect on the proportion of cleaved embryos that developed to morula and blastocyst stages.

Longer incubation period i.e. 48 h resulted in poor cleavage even using less than 1 million sperm/mL (Chuangsoongneon and Kamonpatana, 1991). This was also

observed in the trials made on 12 to 16 h incubation period with 10^7/mL spermatozoa (Bacci *et al*., 1991).

However, the occurrence of polyspermy can be overcome by reducing the duration of co-incubation when high sperm-concentration is employed (Gasparrini, 2002). Determining the desired sperm concentration needs to consider the bull effect because of high variability in the fertilizing ability of different bulls (Totey *et al.*, 1993b; Chauhan *et al.*, 1998d; Yadav *et al*., 2001). This implies there is no single optimal concentration of sperm and supplements for buffalo IVF. This requires necessity of screening sperm from individual bulls before regular use of supplements and desired sperm concentration on the IVF medium. Misra *et al*. (1999a) confirmed bull-specific effect on fertilization rate and viable embryo recovery in superovulated buffalo *in vivo*. Thus further studies need to ascertain the factors which contribute to such bull's specific effect and develop tools to improve reproduction potentials of bulls with low sperm motilities.

To identify the best system for the production of buffalo embryos, fertilized buffalo oocytes were cultured in different media. Some of these were *in vivo* in ligated rabbit oviduct (Totey *et al.*, 1992), in sheep (Galli *et al*., 1998), *in vitro* in complex medium (TCM 199, Madan *et al*., 1994a; Yadav *et al.*, 2000), simple defined media (Totey *et al.*, 1996; Boni *et al*., 1999; Abdoon *et al*., 2001) and semi-defined media (Raghu *et al*., 2002b) supplemented with serum or somatic cells (cumulus cells or oviductal epithelial cells). Totey *et al*. (1992) found out that development rate was enhanced when fertilized ova were cultured in ligated rabbit oviduct (28.0%) than when co-cultured in oviductal cell monolayer (8.2%) Galli *et al*. (1998) reported 29.8 percent cleavage and 44.0 percent blastocyst development out of cleaved embryos after *in vivo* culture in rabbit oviduct.

Studies on the culture of buffalo embryo *in vivo* are limited because its application for large-scale embryo production is unsuitable due to invasive considerations. Very few studies were done using defined media to culture buffalo embryos (Totey *et al.*, 1996; Boni *et al.*, 1999; Abdoon *et al*., 2001). Most investigators have used complex media containing serum and somatic cells (Chauhan *et al*., 1998a-e; Chauhan *et al*., 1997a-d; Nandi *et al*., 1998, 2000, 2001a). Culturing embryos in somatic

cell monolayer facilitated their development through the 8- to 16- cell block.

Nandi *et al*. (2003) examined the developmental competence of cleaved embryos cultured in either a complex co-culture system (TCM 199+10% FCS+oviduct cell monolayer) or defined media in a modified form of synthetic oviductal fluid (mSOF, Takahashi and First, 1992b). They found out that blastocyst yields were significantly higher in a complex co-culture system than in defined media when oocytes were matured in the presence of EGF either alone or in combination with fibroblast growth factor and vasointestinal peptide (34.0% versus 22.0%).

Their results contradicted with the reports of Boni *et al*. (1999) that showed a higher blastocyst rate when embryos were cultured in mSOF (Tervit *et al*., 1972) compared to the co-culture system (13.5% versus 7.0%). Nandi *et al*. (2003) explained that the difference might be due to a different synthetic oviductal fluid formulation, to the use of a different cell type for the co-culture (oviduct cell versus buffalo rat liver cells) or to the use of a different gaseous environment (5% CO_2 in air instead of the widely recommended 5% CO_2, 5% O_2 and 90% N_2). After thawing, *in vitro*-derived embryos in mSOF had significantly higher morphologically normal blastocysts than those cultured in complex co-culture system indicating potential use of the defined media for IVC.

On the supplementation of the embryo culture media with growth factors, insulin growth factor-1 (Narula *et al*., 1996), insulin (Chauhan *et al*., 1998b) and a combination of BSA, epidermal growth factor and insulin-transferrin-selenium (Raghu *et al*., 2002b) were found to increase the blastocyst production and quality. The morphology and number of cells in the trophectoderm and inner cell mass of buffalo blastocysts derived in the presence of insulin growth factor-I were found to be significantly higher than blastocysts developed without insulin growth factor-I (Narula *et al*., 1996). This suggests that growth factors have beneficial effects when added to IVC medium.

New concepts of *in vitro* cattle and human embryo culture that can be applied in buffalo IVP are the use of sequential media systems (Gardner and Lane, 1998), use of autocrine and paracrine factors and the regulation of energy metabolism (Thompson, 2000). In sequential media system, the media components and physical components could be alerted during the culture. Further development of sequential media is the use

of perfusion culture as the vehicle to introduce changes in media composition (Lim *et al*., 1996).

Sub-optimal culture conditions may lead to the production of embryos with developmental abnormalities and reduced viability. Thus, renewed research on the interaction of factors contributing to the development of a viable embryo and signal transduction mechanisms influencing embryo development may assist in understanding the relationship between the embryo and culture environment.

Studies on cryopreservation of water buffalo embryos are limited. Preliminary attempts to cryopreserve water buffalo embryos was reported by Techakumphu *et al*. (1989) using slow freezing method by either manual or automatic technique and using 10% glycerol with 20% FBS in PBS as cryoprotectant. However, success rate was low at 22.2 percent (2/9) expressed in terms of morphology of embryos post-thawing. On their second attempt, Techakumphu *et al*. (2001) transferred 28 *in vivo* derived embryos frozen by the same technique to 17 recipients of which none developed to term. Nevertheless, following 2-step cryopreservation protocol, Ullah *et al*. (1992) were able to achieved 7.0 percent (1/15) fullterm development in Nili Ravi buffaloes. Also, Kasiraj *et al*. (1993) were able to produce live birth after cryopreservation of *in vivo* derived embryos using slow-freezing method with 1.4 M glycerol as cryoprotectant.

These reports suggest that success rate of buffalo embryo cryopreservation is low and requires improvement. Similarly, the literature demonstrates that the field of embryo cryopreservation in buffalo has not been explored thoroughly. The use of slow-freezing method, that has demonstrated a modest rate of success of 7 percent full-term development, requires sophisticated equipment while the vitrification system does not. Hence, a practical approach for cryopreservation needs to be explored.

On the viability of *in vitro*-produced buffalo embryos after transfer, successful pregnancies and calf birth were obtained after transfer of fresh embryos to recipient animals (Madan *et al*., 1991; Chauhan *et al*., 1997a; Ocampo *et al*., 1996; Mattapallil *et al*., 1998; Galli *et al.*, 2001). However, success rate in terms of birth of calves has been low (1.0 to 10.0%).

In vitro embryo production systems in buffalo are sub-optimal and require substantial improvements. Studies conducted to improve the IVC systems to optimize

production of pre-implantation stage embryos are limited. As such, refinement of the system of buffalo oocytes/embryos handling and culture requirements is critical to optimize the efficiency of *in vitro* embryo production.

1.6. Objectives and Scope of this Research

The general objective of this dissertation was to improve the IVC systems for buffalo oocytes and embryos for efficient production of genetically superior buffalo, develop a cryopreservation method for preimplantation stage embryos, and establish a tool for water buffalo genetic improvement programs in the Philippines. The specific objectives were to;

1. Improve *in vitro* maturation, fertilization and *in vitro* culture systems for efficient production of water buffalo embryos *in vitro*.
2. Establish a cryopreservation method for water buffalo embryos by vitrification techniques.
3. Examine the viability of *in vitro*-derived-vitrified embryos both in river and in swamp buffalo recipients.
4. Examine the effects of ovary storage, length of *in vitro* maturation of oocyte, and supplementation in the culture medium on the developmental competence of oocytes and production of water buffalo embryos *in vitro*.
5. Determine the selection parameters of developmentally competent oocytes for efficient *in vitro* production of embryos.
6. Examine the effects of sperm separation by density gradient of silica particles in improving the success rate of *in vitro* fertilization in water buffalo.

This study focused on examining factors that can improve the production of preimplantation stage embryos *in vitro*, test vitrification procedures that can be used as efficient method for cryopreservation of preimplantation stage embryos, and test the viability of these embryos for ET.

The use of the embryo IVP-vitrification-ET as potential tool for water buffalo

genetic improvement was examined by using swamp buffaloes as recipients of river buffalo embryos under field condition. Development of the techniques of embryo IVP and cryopreservation were conducted at the Reproductive Biotechnology Laboratory of the Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Muñoz, Nueva Ecija 3120 Philippines. The production of river buffalo embryos and studies on IVC systems were conducted at the Satellite Embryo Biotechnology Laboratory of the Philippine Carabao Center in India due to the availability of sufficient number of oocytes for comparative studies.

CHAPTER II. Development of Systems for Embryo *in vitro* Production and Cryopreservation in Water Buffalo and Viability of the *in vitro*-Produced-Cryopreserved Embryos

2.1. Introduction

In vitro production of embryos in buffaloes was initiated in other countries like India, Italy, Thailand, China and Bulgaria. However, success rate has been sub-optimal as reviewed in Chapter I. Results were high rate of maturation (70 to 90%), fertilization (60 to 70%) and cleavage (40 to 50%) with a moderate to low rate of blastocyst formation (15 to 30%) and calf production (3 to 10.5%) (Nandi *et al.*, 2002; Gasparrini, 2002). The systems need further refinement before the technology can be applied to buffalo breeding.

From the standpoint of long-term animal improvement programs, cryopreservation of embryos produced using *in vivo* or *in vitro* methods is important. The success of conventional freezing technique for bovine and buffalo embryos has been well documented while the recently developed vitrification system has generated interest. While vitrification method was found to be a very practical approach for cryopreservation in mice (Kasai *et al*., 1990a) and rabbits (Kasai *et al*., 1990b), the efficiency of vitrification technique in the cryopreservation of *in vitro*-derived buffalo embryos has yet to be proven. Hence, studies to understand and refine the systems for water buffalo *in vitro* embryo production and development of the vitrification techniques were carried out.

Viability of the resultant river buffalo embryos was assessed by non-surgical ET to both the river and swamp buffaloes. Swamp buffaloes under field condition were used to tests the applicability and efficiency of embryo IVP-cryopreservation techniques as potential tool for the propagation of genetically superior buffaloes.

2.2. Materials and Methods

To develop the systems for embryo IVP and cryopreservation in buffalo and test the viability of the resultant embryos, three experiments were conducted. Experiment 1-A aimed to establish the methods of embryo IVP in buffalo utilizing techniques developed in cattle. Experiment 1-B was carried out to develop and establish vitrification techniques for the cryopreservation of *in vitro*-produced embryos. Experiment 1-C was designed to examine the viability and full term development of *in vitro*-produced-cryopreserved embryos and test the potential application of this technique for swamp buffaloes as recipients and surrogate mothers of river buffalo embryos under field condition.

2.2.1. Experiment 1-A: Embryo *in vitro* production in water buffaloes following the techniques used in cattle

To produce water buffalo embryos *in vitro* and test the applicability of techniques developed in cattle, IVM of oocytes and culture of embryos were carried out following the IVM and IVC methods described by Hamano and Kuwayama (1993). This meant IVM using TCM 199 with serum and IVC using the same IVM medium with cumulus cell co-culture and IVF method as described by Hufana-Duran (1996) on bovine with few modifications. Modification on the method was storing ovaries for 3 to 6 h due to the distance between the slaughter house and the laboratory. Cleavage and blastocyst development were examined and the speed of embryo development *in vitro* was recorded. Below are the details of the procedure.

2.2.1.1. Culture medium

Unless specified, the medium used to wash and culture the oocytes for IVM and development to preimplantation stage embryos after IVF was TCM 199 (Earle's salts with 25 mM HEPES, Gibco-BRL, Life Technologies, Inc., Grand Island, NY, USA). It

contained 10% FBS (Gibco-BRL) and antibiotics (100 units penicillin/mL and 100 mg streptomycin/mL, both from Sigma Chemical Co., St. Louis, MO, USA).

Culture medium was prepared in 100 µL droplets in Nunc tissue culture dishes (35mm x 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) covered with mineral oil (Embryo tested, Sigma Chemical Co.) and equilibrated in a humidified incubator (Forma Scientific 3111 Series, Forma Scientific Inc., Marietta, OH, USA) gassed with 5% CO_2 in air at 39.0ºC.

2.2.1.2. Oocyte collection and *in vitro* maturation

To establish the methods for embryo IVP (Experiment 1-A) and cryopreservation (Experiment 1-B) in buffaloes, collection of ovaries from slaughtered swamp buffaloes in the Philippines was done. Tests the viability of the *in vitro*-derived cryopreserved embryos (Experiment 1-C), used ovaries from river buffaloes in India. Ovary donors were animals of unknown ages and reproductive status.

The ovaries were transported to the laboratory in 0.9% sodium chloride solution with 100 units penicillin/mL and 100 mg streptomycin/mL (both from Sigma Chemical Co.) at 30 to 33ºC in a sealed container. Immature cumulus-oocyte complexes (COCs) were collected by aspiration from 2 to 8 mm surface follicles using an 18 gauge needle attached to a 10 mL sterile disposable plastic syringe.

Oocytes surrounded by multi layers of compact cumulus cells and evenly granulated cytoplasm graded as A and B (Chauhan *et al*., 1998a) were selected and washed two times in 37ºC pre-warmed PBS with 3% FBS. Then, these were washed two times in pre-equilibrated maturation medium. Oocytes were randomly distributed, 10 in each 100 µL of culture droplets and matured *in vitro* in a water-jacketed incubator for 22 to 24 h at 39ºC and 5% CO_2.

2.2.1.3. Nuclear examination

To determine the IVM rate of the oocytes, oocytes matured *in vitro* for 24 h were freed from cumulus cells, then fixed in 1:3 aceto-ethanol solution and dehydrated with

absolute ethanol, then stained with 1% aceto-orcein (Merck & Co., Inc., Whitehouse Station, NJ, USA) and de-stained with aceto-glycerol (Glycerol: acetic acid: distilled water = 1:1:3 v/v) as described by Hufana-Duran (1996). Oocyte nuclear maturation was evaluated with a compound microscope at x 200 to x 400 following the method described by Hunter and Polge (1966).

2.2.1.4. *In vitro* fertilization

In vitro fertilization droplets made up of BO solution (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine and 4 units heparin/mL were prepared on 35 mm x 10 mm tissue culture dishes as 50 μL droplets covered mineral oil. Then, this was equilibrated inside a water jacketed incubator at 39ºC and 5% CO_2.

Frozen semen from Murrah buffaloes were thawed at 37ºC for 15 sec, dispensed in a sterile centrifuge tube and layered with 6 mL of freshly prepared 37ºC pre-warmed BO solution containing 10 mg BSA Fraction V (Wako Pure Chemical Industries, Osaka, Japan)/mL (BO medium, sperm washing medium). Semen suspensions were washed two times with BO medium by centrifugation at 800 x *g* for 8 min discarding the supernatant after each wash.

The sperm concentration was determined in a sperm counting chamber (Neubauer chamber) and the sperm suspension was adjusted to $2x10^6$ cells/mL by adding BO medium (sperm dilution solution). Then the IVF droplets were diluted 1:1 v/v with sperm dilution solution to form an IVF medium with final sperm concentration of $1x10^6$ sperm cells/mL, 5 mM caffeine, 2 units heparin/mL and 5 mg BSA/mL. Subsequently, *in vitro* matured oocytes were partly removed from cumulus cells. Detached cumulus cells were retained on the droplets to develop cumulus cell monolayer for embryo co-culture.

The partly denuded oocytes were washed two times in freshly prepared pre-incubated oocyte washing medium and one time in a dish of IVF medium. Ten oocytes were transferred to each IVF droplet. Sperm-oocyte co-culture for IVF was done for a period of 6 to 8 h inside a humidified incubator gassed with 5% CO_2 in air at 39ºC.

2.2.1.5. Embryo culture and blastocyst development

Sperm and oocyte were co-cultured approximately for 6 to 8 h for IVF. The oocytes were removed from the fertilization dish, washed four times in pre-incubated culture medium and transferred into the former IVM droplets containing cumulus cells and cultured *in vitro* for embryo development. After 36 h of IVC, cleaved embryos were separated from the uncleaved ones. Embryo development was monitored daily and the culture medium was renewed every 2 days. Embryos that developed to morula, early blastocyst, blastocyst and expanded blastocyst stages, respectively, were cryopreserved by vitrification method. Embryos were classified and evaluated according to the description of Lindner and Wright (1983).

2.2.2. Experiment 1-B: Cryopreservation of *in vitro*-derived water buffalo embryos by the vitrification techniques

Embryos produced *in vitro* following the methods described in Experiment 1-A were used. The cryoprotectant used was EFS40 (Kasai *et al*., 1990a) made up of ethylene glycol (Wako Pure Chemical Industries, Osaka, Japan) diluted at 40% (v/v) in modified phosphate buffered solution (PB1) containing 30% (w/v) ficoll 70 (average molecular weight 70,000; Pharmacia Biotech AB, Uppsala, Sweden) as non-permeating agent plus 0.5 M sucrose (S-PB1) (Sigma Chemical Co.) a low molecular compound which causes embryo shrinkage by osmosis.

The final concentration of the vitrification solution was 40% v/v ethylene glycol, 18% w/v ficoll, 0.3 M sucrose. The precise protocol for preparing this solution was described elsewhere (Kasai, 1995). Ethylene glycol 10% v/v was used as pre-equilibration medium. The respective solutions were then designated as EFS40 and 10% ethylene glycol, respectively. PB1 medium was Dulbecco's phosphate-buffered saline containing 5.56 mM glucose, 0.33 mM pyruvate, 100 unit penicillin/mL and 3 mg BSA fatty acid free/mL.

To develop the vitrification techniques for cryopreservation of water buffalo embryos, two methods were examined; A) in-straw vitrification following the techniques described by Zhu *et al*. (1993) and Kasai *et al*. (2002) (Fig. 2-1) and B) by the ultra-rapid vitrification as described by Vajta *et al*. (1998) with modifications by using an open-poled straw (Fig. 2-2). Exposure time of 0.5 and 1.0 min and effect of embryo stage such as morula, early blastocysts, blastocysts, and expanded blastocysts were assessed. Also, fracture damage, intactness of the zona pellucida and re-expansion rate were assessed at post-warming, and hatching rate within 72 h of further IVC were evaluated to assess the efficiency of the vitrification protocol.

2.2.2.1. Vitrification by in-straw method

Early-, mid-, and expanded-blastocysts stage swamp buffalo embryos developed on the 6^{th} to 7^{th} day of IVC were vitrified essentially by a two-step method described for mice (Zhu *et al*., 1993; Kasai *et al*., 2002). EFS40 solution, 0.5 M sucrose and PB1 medium were prepared. In a 0.25 mL plastic straw (A-201, Instruments de Medicine Veterinaire, France), a column of 0.5 M sucrose (~55 mm) and two columns of EFS40 solution (~3 and ~13 mm) were aspirated successively, separated by air with a 1 mL syringe connected to a small silicone tube as demonstrated in Figure 2-1. The straw was held horizontally at 28 to 30ºC. The 3 mm EFS40 column was used to prevent contamination of the 13 mm EFS40 solution with the 0.5 M sucrose medium adhering to the wall of the straw.

Early-, mid-, and expanded-blastocysts were pre-equilibrated with 10% ethylene glycol at 28 to 30ºC in a watch glass for 5 min then exposed to EFS40 solution on a watch glass washed in the solution twice within an exposure time of 0.5 or 1.0 min. These were transferred into the 13 mm EFS40 column, and the columns of EFS40 solution (~3mm) and S-PB1 medium (~12mm) were aspirated and separated by air before being heat-sealed or sealed with the powder.

The third EFS40 solution (~3mm) was used to catch embryos that might have adhered to the wall of the straw during aspiration. Half of the sealed straw included the

EFS40 columns was rapidly immersed into the liquid nitrogen followed by slow immersion of the rest of the straw in order to prevent bursting of the straw.

2.2.2.2. Ultra-rapid vitrification using open-poled straw

In this method, the technique of open pulled straw method (OPS, Vajta *et al*., 1998) was modified by using an open-poled straw. The front tip of a 0.25 mL French straw was slightly sharpened diagonally with a blade and with the aid of a microscope (Fig. 2-2). This part served as loading area for the embryo. This allowed direct contact of the liquid nitrogen with the vitrification solution containing embryos. By using a steel rod, the natural plug at the opposite end of the straw was pushed to make both ends open making it an open-poled straw.

Subsequently, temperature inside the working room was adjusted to 25ºC and all solutions used were equilibrated in the same temperature for at least 3 h. The open-poled French straws were marked at one end with pens of various colors for embryo identification which included embryo grade, age (day) *in vitro*, developmental stage, date of production, and bull identification.

Embryos were pre-equilibrated with 10% ethylene glycol in PB1 medium for 2 min, and then within 0.5 min, embryos were washed in EFS40, transferred to new EFS40 and loaded to the tip of the pointed-shaped open-poled straw, and the tip of the straw was quickly plunged in liquid nitrogen. To prevent the embryo from dropping, straw was held for a few seconds before slowly submerging it completely into the liquid nitrogen. Thereafter, straws were kept in the liquid nitrogen tank.

2.2.2.3. *In vitro* viability test of *in vitro*-derived-vitrified embryos

Post-warming hatching rate was monitored to assess the viability of the embryos *in vitro*. For embryos vitrified on a sealed straw, straws containing embryos kept in liquid nitrogen for 1 to 120 days were taken out in the air and held for 15 sec to prevent fracture damage (Kasai *et al*., 1996). The lower half of the straw was submerged in a 25ºC water bath for 8 sec. In order for the heavy EFS40 to sink to the 0.5 M sucrose solution, the

whole straw was submerged on the water upside down. As the crystallized 0.5 M sucrose solution in the straw began to melt (~5 sec), the opposite of the natural plug was cut by a scissor or straw cutter. The contents of the straw were expelled in a watch glass by pushing the plug through a small steel rod and agitating gently.

For embryos vitrified by open-poled straw, embryo straws were recovered from liquid nitrogen tank and the open pole where embryos were loaded was directly warmed in 0.5 M sucrose solution at 25ºC for re-expansion within 5 min. Embryos were then pipetted into fresh 0.5 M sucrose solution at room temperature.

The survival of vitrified-thawed embryos was assessed by washing twice the recovered embryos with PB1 medium followed by another wash in a pre-warmed culture medium. Embryos were then cultured for further development for 72 h in IVC medium with 6-day old cumulus cell monolayer at 39ºC and 5% CO_2 in a water-jacketed incubator. The embryos were examined every 24 h under a stereo microscope. The embryos that had a re-expanded blastocoel within 48 h of IVC and hatched from the zonae within 72 h were recorded and removed from the culture dish.

2.2.3. Experiment 1-C: Viability of *in vitro*-derived-vitrified embryos

The viability of *in vitro*-derived-vitrified embryos was examined. *In vitro*-derived vitrified river buffalo embryos produced and cryopreserved following the embryo IVP system and the ultra-rapid vitrification technique described above were transferred to river and swamp buffalo recipients undergoing natural or synchronized estrus. Since the embryos produced *in vitro* were from river buffaloes, viability after ET was examined using the same species to eliminate the possible effect of breed differences. The number of transferred embryo, nature of estrus of the recipient animal, stage and age of embryo during ET, and the weight of the calf at birth were recorded.

Viability of the river embryos using a swamp buffalo as a recipient was also examined to tests the full-term development of this embryo on a host of different breed and test the possibility of using swamp buffaloes to propagate river buffaloes. Pregnancy and fullterm development by calving rate were assessed.

2.2.3.1. Preparation of recipient animals

River buffalo recipients. River buffaloes, institutional herd of the Philippine Carabao Center with a complete confinement system of management were either treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (2 mL intramuscular, Prostavet, Virbac Laboratories, France) to induce estrus as described by Cruz *et al*. (1988) or these were made to receive the embryo after detection of natural estrus. For estrus synchronized recipient animals, functional corpus luteum was checked manually by palpation per rectum prior to $PGF_{2\alpha}$ administration. From the 36^{th} to the 72^{nd} h after the prostaglandin injection, recipient animals were carefully observed every 4 h for estrus symptoms and particularly for the moment of standing heat, using a teaser bull.

Signs of estrus considered were either standing-still while being mounted, tail raining, mucus discharge, frequent urination, bellowing and mounting or a combination of all these manifestations. Symptoms of estrus were confirmed by palpation per rectum of the genitalia. Upon estrus confirmation, embryos were transferred according to their developmental age in the recipient animals on Day 7±1 post-estrus (Day 0=onset of observed estrus/onset of IVF). Recipient animals were treated with lidocaine hydrochloride (2% intramuscular, Ethical Pharmaceutical Co. Pvt. Ltd., India) at varying levels depending on the response while in the chute.

Swamp buffalo recipients. Swamp buffaloes were Philippine carabaos owned by farmers. Selection of recipients and transfer of embryos were all performed at the villages. Due to lack of recording practice among rural farmers, the Philippine carabaos were of known (n=28, 5 to 12 years old) and unknown (n=12) ages. None of the farmers have a record of estrous cycle or accurate data on the latest occurrence of estrus of their animals. Therefore, the candidate animals were subjected to palpation per rectum to examine the reproductive system for any abnormalities and assess suitability for ET.

Animals with palpable functional corpus luteum and active ovarian structures and of at least one calving, and 3 to 4 body scores were selected as recipients. Selected

animals were either treated with $PGF_{2\alpha}$ to induce estrus or otherwise embryo was transferred on Day 7±1 after detection of spontaneous estrus (Day 0=day of standing estrus).

Signs of estrus were the same as described above and were confirmed by palpation per rectum of the genitalia and by examining the turgidity of the uterus. For estrus synchronized recipient animals, functional corpus luteum was checked manually by palpation per rectum prior to $PGF_{2\alpha}$ administration and prior to ET. Synchronized animals that exhibited estrus symptoms were transferred with embryos on Day 7±1 after estrus manifestation.

Synchronized animals that did not exhibit estrus symptoms after the first $PGF_{2\alpha}$ injection were subjected to a second $PGF_{2\alpha}$ injection on Day 11 after the first injection. Transfer of embryo was done on the 10^{th} day after the second $PGF_{2\alpha}$ injection.

2.2.3.2. Warming and transfer of embryos

For ET performed on the institutional herd, warming of embryos was carried out as described for embryos vitrified by open-poled straw in Section 2.2.2.3 above. Embryo straws were recovered from liquid nitrogen tank and the pointed pole where embryo(s) were loaded was directly warmed in 0.5 M sucrose solution in PB1 medium at 25ºC for re-expansion within 5 min. Re-expanded embryos were washed in PB1 medium and either 1, 2 or 3 embryos were loaded in 0.25 mL French straws between two air pockets and two columns of loading medium for non-surgical ET. Embryos that did not re-expand within 5 min were used for another experiment. The straw was inserted into an ET gun (Fujihira Instruments, Tokyo, Japan), covered with an ET sheath (NFA 490, Fujihira Instruments) fixed at the end. A sheath protector cap (NFA 493, Fujihira Instruments) was placed over the ET sheath to prevent contamination during vaginal insertion.

Before the transfer of the embryo(s), the presence of corpus luteum in the ovaries was checked by palpation per rectum. To enhance easy penetration of ET gun into the cervix, a cervix expander (Fujihira Instrument) was first inserted into the vagina of the recipient animals. After expanding the cervix, the expander was removed and the tip of the ET gun was placed in the external os of the cervix and pushed through the sheath

protector cap before entering the uterus. The ET gun was guided and pushed gently until it reached the uterine horn ipsilateral of the ovary bearing the corpus luteum. The tip of the gun was inserted up to 5 to 10 cm beyond the external bifurcation where embryo was deposited into the uterine horn.

At the villages, warming of embryos was modified. Warming of embryos was carried out inside a vehicle (Revo, Toyota, Japan) equipped with air-condition facility. Temperature inside the vehicle varied from 20 to 27°C depending on the location and outside environmental factors. Warming solution (0.5 M sucrose in PB1) and embryo-loading medium (PB1 medium) were kept on ice during transport from laboratory to the villages but removed from ice prior to use. Warming of embryos was done in 0.5 M sucrose solution at a temperature of 15 to 27°C. Thereafter, the embryos were transferred to PB1 medium and processed for ET as described above.

2.2.3.3. Pregnancy diagnosis and calving rate

Recipient animals were subjected to palpation per rectum for at least 30 days after ET to check the persistency of the corpus luteum present during the transfer of the embryos. Thereafter, confirmation of pregnancy was done by another palpation per rectum at least 45 and 180 days after the transfer. The number of calves born out of the diagnosed pregnant recipient animals was recorded.

2.2.4. Statistical analysis

Results were expressed as percentage. Statistical comparison of the cleavage and development rates up to the blastocyst stage was performed using Chi-square with Yate's correction (Snedecor and Cochran, 1980) or Fisher's Exact Test for any significant differences.

2.3. Results

Results for each of the conducted experiments are presented by sections below;

2.3.1. Experiment 1-A: Embryo *in vitro* production in the water buffaloes following the techniques used in cattle

Cleavage and blastocyst development rates are presented in Table 2-1. Out of 183 oocytes, cleavage rate was 53.5 percent and blastocyst development was 11.5 percent out of the number of oocytes subjected to IVM/IVF. Cleavage to different cell stages significantly differed ($P<0.01$) among oocytes with 39.3 percent cleaved to the 2- to 4-cell stage, 10.9 percent cleaved to the 5- to 6- cell stage, and 3.3 percent cleaved to the 7- to 8- cell stages 24 h after IVF (or 32 h after onset of IVF).

Of the cleaved zygotes, 2- to 4- cell stages were highest (73.5%), followed by 5- to 6- cell stage (20.4%) and 7- to 8- cell stage (6.1%). These showed lower number of fast developing zygotes than the slow developing ones.

Development to expanded or hatched stages on 7th day of IVC did not differ significantly with 8.2 percent expanded blastocyst and 3.3 percent hatched blastocyst. Computed out of the total number of cleaved zygotes, blastocyst development was 21.4 percent. The block of development was observed from 2- cell stage with the highest incidence in 8- to 16- cell stage embryos.

Speed of *in vitro* development is presented in Table 2-2. Morula stage embryos were observed in 10.5 percent of fertilized oocytes on the 4th day of IVC (96 to 100 h after IVF) while 15.6 percent was in blastocysts on the 5th day (120 to 135 h after IVF), 6.1 percent turned out to be in expanded blastocysts on the 5th day (140 h) and 6.1 percent became hatched blastocyst on the 6th day (152 h).

2.3.2. Experiment 1-B: Cryopreservation of *in vitro*-derived water buffalo embryos by the vitrification techniques

Result on vitrification of water buffalo embryos by in-straw vitrification technique is presented in Table 2-3. After warming, all embryos (100%, 34/34) were recovered. Four embryos had injured zona, and 30 (88.2%) had intact zona. This suggested fracture damage in some embryos.

Re-expanded blastocoels were found in 77.8 percent (7/9) of early blastocyst exposed for 0.5 min in EFS40, and 55.5 percent (5/9) had the ability to hatch from their zona pellucida within 72 h of IVC. At 1.0 min exposure, 66.7 percent (4/6) of early blastocyst stage embryos had re-expanded blastocoel, and 16.7 percent (1/6) had the ability to hatch. For mid-blastocysts and expanded blastocyst stage embryos, only 40.0 to 42.8 percent embryos exposed for 0.5 min in EFS40 had re-expanded blastocoel and only 14.3 to 20.0 percent hatched after further IVC.

By ultra-rapid vitrification using open-poled straw, improved results were obtained with 82 to 88 percent post-warming hatching rate for morula to expanded blastocyst stage embryos (Table 2-4). During warming all embryos (100%, 94/94) were recovered and 94.0 to 100 percent had an intact zona pellucida. The re-expansion rate (82.0 to 94.0%) of all stages was also similar. High proportions (82.0 to 88.0%) of embryos were able to hatch out of the zona pellucida within 72 h of IVC.

2.3.3. Experiment 1-C: Viability of *in vitro*-derived-vitrified embryos

Results of examining the viability of the *in vitro*-derived-vitrified water buffalo embryos to river and swamp buffalo recipients showed that the *in vitro*-derived-vitrified embryos were viable resulting in normal healthy calves after non-surgical ET.

In river buffaloes as recipients, 166 *in vitro*-derived river buffalo embryos vitrified by ultra-rapid vitrification utilizing an open-poled straw technique were used. Of this member, 71 were warmed for IVC to assess hatching rate within 72 h while 95 were transferred non-surgically to the river buffalo recipients to assess full term development. Eighty-three percent (59/71) of the embryos hatched during the incubation period (Table 2-5).

No significant difference was observed in *in vitro* survival rates after vitrification and warming in embryos of different developmental stages thereby confirming the efficiency of the vitrification technique. Of the embryos transferred to 55 recipient animals (1-3 embryos per animal) as shown in Table 2-6, nine (16.4%) of the recipients were diagnosed pregnant 45 days after ET. Of the pregnant animals, six (10.9%) gave birth to live healthy normal calves (Fig. 2-3) after an average of 312.8±3.0 days of

gestation. An increased pregnancy rate and live birth rate were observed when recipients received 2 or 3 embryos during transfer although these did not differ significantly.

Similarly, calving rate was higher when recipients are in natural estrus (16.1%) than in synchronized estrus (4.2%) (Table 2-7). Average birth weights of calves born are presented in Table 2-8. Birth weights of resultant calves ranged from 33 to 44 kg. No signs of large calf syndrome were observed.

Morula stage embryos, early blastocysts and expanded blastocysts that developed on Days 5, 7 and 8 of IVC, respectively, were capable of undergoing full-term development and birth of live calves after vitrification and ET (Table 2-9). However, no pregnancies resulted after transfer of Day 8- early blastocyst and blastocyst stages embryos suggesting a compromised viability among late-developed embryos.

In swamp buffaloes as recipients, a total of 40 animals served as recipients for 80 *in vitro*-derived-vitrified river buffalo embryos (Table 2-10). Of the 40 recipient swamp buffaloes, 35 (87.5%) were treated with prostaglandin to synchronized estrus while five (12.5%) received embryos after observation of natural estrus.

Under field condition, gathering of animals by natural estrus is difficult. Nevertheless, pregnancy rate was 12.5 percent (5/40), of which 8.6 percent (3/35) was diagnosed in recipients with synchronized estrus, and 40.0 percent (2/5) in recipients with natural estrus. Four healthy calves were delivered normally, presenting a 5.0 percent (4/80) full-term development rate and 10.0 percent (4/40) calving rate. Of the calves delivered to term, three (75.0%) were delivered alive and healthy, while one (25.0%) was a case of stillbirth caused by dystocia. The live calves were all healthy and delivered normally after an average 302.3±10.4 days gestation period (range: 294–314 days). Figure 2-4 presents one of the river buffalo calf with its surrogate mother, a swamp buffalo.

2.4. Discussion

In the present experiment, pre-implantation stage embryos were produced in water buffaloes following the *in vitro* systems developed for cattle. However, the success rate in water buffalo (53.5% cleavage and 11.5% blastocysts), was sub-optimal compared to

cattle. Hamano and Kuwayama (1993) reported 66.1 percent cleavage and 36.4 percent blastocyst development following the same culture system. The lower success rate in water buffalo which could be due to the inadequacy of the culture conditions or the inherent character of the water buffalo oocytes is a subject for further study.

One factor that differed between the present experiment and that of Hamano and Kuwayama (1993) was the duration of storage of the ovaries after excision from the peritoneal cavity of the donor (3 to 6 h versus < 3 h). It is possible that the longer storage of the ovaries in the present experiment may have affected the developmental competence of the oocytes caused by cellular damage due to autolytic processes after residing for prolonged period in excised ovaries. Thus, the effect of ovary storage on the developmental competence of the oocytes is a subject for follow-up research to elucidate the present findings.

However, results of the present study also conformed to the observations in other studies following different culture systems (Totey *et al*., 1992; 1993a, b; Madan *et al*., 1994b). These indicated that lower success rate of embryo IVP in water buffaloes might be attributed to fragility of buffalo semen (Madan *et al*., 1994b), higher lipid content of oocytes of water buffaloes than cattle (Boni *et al*., 1992) and higher sensitivity to stress of buffalo oocytes than cattle oocytes (Neglia *et al*., 2003).

The development of water buffalo embryos was a day earlier when compared to that of cattle: morula at 141 h and blastocysts at 166 h (Hufana-Duran, 1996; Van Langendonckt *et al*., 1996). This observation confirmed the review by Gasparrini (2002) and reports of other authors indicating that buffalo embryos develop 24 to 36 h earlier than domestic cattle embryos (Drost *et al*., 1986; Karaivanov *et al*., 1987; Misra *et al*., 1990) although the pattern of embryonic development up to the blastocyst stage is similar (Jian *et al*., 1998).

In fact, using confocal microscopy, Neglia *et al*. (2001) showed that the highest proportion of MII oocytes occurred at 19 h in buffalo compared to 24 h in cattle and reach the early- and mid-blastocyst stages at 141 and 156 to 176 h after estrus (Anwar and Ullah, 1998; Narula *et al*., 1996).

Similarly, a study by Roy and Atreja (2008) demonstrated a differential capacitation and protein tyrosine phosphorylation pattern in cattle and buffalo

spermatozoa. They showed that buffalo sperm took more time than cattle for capacitation but its associated protein tyrosine phosphorylation event started very early, 0 h of incubation, as compared to 4 h in cattle.

Buffalo sperm membrane was also found to have more ether phospholipids compared to cattle (Jain and Anand, 1976). Ether phospholipids, more commonly known as platelet activating factor released from the sperm membrane in some species when diluted in capacitating medium, is responsible for an autocrine induction of spontaneous capacitation in mammalian spermatozoa (Wu *et al*., 2001). These differences between cattle and buffalo may indicate that the requirements for successful maturation, fertilization and development are also different between these two animal species. These findings suggest a need to refine the IVC systems to suit the water buffalo requirements.

Developmental speed of fertilized oocytes varied among oocytes with some oocytes developing to 7- to 8- cell stage while others were at 2- to 4- cell stage at 24 to 30 h of IVC. Whether this variability was due to the inherent quality of the oocyte, effect of the culture condition or the timing of fertilization by the sperm cell was not accounted for in the present experiment.

However, it was demonstrated (Totey *et al*., 1996) that the time of the first cleavage post insemination *in vitro* and the developmental competence are related with those oocytes cleaving earliest after IVF being more likely to reach the blastocyst stage than their late-cleaving counterparts. This phenomenon was indicated as common to many species (mouse, Warner *et al*., 1998 and Dinnyes *et al*., 1999; bovine, Lonergan *et al*., 1999; human, Fenwick *et al*., 2002). Concomitantly, recent data have demonstrated that the post fertilization culture system used impacts considerably on the mRNA expression of fast-cleaving embryos that are more likely to develop to blastocyst stage (Gutierrez-Adan *et al*., 2004).

In the present experiment, the *in vitro* matured and fertilized buffalo COCs were cultured *in vitro* in the presence of cumulus cell and 10% FBS in IVC medium and 11.5 percent blastocyst development were achieved. This blastocyst development rate was higher than that obtained by Madan *et al*. (1994b). They found out that using the same media formulation over an IVC period of 3 to 9 d, 4.0 percent of the inseminated oocytes developed to the morula stage when cultured with cumulus cells alone and 17.8 percent

when cumulus cells plus oviductal epithelial cells were used. The difference on the success rate between these studies could not be accounted because of differences in source of oocytes, sperm preparation techniques, oocytes maturation and fertilization conditions, and embryo culture environments as well as other factors that have major effects on embryo development rates, yield and quality. Also, conditions influencing embryo production and the generation of pregnancies following ET vary between laboratories. However, this observation suggests that the nature of products secreted by the somatic cells and their specific roles in embryonic development merit further investigations.

Around 80 percent of the cleaved zygotes failed to further develop *in vitro* with block of development observed from 2- cell stage with the highest incidence in 8- to 16-cell stage embryos. This development block was also observed in other mammalian species (mouse, Legge and Sellens, 1991; bovine, Takahashi *et al*., 1993; cat, Swanson *et al*., 1996). This was also found to be influenced by the genetic background of the embryo (Dinnyes *et al*., 1995) and the IVC system (Gutierrez-Adan *et al*., 2004). The biological reason behind this arrest was indicated to be the maternal-zygotic transition of gene activities which results in a drastic change in embryo protein synthesis (Bagis *et al*., 2003). Studies to improve the IVC system, especially in catering the metabolic requirements of the oocytes and embryos may help overcome this development block. Therefore, this is a potential subject of further research.

On the vitrification of the resultant *in vitro*-produced embryos, vitrification by in-straw methods was sub-optimal. However, ultra-rapid vitrification by open-poled straw method was successful resulting in the development of cryopreservation of pre-implantation stage embryos of water buffalo by the vitrification technique.

The in-straw vitrification technique was not efficient as shown by decreased survival of embryos post-warming. This could be due to slow cooling that resulted in longer exposure or more permeation of the embryos to the cryoprotectant. This was detrimental as shown by the low survival rate of post-warming when the exposure time of embryos to EFS40 was extended to 1.0 min. This confirmed the observations in mouse by Miyake *et al*. (1993) and Shaw *et al*. (1991) indicating that more permeation is harmful to

embryos. The blastocoelic cavity was likely to be frozen which might have damaged the cells suggesting a need of rapid cooling to avoid osmotic injury.

In ovine, viable lambs were produced at the rate of 7.4 percent survival by this method of vitrification using 25% glycerol and 25% ethylene glycol solution (Okada, *et al*., 2002). In bovine, Kuwayama (1994) also reported a successful in-straw dilution method by vitrification. The present result indicates that direct transfer with the in-straw dilution method seemed possible; however, refinement and modification of the technique to improve the viability of vitrified *in vitro*-produced buffalo embryos is recommended.

On the other hand, the advantage of the ultra-rapid vitrification technique that resulted in high survival rate of embryos post-warming might have been on the high cooling and warming speed. The reduced volume of solutions (approximately 0.5 µl in the present technique and more than 5µl in in-straw method) and the increased rate of cooling and warming might have considerably decreased the chilling injury, thus, avoiding fracture damage. This was shown by the intact zona pellucida and high re-expansion rate.

The beneficial effect of cooling and warming is a common phenomenon when embryos are rapidly cooled or warmed in normal straws (Kasai *et al*., 1996). Another reason for the high survival in the ultra rapid vitrification can be the immediate dilution of the cryoprotective additives during the warming process. Because the total exposure time of embryos to the concentrated cryoprotectant solutions over -180ºC was less than 30 sec, the toxic and osmotic effect of these solutions might have been minimized (Vajta *et al*., 1998) thus, higher survival rate of the embryos was recorded.

After non-surgical ET, the *in vitro*-derived vitrified embryos were found viable. This was evidenced by 10.9 percent and 10.0 percent calving rates after ET in river and swamp buffalo recipients, respectively. The data shows that *in vitro*-derived buffalo embryos can withstand the additional stress imposed by the vitrification technique and results in birth of live healthy calves after ET.

The high percentage of hatchability of the vitrified-warmed embryos likewise indicates that the *in vitro*-produced embryos are viable. Also, vitrification and warming system applied to *in vitro*-derived embryos is effective for cryopreservation of buffalo

embryos and the technology of embryo IVP-vitrification-transfer could be an efficient tool for the water buffalo genetic improvement.

A tendency of higher calving rate was observed when embryos were transferred in twos and threes to a recipient animal (Table 2-6) suggesting quality of embryo and synchrony of the recipients as factors affecting success rate. In early trials, pregnancy rates following non-surgical transfer of *in vivo*-derived buffalo embryos were 9.2 percent (Kurup, 1988), 17.9 percent (Drost *et al*., 1988), 18.3 percent (Alexiev *et al*., 1988) and 17.0 percent with 9.8 percent success rate (Misra *et al*., 1994). These findings were confirmed by this study using *in vitro*-produced vitrified embryos.

On the other hand, Galli *et al*. (1998) reported three live calves (33.3%) born after transfer of embryos cryopreserved by slow-freezing, using OPU-recovered oocytes, matured and fertilized *in vitro* and cultured *in vivo* in the oviducts of ewes. Madan *et al*., (1994b) reported 12.5 percent and Techakumphu *et al*. (2001) found out a 14.3 percent (2/14) calving rate in the transfer of *in vitro*-derived buffalo embryo transferred fresh to the recipient animals.

The low success rate in the present study may be associated with the *in vitro* embryo production per se considering that buffalo embryos produced *in vivo* and cryopreserved by slow-freezing method resulted in 23.0 percent calving rate (Kasiraj *et al*., 1993). Nevertheless, *in vitro*-derived embryos that reached morula, early blastocyst, and expanded blastocyst stages all resulted in birth of live calves. Previous study (Totey *et al*., 1996) noted a high tendency of reduced number of blastomeres in buffalo embryos that developed late *in vitro*.

Though data in the present report are limited, early blastocyst stage embryos developed on Day 7 of IVC (Table 2-9), which was 1 to 2 days late nonetheless resulted in birth of live calf after transfer. This suggested that though viability was compromised among late-developed embryos *in vitro*, live calves can still be produced as long as the morphology is within the acceptable quality and have developed to the advanced preimplantation stages.

In conclusion, water buffalo embryos could be produced *in vitro* following methods described in cattle although success rate was sub-optimal. There is a need to refine the techniques to suit the water buffalo requirement. Speed of development in

water buffalo embryos is a day earlier compared to cattle, suggesting that culture requirement may differ implying further study.

The two-step ultra-rapid vitrification technique, using open-poled straw with 10% ethylene glycol for pre-equilibration and EFS40 as cryoprotectant solution at 0.5 min exposure time, is an effective method for cryopreservation of water buffalo pre-implantation stage embryos. The *in vitro*-produced-vitrified water buffalo embryos are viable. To overcome embryo-recipient asynchrony and improve success rate, the transfer of two different stage embryos is recommended. Differences in chromosome numbers between the embryo (2n=50) and the recipient (2n=28) does not hinder the success of ET in water buffalo; hence, swamp buffaloes can be used as potential recipients and surrogate mothers for the propagation of river buffaloes. However, further studies to improve the current success rate are necessary before the technology can be routinely used for buffalo breeding. Factors that can improve production of good quality embryos produced *in vitro* need to be investigated.

Table 2-1. Cleavage and blastocyst development rates of swamp buffalo oocytes matured and cultured in serum supplemented culture medium after *in vitro* cross-fertilization with river buffalo semen

Parameters	**Out of oocytes (%)**	**Out of zygotes (%)**
Number of oocytes	183	98
Cleaved embryos[1)]		
2 to 4 cell stage	72 (39.3)[a]	72 (73.5) [a]
5 to 6 cell stage	20 (10.9)[b]	20 (20.4) [b]
7 to 8 cell stage	6 (3.3) [c]	6 (6.1) [c]
Cleavage rate	98 (53.5)	
Developed to blastocysts[2)]		
Expanded blastocysts	15 (8.2)	15 (15.3)
Hatched blastocyst	6 (3.3)	6 (6.1)
Blastocyst development rate	21 (11.5)	21 (21.4)

[1)] *Observations carried out approximately 24 to 30 h after IVF or 30 to 36 h upon start of IVF.*

[2)] *Observations carried our on the 7th day (168 to 170 h) of in vitro culture after IVF.*

Values in the same column with different superscript are significantly different (P<0.01) by Chi-square.

Table 2-2. Speed of development of *in vitro* matured swamp buffalo oocytes cross-fertilized *in vitro* with river buffalo semen

Developmental stages	**IVC period, day (h after onset of IVF) ***	**Developed embryos, % Mean ± SD, n=98**
Morula	4 (96-100)	10.5 ± 2.0
Blastocyst	5 (125-130)	15.6 ± 2.4
Expanded blastocyst	5 (140)	6.1 ± 1.5
Hatched blastocyst	6 (152)	6.1 ± 1.5

**Onset of IVF is the time sperm-oocyte co-culture started.*

Table 2-3. Survival of *in vitro* produced swamp buffalo embryos after vitrification by in-straw vitrification method with EFS40 as cryoprotectant solution

Developmental stages	Exposure time, min	No. of embryos	No. of embryos recovered (%)	No. of embryos with intact zona (%)	Re-expanded within 48 h (%)	Hatched within 72 h (%)
Early blastocyst	0.5	9	9 (100)	7 (77.8)[a]	7 (77.8)[a]	5 (55.5)[a]
	1.0	6	6 (100)	4 (66.7)[a]	4 (66.7)[a]	1 (16.7)[b]
Mid-blastocysts	0.5	7	7 (100)	7 (100)[b]	3 (42.8)[a]	1 (14.3)[b]
	1.0	3	3 (100)	3 (100)[b]	0 (0)[b]	0 (0)[c]
Expanded blastocyst	0.5	5	5 (100)	5 (100)[b]	2 (40.0)[a]	1 (20.0)[b]
	1.0	4	4 (100)	4 (100)[b]	0 (0)[b]	0 (0)[c]
Total		**34**	**34 (100)**	**30 (88.2)**	**15 (44.1)**	**8 (23.5)**

[a, b] *Values are significantly different (P<0.05) by Chi-square.*

Table 2-4. Survival of *in vitro* produced swamp buffalo embryos vitrified in EFS40 by ultra-rapid vitrification using open-poled straw at 0.5 min exposure time after pre-equilibration with 10% ethylene glycol at 25 to 26ºC

Developmental stages	No. of embryos recovered (%)	No. of embryos with intact zona (%)	Reexpanded within 48 h (%)	Hatched within 72 h (%)
Morula	22/22 (100)	22 (100)	18 (82)	18 (82)
Early blastocyst	20/20 (100)	20 (100)	17 (85)	17 (85)
Mid-blastocyst	18/18 (100)	18 (100)	17 (94)	16 (88)
Expanded blastocyst	34/34 (100)	32 (94)	32 (94)	30 (88)

No significant difference was observed among values on each column.

Table 2-5. *In vitro* hatching rate of *in vitro*-derived river buffalo embryos after vitrification and warming

Embryo stages	**No. embryos examined**	**No. embryos hatched, (%)**
Morula	11	10 (90.9)[a]
Early blastocyst	20	16 (80.0) [a]
Mid-blastocyst	20	15 (75.0) [a]
Expanded blastocyst	20	18 (90.0) [a]
Total	**71**	**59 (83.1)**

[a] *Values are not significantly different (P>0.05) by Chi-square or Fisher's Exact Test.*

Table 2-6. Live birth rate of *in vitro*-derived vitrified river buffalo embryos after non-surgical embryo transfer to river buffalo recipients

Number of transferred embryos	Number of recipient animals	Number of pregnant, (%)	Number of recorded abortions*	Number of live births (%)
1	17	1	0	1 (5.88) [a]
2	36	7	3	4 (11.11) [a]
3	2	1	0	1 (50.00) [a]
Total	**55**	**9 (16.4)**	**3 (5.5)**	**6 (10.9)**

** Abortions were all recorded during the early trimester of pregnancy.*
[a] Values are not significantly different (P>0.05) by Fisher's Exact Test.

Table 2-7. Live births of *in vitro*-derived vitrified river embryos after non-surgical embryo transfer to recipient river buffaloes with either natural or synchronized estrus

Nature of estrus	Number of recipients	Resultant live births (%)
Natural	31	5 (16.1) [a]
Synchronized	24	1 (4.2) [a]
Total	**55**	**6 (10.91)**

[a] *Values are not significantly different (P>0.05) by Chi-square or Fisher's Exact Test.*

Table 2-8. Live births and average birth weights of river buffalo calves resulting from *in vitro*-derived vitrified river buffalo embryos at various stages of development after embryo transfer

Embryo stages at transfer	**Number of recipient**	**Number of live births, (%)**	**Number and age(s) of loaded embryo(s)**	**Average birth weights of calves, kg±SD**
a. Morula	7	1 (14.3) [a]	1, 5-day	44.0
b. Early blastocyst	12	1 (8.3) [a]	2, both 7-day	33 .0
c. Mid-blastocyst	11	0 [a]	0	-
d. Expanded blastocyst	17	3 (17.6) [a]	A	38.8±1.04
e. Combination of b&c	4	0 [a]	0	-
f. Combination of b&d	2	1 (50.0) [a]	2, both 6-day	39.0
g. Combination of c&d	2	0 [a]	0	-
TOTAL	**55**	**6 (10.9)**		**38.8±3.55**

A=Number and ages of loaded embryos are: for the first calf, 2 embryos, 8-day; for the second calf, 2 embryos, 7-day; and for the third calf, 3 embryos all developed on 7-day of IVC.

[a] *Values are not significantly different (P>0.05) by Fisher's Exact Test.*

Table 2-9. Live births in relation to *in vitro* developmental ages (expressed by number of days) and stages of embryos during vitrification and transfer

Embryo stages at transfer	**5 Days**		**6 Days**		**7 Days**		**8 Days**		**Total**	
	n	**LB**	**n**	**LB**	**n**	**LB**	**n**	**LB**	**n**	**LB**
a. Morula	7	1	-	-	-	-	-	-	7	1
b. Early blastocyst	2	0	4	0	5	1	2	0	13	1
c. Mid-blastocyst	-	-	3	0	4	0	2	0	9	0
d. Expanded blastocyst	1	-	8	0	7	2	1	1	17	3
e. Combination of b&c	1	-	-	-	1	0	1	0	3	0
f. Combination of b&d	-	-	4	1	-	-	-	-	4	1
g. Combination of c&d	-	-	1	0	1	0	-	-	2	0
Total	**11**	**1**	**20**	**1**	**18**	**3**	**6**	**1**	**55**	**6**

n= No. of recipient animals; LB= Live births.

Table 2-10. Pregnancy and calving rates after non-surgical transfer of *in vitro* derived vitrified river buffalo embryos (2n=50) to swamp buffalo recipients (2n=48)

Nature of estrus	Number of recipients	Pregnancy rate (%)	Calving rate (based on number of recipients)		
			Total	Normal	Stillbirth/ dystocia
Synchronized	35	3 (8.6)[a]	2 (5.7)[a]	2	0
Natural	5	2 (40.0)[b]	2 (40.0)[b]	1	1
Total	**40**	**5 (12.5)**	**4 (10.0)**	**3 (75.0)**	**1 (25.0)**

Figures in the same column with different superscript are significantly different (P<0.05) by Fisher's Exact Test.

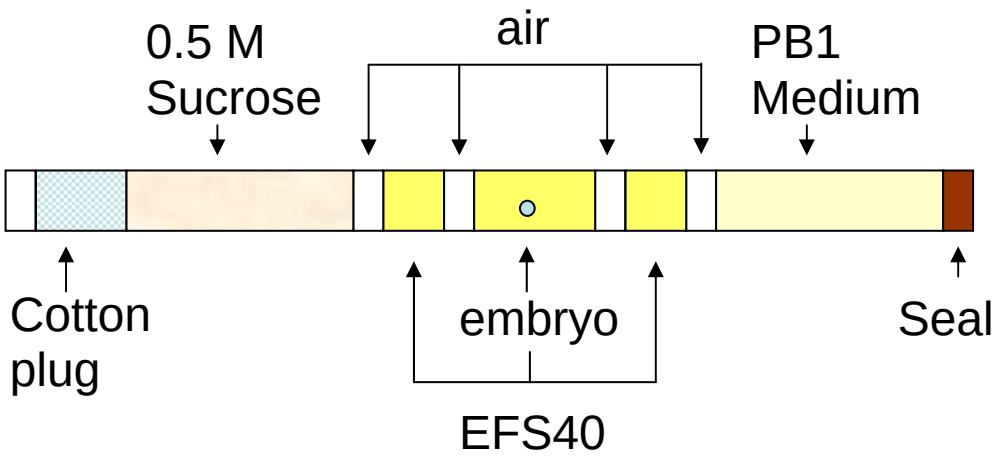

Figure 2-1. Configuration of a 0.25 mL French straw containing the embryo in in-straw vitrification technique. A 0.5 M Sucrose solution (~55 mm) was first aspirated followed by a space of ~2 mm air, then ~3 mm and ~13 mm EFS40 solution successively aspirated and separated by a space for ~2 mm air. The ~13 mm EFS40 solution is where the embryo was loaded. After loading the embryo, a space for ~2 mm air was created then ~3 mm EFS40 was again aspirated followed by and air space then PB1 medium before completely sealed by heat or a powder.

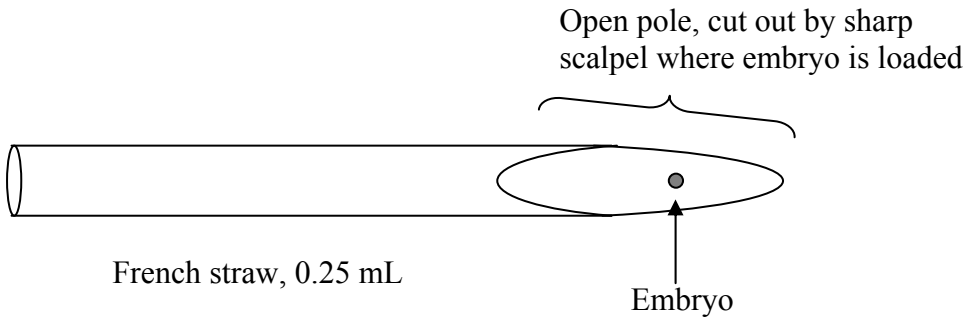

Figure 2-2. Illustration of a French straw prepared as pointed-shaped open-pole where embryos are loaded to allow ultra-rapid vitrification.

Figure 2-3. River buffalo calves born out of *in vitro*-derived-vitrified-warmed embryos after non-surgical embryo transfer to river buffalo recipients at the institutional herd.

Figure 2-4. River buffalo (2n=50) calf (right) born from swamp buffalo (2n=48) surrogate mother (left) after non-surgical transfer of *in vitro*-derived vitrified embryo.

CHAPTER III. Factors Affecting Production of Water Buffalo Embryos *in vitro*.

3.1. Introduction

Viability of *in vitro*-produced-vitrified buffalo embryos and the potential application of embryo IVP and vitrification techniques for production and transport of buffalo embryos from germplasm-rich sources to guarantee genetic improvement in many parts of the world have been demonstrated. However, while the success of vitrification is high, the overall efficiency of IVP of embryos is sub-optimal as shown in Chapter II. Improvement of IVP systems requires serious attention specifically in increasing the success rate in yield of transferable quality embryos to optimize the reproduction performance of genetically superior animals and enable efficient production and birth of calves after ET.

Several factors may affect the production and quality of embryos. Some of these factors may be the handling of ovaries as sources of oocytes, the duration and culture conditions during IVM. These factors could be evaluated by the nuclear maturation of the oocytes after IVM, the rate of blastocyst development and the number of blastomeres of the resultant embryos after culture. The incidence of chromosome abnormalities among early stage embryos may also be used to determine the factors that cause cellblock and low blastocyst yield.

The effects of hormones and growth factors on IVM of oocytes are well established in bovine (Lonergan *et al*., 1996; Sakaguchi *et al*., 2000, Kobayashi *et al*., 1994) although with few reports in buffaloes (Chauhan *et al*., 1999; Purohit *et al*., 2005). So far, very little information is available on the influence of hormones and EGF supplementation during IVM on the subsequent development of buffalo oocytes. Hence, a study was carried out to examine the effects of FSH, E_2 and EGF or their combination as supplements in IVM medium. To determine points for improvement in the water buffalo embryo IVP, the incidence of chromosome abnormalities was also examined.

3.2. Materials and Methods

Four studies were conducted to determine factors affecting blastocyst development of buffalo embryo *in vitro*. Experiment 2-A examined the effect of ovary storage, Experiment 2-B, the effect of duration of IVM of oocytes, Experiment 2-C, the effect of supplementing hormones and growth factor in IVM medium, and Experiment 2-D, the incidence of chromosome anomalies in early stage embryos. The experimental procedures in each experiment are discussed below;

3.2.1. Experiment 2-A: Effect of ovary storage on cleavage and blastocyst development

The effects of ovary storage on cleavage and blastocyst development were examined using swamp buffalo ovaries obtained from a local abattoir. These were divided into two groups. Group 1 was stored and transported to the laboratory within 3 to 4 h, and Group 2 was stored and transported to the laboratory within 5 to 6 h. Storage condition was in a normal saline (0.85% sodium chloride) at 30 to 33ºC.

In each group of ovaries, cumulus-oocyte complexes were aspirated from 2 to 8 mm diameter follicles using 18-gauge needle fitted to a 10 mL syringe and processed for IVM, IVF and IVC as described in Experiment 1-A. Briefly, oocytes surrounded by multi-layers of compact cumulus cells and evenly granulated cytoplasm (Grades A and B oocytes) were selected. Then these were washed two times in 37ºC pre-warmed PBS with 3% FBS (Gibco-BRL) and two times in pre-equilibrated maturation medium.

IVC medium for oocyte maturation and development to preimplantation stage embryos was TCM 199 (Earle's salts with 25 mM HEPES, Gibco-BRL) containing 10% FBS and antibiotics (100 units penicillin/mL and 100 mg streptomycin/mL, both from Sigma Chemical Co.). Culture medium was prepared in 100 µL droplets in Nunc tissue culture dishes (35mm x 10 mm, Nunclon 153066, Inter-med., Roskilde, Denmark) covered with mineral oil (Embryo tested, Sigma Chemical Co.). This was equilibrated in a humidified incubator (Forma Scientific 3111 Series, Forma Scientific Inc., Marietta, OH) and gassed with 5% CO_2 in air at 39.0ºC.

Oocytes were randomly distributed, 10 in each 100 µL of culture droplets and matured *in vitro* in a water-jacketed incubator for 22 to 24 h at 39ºC and 5% CO_2. After IVM, oocytes were *in vitro* fertilized with frozen-thawed semen processed for IVF as described in Experiment 1-A. The IVF medium droplets contained a final sperm concentration of $1x10^6$ sperm cells/mL, 5 mM caffeine, 2 units heparin/mL and 5 mg BSA/mL. Ten oocytes were transferred to each IVF droplet.

Sperm–oocyte co-culture for IVF was done for a period of 6 to 8 h inside a humidified incubator gassed with 5% CO_2 in air at 39ºC. The culture medium in IVM droplets was renewed without removing the debris of cumulus cells and kept inside the incubator. After sperm-oocyte co-culture for IVF, the oocytes were removed from the fertilization dish, washed four times in pre-incubated culture medium and transferred into the former IVM droplets containing cumulus cells and cultured *in vitro* for embryo development.

After 36 h of IVC, cleaved embryos were recorded and separated from the uncleaved ones. Embryo development of the cleaved zygotes was monitored daily while the culture medium was renewed every 2 days. Blastocyst development was assessed on Day 7 of IVC.

3.2.2. Experiment 2-B: Effect of the duration of oocyte *in vitro* maturation on meiotic competence

To assess the effects of the duration of IVM on meiotic competence of buffalo oocytes, ovaries were collected from slaughtered swamp buffaloes and transported to the laboratory within six hours as described above. Collected Grades A and B oocytes were randomly divided into five groups and cultured for IVM for 16, 19, 22, 25 and 28 h in TCM 199 containing 10% FBS and antibiotics (100 units penicillin/mL and 100 mg streptomycin/mL).

After the designated IVM period, the oocytes were denuded from cumulus cells by brief exposure in 0.2% hyaluronidase, fixed in 1:3 aceto-ethanol, dehydrated with absolute ethanol, stained with 1% aceto-orcein (Merck & Co., Inc.) and de-stained with

aceto-glycerol (Glycerol: acetic acid: distilled water = 1:1:3 v/v) as described by Hufana-Duran (1996).

Oocytes nuclear maturation was evaluated over a compound microscope at x 400 following the method described by Hunter and Polge (1966). Figure 3-1 presents the different nuclear stages of water buffalo oocytes. Oocytes that reached the MII stage were considered matured and the percentage of this nuclear stage was compared in each IVM time.

3.2.3. Experiment 2-C: Effect of hormones and growth factors in the *in vitro* maturation medium on the production of water buffalo embryos *in vitro*

The effects of hormones and growth factors during IVM on nuclear, cytoplasmic, and *in vitro* development to blastocysts after IVF were assessed. Swamp buffalo ovaries were obtained at a local abattoir and transported to the laboratory in normal saline (0.85% sodium chloride) within six hours as described above. COCs were aspirated from 2 to 8 mm diameter follicles using 18-gauge needle fitted to a 10 mL syringe. Collected Grades A and B oocytes were held and washed three times with HEPES-buffered modified Tyrode's medium supplemented with 3 mg/mL BSA, 0.2 mM pyruvate and 50 μg/mL gentamycin sulfate (TALP-HEPES, Bavister *et al*., 1988) modified as described by Takahashi and First (1992b).

Oocytes were randomly divided into four groups and matured *in vitro* in IVM medium made of 10% FCS in TCM 199 with a) no supplement (control), b) hormones (0.02 units FSH/mL and 1 μg E_2/mL); c) EGF (10 ng/mL), d) hormones and EGF. After IVM of 24 h, sample oocytes were taken for examining the nuclear development while the rest were fertilized *in vitro*.

Nuclear examination was carried out as described earlier (Experiment 2-B). For IVF, spermatozoa were separated using the discontinuous 45 percent and 90 percent Percoll gradient method by centrifugation at 800 x *g* for 20 min.

The separated sperm cells were washed with 6 mL BO medium and centrifuged at 800 x *g* for 5 min. Semen concentration was adjusted to 2 x 10^6 sperm cells/mL and equal

volume of the sperm suspension was added to pre-equilibrated IVF medium containing 3 mg/mL BSA and 2.5 mM theophylline.

Ten to twelve *in vitro* matured oocytes were placed on a 100 µL IVF drop. Sperm-oocyte co-culture for IVF was done for 22 h at 38.5ºC under 5% CO_2. After 22 h of sperm-oocyte co-culture for IVF, the oocytes were removed from IVF drops and stripped of cumulus cells by repeated pipetting.

Presumptive zygotes were washed several times in TCM 199 with 10% FCS and in IVC medium: a modified synthetic oviductal fluid containing 20 amino acids and 10 µg/mL fatty acid free BSA (mSOFaa) with cumulus cell co-culture. Embryos in culture dishes were cultured for 9 to 10 days and examined under the microscope every other day. Culture medium was replaced at 48 h interval during IVC.

Cytoplasmic maturation was assessed by the rate of cleavage and blastocyst development. Quality of the embryos was assessed by the number of blastomeres of the resultant blastocyst determined by staining with fluorescent dye, Hoechst 33342 and examined under the epi-flourescent microscope (Eclipse TE 300, Nikon).

3.2.4. Experiment 2-D: Incidence of chromosome abnormalities as a factor of low blastocysts yield in water buffaloes

Factors leading to cellblock that result in low development to blastocyst were assessed by examining incidence of chromosome abnormalities among early stage (2 to 8 cell stage) embryos. Cleaved zygotes were treated with vinblastin sulfate and metaphase plates were analyzed. Metaphase plates with chromosome abnormalities were recorded.

Briefly, river buffalo oocytes derived from slaughter house ovaries were processed and *in vitro* matured and fertilized with river buffalo sperm cells as described in Experiment 2-C. At 36 to 40 h of IVC, developed 2- to 8- cell stage embryos were treated with 100 ng vinblastin/mL (Sigma Chemical Co.) for 16 to 18 h under incubation in IVC medium containing cumulus cells for co-culture. Thereafter, embryos were treated with hypotonic solution 1% sodium citrate for 15 min on a hollow slide (washing on the same solution once) and mild fixed by addition of 10 to 20 µL of aceto-methanol (1:1) in hypotonic solution for a few minutes. Then the embryo was loaded on slide glass (1

embryo/glass slides) and spread by a droplet of acetic acid followed by 5 to 6 drops of fixative (1:3 aceto-methanol).

Slides were dried in high humidity and stained with 2% Giemsa solution for 10 to 15 min. Chromosome samples were analyzed at x 400 to x 1000.

3.2.5. Statistical analysis

The percentage of oocytes at MII stages, as well as the proportion of cleaved, morula and blastocysts stage embryos after testing them on their different maturation conditions were analyzed and compared by analysis of variance (ANOVA) using Stat View Software (Abacus Concepts Inc., Berkeley, CA, USA). When significant f-ratio was defined by ANOVA, groups were compared using the Fisher's Protected Least Significance Difference (PLSD) as post hoc test.

3.3. Results

3.3.1. Experiment 2-A: Effect of ovary storage on cleavage and blastocyst development

The duration of storage of the donor ovaries was found to affect the developmental competence of the water buffalo oocytes. Storage at 30 to 33ºC for 3 to 4 h is better than storage for 5 to 6 h as evidenced by higher cleavage rate (58.6% versus 46.8%) and blastocyst development rate (21.4% versus 12.9%) in 3 to 4 h compared to 5 to 6 h storage duration (Table 3-1).

3.3.2. Experiment 2-B: Effect of the duration of oocyte *in vitro* maturation on meiotic competence

In examining the effect of duration of IVM and determining the optimum IVM time, results showed varying nuclear status among oocytes with 15.0 percent MII stage at 16 h increased with the duration of IVM and reached peak (85.0%) at 25 h but decreased

at 28 h (Table 3-2). The highest progression to MII was observed in oocytes cultured for 25 h followed by 28 h. However, very small or shrunken MII plates and polar bodies were found highest in oocytes examined at 28 h of IVM (27.7%) Fewer incidences were observed at 25 h IVM (11.8%) and none at shorter IVM times.

3.3.3. Experiment 2-C: Effect of hormones and growth factors in the *in vitro* maturation medium on the production of water buffalo embryos *in vitro*

Results on the effects of hormones (FSH and E_2), EGF and combination of hormones and EGF are summarized in Tables 3-3 and 3-4. Hormones and EGF alone increased the proportion of oocytes reaching MII compared with control, 78.3±1.4 percent and 74.7±0.7 percent versus 65.0±0.8 percent. However, significant difference was obtained when the hormones and EGF were combined as supplements on the IVM medium, 81.9±0.4 percent ($P<0.05$) (Table 3-3). Similar results were observed on development to morula after IVF (Table 3-4). Addition of hormone (59.1±0.3%), EGF (57.5±0.8%) and combination of hormones and EGF (62.3±1.1%) had higher morula than the control group (44.3±0.4%) ($P<0.05$) which was consistent even on the development to the blastocysts; 25.0±0.3 percent, 24.9±0.8 percent, and 27.1±0.4 percent versus 17.5±0.4 percent, and on the number of blastomeres of the resultant blastocysts; 75.1±0.8 cells, 73.6±1.0 cells, and 78.9±0.5 cells versus 67.5±0.8 cells.

3.3.4. Experiment 2-D: Incidence of chromosome abnormalities as a factor of low blastocysts yield in water buffaloes

On examining the incidence of chromosome abnormalities among early stage embryos, 206 embryos were examined. Of these oocytes, 51.0 percent displayed metaphases (105/206) and 44.7 percent was successfully analyzed (92/206). Of the 92 analyzable embryos, the incidence of chromosome abnormalities reached 47.7 percent. Polyploidy was the most frequent abnormality, (23.9%), while incidences of mixoploidy and haploidy were the same (11.9%). Among mixoploidy, embryos of haploid/diploidy

were found as the most frequent abnormality (54.5%). Triploidy (Fig. 3-2) was observed the most frequent among polyploidy embryos.

3.4. Discussion

As recommended in Experiment 1-A, studies on the effect of ovary storage on the developmental competence of the oocytes after IVM-IVF-IVC was carried out. Shown were longer storage of ovaries, 5 to 6 h at 30 to 33ºC, reduced both the cleavage and blastocyst development when compared to 3 to 4 h. In bovine, longer storage of ovaries was also found to significantly reduce the percentage of morphologically normal follicles (Lucci *et al*., 2004). In fact, storage of ovaries at 37 to 39 °C for 5 to 8 h decreases the maturation rate of follicular oocytes and the potential to develop into blastocysts after IVF (Yang *et al*., 1990; Nakao and Nakatsuji, 1992; Abe and Shioya, 1996).

However, storage of ovaries at 20°C (Abe and Shioya, 1996) or 25 °C (Yang *et al*., 1990) for 8 h did not reduce the maturation rate or the potential of *in vitro*-fertilized oocytes to develop into blastocysts showing that the storage of ovaries without cooling had a direct effect on the developmental competence of the oocytes as observed in this study. Recently, Neglia *et al*. (2003) also observed that cleavage and blastocyst development was higher in OPU-derived oocytes than in oocytes from slaughterhouse-derived ovaries. This suggests that the duration of storage and transport of the ovaries from the slaughter house to the laboratory can affect the development potential of the oocytes. Also, prolonged storage of ovaries may cause degeneration in some of the accumulated mRNA or oocyte proteins that are necessary for early development resulting in reduced developmental potential.

These findings supported the observations in mouse (Snow *et al*., 2001) showing that the rate of follicle loss over time was very rapid, with approximately 50 percent fewer follicles in grafts derived from ovaries stored for only 3 h compared to non-stored fresh grafts (0 h). Schroeder *et al*. (1991) also observed that the yield of intact, cumulus-oocyte-complexes decreased as the interval between death of the animal and removal of the ovary increased. Likewise, follicles of 6 h postmortem ovaries showed degeneration manifested by prominent crystal-line inclusions within the oocytes and many apoptotic

granulosa cells. The above findings show that the length of ovary storage is an important consideration to optimize production of embryos *in vitro*.

On the other hand, studies on the effect of the duration of IVM showed that the length of IVM significantly ($P<0.01$) varies among oocytes. Oocytes selected based on the universally accepted classification system i.e. surrounded by multi-layers of cumulus cells with evenly granulated ooplasm (categorized as Grades A and B), have varying speed of development in the MII stage with some developed to MII as early as 16 h and as late as 28 h as shown in this study.

The highest incidence of MII was observed at 25 h but incidence of shrunken MII plates and polar bodies was observed. In humans, the small metaphase plate indicates aging among oocytes and is characterized by a shrinking of the spindle (Eichenlaub-Ritter *et al*., 1986). This means that at 25 h IVM, the incidence of aging can occur in some of the water buffalo oocytes. These aging oocytes could be the oocytes that have reached the MII stage at 16 (about 15.0%) to 19 h (about 20.0%) as observed in the present experiment.

In very recent reviews, Gasparrini (2002) and Nandi *et al*. (2002) reported that 24 h was considered an optimum time for IVM of water buffalo oocytes. This claim was supported by the results of this study. However, the varying speed of progression to the MII stage of the oocytes affected the success rate of IVF. This observation was observed in buffalo following different culture system (Ocampo *et al*., 2000; Gasparrini *et al*., 2008), in cattle (Khatir *et al*., 1998: Adona *et al*., 2008) and other mammalian species (canine, Hanna *et al*., 2008; human, Trounson *et al*., 2001).

These results showed differences on the meiotic progression among oocytes as influenced by the growth stage. This means that though oocytes were selected and graded as A and B, they are still heterogeneous. This heterogeneousity of the oocyte population could be another factor that have led to the optimal rate of success in the production of blastocysts in Experiment 1-A. Although majority of the oocytes were at the MII stage at 25 h and that incidence of aging was observed at this time, pre-determined IVF at 24 h could have enhanced successful fertilization to the oocytes that are at MII stage but failed in those that were still maturing and aging. It was indicated that the success of

fertilization lies on the perfect timing of introduction of sperm to oocyte for insemination (Grondahl, 2008).

To overcome this problem, synchronization of oocyte development to enhance development to MII of oocytes at the same time has been done using roscovitine (Romar and Funahashi, 2006) or butyrolactone I combined or not with roscovitine (Adona *et al*., 2008). The system required additional pre-maturation time and was able to synchronize the meiotic progression of the oocytes.

Although improvement in blastocyst rate was achieved, the efficiency of production still remains relatively low which could a due to side effects of the treatment used. Apparently, the inherent quality of the oocytes dictates its development potential and its response to the IVC environment. Selection of the homogeneous population of the oocytes may minimize asynchrony and help improve the IVC systems for efficient production of blastocysts.

Studies to determine the effects of the IVM medium on blastocyst development showed that addition of hormones and EGF alone or in combination promoted nuclear maturation, cleavage and subsequent development of embryos to morula and blastocyst stage.

These results suggest that the nature of supplements in the IVM medium has a significant effect on the development potential of the oocytes. The data were comparable with the maturation rate using FSH (Chauhan *et al*., 1996) and EGF (Chauhan *et al*., 1999), maturation and fertilization rates by Totey *et al*. (1993a), and the subsequent cleavage and developmental rates obtained by Abdoon *et al*. (2001) using equine chorionic gonadotrophins. The present findings also agreed with the results reported by Fukushima and Fukui (1985) indicating that the maturation and fertilization rates of bovine oocytes were improved when FSH, LH and E_2 were added to the maturation medium.

In this study, changes in morphological characteristics in oocyte-cumulus cell complexes were observed after IVM of buffalo oocytes in different conditions. In *in vivo*, the pre-ovulatory gonadotrophin surge provides stimulus for ovulation wherein the cumulus oophorus undergoes mucificcation and becomes embedded in a glucosaminoglycan matrix, a process called cumulus expansion.

In the present study, EGF supplementation resulted in the expansion of cumulus cells surrounding the oocytes. A maximal cumulus expansion was observed after the combined stimulus provided by EGF and FSH.

Earlier studies have shown that while EGF was significantly more effective than other growth factors in promoting oocyte maturation, its effects on cumulus cell expansion were even more striking (Downs, 1989). Even FSH, a well-known stimulator of cumulus expansion *in vitro* was not as effective as EGF in inducing expansion of cumulus cells as what was observed in the present study. Although, it was demonstrated that mouse oocytes with excellent cumulus resulted in high percentage of oocyte undergoing meiotic progression, it was concluded that these two physiological events were not casually related and therefore can occur independently (Downs, 1989).

Epidermal growth factor alone increased the number of oocytes reaching MII (74.0% versus 64.9%), cleaved (69.9% versus 60.1%), and developed to morula (57.5% versus 44.3%) and blastocyst (24.9%). These results suggest that EGF promoted nuclear and cytoplasmic maturation which was also observed in mouse (De la Fuente *et al*., 1999), bovine (Kobayashi *et al*., 1994; Lonergan *et al*., 1996), bubaline (Chauhan *et al*., 1999; Kumar and Purohit, 2004; Purohit *et al*., 2005), and cat (Merlo *et al*., 2005) oocytes. Although the physiological significance of EGF is not yet fully understood, it is possible that EGF plays a regulatory role in follicular development in a paracrine/autocrine manner, or it might be one of the signaling factors for resumption of meiosis in oocytes (Coskun *et al*., 1991).

As observed in this study, the most effective treatment was the combination of hormones (FSH and E_2) and EGF, in which the frequency of oocytes reaching MII (81.9%), cleaved after fertilization (72.5%), developed to morula (62.3%) and blastocyst (27.1%) were significantly higher than those of the control (64.9%, 60.1%, 44.3% and 17.5%, respectively). More importantly, the cell number per blastocyst obtained from the combination was significantly higher than those from hormones or EGF cultured alone.

The improvement in the developmental competence after addition of this combination was also observed in mouse cumulus-oocyte complexes *in vitro* (Downs, 1989). Similar information were obtained in oocytes from the mouse follicles using a whole follicle culture system (Boland and Gosden, 1994), as well as in sheep oocytes

(Guler *et al*., 2000). The possibility that FSH caused the biological activity of EGF, thus an enhancing development, cannot be ruled out in the present study. However, De la Fuente *et al*. (1999) showed that gonadotrophins *in vivo* increased the sensitivity of responsiveness of cumulus cell-enclosed oocytes to EGF, thereby promoting both nuclear and cytoplasmic maturation.

Similarly, cumulus expansion was the distinct morphological effect exhibited by the addition of either the hormones or EGF and was more striking when the combination of these compounds was used in the IVM medium. This means that cumulus expansion plays an important role in the process of fertilization by enhancing sperm capacitation and increasing sperm motility (Schroeder and Eppig, 1984) which may explain the improved cleavage rate in the present experiment.

Although evidences has accumulated concerning the ability of gonadotrophins and growth factors to increase proportions of buffalo oocytes developing until blastocyst stage, not much is known about the validity of the cell number as indicator of the viability of *in vitro* developed pre-implantation embryos. Totey *et al*. (1996) reported that slow developing blastocysts are of lower quality and have fewer cell numbers than their fast developing counterparts.

In this present work, those that formed blastocysts on the 7^{th} day of IVC were classified as fast developing, whereas those blastocyst formed after were classified as late developing embryos. It was recorder that the blastocysts derived from oocytes matured in the presence of both the EGF and hormones formed blastocysts as early as 6.5 days of IVC. The data on the present average cell count were obtained from embryos considered to be fast developing embryos. The reason for an increase in cell number of blastocyst from IVM protocol with hormones and EGF together is not clear but previous results have indicated that the mitogenic actions of FSH and EGF have been documented during follicular development in mice (Boland and Gosden, 1994) and bovine (Spicer and Stewart, 1996). They have associated these observations to in DNA synthesis which culminates in DNA duplication then cell proliferation.

However, in spite of the improved blastocyst yield and quality of the blastocyst after supplementation of hormones and EGF in the culture medium, about 45 percent of the cleaved zygotes failed to develop to reach the blastocyst stage. Examination on the

incidence of chromosome abnormalities among early stage embryos showed 47.7 percent incidence of chromosome abnormalities. This incidence of chromosome abnormalities is one of the major problems on *in vitro* embryo production in water buffaloes.

This same problem was also observed in other species (rabbit, Shaver *et al*., 1967; mouse, Fraser *et al*., 1976; Maudlin *et al*., 1978; murine models, Santalo *et al*., 1992; cattle, Viuff *et al*., 1999; 2001; porcine, Ulloa Ulloa *et al*., 2008; and humans, Edwards, 1986; Bongso *et al*., 1988; Plachot *et al*., 1988). Polyploidy was the most frequent abnormality, (23.9%) while incidences of mixoploidy and haploidy were the same, (11.9%). Trisomy was is the most frequent case of polyploidy and haploidy the most frequent case of mixoploidy.

This factor could be the cause of cellblock resulted to failure of zygotes to develop to the blastocysts. Chromosome abnormalities was said to be associated by the *in vitro* condition and is due to maternal age of donors (Maudlin *et al*., 1978; Bongso *et al*., 1988). In a slaughter house derived oocytes, this could not be avoided due to high incidence of retired work or dairy animals slaughtered. The above findings suggest that the IVF and culture system needs to be refined to minimize the risk of chromosome abnormalities and improve blastocyst yield in water buffaloes.

In summary, several factors were found critical for the production of embryos *in vitro*. Long storage of ovaries at 30 to 33ºC compromises the developmental competence of the oocytes; hence, it is necessary to consider the duration of storage of ovaries to optimize the success rate of embryo development. The development to MII of Grades A and B oocytes varies so there is a need to develop selection parameters of homogeneous populations of oocytes to optimize development potentials. To improve the IVM environment and achieve higher blastocyst development and produce better quality embryos, IVM medium has to be supplemented with hormones such as FSH and E_2, and EGF. To avoid cellblock problems, studies on the quality of oocytes and sperm cells used for IVM and IVF, and the IVC environment for the development of the embryos *in vitro* need to be improved.

Table 3-1. Cleavage and blastocyst development rates of water buffalo oocytes derived from ovaries stored for 3 to 4 and 5 to 6 h at 30 to 33ºC

Ovary storage, (h)	Number of ovaries	Number of oocytes (Grade A & B/ovary)	Cleavage (%)	Blastocysts (%)
3-4	130	70 (0.5)	41 (58.6)	15 (21.4)
5-6	110	62 (0.6)	29 (46.8)	8 (12.9)

Statistical analysis showed no significant difference.

Table 3-2. Development to metaphase II of water buffalo oocytes matured *in vitro* at different time period

IVM period, h	16	19	22	25	28
No. of oocytes	20	20	20	20	17
Metaphase II (%)	3 (15.0)^{A}	4 (20.0) ^{A}	6(30.0) ^{Aa}	17(85.0) ^{BC}	11(64.7) ^{Cb}
Shrunken MII* (%)	0	0	0	2 (11.8)	3 (27.3)

**Shrunken MII are oocytes with metaphase plate and polar body present but are very small and have undergone shrinkage. Percentage value was computed out of the total number of examined MII oocyte from the same group.*

^{A,B,C} *Values are significantly different at P<0.01;* ^{a,b} *Values are significantly different at P<0.05 by Chi-square or Fisher's Exact Test.*

Table 3-3. Effect of FSH, E_2 and EGF in IVM medium on the proportion (%) of swamp buffalo oocytes attaining nuclear maturation *in vitro*

Supplement to TCM 199 with 10% FCS	**No. of oocytes matured *in vitro***	**Percent of oocytes reaching**		
		GVBD	**Metaphase I**	**Metaphase II (Mean±S.E)**
Control	60	18.3	16.7	65.0±0.8[a]
FSH and E_2[1)]	70	7.1	14.3	78.3±1.4[ab]
EGF[2)]	64	6.3	18.7	74.7±0.7[ab]
Hormone+EGF	66	3.0	15.1	81.9±0.4[b]

Supplements;

[1)]*Hormones: FSH, 0.02 units /mL, E_2, 1μg /mL*

[2)]*EGF, 10 ng/mL*

[a,b] *Values in the same column are significantly different at P<0.05 by ANOVA.*

Table 3-4. Effect of FSH, E_2 and EGF in IVM medium on the proportion of swamp buffalo oocytes cleaved and reaching blastocysts stage under *in vitro* culture

Supplement to TCM 199 with 10% FCS	No. of oocytes fertilized	Cleavage, % Mean±S.E.	Embryo development to		Blastocyst cell number, Mean±S.E. (n)
			Morula, % Mean±S.E	Blastocyst, % Mean±S.E.	
Control	93	60.1±0.4	44.3±0.4[a]	17.5±0.4	67.5±0.8 (16)
Hormone[1)]	119	68.7±0.2	59.1±0.3 [b]	25.1±0.3	75.1±0.8 (18)
EGF[2)]	87	69.9±0.5	57.5±0.8 [ab]	24.9±0.8	73.6±1.0 (16)
Hormone+EGF	107	72.5±0.6	62.3±1.1[b]	27.1±0.4	78.9±0.5 (20)

Supplements;

[1)]*FSH, 0.02 units /mL, E_2, 1µg /mL*

[2)]*EGF, 10 ng/mL*

[a,b] *Values in the same column with different superscripts are significantly different at P<0.05 by ANOVA and Fisher's Protected Least Significance Difference.*

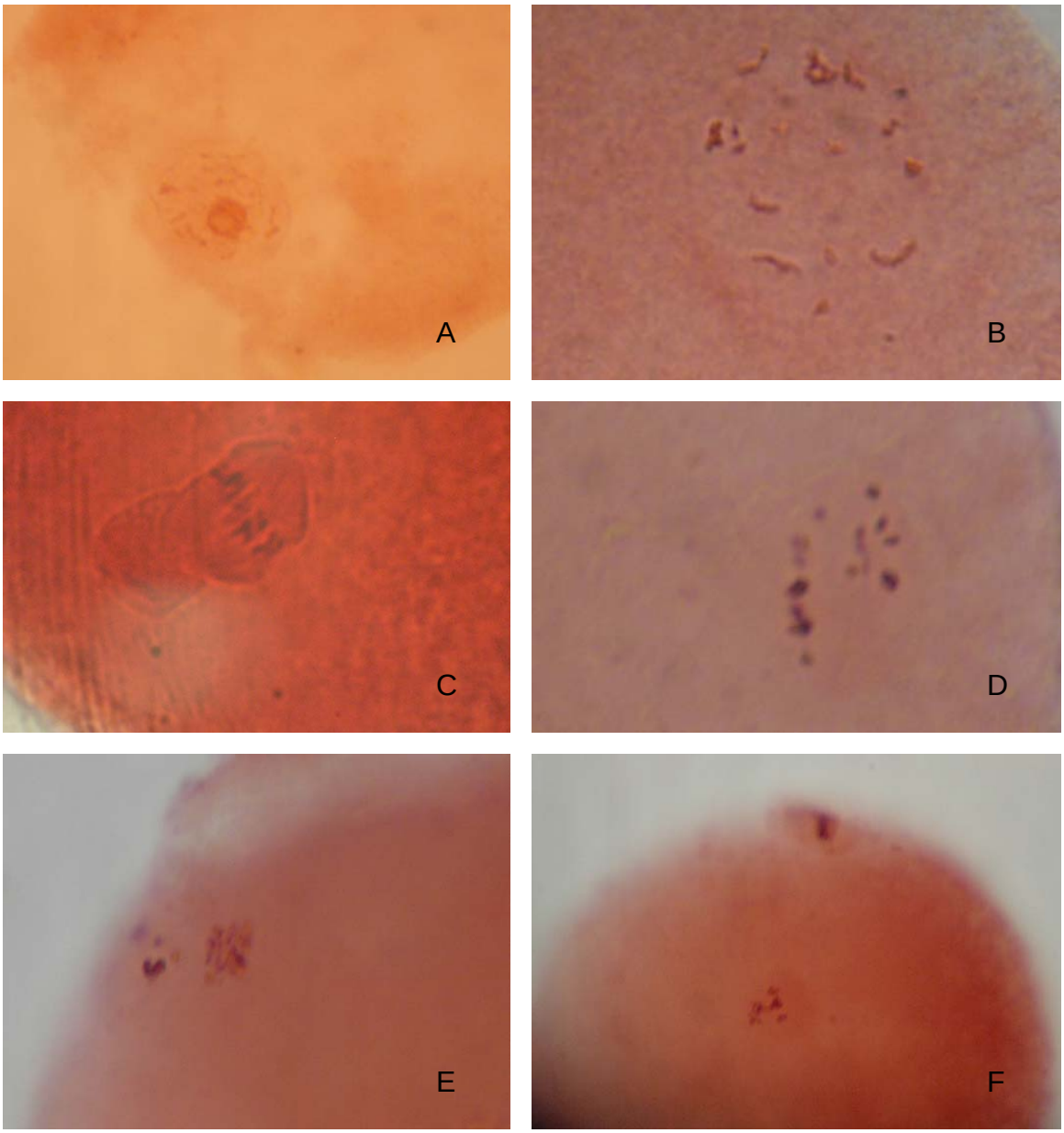

Figure 3-1. Nuclear appearance and classification of water buffalo oocytes: A, Germinal vesicle, nucleus enclosed by a membrane, x400. B, Germinal vesicle breakdown, condensed chromosomes and disappearance of the membrane, x1000. C, Metaphase 1, spindle formation, x400. D, Anaphase I, separation of the homologous chromosomes, x400. E, Telophase, extrusion of the first polar body, x400. F, Metaphase II, x400.

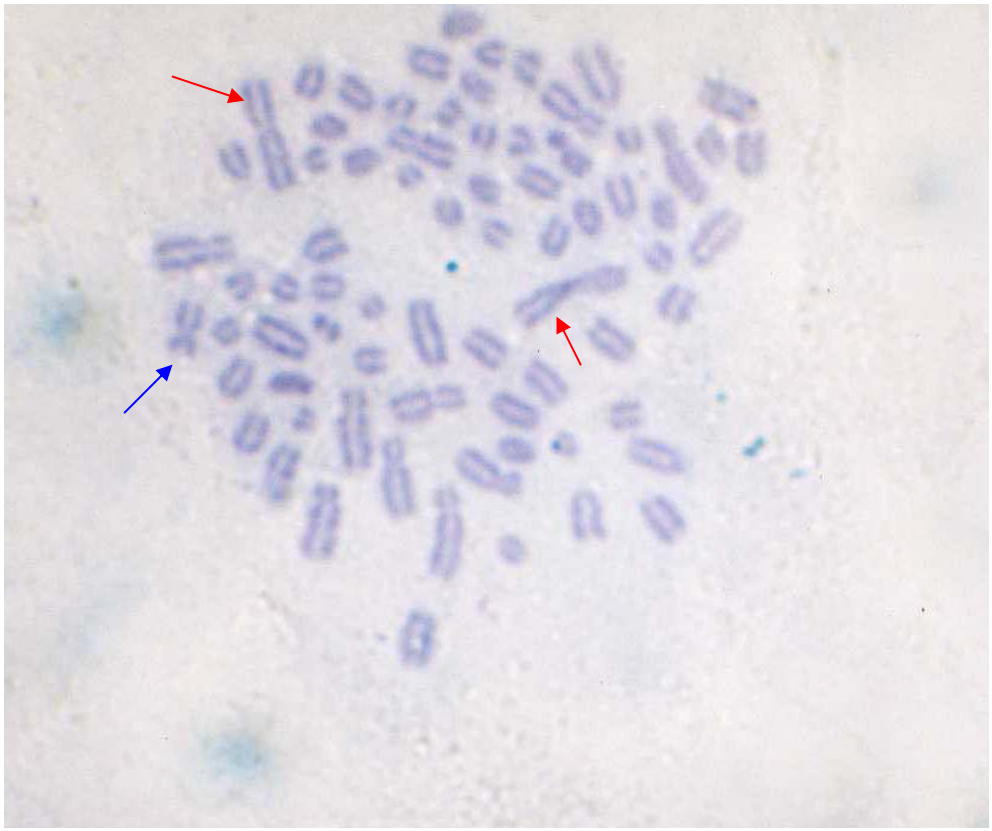

Figure 3-2. A chromosome anomaly (triploidy) in early stage *in vitro* produced water buffalo embryo. The metaphase plate contains three haploids as shown by the presence of one Y chromosome and two X chromosomes, x1000.

CHAPTER IV. Improving the *in vitro* Culture Systems for Efficient Production of Viable Water Buffalo Embryos

4.1. Introduction

Chromosome anomalies were observed as factors affecting blastocyst development. Chromosome abnormalities can be due to several factors; 1) polyspermy, triploidy was found as a prominent abnormality which meant more sperm cells had penetrated the oocyte. Hence, oocyte quality needs to be addressed; 2) quality of sperm cells, haploidy was found as the most prominent mixoploidy so quality of sperm cells for IVF must be improved; and 3) failure of successful chromosome segregation as observed by the presence of diploidy which may be due to poor or inherent quality of the oocytes or sperm cells and inadequate or complicated culture systems.

In view of the above, selection of developmentally competent oocytes for IVM and IVF is important. The quality of the oocyte and the donor follicle dictates the developmental competence of oocytes (Hagemenn *et al*., 1999; Raghu *et al*., 2002a), so selection of homogeneous populations of developmentally competent oocytes for IVM is necessary.

Brilliant cresyl blue (BCB) was proven effective in identifying developmentally competent oocytes in bovine (Alm *et al*., 2005). BCB is a vital blue dye which determines the intracellular activity of glucose 6-phosphate dehydrogenase, an enzyme synthesized during the oocyte growth phase but with decreased activity in oocytes have finished their growth phase (Wassarman, 1988). Therefore, the cytoplasm of developmentally competent oocytes turns blue (BCB positive) because it does not reduce BCB to a colorless compound. By this technique, the use of heterogeneous populations of oocytes during IVM may be controlled.

On the other hand, quality and quantity of live sperm cells in the fertilization medium were shown to greatly affect the success of fertilization. Also, fertility was reported to increase with increasing number of quality sperm present at the insemination site (Salisbury *et al*., 1961) and that presence of low quality sperm cells may favor fertilization by less competent sperm that causes abnormalities and embryonic death

(Saacke *et al*., 1994). More recently, observations in a variety of species indicated that factors associated with lowered sperm motility and quality resulted in very early embryonic death prior to maternal pregnancy due to fertilization out of low quality sperm resulting to abnormalities in the resultant embryo (Barth, 1992; Courot and Colas, 1986; De Jarnette *et al*., 1992; Setchell *et al*., 1988). To ensure quality semen for IVF, a density gradient separation technique can be used. Initially, a polyvinylpyrolidone-coated silica medium (Percoll, Pharmacia Biotech AB) was used as the density medium of choice (Totey *et al*., 1996). However, Soderlund and Lundin (2000) reported high levels of endotoxins in Percoll and Nagata and Shirakawa (1996) and Fishel *et al*. (1988) showed these endotoxins reduce the implantation potential of human embryos.

To address this problem, the density gradient sperm separation technique using silica particles was tested. Silica particles have been found effective in processing human sperm (Perez *et al*., 1997). These contain low levels of endotoxins with performance comparable with Percoll in isolating live sperm cells (Claassens *et al*., 1998; Centola *et al*., 1998; Makkar *et al*., 1999).

On the other hand, the energy requirements of water buffalo embryos have not yet been fully defined. In cattle, determination of the energy requirements was difficult due to the 8- to 16- cells block (Camous *et al*., 1984), a problem also observed in water buffalo (Experiment I-A, Experiment 2-C). Several researchers were able to culture bovine embryos past the "block" stages by co-culture with oviductal cells (Eyestone and First, 1985), trophoblastic vesicles (Heyman *et al*., 1987), cumulus cells (Goto *et al*., 1989) or by addition of growth factors (Larson *et al*., 1990).

Somatic cell support was found beneficial to the development of the embryos in culture. Secretions of these cells provide nutrients for the metabolic requirements of the developing embryo (Eyestone and First, 1985; Goto *et al*., 1989). However, these cells may also use the energy substrates present in the culture medium that may limit the utilization of the embryos. Addition of energy substrates to basic medium in a co-culture system may improve development of the embryos.

Recently, a sequential media system in which embryos are grown in a mixture of simple and complex media (Scholtes and Zeilmaker, 1996; Jones *et al*., 1998) was developed and found to be a good substitute for a co-culture system (Fong and Bongso,

1998). This was designed based on the fact that the nutrient requirement of embryos changes as they develop. In fact, the first two days after IVF are the prerequisite stage for embryonic genome activation (Thompson and Peterson, 2000). Also, Leese (2003) observed that the presence of serum during early cleavage inhibited development but development could be accelerated when serum is present from the initiation of compaction. Earlier studies on the development of pre-implantation mouse embryos in culture showed that the development of the zygote to the 2-cell stage had an absolute requirement for pyruvate (Biggers *et al*., 1967) while development from the 2-cell stage is supported by both pyruvate and lactate, and glucose supports embryo development from the late 4-cell/8-cell stage (Takahashi and First, 1992a).

In support of these early studies, subsequent analysis of nutrient uptake in mouse embryos showed that initially the early mouse embryo preferentially takes up pyruvate. After the 8-cell stage, pyruvate uptake declined, and by the blastocyst stage, glucose was the preferred nutrient (Brinster, 1965; Leese and Barton, 1984; Gardner and Leese, 1986). In bovine, Rosenkrans *et al*. (1993) reported that the lactate:pyruvate ratio is less important than the total lactate or pyruvate concentration, a case that appears as well in hamster embryos (McKiernan and Bavister, 1990).

With the above considerations, this experiment was designed to select developmentally competent oocytes for IVM, separate quality sperm cells for IVF and improve the IVC of embryos using stage dependent culture system. Specifically, Experiment 3-A aimed to determine the important selection parameters for developmentally competent oocytes. Experiment 2-B aimed to identify the best combination of colloidal suspension of silica particles for isolation and separation of motile and functional sperm cells, examine the effects of using density gradient sperm-separation techniques in improving IVF rates in water buffalo, and examine the effect of the system in improving the IVF potentials of bulls with low sperm motility. Experiment 3-C addressed the changing needs of the embryos by supplementing energy substrates and increasing the concentration of serum to cater the changing requirement of the embryos for IVC.

4.2. Materials and Methods

This experiment was conducted at the Reproductive Biotechnology Laboratory of the Philippine Carabao Center in the Philippines and at the Satellite Embryo Biotechnology Laboratory in India. To determine the selection parameters for developmentally competent oocytes and examine the effects of sperm separation technique utilizing density gradients of silica particles, sufficient number of oocytes was needed, As such, the study was conducted in the India laboratory.

To assess the effects of supplementation of pyruvate and lactate and increasing concentration of FBS in the IVC medium, swamp buffalo ovaries were used for experimental purposes and it was conducted in the Philippines. Tests of the viability of the embryos produced out of the improved system, i.e. a system where homogeneous populations of oocytes were used for embryo IVP, IVM were done with hormones and growth factors added in the IVM medium, use of sperm cells purified by the use of density gradients of silica particles, and IVC with energy substrates and stage-dependent culture system, river oocytes were used and the embryos were produced in the India laboratory. Transfer of the embryos was done in the Philippines using river buffaloes as recipient animals. The details of the experimental procedure are explained below.

4.2.1. Experiment 3-A: Selection of developmentally competent oocytes

River buffalo ovaries were used in this study. Ovary storage to aspiration of oocytes was within 4 h while IVM, IVF and IVC of embryos were carried out as described in Experiment 1-A with modification by supplementing a combination of hormones (0.02 units FSH/mL and 1 µg E_2/mL) and EGF (10 ng/mL) in the IVM medium as described in Experiment 2-C. Nuclear examination was carried out as described in Experiment 2-B.

Briefly, ovaries were collected from slaughtered river buffaloes and brought to the laboratory in 30 to 33ºC physiological saline. At the laboratory, ovaries were washed several times. To determine the morphology and structure of water buffalo oocytes with developmental competence *in vitro*, two studies were conducted and factors inherent of

the oocyte were evaluated: Study 1; quality of the oocytes based on the a) morphology and b) compactness of the surrounding cumulus cells, c) granulation and d) size of ooplasm; Study 2; the size of the donor follicle.

Meiotic competence, cleavage and blastocyst development rates were assessed. Homogeneousity of the oocytes was examined by BCB staining techniques (Alm *et al*., 2005) and efficiency of this staining technique was evaluated by the subsequent blastocyst development of the oocytes. Characteristics of the ultrastructure of the oocytes were defined by transmission electron microscopy (TEM).

4.2.1.1. Study 1. Effect of the quality of the cumulus-oocyte-complexes (COCs)

Based on morphology of the surrounding cumulus cells. The effect of oocyte quality was analyzed based on the morphology of the surrounding cumulus cells. COCs were classified and grouped based on the nature of the surrounding cumulus cells as shown in Figure 4-1: a) Grade A, oocyte is surrounded by ≥5 attached layers of dense cumulus cells; b) Grade B, surrounded by 2<5 dense layers of cumulus cells; c) Grade C, surrounded by irregular and <2 layers of cumulus cells; d) Grade D, denuded/free from cumulus cells; e) Grade E, surrounded by expanded cumulus cells. After classification, oocytes were subjected to IVM, IVF and IVC to assess the embryo development potential.

Based on compactness of the surrounding cumulus cells. Analysis the effect of oocyte quality was based on the compactness of the surrounding cumulus cells. Grades A and B COCs were re-classified and grouped as; a) Compact, when the surrounding cumulus mass is tightly connected; and b) Loose, when the cumulus mass is slightly expanded (Fig. 4-2). To tests the hypotheses that oocytes with loosen cumulus cells have resumed meiosis, each group of oocytes was divided into two groups and fertilized at either shorter period (20 to 22 h) or longer period (24 to 26 h) of IVM. Cleavage rate was assessed approximately 36 h after IVF and blastocyst development rate was assessed on Day 7 of IVC.

Based on granulation of the ooplasm. To assess if granulation of ooplasm affects the developmental competence of the oocytes, Grades A and B COCs with compact cumulus cells as described earlier were re-classified based on the granulation of the ooplasm as follows; a) Homogeneous, when ooplasm is evenly granulated and b) Heterogeneous, when ooplasm is unevenly granulated (Fig. 4-3). Each group was matured and fertilized *in vitro* at 24 h of IVM. Cleavage and blastocyst development rates were recorded.

Based on the diameter of the ooplasm. To assess if size of ooplasm affects the oocytes developmental competence, Grades A and B COCs with compact cumulus cells and evenly granulated ooplasm were re-classified based on the size of their ooplasm using a calibrated eyepiece micrometer and grouped as; <100, 100 to 119, 120 to 139, >140 µm oocytes.

After grouping, digital image of each oocyte groups magnified at x 20 was captured by a MicronCam (Focused Enterprise, Los Baños, Laguna, Philippines), a compact color video camera designed as an electronic or digital eyepiece of compound microscopes (23 mm standard eyepiece) and stereomicroscopes (30 mm standard eyepieces) for faster computer-based recording of images and videos (640 x 480 max resolution). The images were saved in the computer. The exact diameter of the oocytes was measured by ImageJ software.

To assess the nuclear status, sample of oocytes were subjected to nuclear examination. The rest were allowed to mature and fertilized *in vitro* to assess the *in vitro* meiotic competence and developmental potential.

Nuclear status and meiotic competence of the oocytes were examined following the methods described earlier (Hufana-Duran, 1996). Oocytes were exposed to 0.2% hyaluronidase and surrounding cumulus cells were removed by a vortex for 1 min. Denuded oocytes were retrieved and washed in Dulbecco's PBS with 7.5 mg BSA/mL followed by fixation in 1:3 aceto-ethanol solutions 24 to 48 h at 4 to 5°C. After fixation, oocytes were mounted on a slide glass and dehydrated with absolute ethanol, stained with 1% aceto-orcein (Merck & Co., Inc.), and de-colored with aceto-glycerol (glycerol: acetic

acid: distilled water = 1:1:3). Nuclear status of oocytes was judged as described in Figure 3-1 over a compound microscope at x 200 to x 400.

Development potential was assessed by examining the cleavage rate at 36 to 48 h of IVC, development to morula on 5th day of IVC, and development to blastocyst on the 7th day of IVC.

4.2.1.2. Study 2. Effect of size of the donor follicles

To assess the effect of size of the donor follicle on the developmental competence of the oocyte, ovaries of swamp buffaloes were dissected using sterile surgical scalpel blades, scissors and fine forceps over a warming plate on a sterile 100 x 20 mm plastic dish containing TALP-HEPES to isolate the antral follicles. Isolated antral follicles were grouped by sizes; <2, 2 to 3.9, 4 to 5.9, 6 to 7.9, ≥8 mm and the following parameters were examined; 1) oocytes quality based on the surrounding cumulus cells, 2) size of ooplasm, and 3) *in vitro* development potential after IVM-IVF.

Abnormal follicles (cystic and highly atretic) were recorded but excluded in the analysis. Analysis of the relation of follicle size with the diameter and nuclear status of oocytes was done using Grade A oocytes to minimize the variation caused by the quality of the oocytes. Analysis of *in vitro* development potential after IVM and IVF was done using Grades A and B oocytes since *in vitro* development potential of these oocytes were not significantly different. To minimize the effect of time on the viability of the oocytes, each parameter was examined within 2 h from follicle isolation.

4.2.1.3. Study 3. Use of brilliant cresyl blue for selection of homogeneous population of oocytes

In another batch of oocytes, immediately after oocyte collection and morphological classification, oocytes from each oocyte category were exposed to BCB stain (B-2002, Sigma Chemical Co.) as described by Alm *et al*. (2005). This was done to test the use of the stain in selecting homogeneous population of developmentally

competent water buffalo oocytes and check the uniformity of the categorized oocytes on the basis of compactness of cumulus cells and granulation of ooplasm.

BCB were diluted in mPBS at a concentration of 26 µM and each group of oocytes was exposed on the stain for 90 min at 38.5 °C in a humidified atmosphere. After exposure to BCB, each group of oocytes was washed three times in mPBS and classified into two sub-groups, depending on the coloration of their cytoplasm: a) BCB positive group, oocytes with blue cytoplasm and b) BCB negative group, oocytes without blue coloration of the cytoplasm.

The numbers of BCB positive and BCB negative in each oocyte categories were recorded. Oocytes were matured and fertilized *in vitro* to assess the blastocyst development. Oocytes not exposed to BCB were used as control.

4.2.1.4. Study 4. Electron microscopy of the oocytes under different categories

To define the differences between oocytes categories, ultrastructural features of water buffalo oocytes grouped according to the size of ooplasm (Small versus Big), nature of the surrounding cumulus cells (Compact versus Swell/Loose). Granulation of ooplasm (Evenly/Homogeneous versus Uneven/Heterogeneous) was examined by TEM (JEM-1210; JEOL, Tokyo, Japan). Morphology of the cumulus cells, arrangement of the organelles and the other distinct features of the oocytes were recorded.

To prepare oocytes for TEM examination, oocytes were fixed in 2.5% glutaraldehyde in 0.1 M PBS at pH 7.3, and post-fixed in 0.1% osmium tetroxide in the same buffer. Fixed oocytes were then dehydrated in a graded series of ethanol, treated with propylene oxide, embedded in epoxy resin and sectioned.

The semi-thin sections (0.5 to 1.0 µm) stained with 0.5% toluidine blue at pH 7.5 were observed under a light microscope at x 200 to x 400. The ultra-thin sections (60 to 100 nm) stained with ethanolic uranyl acetate and lead citrate were examined under a TEM.

Differences on the nature and structure of the cumulus cells were performed on sections at the area of the cumulus cells, at the zona pellucida, equatorial region of the ooplasm and at the section of the nucleus. Evaluations of ultra-structural features in the

semi-thin and ultra-thin sections were performed on sections at the equatorial plane of the oocytes.

4.2.2. Experiment 3-B: Effect of discontinuous density gradients of silica particles in separating live, motile sperm cells for *in vitro* fertilization

To assess the efficiency of density gradients of silica particles in separating motile sperm cells, three colloidal suspensions (45%, 65% and 95%) developed to form discontinuous density gradients as presented in Figure 4-4 were used. These layers were prepared on a sterile 15 mL centrifuge tube. Five straws of frozen semen from a genetically superior river bull previously recorded to have a low post-thaw motility, 10 to 20 percent, were thawed and pooled on a sterile test tube. Post-thaw motility and sperm concentration were examined and recorded. Each tube of the three density gradient treatments was layered at the top with 0.5 mL of the semen sample and another 0.5 mL of the semen sample was processed under conventional washing method as described in Experiment 1-A to serve as control.

To examine the bull-factor effect and assess the effect of the system in improving IVF of bulls with low post-thaw motilities, semen from three bulls (Bulls A, B, and C) with known post-thaw motilities was processed using the best separation technique and replicated three times. Sperm motility and concentration before (C: control) and after density gradient sperm separation by the discontinuous colloidal suspension of silica particles (T: treated) were recorded.

To examine the IVF potential of the separated sperm cells, oocytes were sourced-out from ovaries of slaughtered river buffaloes. These were processed following the established method for the collection of ovaries, aspiration and selection of oocytes, and IVM as described in Experiment 1-A. Cleavage and embryo development rates after IVF were recorded and assessed.

To separate motile sperm cells by density gradients of silica particles, frozen-thawed semen, 0.5 mL, was layered gently on the top of discontinuous layers (Fig. 4-4). The sperm cells were separated by equilibrium by subjecting the tube to centrifugation at 800 x *g* for 10 minutes.

The supernatant was discarded leaving only the sperm pellet at the bottom of the 95 percent fraction and layered with pre-incubated modified BO medium without BSA (mBO medium; Hufana-Duran, 1996) (sperm washing medium) and centrifuged again for another 800 x *g* for 5 min. The supernatant was discarded leaving the sperm pellet.

A desired volume of sperm washing solution was added to sperm pellet to make 1 mL sperm suspension. It was kept on a water bath at 37°C, and desired volume was taken for assessment of sperm motility and concentration.

Sperm motility was examined and analyzed using sperm counting chamber (SpermMeter, Sperm Processor, Aurangabad, India). Motile sperm cells characterized by forward movement were counted under a compound microscope. Motility was expressed as the number of mobile sperm cells over the total number of dead and live sperms on the four major squares, multiplied by 100.

Sperm concentration was determined by taking 50 µL of the sperm suspension and diluted with 4,950 µL of 2% sodium chloride. Sperm cells were counted in a Neubauer chamber (LO-Laboroptik GmbH, Germany) and sperm concentration was estimated using the following formula:

Sperm concentration = sperm count x 5000 uL ÷ 0.1 uL x 1000 uL ÷ 50 uL

Where: 5000 µL as the volume of the sperm suspension i.e. 50 µL sperm + 4,950 µL 2% sodium chloride

0.1 µL as the volume of semen examined i.e. based on the area of the counting chamber

1000 µL as the volume where the concentration is estimated i.e. for sperm cell mL-1

50 µL as the volume taken from the pellet for examination

Upon determining the sperm concentration, sperm suspension was diluted with the desired volume of sperm washing solution. This was further diluted to 1:1 (v:v) with BO solution containing 10 mg BSA/mL (Fraction V, Wako Pure Chemical Ind., Osaka, Japan) (sperm dilution solution) to make a final sperm concentration of 1 x 10^6 sperm cells/mL, 5 mM caffeine, 2 units heparin/mL, and 5 mg BSA/mL for IVF medium.

Droplets of IVF were prepared in 100 µL droplets in a culture dish and covered with equilibrated mineral oil. *In vitro* matured oocytes were co-cultured with the sperm cells for IVF at 39°C, 5% CO_2 for 6 to 8 h. After sperm-oocyte co-incubation, fertilized oocytes were washed four times and transferred to a culture dish containing cumulus cells.

The culture medium was renewed every 48 h thereafter. Cleavage and embryo development to morula and blastocysts stages were assessed at 24 to 36 h and 5 d past fertilization.

4.2.3. Experiment 3-C: Embryo development of water buffalo oocytes in stage-dependent culture system and in co-culture system with or without pyruvate and lactate

To assess the effects of supplementation of energy substrates in the IVC medium and the stage-dependent culture system on the efficiency of blastocyst production, four culture media were prepared to culture pronucleate zygotes: 1) Fixed dose of FBS (10%) without pyruvate and lactate; 2) Fixed dose of FBS with pyruvate and lactate;, 3) stage-dependent culture system without pyruvate and lactate, and 4) stage-dependent culture system with pyruvate and lactate. IVC using these media were carried out with cumulus cells co-culture as described in Experiment 1-A and briefly discussed below.

Stage-dependent culture system involved the use of increasing concentration of FBS in the IVC medium (Day 0 to 3 of IVC: 1% FBS, Day 3 to 7 of IVC: 15% FBS). The pyruvate and lactate containing medium has 0.4 mM pyruvate and 5 mM lactate. Each medium was covered with antibiotics (100 unit penicillin/mL and 100 µg streptomycin/mL).

Ovaries were sourced out from slaughtered swamp buffaloes and transported to the laboratory in a saline solution. Oocytes surrounded by ≥3 or more layers of cumulus cells were re-classified based on the compactness of the cumulus cells and grouped as: 1) Compact group and 2) Loosen group.

Each group of oocytes was matured *in vitro* in IVM medium containing a combination of hormones and growth factor as described in Experiment 2-C; TCM 199 (with Earle's salts, 25 mM HEPES, Gibco-BRL), with 10% FBS (Gibco-BRL), 0.02 units

FSH/mL, 1 µg E_2/mL and 10 ng EGF/mL and antibiotics (100 units penicillin/mL and 100 µg streptomycin/mL, both from Sigma Chemical Co.).

Oocytes classified as Compact group was *in vitro* fertilized at 24 h of IVM while oocytes in Loosen group was *in vitro* fertilized at 20 h. After 12 h of sperm-oocyte co-incubation, excess sperm cells were removed by washing and pronucleate embryos were divided into four groups and allotted into the four IVC treatment groups described above.

Medium in each treatment group was renewed simultaneously with the replacement of medium in stage-dependent culture system. All media used for medium replacements and renewal were pre-incubated at least 3 h prior to use. Embryos that reached morula on Day 5 and blastocysts on Day 7 of IVC were recorded.

To prepare the IVC dishes for cumulus cell co-culture, *in vitro*-matured oocytes were slightly freed from their cumulus cells before IVF. The detached cumulus cells were plated on IVC dish (Nunclon, Roskilde, Denmark) containing either medium for stage-dependent culture system or by co-culture system both under oil.

The viability of the *in vitro*-produced embryos out of the improved system was tested. Used were the oocytes selected and matured *in vitro* following the recommendations obtained in Experiment 3-A and Experiment 2-C, and fertilized as recommended in Experiment 3-B and cultured for *in vitro* development following the recommendation in Experiment 3-C. Embryos were vitrified following the ultra-rapid vitrification using open-poled straw described in Experiment 1-B and transferred to recipient river buffaloes as described in Experiment 1-C. Success rate was recorded by the fullterm development and calving rate after ET.

4.2.4. Statistical analysis

Data for oocytes quality, nuclear stages, cleavage rate and blastocyst development rate were expressed by percentages and significant differences were analyzed by Chi-square or Fisher's Exact Test. Significant differences between the mean diameters of oocyte groups were analyzed using Duncan's Multiple Range Test for variability.

On the selection of developmentally competent oocytes using BCB test, preliminary analysis on the relationship between oocyte groupings was done by Chi-

square test of independence. Comparison of the different proportions of BCB positive between pairwise oocyte groups was analyzed by 2 x Chi-square test of independence and comparison of BCB positive and BCB negative within each oocyte group was analyzed by Z test for a binomial population.

Differences between the mean values were considered significant when the *P* values were less than 0.05. All analyses were performed using SAS software (JMP version 9, SAS Institute, Cary, NC, USA).

In the study of the effect of density gradients, data were gathered in three replicates. Results were expressed in averages and percentages. Treatment means were analyzed using Randomized Complete Block Design and Least Significant Difference to examine significant differences among treatment means. Linear contrast was used to compare the means of the treatment and control and Levene's Test was used for equality of variances and interactions. Results on cleavage and embryo development rates were expressed in percentages which were analyzed using Chi-square or Fisher's Exact Test for any significant difference.

4.3. Results

4.3.1. Experiment 3-A: Selection of developmentally competent oocytes

4.3.1.1. Study 1. Effect of oocyte quality

Based on morphology and compactness of the surrounding cumulus cells. A total of 96 ovaries (Table 4-1) were used. From these ovaries, 345 cumulus-oocyte complexes were retrieved presenting a 3.6 oocyte/ovary irrespective of quality. Grade A oocyte accounted an average of 1.0/ovary. Proportions of Grades A, B, C, D, and E oocytes were not significantly different. Nuclear analysis showed highest germinal vesicle stage in Grade A (55.3%) oocytes while germinal vesicle breakdown stage was highest in Grade B (35.8%). Advance nuclear stage was highest in Grade C (28.0%) whereas degenerating oocytes were highest in Grade D (58.8%).

Cleavage and blastocyst development rates were significantly ($P<0.01$) highest in Grade A oocytes at 72.6 percent and 22.3 percent, respectively with decreasing trend from Grades B to E. Grade D oocytes had the lowest cleavage rate (11.1%) but none developed to blastocysts. Similar results were observed in Grade E oocytes.

The analysis of re-classifying the Grades A and B oocytes based on the compactness of the surrounding cumulus cells (Table 4-2) showed that oocytes with loosen cumulus cells had optimal development at shorter duration of IVM, 20 to 22 h. This was shown by the significantly higher ($P<0.01$) blastocyst development (32.8%) than when matured for 24 to 26 h (11.6%).

On the contrary, oocytes with compact cumulus cells required longer duration, 24 to 26 h, of IVM as evidenced by significantly ($P<0.01$) highest cleavage (72.5%) and blastocyst development (27.1%) than when matured for 20 to 22 h (55.3 and 9.7% cleavage and blastocyst development, respectively).

***Based on the granulation and size of ooplasm*.** Results in examining the developmental competence of oocytes classified based on the granulation of the ooplasm showed that cleavage and blastocyst development rates were not significantly different between the two groups, 72.6 versus 74.4 percent and 32.9 percent versus 24.4 percent, respectively (Table 4-3) although blastocyst development was higher in oocytes with homogeneous ooplasm. Yet, diameter of oocytes was found to be a positive indicator of developmental competence ($P<0.01$, Table 4-4).

Meiotic competence assessed by the nuclear status both at the beginning and at the end of IVM showed significant effect of oocytes diameter. Resumption of meiosis evidenced by germinal vesicle breakdown increased as oocyte diameter increased. High incidence of degenerating oocytes (28.9%) was observed in <100 μm oocytes and after IVM, oocytes in this category neither develop to MII stage or cleave after IVF while about 70 percent (46 to 97%) oocytes in ≥100 μm size groups reached MII.

Of the 100 to 119 μm size group, 25 percent of zygotes (14% of *in vitro*-fertilized oocytes) developed to blastocysts after IVF. In oocytes 120 to 139 μm diameters, significantly high MII (96.6%, $P<0.01$), cleavage (96.8%, $P<0.01$) and blastocysts (27.0%, $P<0.05$) developments were observed. High incidence of degenerating oocytes

(39.1%) was found after IVM in ≥140 μm size group and the remaining 60.0 percent of these oocytes reached MII, cleaved, and 30 percent of zygotes (20.0% of *in vitro*-fertilized oocytes) developed to blastocysts. IVC of oocytes according to sizes showed that oocytes with ooplasm diameter ≥107 μm cleaved after IVF and those with ≥113 μm developed to blastocysts.

4.3.1.2. Study 2. Effect of size of the donor follicle

Results on relation of follicle size and developmental competence of oocytes are shown in Table 4-5. Follicles <4 mm diameter (67.8%) was higher (P<0.01) than ≥4 mm follicles (32.2%). Small follicles (<2 mm, 2 to 3.9 mm) contained more Grades C and D oocytes. Medium size follicles (4 to 5.9 mm, 6 to 7.9 mm) contained highest good quality oocytes (Grades A and B) while big follicles (6 to 7.9 mm) contained highest Grade E oocytes (P<0.05) suggesting that change on morphology of cumulus cells goes with the diameter of the follicles.

Mean diameters of oocytes increased as their donor follicle size increased (P<0.01) although there was no increase in the average diameters between the oocytes retrieved from the follicles of categories 2 to 3.9 mm and 4 to 5.9 mm. Majority (51%) of oocytes from <2 mm follicles were <100 μm oocytes; from 2 to 5.9 mm follicles, >40 percent of oocytes were ≥120 μm; and from follicles 6 to 7.9 mm and >8 mm, 70 and 80 percent were >120 μm oocytes.

Frequencies of germinal vesicle and germinal vesicle breakdown were not different in all follicle size groups. Degenerating nuclei was high in oocytes from small (<2 mm and 2 to 3.9 mm) follicles. The cleavage and blastocyst development rates increased as their donor follicle-size increased (P<0.05).

4.3.1.3. Use of brilliant cresyl blue in selecting homogeneous population of water buffalo oocytes

Table 4-6 presents the results of BCB test. Oocytes surrounded by loose cumulus cells are developmentally competent oocytes as evidenced by significantly highest BCB

positive oocytes (91.4±0.8%) and lower BCB negative oocytes (8.6±0.8%) compared to oocytes with compact cumulus cells, 60.9±14.4 percent BCB positive and 39.1±1.5 percent BCB negative, respectively. On the oocytes classified based on the granulation of ooplasm, no significant difference was observed although oocytes with evenly granulated ooplasm had the tendency of having higher BCB positive and lower BCB negative oocytes.

When the combination of compactness of the surrounding cumulus cells and the granulation of ooplasm was used as selection criteria and compared, oocytes with loose cumulus cells and evenly granulated ooplasm had significantly higher (96.7±0.5%) BCB positive oocytes. The lowest was the oocytes with compact cumulus cells and unevenly granulated ooplasm (87.9±1.8%) ($P<0.01$). Oocytes with uneven granulation of ooplasm had consistently the lowest BCB positive and highest BCB negative. IVM-IVF-IVC of the BCB treated oocytes showed that blastocyst development was significantly higher in BCB positive group than the control (unselected) group (Table 4-7). Oocytes surrounded by loose cumulus cells had the tendency of having higher cleavage and blastocyst development than oocytes with compact cumulus cells. Oocytes with loose cumulus cells and evenly granulated ooplasm had significantly the highest cleavage (90.5±0.2%) and blastocyst development rate (34.9 ± 0.1%). The oocyte with compact cumulus and unevenly granulated ooplasm had lower cleavage and blastocyst developmental, though the difference was not statistically different. BCB negative oocytes with compact cumulus cells had poor cleavage (26.1 to 42.4%) but did not develop to the blastocyst stage. BCB negative oocytes from loose cumulus cell group had consistently no cleavage and blastocysts development.

4.3.1.4. Ultrastructure of the oocytes under different morphological categories

Five to twelve oocytes were examined under each oocyte category. Figure 4-7 presents the ultrastructure of the oocytes. Of the twelve oocytes with small ooplasm, eight showed active growing structures characterized by numerous organelles distributed throughout the ooplasm and cuboidal shape cumulus cells (a, b). Four were characterized

by a half-moon shape (d). Of the five oocytes with big ooplasm, all had spherical shape and plenty of vesicles (c, i, j) distributed throughout the cytoplasm and one oocyte showed a large cavity near the nuclei (j).

All oocytes in these categories had nuclei in the germinal vesicle stage. These were located at the peripheral region (a, g, j). Interestingly, three of the oocytes examined under the category of small ooplasm and two of those with big ooplasm had no micro villi protruding towards the surrounding corona radiata (c, l, arrows) except for occasional area with few gap junctions present (l, arrow head). This feature was obvious when compared with the oocyte with small ooplasm (b) and medium size ooplasm (e, h, k).

In all oocyte categories, different sizes and shapes of the surrounding cumulus cells (b, f) with processes penetrating the zona pellucida (h, arrow) and directed towards the oolemma where prominent intermediate and small gap junctions (h, diamond arrow) were observed between oocyte and granulosa cell membranes. Organelles within the oocyte were located at the periphery of the ooplasm and had the tendency of being clustered and associated with each other (i, arrow heads).

In all the oocytes, a perivitelline space was not present but numerous slender villi protruded from oolemma and directed towards the surrounding corona radiata (b, e, h, k, arrows). Pinocytotic vesicles and multivesicular bodies were present in various sizes. The mitochondria were located in clusters (e) and are randomly positioned at the periphery of the cytoplasm (e, i, arrows). Golgi bodies, smooth endoplasmic reticulum (k, stealth arrow) and rough endoplasmic reticulum (k) were present and can be found in peripheral positions with few found towards the central part of the cytoplasm and also at the region of the nucleus.

Lipid droplets were also present and in big sizes positioned in one side of the cytoplasm (i). Cortical granules were present but in solitary position and were very few (k, long arrow).

Ribosomes were attached with the endoplasmic reticulums. Free ribosomes were not conserved but appeared as dark droplet-like materials present and scattered along with the lipid droplets (e, i). Lipid droplets (i, n, arrow) are the main contributor of the even or uneven granulation of the ooplasm.

Light microscopy showed distinct difference between oocytes with compact (m) and loose cumulus cells (p) on the basis of the distances and shape of the cumulus cells. Electron microscopy revealed that shapes of cumulus cells in oocytes with compact cumulus cells were circular to cuboidal while those with loose cumulus cells mostly were elongated with some oocytes showing dissociations (o).

Oocytes with compact cumulus cells had cumulus cells processing endings penetrating the zona pellucida (b, f). The surface of the oolemma had distinct bodies and prominent gap junctions. In contrast those with loose cumulus cells, had less processing endings penetrating the zona pellucida and with slender micro villi at the surface of the oolemma. Organelles of the oocytes with loose cumulus cells had a tendency of being positioned towards the center of ooplasm while oocytes with compact cumulus cells were at the periphery.

Between oocytes with homogeneous and heterogeneous ooplasm, numerous slender micro villi were present at the surface of the oolemma. Clusters of mitochondria and other cell organelles were positioned in the periphery of the ooplasm. Vesicles were prominent throughout the ooplasm and lipid droplets were present and located in one side of the ooplasm constituting the biggest cytoplasmic material. In oocytes with evenly granulated or homogeneous ooplasm (q), vesicles and cell organelles were evenly positioned in the ooplasm while the heterogeneous, had the position of organelles toward the center or one side of the ooplasm.

4.3.2. Experiment 3-B: Effect of discontinuous density gradients of silica particles in separating live, motile sperm cells for *in vitro* fertilization

Separation of motile spermatozoa by density gradients of silica particles. The sperm motility and concentration of frozen semen (Table 4-8) after thawing were 23.9±1.2 percent and 41.8±0.4 million/mL, with 80 to 90 percent of the spermatozoa dead and abnormal. Sperm separation by the three combinations of discontinuous colloidal suspensions of silica particles significantly ($P<0.01$) improved the semen motility with motility rate of 96.4±1.9 percent after centrifugation on three layers of

colloidal suspensions (45/65/95) followed by 87.4±7.9 percent (65/95) and 77.1±7.9 percent (45/95). This means a 50 to 72.5 percent improvement in motility for IVF.

IVF using spermatozoa separated by density gradients of silica particles. An examination of the cleavage and embryo development rates of *in vitro*-matured water buffalo oocytes fertilized with spermatozoa separated by density gradients of silica particles revealed a significantly ($P<0.01$) improved cleavage and blastocyst development rates compared to those fertilized by spermatozoa prepared by the conventional method (control) (Table 4-9). Cleavage rates were 63.6±1.7 percent, 65.1±2.3 percent, and 69.1±2.0 percent for 45/95, 65/95, 45/65/95, respectively as compared to only 28.9±1.4 percent in the control group.

No significant difference was observed on the cleavage rate between two discontinuous colloidal suspensions (45/95 and 65/95). However, significantly higher ($P<0.01$) cleavage rate was recorded in the three discontinuous colloidal suspensions (45/65/95).

On embryo development rate, significantly higher ($P<0.01$) blastocyst stage embryos were produced when treated sperm cells were used. Increasing concentration of density gradients gave higher efficiency rate; 20.4±2.5 percent, 21.8±3.0 percent, 23.6 ±1.0 percent for 45/95, 65/95 and 45/65/95 density gradients against 10.0±1.3 percent in the control group, respectively. Out of the cleaved zygotes, no significant difference was observed on the blastocyst development rate; 32.2±4.0 percent, 33.4±4.3 percent, 34.0±1.0 percent for 45/95, 65/95 and 45/65/95 density gradients against 34.6±4.5 percent in the control group, respectively.

Efficiency of density gradient sperm separation techniques using silica particles in IVF of bulls with low post-thaw motilities. Results showed variation on sperm motility of individual bulls even at pre-treatment (post-thaw) after conventional processing and after separation by colloidal suspension of silica particles (Table 4-10). Significantly ($P<0.01$) higher post-thaw motility was observed in bulls B (22.6±8.9%) and C (25.5±8.8%) than bull A (12.0±3.2%) at pre-treatment and after processing with

the conventional method; 26.8±3.0 percent and 27.4±1.1 percent for Bulls B and C against 22.8±0.3 percent for Bull A, respectively.

Separation of motile spermatozoa by colloidal suspension of silica particles resulted in improved motility in all bulls but Bull C (87.8±8.3%) had a significantly ($P<0.01$) higher motility rate than to Bull A (80.4±12.4%) and B (80.4±12.4%). Analysis of the differences in sperm concentration revealed no significant differences among bulls at pre-treatment: 41.6 to 43.3 million sperm cells/mL (Sperm concentration in each straws was standardized during semen processing) and after processing by conventional method, 37.3 to 39.0 million sperm cells/mL, but a significantly ($P<0.01$) higher sperm concentration in Bull B (10.2 million sperm cells/mL) than Bull A (5.8 million sperm cells/mL) was observed after sperm separation.

The use of separated motile spermatozoa for IVF showed that the system had significantly improved the efficiency of each bull for IVF. Irrespective of the bull, cleavage and blastocyst development were consistently higher ($P<0.05$) when the spermatozoa used for IVF were separated by the density gradients of silica particles. Cleavage rate was improved from 8.0 to 54.0 percent in Bull A, from 45.5 to 70.0 percent in Bull B, and from 43.0 to 73.8 percent in Bull C, respectively. Blastocyst development was improved from 1.3 to 12.4 percent in Bull A, 16.0 to 23.8 percent in Bull B, and from 12.7 to 29.5 percent in Bull C, respectively. Similarly, blastocyst development out of the cleaved zygotes was significantly improved ($P<0.05$) by the system. Bull differences existed but the use of density gradient sperm separation using discontinuous gradients of silica particles improved the fertilization potential of the bulls and the subsequent development of pre-implantation stage embryos.

4.3.3. Experiment 3-C: Embryo development of water buffalo oocytes in stage-dependent culture system and in co-culture system with or without pyruvate and lactate

A total of 267 water buffalo oocytes matured and fertilized *in vitro* was used. Of this total, 129 oocytes were used to study the effects of fixed-dose of FBS with or without pyruvate and lactate while 238 oocytes were used to study the effects of stage-dependent

culture system by an increasing dose of FBS with or without pyruvate and lactate (Table 4-12).

No significant difference was observed on the cleavage rate between the four treatment groups. However, in both the stage-dependent culture system and the fixed-dose of FBS systems, development to morula on 5th day (42.6 and 43.4%) and blastocyst on 7th day (36.1 and 35.7%) was consistently higher in the pyruvate and lactate containing medium than in the absence of pyruvate and lactate, 21.8 and 31.1 percent cleavage and 14.7 and 23.5 percent blastocyst development, respectively.

Similar trend was observed in the hatching rate examined on the 10th day of IVC. The presence of pyruvate and lactate was found beneficial in enhancing blastocyst development while increasing the dose of FBS from 1 to 15 percent had no beneficial effect in improving the production rate of preimplantation stage embryos in buffaloes.

Viability of the embryos *in vitro*-produced out of the improved system i.e. oocytes with compact cumulus *in vitro* matured in IVM medium containing hormones and EGF and fertilized *in vitro* using semen separated by three discontinuous layers of silica particles and cultured for *in vitro* development in stage-dependent culture system with pyruvate and lactate, vitrified using open-poled straw and transferred to recipient animals, revealed 23.1 percent (6/26) calving rate that includes a twin (Fig. 4-8) while fullterm development was 26.9 percent (7/26) out of the recipient animals or 13.5 percent (7/52) out of the transferred embryos (Table 4-13).

4.4. Discussion

Studies on developing the selection parameters to identify a homogeneous population of oocytes showed that aside from the density of the surrounding cumulus cells that was universally accepted as basis for oocyte classification (Chauhan *et al*., 1998a; De Loos *et al*., 1989), the compactness of the surrounding cumulus cells, the diameter of the ooplasm, and the size of the follicles were positive indicators of developmentally competent oocyte. Oocytes surrounded by compact cumulus (Grade A) were mostly at germinal vesicle stage while those with loose cumulus were mostly at germinal vesicle breakdown stage, and those denuded from cumulus were degenerating.

These findings conformed to the observation in cattle (De Loos *et al*., 1989). Germinal vesicle breakdown was observed in all oocyte categories confirming the report that mammalian oocytes removed from their follicular environment spontaneously resume meiosis (Pincus and Enzmann, 1935). Also, cumulus cells are clearly in control of meiosis since compounds which elevate intracellular cAMP levels delay meiotic resumption in cumulus-enclosed oocytes, but not in cumulus-denuded oocytes (Dekel *et al*., 1984).

Cleavage and blastocyst development rates were significantly ($P<0.01$) highest in Grade A oocytes with decreasing trend from Grades B to E. These suggest that oocytes morphology is a positive indicator of developmental competence.

In bovine, De Loos *et al*. (1989) described a four level system for classifying oocytes based on compact multi-layered cumulus investment and homogenous ooplasm: (1) Compact multi-layered cumulus of greater than three layers with homogenous ooplasm; (2) compact cumulus with one to two layers with homogenous ooplasm having a coarse appearance and a darker zona pellucida; (3) oocytes with less compact cumulus and irregular ooplasm containing dark clusters; and (4) nude oocyte or expanded cumulus, irregular ooplasm and jelly-like matrix.

They found out that level 1 and 2 oocytes had the highest cleavage and blastocysts development. On the other hand, Smith *et al*. (1996) classified the bovine oocytes as Class 1 for those with compact cumulus, Class 2 for those with less than three layers of cumulus, Class 3 for those that are partially nude from cumulus, and Class 4 for those that are nude. They found significantly more Class 1 oocytes cleaved (63.7% compact) than Class 3 (29.5% partially nude) and Class 4 (17.7% nude) oocytes confirming the observations in water buffalo.

Similarly, Chauhan *et al*. (1998b) obtained 37.4 percent blastocyst development on 7^{th} day of IVC of cumulus oocyte complexes co-cultured with buffalo oviductal epithelial cells that commenced to 41.7 percent blastocyst development at the 10^{th} of IVC. This suggests that the presence of somatic cells has beneficial effect on the IVC of oocytes. This could be the reason why oocytes surrounded by greater than three layers of cumulus cells had higher developmental competence than those with less than three layers.

The study on the compactness of the surrounding cumulus cells demonstrated that to optimize development rate, oocytes must be re-classified and cultured depending on the morphology of the cumulus i.e. shorter period of IVM for those with compact cumulus and longer for those with loose cumulus. These results explain the observations in Experiment 2-B and reports (Yadav *et al*., 1997; Singh and Majumdar, 1992; Nandi *et al*., 2001a; Neglia *et al*., 2001; Gasparrini *et al*., 2008) claimed that water buffalo oocytes cultured for maturation *in vitro* had varying degree of development potential over time from 15 to 28 h with 18 to 24 h, the most optimal for IVM.

The findings clearly demonstrate that the oocytes are developmentally competent but due to the different degree of developmental stages, some oocytes may be at the late stage of development while others are at the early stage. A pre-determined time of IVF may mean fertilization of under maturation, on time maturation or over maturation/aging among oocytes that may result in varying developmental rate as well as chromosomal abnormalities in some of the resultant embryos as observed in human (Bongso *et al*., 1988). These clearly suggest a need to select homogeneous population of oocytes and implement IVM following the desired duration to optimize development rate.

Classification based on the granulation of the ooplasm showed no significant difference which suggests that in buffalo oocyte, both uneven and evenly granulated ooplasm, does not impair developmental competence. In cattle, oocytes with unevenly granulated ooplasm had higher developmental competence (Nagano *et al*., 2006) at 22 to 24 h IVM. Since *in vitro* embryo development is one day earlier in water buffalo compared to cattle (Gasparrini *et al*., 2007), the above results suggest with unevenly granulated ooplasm oocyte is an indication of advance developmental stage.

On the other hand, the diameter of the oocyte was found as a positive indicator of oocytes developmental competence. Nuclear maturation and *in vitro* development potential of the water buffalo oocytes was associated with increase in oocytes diameter. This was evidenced by the failure of oocytes with <100 μm in diameter to develop to the MII stage demonstrating lack of maturational capacity. Oocytes with 100 to 119 μm in diameter acquired maturational capacity with few acquired developmental potential evidenced by the increased MII and blastocyst development after IVM-IVF. Oocytes with 120 to 139 μm diameter acquired full maturational and developmental capacity as shown

by the significant improvement on MII development, cleavage and blastocyst development rate. Oocytes with ≥140 µm have full developmental competence but some are at the transition period of degeneration.

These results showed that in spite of being Grades A and B oocytes, high degeneration was observed in small (<100 µm) oocyte before IVM and in large (≥140 µm) oocytes after IVM which could be a major cause for reduced efficiency rate of IVM-IVF-IVC. The high incidence of degeneration observed in <100 µm oocytes was characterized in cattle as having ceased synthesizing RNA (Crozet *et al*., 1986; Fair *et al*., 1996) that represents a stable storage of macromolecules essential for resumption of meiosis and early embryonic development (De Smedt *et al*., 1994; Crozet *et al*., 1981; Antoine *et al*., 1988; Sirard *et al*., 1992). This could be the reason of failure of oocytes <100 µm diameter to reach the MII stage after IVM. The increased incidence of degeneration among ≥140 µm oocytes could be attributed by the fact that follicles activated to enter the growth phase characterized by increase in size of the oocytes (Gougeon, 2003) may end up to atresia resulting in degeneration and loss of competence.

These were in agreement with the observations in Nili Ravi buffaloes (Yousaf and Chohan, 2003) and in cattle (Fuhrer *et al*., 1989; Otoi *et al*., 1997; Park *et al*., 1998) indicating oocytes acquire full meiotic competence at a diameter of 115 µm and full developmental competence at a diameter of 120 µm but not in goat, 125 µm for meiotic competence (Anguita *et al*., 2007) and 135 µm for full developmental competence (Martino *et al*., 1994; De Smedt *et al*., 1994).

In Indian buffaloes, however, Raghu *et al*. (2002a) reported that meiotic competence was achieved at a diameter of 145 µm in ≥8 mm follicles which is bigger than what we observed. These differences could be attributed by the breed-type and the method used in the measurement of oocyte diameter. In cattle, Crozet *et al.* (1986) and Fair *et al.* (1996) demonstrated that the *in vitro* developmental ability of oocytes to reach MII was directly proportional to their diameter. In order to complete meiotic maturation, it must have reached a diameter of at least 110 µm.

Other studies have shown that oocytes ≥120 µm in diameter have ceased synthesizing ribonucleic acid (Fair *et al*., 1996) of which, despite their meiotic competence, the majority of these oocytes failed to reach the blastocysts stage following

IVM, IVF and IVC (Crozet *et al.*, 1986). These findings have been linked to a number of ultrastructural and molecular changes occurring during oocytes development within the follicle of origin (Assey *et al.*, 1994; Fair *et al*., 2002) which was supported by the observations gathered in the present experiment on the ultrastructure of oocytes. Oocytes with <100 and >145µm had no micro villi protruding towards the surrounding corona radiata except for occasional area with few gap junctions present. This feature indicated the structure of oocytes that were degenerating and have ceased communication with the surrounding corona cells. These oocytes could be those oocytes that failed to mature and develop after IVM and IVF.

These results suggest that the size of the ooplasm, quality of the surrounding cumulus cells and the granulation of the ooplasm signify differences on the developmental status of the oocytes. These differences may imply the need of a different treatment protocol for each category in order to optimize the *in vitro* development potential.

Results in the study of the relation of follicle size on oocyte developmental competence showed that size of follicle was linearly correlated with oocyte quality ($r=0.45$; $P<0.01$), diameter ($r=0.36$; $P<0.01$) and developmental competence ($r=0.37$; $P<0.01$) (Fig. 4-6). Follicular sizes 4 to <8 mm yielded more uniform, high quality oocytes (Fig. 4-5) with the lowest frequency of degeneration (Grades C to E oocytes). This suggests that oocytes at these follicular sizes are more of the developmental phase as evidenced by increased development rate observed in oocytes after IVM-IVF-IVC. Denuded oocytes (Grade D) were high in small follicles but decreased in number as follicle size increased indicating weak cumulus-oocyte connections in small follicles. Also, retrieval of the oocytes by follicular aspiration may enhance removal of the attached cumulus thereby a denuded oocyte.

Oocytes with intact and compact cumulus cells (Grades A and B) increased from >2 mm follicles and was highest at 4 to 5.9 mm indicating that cumulus-oocyte connection in these follicles was established accompanied by developmental competence of the oocytes as evidenced by the increased cleavage and blastocyst development. Grades A and B oocytes decreased in 6 to 7.9 mm follicles while oocytes with expanding cumulus cells increased demonstrating the morphological changes in cumulus-oocyte

morphology and connections as follicle grew and reached atresia or regressed/ovulation phase.

Salamone *et al*. (1999) reported that Grade E oocytes were found from follicles in the regressing phase of the wave which suggested that follicular regression might have occurred at >6 mm follicles sizes in water buffalo. As observed by Gordon and Lu (1990), the first signs of atresia were manifested by the degeneration of the granulosa cells that lose their aromatase activity and undergo apoptosis.

This degeneration of cells is associated with low E_2 or high P_4 levels in follicular fluid (Assey *et al*., 1994), low ATP content (Nagano *et al*., 2006), and increase in the percentage of degenerated oocytes (abdoon *et al*., 2001) which may support the observation that Grade C oocytes were highest in >8 mm follicles and had low development rate (Liu *et al*., 1991; Suzuki *et al*., 1991; Chauhan *et al*., 1998a):

In contrast, the Grades A and B oocytes in these follicles attained the developmental competence, had sufficient exposure to gonadotrophins and distained for ovulation thus possessing a higher blastocyst development rate. These observations conformed to earlier studies indicating that selection of oocytes for IVM on the basis of visual assessment showed positive correlation between oocyte morphology and blastocysts yield (Liu *et al*., 1991; Suzuki *et al*., 1991; Chauhan *et al*., 1998a).

Moreover, between follicular size groups, significant increase ($P<0.01$) in the mean oocytes diameter were observed except in 2 to <4 mm and 4 to <6 mm follicles where oocyte diameter appeared to be stable in these follicle sizes. Developmental potential after IVM-IVF-IVC was significantly lower in oocytes from <2 mm follicular group. This could be attributed to few numbers (2.6%) of oocytes with ≥120 µm of diameter that could develop to the MII stage and acquire developmental capacity after IVM as observed in the earlier study.

There was an increase in the rates of cleavage and blastocyst development as the follicular diameter increased which was attributable to the increase in the proportion of the oocytes with ≥120 µm of diameter suggesting that the developmental competence of the oocytes is acquired within the follicle and supporting the observations in cattle (Blondin *et al*., 1997; Krisher, 2004).

In each follicle-size category, proportion of the oocytes with ≥120 µm of diameter is around 3, 45, 70 and 80 percent for <2, 2 to 5.9, 6 to 7.9 and ≥8 mm follicles, respectively. If 60 percent of ≥120 µm oocytes developed to the blastocysts, the rate of blastocysts in each group could be estimated as 0, 20, 40 and 55 percent, respectively. These values are almost same as the results obtained in this study where the rates of blastocysts were 2.2, 17.2, 23.8, 40.0, and 53.8 percent, respectively. These values were somewhat higher than those in earlier study on the effect of oocyte diameter on blastocyst development. This means that even the oocytes with ≥120 µm diameters have their developmental capacity depend on their donor follicle suggesting that follicle size has positive implication on the oocyte developmental competence.

It could be deduced from these results that the oocytes from <2 mm follicles are at the growing phase of development, those at 2 to >4 mm are at the transition phase of development while those at ≥4 mm have acquired the developmental potential which are in agreement with previous reports (De Wit *et al*., 2000; Raghu *et al*., 2002; Yousaf *et al*., 2003; Vassena *et al*., 2003). The 2 mm follicular size was identified in cattle as the follicular recruitment stage when a cohort of antral follicles begins to grow beyond 4 mm in diameter in the presence of sufficient gonadotrophin to permit their progress toward ovulation (Ginther *et al*., 2001a, b). This is associated with the appearance of aromatase activity within the granulose layer detected in bovine follicles with a diameter of 3 to 4 mm (Ginther *et al*., 1996).

On the other hand, about 46 percent of the oocytes in ≥8 mm follicular group failed to develop to the blastocysts stage while 17 to 40 percent of the oocytes from 2 to <8 mm follicles developed. These variations could be attributed to the degree of atresia or regression of the donor follicle.

Some studies have shown that follicular atresia may promote the acquisition of developmental competence (Blondin and Sirard, 1995; Hagemann *et al*., 1999) but highly atretic follicle encloses oocytes that are degenerating (Abdoon *et al*., 2001). The degree of atresia of the follicles was not considered in the selection and classification of follicles in this study. However, Kruip and Dieleman (Kruip and Dieleman, 1982) observed that 85 percent of follicles found in an ovary at any time in the estrous cycle were atretic. In cattle, the proportion of atretic follicles increased with follicle size (Rajakoski, 1960).

It could be inferred from this experiment that follicles with similar diameters may be in very different physiologic phases and that large follicles developed during luteal phase or at mid cycle failed to ovulate but those developed during follicular phase ovulated (Webb *et al*., 1992) which formed part of oocytes with developmental competence in this study. The incidence of apoptosis may have also contributed to the vanished developmental competence anticipated as the primary mechanism by which cell loss is mediated during follicle degeneration (Kerr *et al*., 1972; Tilly *et al*., 1996).

In testing the use of BCB in identifying and selecting a homogeneous population of developmentally competent oocytes, results showed that BCB test was effective. It is a reliable method as evidenced by the significantly higher cleavage and blastocyst development rates in BCB positive oocytes than in BCB negative oocytes confirming the results obtained in cattle (Pujol *et al*., 2004; Bhojwani *et al*., 2007).

The BCB test showed that BCB positive oocyte was higher in oocytes with swelled/loose cumulus cells than in oocytes with compact cumulus. This suggests that oocytes with loose cumulus are most likely at the developmental phase while some of the oocytes with compact cumulus are still at the growing phase as evidenced by the need of a longer IVM period. In oocytes selected based on the granulation of ooplasm, BCB positive was the same for oocytes with heterogeneous and homogeneous ooplasm implying that the granulation of the ooplasm, at least in water buffalo, is not a positive indicator of developmental competence. Meanwhile swelled/loosened cumulus is a parameter indicative of oocytes advanced developmental status.

The density gradient sperm separation technique using silica particles was an effective method in separating high quality motile sperm cells, enhancing significantly higher cleavage and blastocyst development among *in vitro*-fertilized oocytes and in optimizing the reproduction potential of genetically superior bulls with low sperm motility. The presence of immotile sperm cells is the major reason why production of embryos *in vitro* is difficult in bulls with very few motile spermatozoa in the semen (Madan *et al*., 1996).

Sperm separation by the discontinuous colloidal suspensions of silica particles significantly ($P<0.01$) improved the semen motility with the three layers of colloidal suspensions (45/65/95) yielding the highest improvement. The same observation was

reported in human indicating that higher concentration of silica particles was superior in separating motile and morphologically normal sperm cells (Chen and Bongso, 1999; Soderlund and Lundin, 2000; Hammadeh *et al*., 2001).

Similarly, separation of motile spermatozoa by colloidal suspension of silica particles resulted in improved reproductive efficiency in bulls with low sperm post-thaw motilities. Blastocyst development of cleaved zygotes revealed no significance difference among bulls. However, standard deviation was too large in control groups and Levene's Test for equality of variances showed significant difference ($P<0.05$) between Bulls A and B. The high variability in the fertilizing ability of different bulls was observed by other scientists (Totey et al., 1993b; Chauhan et al., 1998d; Yadav et al., 2001). Individual bulls affected the subsequent development of the embryo they produced as reported by Hamamah *et al*. (1997). These results suggest that the separation of the motile sperm cells by density gradients of silica particles improved the potential of these bulls for use in an IVF program.

On the other hand, studies on improving the IVC system by supplementation of energy substrate (pyruvate and lactate) and catering to the changing requirements of the embryos during IVC by increasing the dose of FBS showed that supplementation of pyruvate and lactate were effective but increasing the concentrations of FBS had no beneficial effect in enhancing blastocyst development. Results demonstrated that the absence of pyruvate and lactate and low concentration of serum during the initiation of zygotic stage of development may limited the energy requirements of the embryos resulting in failure of development as reported in other animals (sheep and cattle, Thompson, 2000).

It has been proven and accepted for many years that the pre-implantation embryo has an initial preference for pyruvate at the cleavage stages (Leese and Barton 1984; Gardner and Leese, 1986) with division to the 2- cell stage is supported only by pyruvate or phosphoenolpyruvate, while from the 2- cell stage, development can be supported by both pyruvate and lactate (Rosenkrans *et al*., 1993). In fact, zygotes failed to cleave when lactate was the sole energy substrate despite the observation that the zygote can oxidize lactate from the medium (Biggers *et al*., 1967).

In porcine, Kikuchi *et al*. (2002) and Medvedev *et al*. (2004) demonstrated the importance of pyruvate and lactate as energy supplements, especially for an early embryonic development *in vitro*. On the other hand, blastocysts formation required much energy level due to increased protein synthesis levels (Morales *et al*., 1992) and increased activity of the Na+/K+-ATPase to create the blastocoel cavity (Fleming *et al*., 2004). These findings supported the present observation that increased concentration of serum (15%) in the presence of lactate and pyruvate during the late cleavage stages enhanced higher development of blastocysts stage embryos as observed in Treatment 4. Transfer of the resultant embryos to recipient animals revealed 23.1 percent calving rate which was higher than the success rate obtained in the Experiment 1-C (10.9%) and transfer of *in vivo*-derived embryos (pregnancy rate of 9.2%, Kurup *et al*., 1988; 17.9%, Drost *et al*., 1988; 18.3%, Alexiev *et al*., 1988; 17.0%, Misra *et al*., 1994; and calving rate of 9.8%, Misra *et al*., 1994; 14.3%, Techakumphu *et al*., 2001).

The success rate was comparable to that obtained in cattle using *in vivo*-derived embryos frozen in ethylene glycol and placed in straws for direct transfer (pregnancy rate of 28.8% in Brazil, 22.2% if recipient has Day 5 CL, and 25.0% in +24 h embryo synchrony (Benyei *et al*., 2006).

Based on the above findings, heterogeneous population of oocytes is one of the major reasons of the low blastocyst development rate after IVC in water buffalo. This could be due to the use of oocytes with small diameters or from small or degenerating follicles that have not achieved the developmental competence or big oocytes or oocytes from big follicles that have lost their developmental potential at the time when they are subjected to mature *in vitro*. To optimize blastocyst development, it is recommended to collect grades A and B oocytes from ≥6 mm follicles or with >120 µm diameter of ooplasm and re-select based on the compactness of surrounding cumulus and apply the desired IVM period. BCB staining technique is also recommended for use in the determination and selection of developmentally competent oocytes for IVM.

For IVF, the use of three discontinuous layers of colloidal suspension of silica particles is effective in separating motile sperm cells. The use of these sperm cells is effective in improving the rate of cleavage, blastocyst development and the fertilization potential of semen from bulls with low sperm motilities. For IVC, the supplementation of

energy substrates such as pyruvate and lactate is important because it supports the metabolic requirements of the growing embryos and ensures higher blastocyst development. Following the above recommendations would yield an improved fullterm development and calving rate after ET indicating production of viable embryos *in vitro*.

Table 4-1. Nuclear status of water buffalo oocytes before *in vitro* maturation and subsequent development after *in vitro* maturation and fertilization, 96 ovaries

COC Grade	*n* (/ovary)	Nuclear status of oocytes before IVM (Mean±S.E.)				*In vitro* development (Mean±S.E.)		
		GV	GVBD	Adv	Deg	*n*	≧2-cell	BL
A	94 (0.98)	55.3±0.7^{a}	28.0±0.6^{ad}	9.6±0.6^{a}	6.4±0.7^{a}	99	72.6±0.0^{a}	22.3±0.5^{a}
B	73 (0.76)	37.3±1.4^{b}	35.8±1.4^{ab}	15.5±1.1^{ab}	11.2±1.2^{a}	76	41.6±0.5^{b}	19.4±0.1^{a}
C	73 (0.76)	27.8±0.9^{c}	16.7±0.4^{cd}	28.0±3.7^{b}	34.0±1.4^{b}	89	29.6±0.4^{c}	9.0±0.6^{c}
D	63 (0.66)	5.0±1.3^{d}	22.1±1.2^{d}	14.0±1.7^{a}	58.8±2.5^{c}	105	11.1±0.5^{d}	0^{d}
E	42 (0.44)	7.1±1.9^{d}	23.1±3.6^{d}	21.6±0.7^{b}	32.2±5.7^{b}	114	13.7±0.4^{d}	0^{d}

Cumulus-oocyte-complexes (COC) grade: Grade A, oocyte is surrounded by ≥5 attached layers of dense cumulus cells; Grade B, oocyte surrounded by 2<5 dense layers of cumulus cells; Grade C, surrounded by irregular and <2 layers of cumulus cells; Grade D, denuded/free from cumulus cells; Grade E, surrounded by expanded cumulus cells.
Abbreviations: IVM= in vitro maturation; GV=germinal vesicle; GVBD= germinal vesicle breakdown, Adv= advance meiotic stages that includes methaphase I, Anaphase I, and Telophase I, Deg=degeneration nuclear material characterized by fragmentation, no distinct material, presence of dotted tiny materials; BL= blastocyst development rate. Ten replicated trials were carried out. Figures in the same column with different superscripts are significantly different (P<0.05) by Fisher's Exact Test.

Table 4-2. Cleavage and blastocyst development of water buffalo Grades A and B oocytes re-classified based on the compactness of the surrounding cumulus cells and *in vitro* fertilized at shorter (20 to 22 h) or longer (24 to 26 h) IVM periods

Oocyte group	**IVM length, h**	**n**	***In vitro* development (%)**	
			≧2-cell	**BL**
Loosen	20-22	114	76.5 [ab]	32.8 [a]
	24-26	156	68.3 [b]	11.6 [b]
Compact	20-22	117	55.3 [c]	9.7 [b]
	24-26	131	72.5 [a]	27.1 [a]

Oocyte grouping was based on the nature of the cumulus cells surrounding the oocyte; Loosen when the cumulus mass surrounding the oocyte is slightly expanded and Compact when the surrounding cumulus mass is tightly connected.
BL is blastocysts.
Values in the same column with different superscript are significantly different (P<0.01) by Fisher's Exact Test:

Table 4-3. Cleavage and blastocyst development of water buffalo Grades A and B oocytes re-classified based on the granulation of the ooplasm and *in vitro* fertilized at 22 to 24 h IVM period

Oocyte group	IVM length, h	n	*In vitro* development (%)	
			≧2-cell	BL
Homogeneous	22-24	164	72.6	32.9
Heterogeneous	22-24	180	74.4	24.4

Oocyte group was based on the granulation of the ooplasm; Homogeneous, when ooplasm is evenly granulated and Heterogeneous, when ooplasm is unevenly granulated. BL is blastocyst development rate.

Table 4-4. Maturational and developmental competences of oocytes with different diameters

Size of ooplasm diameter, μm	Oocyte categories			
	<100	100 to 119	120 to 139	≥140
Nuclear status before IVM				
(n)	(38)	(103)	(72)	(41)
Diameter, μm (Mean±S.E.)	86.2±1.3	111.7±0.5	126.9±0.5	149.3±1.2
% GV	42.1^{a}	65.1^{bA}	63.9^{b}	39.0^{aB}
% GVBD	28.9^{ab}	26.2^{a}	36.1^{ab}	46.3^{b}
% MI to MII	0.0^{ab}	4.9^{ab}	0.0^{a}	9.8^{b}
% degenerated	28.9^{A}	3.9^{B}	0.0^{B}	4.9^{B}
Nuclear maturation after IVM				
(n)	(19)	(69)	(29)	(23)
Diameter, μm (Mean±S.E.)	94.3±0.9	110.7±0.8	125.1±0.8	155.4±2.6
% GVBD to TI	84.2^{aA}	53.6^{bB}	3.5^{cC}	0.0^{cC}
% MII	0.0^{A}	46.4^{B}	96.6^{C}	60.9^{B}
% degenerated	15.8^{aA}	0.0^{bB}	0.0^{bB}	39.1^{aA}
Development after IVF-IVC				
(n)	(30)	(140)	(63)	(20)
Diameter, μm (Mean±S.E.)	83.4±1.8	112.8±0.6	124.9±0.1	146.8±1.8
% cleaved	0.0^{A}	57.1^{B}	96.8^{C}	60.0^{B}
% blastocysts	0.0^{aA}	14.3^{b}	27.0^{cB}	20.0^{bc}

Abbreviations: GV=germinal vesicle; GVBD=germinal vesicle breakdown; MI=metaphase I; TI=telophase I; MII=metaphase II.

Meiotic and developmental competences were analyzed by Chi-square or Fisher's Exact Test.

^{a,b,c} *Values in the same row with different lower class superscript are significantly different at P<0.05.*

^{A,B,C} *Values in the same row with different upper class superscript are significantly different at P<0.01.*

Table 4-5. Quality (Grade), diameter, nuclear status and developmental competence of oocytes retrieved from follicles of different size categories

Follicle size diameter, mm	Follicle categories				
	<2	2 to 3.9	4 to 5.9	6 to 7.9	≥8
COC grade					
(n)	(244)	(221)	(116)	(61)	(46)
% grades A+B	53.3 ^{A}	54.3 ^{A}	70.7 ^{B}	62.3 ^{AB}	43.5 ^{A}
% grades C	5.3 ^{aA}	17.2 ^{bB}	15.5 ^{bB}	8.2 ^{ab}	30.4 ^{cC}
% grades D	25.4 ^{a}	16.7 ^{b}	0.0 ^{dA}	4.9 ^{c}	8.7 ^{bcB}
% grades E	16.0 ^{ab}	11.8 ^{b}	13.4 ^{ab}	24.6 ^{a}	17.4 ^{ab}
Oocyte diameter (Grade A)					
(n)	(39)	(99)	(65)	(24)	(24)
Diameter, µm (mean±S.E.)	99.7±2.3^{A}	115.3±1.4^{B}	115.7±2.0^{C}	125.8±4.1^{C}	134.9±4.6^{D}
% <100 µm	51.3 ^{A}	25.3 ^{B}	23.1 ^{B}	16.7 ^{B}	8.3 ^{B}
% 100 to 119 µm	46.2 ^{aA}	31.3 ^{ab}	32.3 ^{ab}	12.5 ^{bcB}	8.3 ^{cB}
% 120 to 139 µm	2.6 ^{A}	35.4 ^{B}	35.4 ^{B}	33.3 ^{B}	37.5 ^{B}
% ≥140 µm	0.0 ^{A}	8.1 ^{A}	9.2 ^{A}	37.5 ^{B}	45.8 ^{B}
Development after IVF-IVC (Grades A & B)					
(n)	(45)	(46)	(42)	(20)	(13)
% cleaved	42.2 ^{aA}	65.2 ^{b}	76.2 ^{bcB}	90.0 ^{cB}	92.3 ^{bcB}
% blastocysts	2.2 ^{aA}	17.4 ^{b}	23.8 ^{bcB}	40.0 ^{bcB}	53.8 ^{cB}

Cumulus-oocyte-complexes (COC) grade: Grade A, oocyte is surrounded by ≥5 attached layers of dense cumulus cells; Grade B, oocyte surrounded by 2<5 dense layers of cumulus cells; Grade C, surrounded by irregular and <2 layers of cumulus cells; Grade D, denuded/free from cumulus cells; Grade E, surrounded by expanded cumulus cells.
Oocyte quality, meiotic and developmental competences were analyzed by Chi-square or Fisher's Exact Test, while oocyte diameter by Duncan's Multiple Range Test.
^{a,b,c}Values in the same row with different lower class superscript are significantly different at P<0.05.
^{A,B,C} Values in the same row with different upper class superscript are significantly different at P<0.01.

Table 4-6. Results of brilliant cresyl blue (BCB) test among oocytes categories

Oocyte	Compactness of CC		Granulation of ooplasm		Compact cumulus		Loosen cumulus	
Classification	Compact	Loosen	Evenly	Unevenly	Evenly	Unevenly	Evenly	Unevenly
No. of oocytes	96	101	124	151	120	141	149	149
BCB positive, Mean±S.E.	60.9±15 ^{dC}	91.4±0.8 ^{ab}	80.8±0.7 ^{cD}	77.1±2.0 ^{cD}	92.4±1.2 ^{bcB}	87.9±1.8 ^{cB}	96.7±0.5 ^{aA}	93.7±0.9 ^{ab}
BCB negative, Mean±S.E	39.1±1.5	8.6±0.8	9.6±0.7	22.9±2.0	7.6±1.2	12.1±1.8	3.3±0.5	6.3±0.9

Figures in the same row with different lower case superscript(s) are different at P<0.05 while those with upper class superscript are different at P<0.01) by Chi-square or Fisher's Exact Test.
Statistical analysis using Z-test for a binomial population showed significant (P<0.01) difference between the BCB positive and BCB negative proportions of each group of oocytes.

Table 4-7. Efficiency of brilliant cresyl blue (BCB) in selecting developmentally competent oocytes.

Treatment	Oocyte Classification	*n*	Cleavage rate, Mean±S.E.	Blastocyst development, Mean±S.E.	
				/IVM oocytes	/cleaved zygotes
BCB positive	Compact-evenly	99	75.9 ± 0.1^{A}	$31.5\pm0.1^{a\,A}$	41.4 ± 0.1^{aA}
	Compact-unevenly	127	74.0 ± 0.3^{A}	$26.1\pm0.1^{a\,A}$	35.5 ± 0.4^{aA}
	Loosened-evenly	141	90.5 ± 0.2^{B}	34.9 ± 0.1^{AC}	39.9 ± 0.1^{aA}
	Loosened-unevenly	142	83.1 ± 0.2^{AB}	33.1 ± 0.1^{AC}	38.5 ± 0.2^{aA}
BCB negative	Compact-evenly	21	42.4 ± 0.5^{E}	0^{B}	0^{B}
	Compact-unevenly	14	26.1 ± 1.8^{C}	0^{B}	0^{B}
	Loosened-evenly	7	0^{D}	0^{b}	0^{B}
	Loosened-unevenly	8	0^{D}	0^{b}	0^{B}
Control	Mixed	61	64.1 ± 1.1^{E}	21.1 ± 0.7^{a}	32.7 ± 1.1^{aA}

Figures in the same row with different lower case superscript(s) are different at P<0.05 while those with upper class superscript are different at P<0.01 by Chi-square or Fisher's Exact Test.

Table 4-8. Motility and sperm concentration before and after discontinuous density gradient sperm separation

Treatment	Motility, % (Mean ± SD)	Sperm concentration, million (Mean ± SD)
Pre-treatment	23.9±1.23 [a]	41.6±0.44 [a]
Control	25.7±4.0 [a]	37.8±1.6 [a]
45/95	77.1±7.9[b]	15.0±0.8 [b]
65/95	87.4±7.9 [b]	10.5±5.6 [c]
45/65/95	96.4 ±1.9[c]	9.0 ±4.9[c]

Treatment 45/95 was two colloidal suspensions, 45% and 95%, of silica particles developed to form a discontinuous density gradient. Treatment 65/95 was two colloidal suspensions, 65% and 95%, while treatment 45/65/95 was three colloidal suspensions, 45%, 65% and 95% of silica particles.
Figures in the same column with different superscript(s) are significant (P<0.01) by Least Significant Difference.

Table 4-9. Embryo development after *in vitro* fertilization with sperm cells separated by discontinuous density gradients of silica particles

Treatment	Number of oocytes	Cleavage rate , % (Mean± SD)	Embryo dev. rate % (Mean± SD) (per IVF ova)	Embryo dev. rate,% (Mean± SD) (per cleavage)
Control	450	28.9±1.39 [a]	10.0±1.33 [a]	34.6±4.5 [a]
A. 45/95	450	63.6±1.70 [b]	20.4±2.52 [b]	32.2±4.0 [a]
B. 65/95	450	65.1±2.34 [bc]	21.8±3.01 [b]	33.4±4.3 [a]
C. 45/65/95	450	69.1±2.04 [c]	23.6±1.00 [b]	34.0±1.0 [a]

Treatment 45/95 was two colloidal suspensions, 45% and 95%, of silica particles developed to form a discontinuous density gradient. Treatment 65/95 was two colloidal suspensions, 65% and 95%, while treatment 45/65/95 was three colloidal suspensions, 45%, 65 & and 95% of silica particles.
Figures in the same column with different superscript(s) are significant (P<0.01) by Chi-square or Fisher's Exact Test.

Table 4-10. Motility and sperm concentration of treated and control semen from different bulls before and after density gradient sperm separation using three colloidal suspensions (45%, 65%, 95%) of discontinuous gradients of silica particles

Bulls	Motility, % (Mean ± SD)			Sperm concentration, M (Mean± SD)		
	Pre-treatment	Control	Density gradient	Pre-treatment	Control	After
Bull A	12.0±3.2[a]	22.8±0.3 [a]	80.4±12.4 [a]	41.6±2.0[a]	37.4 ±4.6[a]	5.8 ±2.1[a]
Bull B	22.6±8.9[b]	26.8±3.0 [b]	80.4±12.4 [a]	43.3±2.3[a]	39.0±5.6 [a]	10.6 ±6.0[b]
Bull C	25.5±8.8[b]	27.4±1.1 [b]	87.8±8.3 [b]	41.8±0.9[a]	37.3 ±8.1[a]	8.0±4.5 [ab]

Figures in the same column with different superscript(s) are significant (P<0.01) by Least Significant Difference.

Table 4-11. Embryo development after *in vitro* fertilization with sperm from different bulls separated by three colloidal suspensions (45%, 65%, 95%) of discontinuous gradients of silica particles

Treatment	Nos. ova	Cleavage rate (Mean± SD)	Embryo dev. rate (Mean± SD) (per IVF ova)	Embryo dev. rate (Mean± SD) (per cleaved ova)
Treated (Sperm separated by density gradients of silica particles)				
Bull A	450	54.0±3.7 [a]	12.4±2.1 [a]	23.0 ±2.8 [aA]
Bull B	450	70.0±4.0 [b]	23.8±1.9 [b]	34.0 ±1.4 [aA]
Bull C	450	73.8±2.8 [b]	29.5±1.5 [c]	40.1±1.5 [aA]
Control (sperm not separated)				
Bull A	150	8.0±2.0[c]	1.3±2.3 [d]	22.2±8.7 [aB]
Bull B	150	45.5±5.6 [a]	16.0±2.0 [a]	36.4±7.6 [aC]
Bull C	150	34.0±4.0 [d]	12.7±2.3 [a]	37.3±8.7 [aC]

Figures in the same column with different superscript denoted by small letter are significantly different at P<0.01 by Least Significant Difference.
For equality of variances, Levene's Test showed significant difference (P<0.05) in the same column with different superscript denoted by upper case letters.

Table 4-12. Development rate of *in vitro* matured and fertilized oocytes in cumulus cells-co-culture system with fixed FBS dose or stage-dependent FBS dose in the presence or absence of pyruvate and lactate, mean±S.E.

Culture group	FBS	PL	*n*	% ≧2-Cells	% Morula at Day 5 /oocyte	% Morula at Day 5 /cleavage	% Blastocysts at Day 7 /oocyte	% Blastocysts at Day 7 /cleavage	% Blastocysts at Day 7 /morula	% Hatched at Day 10 /morula	% Hatched at Day 10 /blastocysts
Fixed dose of FBS											
1	10%	-	73	75.7±1.0^{a}	31.1±0.9^{ab}	41.2±1.3^{ab}	23.5±0.9^{ab}	31.0±1.2^{aB}	79.6±5.1^{ab}	59.2±6.3^{ab}	75.0±9.2^{a}
2		+	56	83.2±1.5^{a}	43.4±1.3^{a}	52.7±1.8^{ab}	35.7±1.1^{a}	43.5±1.7^{aB}	86.3 ± 5.0^{a}	73.8±5. 5^{a}	85.8±4.3^{bB}
Stage-dependent dose of FBS											
3	1 to	-	72	77.9±1.4^{a}	21.8±0.8^{bA}	29.0±1.1^{a}	14.7±0.7^{bA}	19.1±1.1^{aA}	71.7 ± 8.6^{b}	48.3±10.3^{bA}	60.0±13.8^{aA}
4	15%	+	66	81.5±1.7^{a}	42.6±1.2^{aB}	52.8±1.5^{b}	36.1±0.6^{aB}	44.6 ± 0.8^{B}	87.7±3.2^{a}	67.7±4.9^{aB}	76.7±5.1^{bB}

Abbreviations: PL stands for pyruvate and lactate; % ≥ 2- cell examined at Day 2 of IVC.
Stage-dependent FBS dose involves 1% FBS from Day 0 to 3 of IVC and 15% FBS from Day 3 to 7 of IVC.
^{a,b} Different at P<0.05; $^{A, B}$ Different at P<0.01 by Chi-square or Fisher's Exact Test.

Table 4-13. Full-term development of *in vitro*-derived vitrified water buffalo embryos produced in the improved culture system

Post-warming hatching rate, %	Claving rate, %	Full-term development, %		Ave. body weight of calves, kg±SD
		/recipient	/embryo	
87.5 (63/72)	23.1 (6/26)	26.9 (7/26)	13.5 (7/52)	37.0±1.4

Values in the parenthesis are x/n of each parameter.

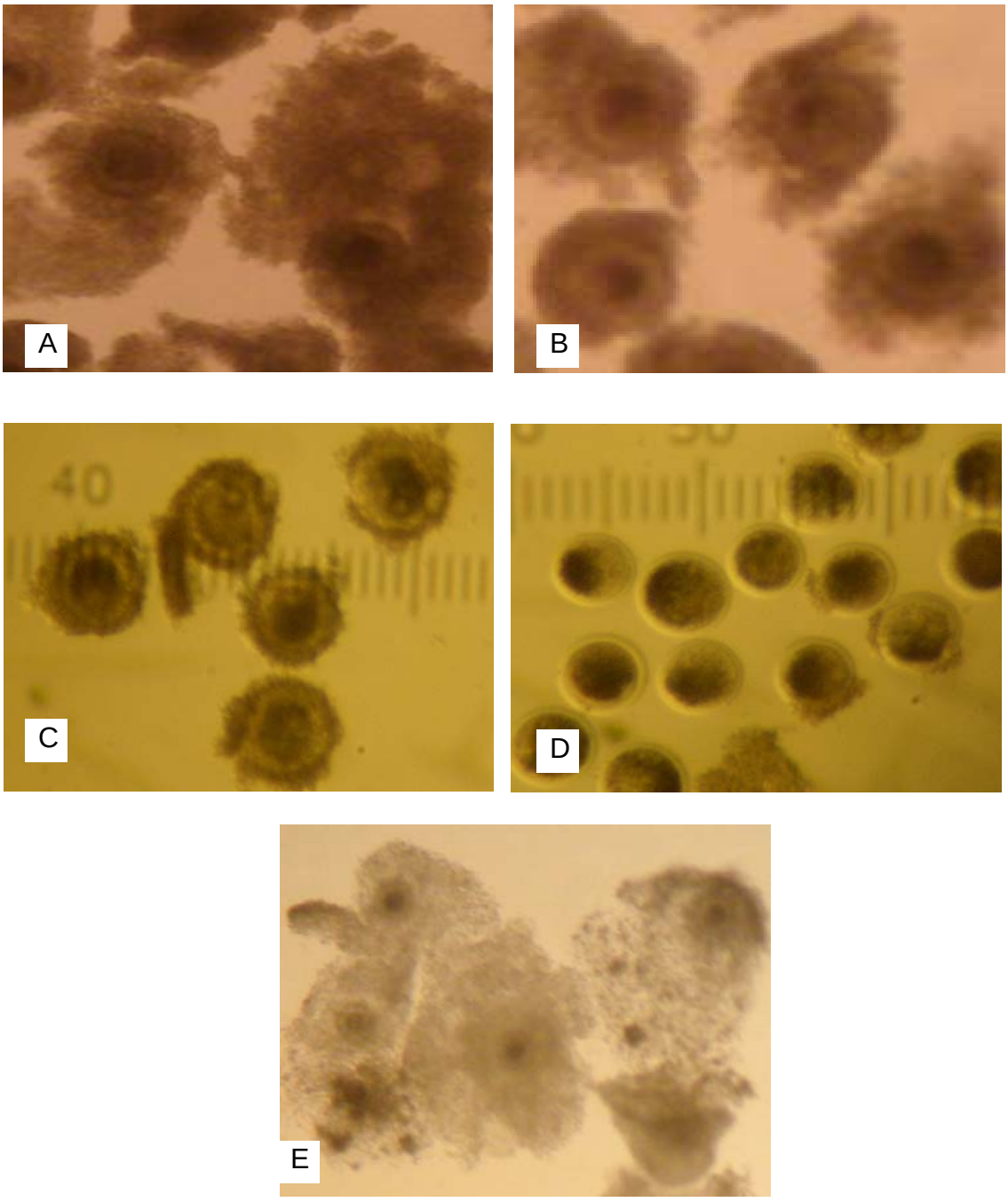

Figure 4-1. Quality of river buffalo oocytes classified based on the morphology of the surrounding cumulus cells. Grade A: surrounded by ≥5 layers of dense cumulus mass. Grade B: surrounded by 2-<5 layers of dense cumulus mass. Grade C: surrounded by <2 layers of irregular cumulus mass. Grade D: denuded or free from cumulus cells. Grade E: surrounded by expanded cumulus mass. Plates A to D at x40, E at x10.

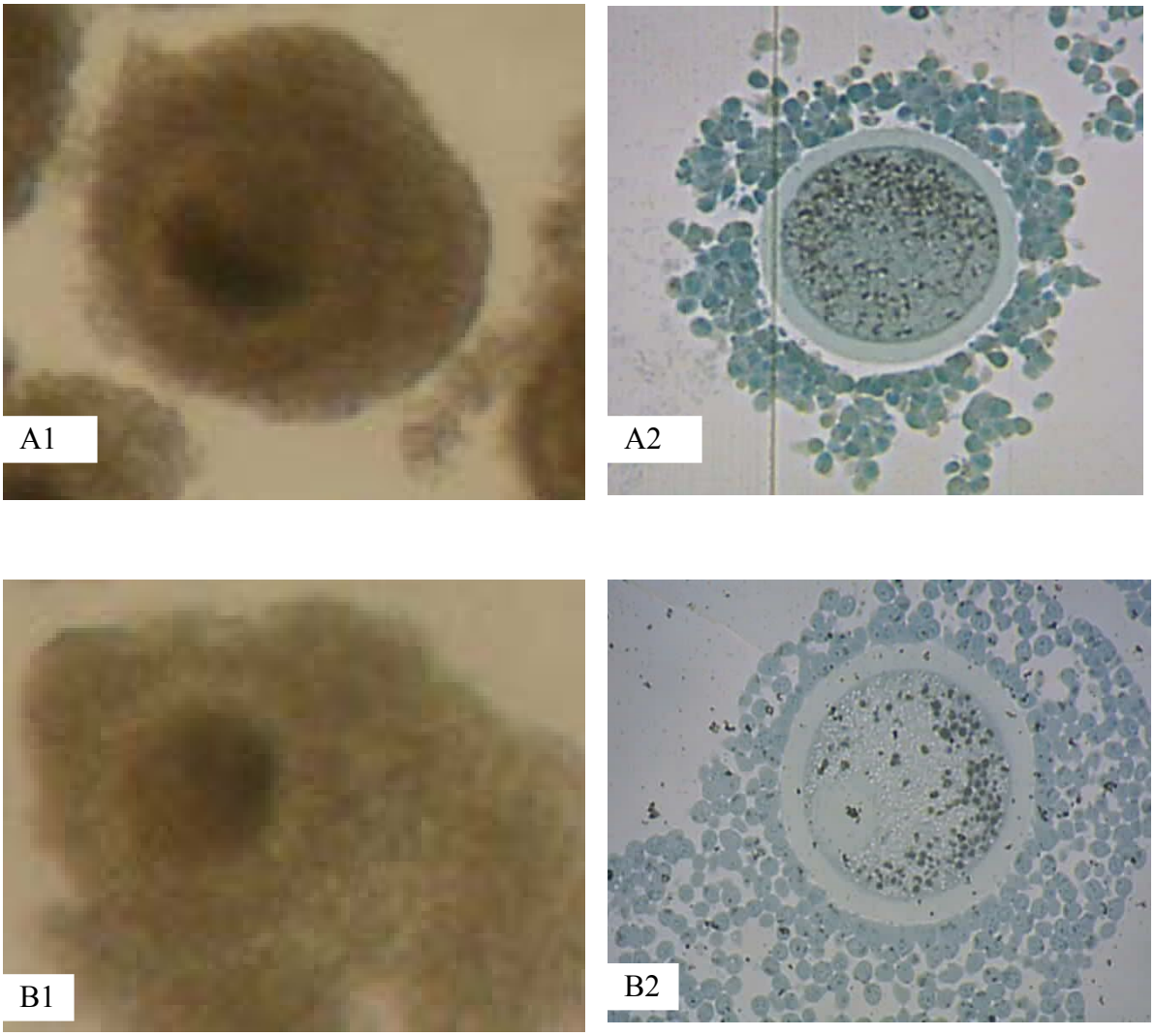

Figure 4-2. Classification of oocytes based on the compaction of the surrounding cumulus cells. A1: Oocyte classified as with compact cumulus cells, the cumulus cells surrounding the oocyte is closely attached, x40. A2: shows a thick section of an oocyte with compact cumulus, x400. B1: Oocyte with loosened cumulus cells, the cumulus surrounding the oocytes are slightly expanded, x40. B2: shows a thick section, x400. Thick sections were stained by 0.5% toluidine blue pH 7.5.

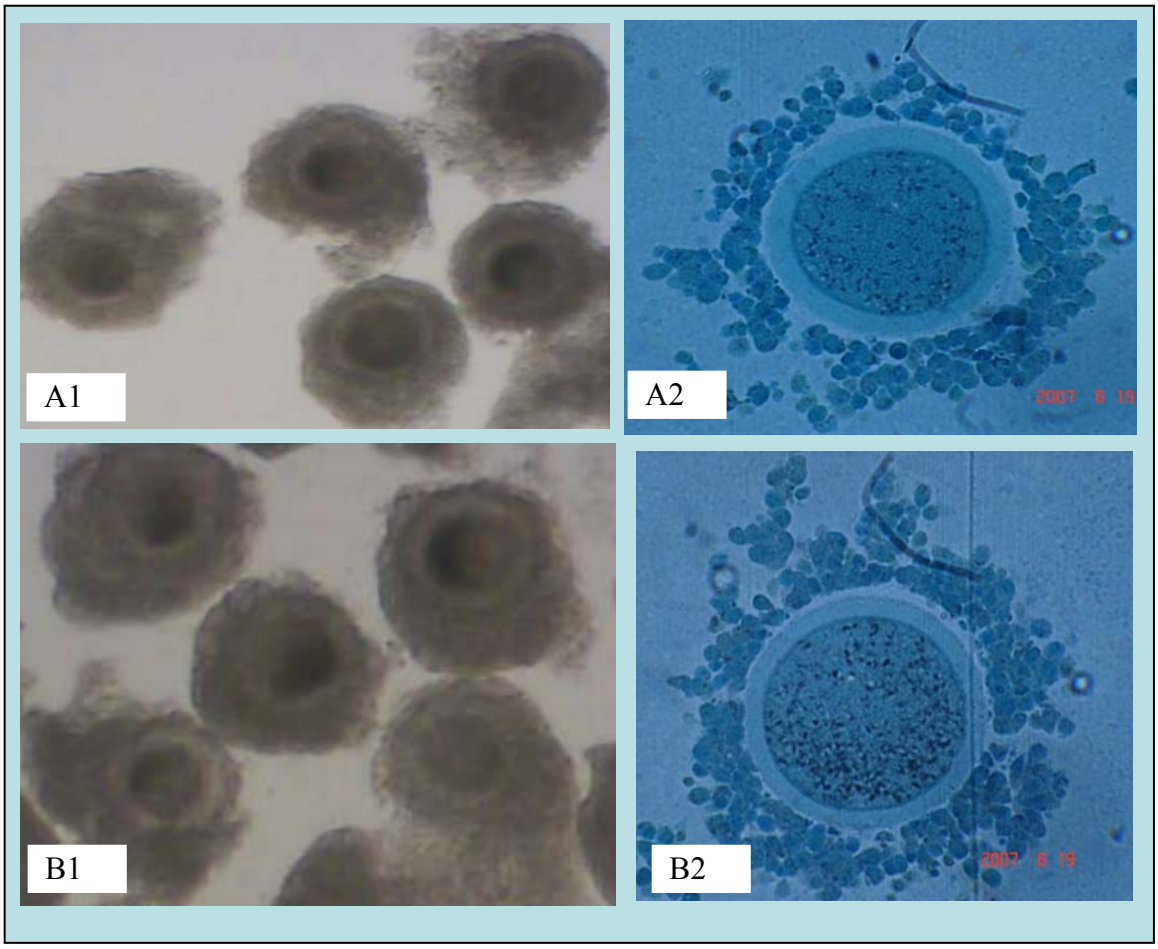

Figure 4-3. Classification of oocytes based on the granulation of the ooplasm.
A1: Oocytes classified as with homogeneous or evenly granulated ooplasm, the color of the ooplasm is even, x40. A2 shows a thick section of an oocyte with homogeneous ooplasm, x400. B1: Oocytes classified as with heterogeneous or unlevenly granulated ooplasm characterized by a darker potion on one side of the ooplasm in oocytes, x40. B2 shows a thick section of an oocyte with heterogeneous ooplasm, x400. Thick sections were stained by 0.5% toluidine blue pH 7.5.

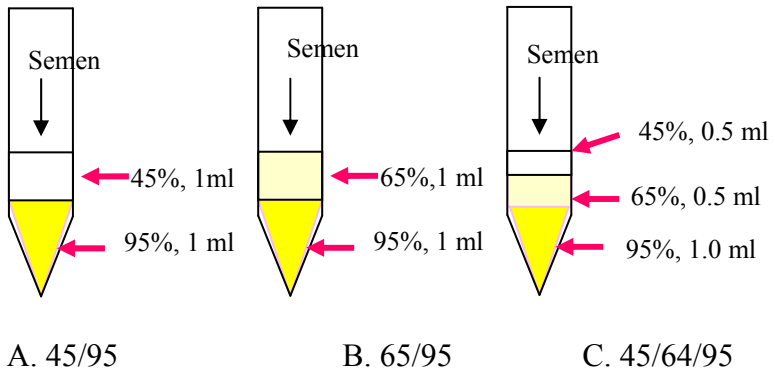

Figure 4-4. Make-up of discontinuous colloidal suspension of silica particle for discontinuous density gradient sperm separation

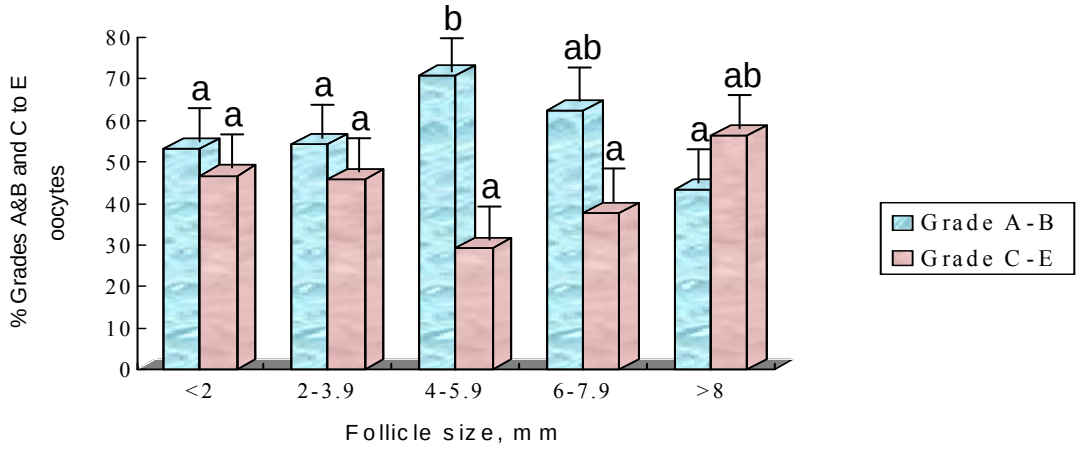

Figure 4-5. Percentage distribution of Grades A and B and Grades C to E COCs from different sizes of antral follicles. Bars with different letters are significantly different ($P<0.01$) by Chi-square or Fisher's Exact Test.

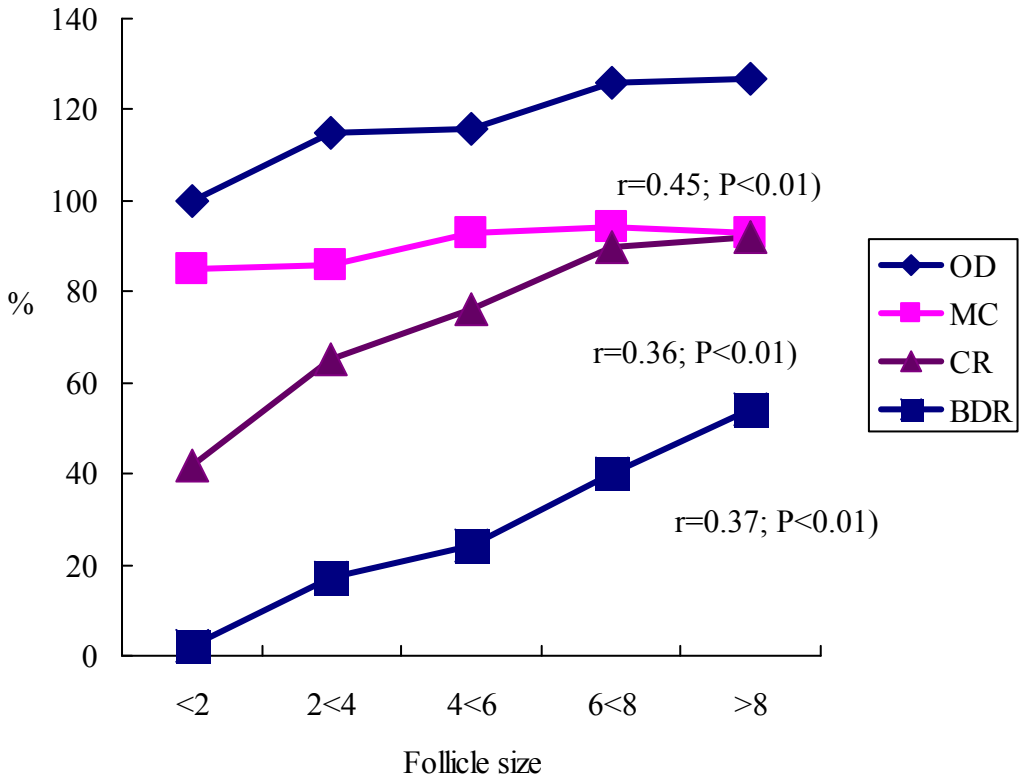

Figure 4-6. Relationship of oocyte diameter (OD), meiotic competence (MC), cleavage rate (CR), and blastocyst development rate (BDR) of oocytes in water buffalo with the sizes of antral follicles. Positive linear correlation was observed in all the parameters.

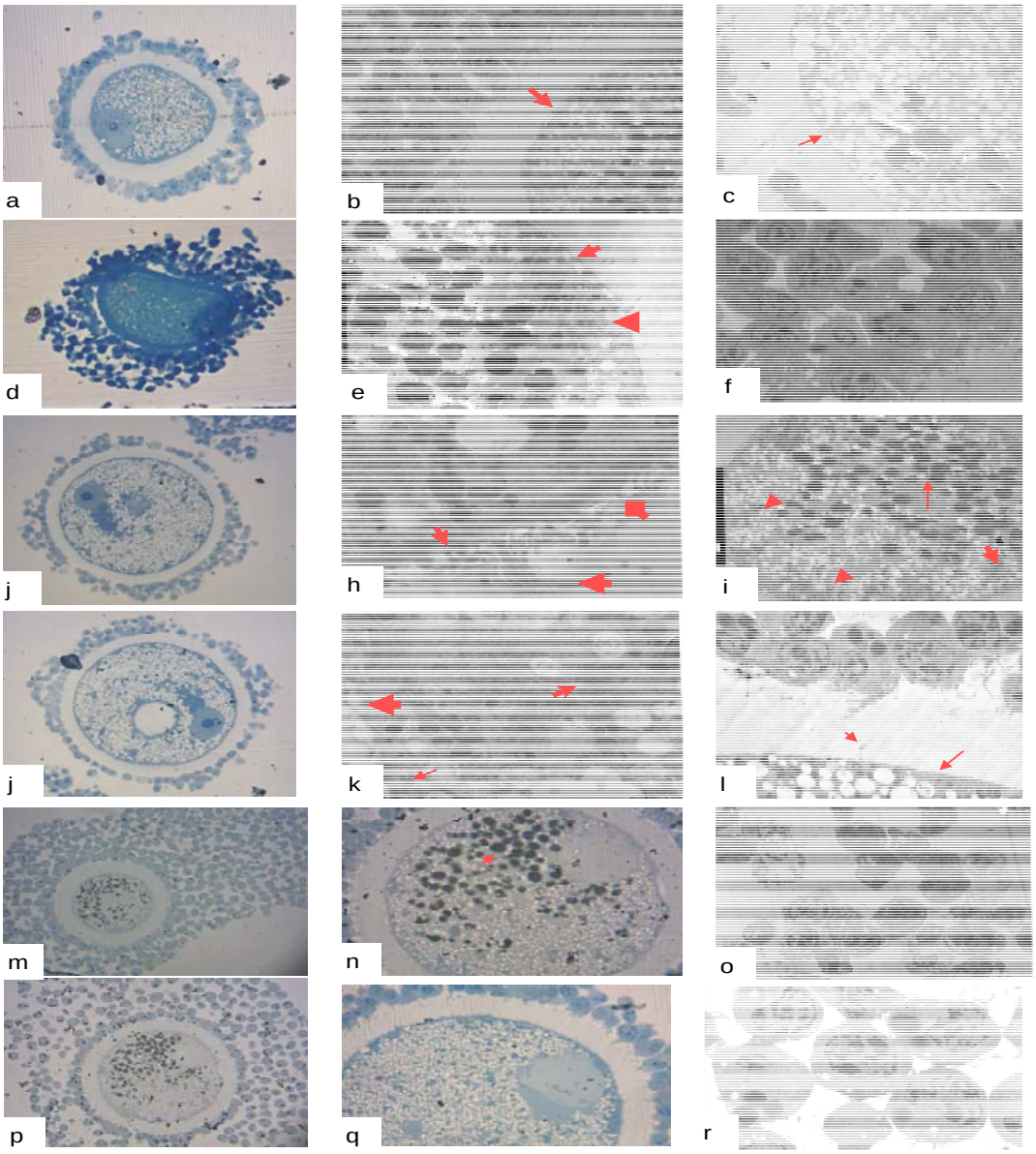

Figure 4-7. Thick and thin sections showing the morphology and ultrastructures of water buffalo oocytes classified by the size and granulation of the ooplasm, and the compactness of the surrounding cumulus cells. a, Oocytes with small ooplasm, x200. b, Structural feature of oocyte with small ooplasm, x1500, and c, big ooplasm, x1500. d, Oocyte with small ooplasm and is bean shape, with loose cumulus cells, x200. e, Clusters of mitochondria located at the periphery of the ooplasm and lipid droplets (dark material), x4000. f, Cumulus cells of circular and cuboidal in shape, x2500. j, Oocyte with big ooplasm, x200. h, Micro villi and cumulus cells processing endings between oolemma and zona pellucida, x12k. i, Arrangement of organelles in heterogeneous ooplasm oocyte, x3000. j, Oocyte with big ooplasm having large cavity on the ooplasm, x200. k, Ultrastructural feature showing the golgi bodies, cluster of mitochondria and other organelles, x10k. l, Smooth oolemma of big-ooplasm oocyte, x3000. m, Oocyte with compact cumulus cells, x100. n, Oocyte with heterogeneous ooplasm, x450. o, Ultrastructural feature of loose cumulus cells, x2000. p, Oocyte with loose cumulus cells, x200. q, Oocyte with homogeneous ooplasm, x450. r, Ultrastructure of compact cumulus cells, x5000.

Figure 4-8. Twin calves born out on *in vitro*-produced vitrified water buffalo embryos. The twins were from embryos derived from oocytes of slaughtered buffalo in India, matured and fertilized using sperm cells separated by density gradients of silica particles and cultured for embryo development in TCM 199 supplemented with 10% FBS, pyruvate and lactate in cumulus cells co-culture. The embryos were cryopreserved by ultra-rapid vitrification technique using open-poled straw with EFS40 as cryoprotectant, stored in liquid nitrogen and transported to the Philippines. The embryos were warmed in 0.5 M sucrose and transferred non-surgically to recipient river buffaloes at the herd of the Philippine Carabao Center.

Chapter V. General Discussion

5.1. Achievements of the Present Study for the Improvement of *in vitro* Culture Systems of Water Buffalo Oocytes and Embryos

The present study showed that extrapolating the systems developed on cattle resulted in sub-optimal success rate in water buffalo. However, by applying several modifications such as the separation of motile sperm for IVF, selection of high quality oocytes for IVM and improvement of culture medium suitable for buffalo oocytes and embryos, success rates can be enhanced to a similar level achieved in cattle. As such, it can demonstrate that the techniques could be an attractive tool for water buffalo genetic improvement.

Experiments conducted to determine the factors affecting the success rate showed that the duration of the ovary storage and the heterogeneousity of the oocytes used for IVM compromised the subsequent success of blastocyst development. Prolonged storage of the ovaries affects the developmental competence of the oocytes which could be due to apoptosis in follicular cells and degeneration of important growth initiating compounds as observed by Hussein (2005). This could be explained by the observations that follicles in postmortem ovaries degenerate overtime (Schroeder *et al*., 1991; Snow *et al*., 2001).

Similarly, the varying rate of progression to MII of buffalo oocytes manifested a heterogeneous population that IVF at one pre-determined time as described in cattle would result in inconsistent rate of blastocyst development due to varying meiotic competence of the oocytes. Re-selection of oocytes classified universally as Grades A and B for IVM of a closely related or homogeneous population of oocytes helped improve the blastocyst development as observed in Experiment 3-A.

Though previous studies also showed that compared with cattle, *in vitro* embryo production in water buffaloes has a lower rate of success (Totey *et al*., 1992; 1993a, b; Madan *et al*., 1994b; Kochlar *et al*., 2002; Neglia *et al*., 2003), the present experiments improved blastocyst development rate by implementing in the IVC systems the following interventions; re-selection of the universally classified A and B oocytes based on the compactness of the surrounding cumulus cells and employing desired length of IVM

(IVM of oocytes with compact cumulus for 24 to 26 h and 20 to 22 h for oocytes with loose cumulus), addition of hormones FSH and E_2 and EGF in the IVM medium (27.1% versus 17.5%), use of purely motile sperm cells separated by density gradients of silica particles for IVF (29.5% versus 12.7%), and addition of energy substrates pyruvate and lactate in IVC medium (36.1% versus 23.5%).

The blastocyst development rates after these modifications were comparable to cattle embryo IVP utilizing 5% CS with cumulus cell co-culture system (36.4%, Hamano and Kuwayama, 1993; 38.8%, Imai *et al*., 2002) and oocytes retrieved from the follicles at growth and the dominant phases (36.7% and 27.8%, Machatkova *et al*., 2004).

These results showed that improving the IVC systems in water buffaloes could lead to a success rate comparable to cattle. Apparently, the requirements for maturation, fertilization and development in these species are similar in components but different in timing and concentration in the culture medium. This could be supported by the findings of Neglia *et al*. (2003) indicating higher development rate in cattle than in buffalo after direct comparative study following the culture system developed in cattle.

Similarly, the viability of the embryos produced out of IVC systems incorporating the interventions mentioned above have led to 34.6 percent (9/26) pregnancy rate, 23.1 percent (6/26) calving rate, and 13.5 percent (7/52) fullterm development presenting a modest improvement of 12.0 percent, 7.0 percent, and 5.4 percent for pregnancy, calving rate and fullterm development, respectively compared to the 22.6 percent (7/31), 16.1 percent (5/31), and 8.1 percent (5/62) obtained in Experiment 1-A, respectively.

The obtained pregnancy rate was higher than *in vitro*-derived-vitrified bovine embryos transferred to unselected recipient animals (27%, n=11, Agca *et al*., 1998). But this was lower when the embryos were selected prior to ET (60.0%, n=20) and after bilateral transfer (63%, n=16) (Agca *et al*., 1998). It was comparable to *in vitro*-derived bovine embryos cryopreserved by slow-freezing technique (37.3%, n=3952, Aoki *et al*., 2004; 41%, n=17, Agca *et al*., 1998). These suggest that the viability of the *in vitro*-derived embryos by the present study has achieved some degree of comparability with that of cattle. Calving and fullterm development rates however, were lower; 67 percent (8/16) and 25 percent (8/32) for vitrified embryos and 77 percent (7/17) and 20.5 percent

(7/34) for conventionally frozen embryos (Agca *et al*., 1998). This implies further research to improve the current success rate.

5.2. Unsettled Factors for Further Improvements of *in vitro* Production Systems of Water Buffalo Embryos

The success rate obtained on embryo IVP in the current experiments was comparable to that obtained in cattle following closely the same culture system. However, fullterm development was low which could be due to embryo quality or problems associated with the recipient animals, i.e., estrus synchronization. Nevertheless, the IVP system has achieved a level of improvement. It was noted however, that cellblock was still a major problem as shown by the failure of the 40 to 50 percent cleaved zygotes to develop to the blastocysts. This problem was also observed by other authors in water buffalo (Abdoon *et al*., 2001; Gasparrini, 2002; Gupta *et al*., 2002; Kumar *et al*., 2004; Purohit *et al*, 2005; Gasparrini *et al*., 2008), in cattle (Avery *et al*., 2003; Krisher, 2004; Nagano *et al*., 2006), sheep (Guler *et al*., 2000), and in pig (Medvedev *et al*., 2004). In fact, it was considered as the major problem in IVP system. In spite of numerous variations of the basic techniques, blastocysts production *in vitro* was only 15 to 45 percent of inseminated oocytes. Factors that caused the failure of the zygotes to further develop are subject of further research. Though studies on the differences in source of oocytes, sperm preparation techniques, oocytes maturation and fertilization conditions, and embryo culture environments as well as other factors that have major effects on embryo development rates, yield and quality have been carried out, much is still unknown.

In mouse, developmental block in embryos was indicated to be caused by three to four times higher oxygen concentration in *in vitro* conditions than *in vivo.* This was due to the production of a variety of reactive oxygen species: superoxide radicals (O_2), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (Ozawa *et al*., 2002). The increased oxidative stress appears to be a major factor impairing *in vitro* mammalian embryo development.

Several studies indicated that the rise in embryonic hydrogen peroxide levels attributable to *in vitro* culture may be a cause of species-specific blocks to embryo

development (Legge and Sellens, 1991; Takahashi *et al*., 1993). In most cells, the effect of oxidative stress can be attenuated by efficient antioxidant systems such as catalase or superoxide dismutase as well as thiol compounds acting as metabolic buffers which scavenge reactive oxygen species (Del Corso *et al*., 1994). In water buffalo, Gasparrini *et al*. (2000) examined the effect of cysteamine in IVM medium and found out that this thiol compound did not improve IVM rate but improved the embryo development rate. The best-known example of metabolic regulation is the use of ethylenediamine tetraacetic acid (EDTA), a non-selective chelator of divalent cations during embryo development. EDTA manipulates the metabolic profiles of embryos by sequestering the toxic effects of contaminating heavy metal cations and depressing glycolytic rates during early embryonic development leading to improved development (Thompson 2000). Use of EDTA in water buffalo IVEP is yet to be explored.

The insufficiency of the culture medium during IVM and IVC could also be a factor that contributed to the block of development. In other species, this was overcome by simple alterations in energy substrates and other media constituents (Chatot *et al*., 1989) and *in vivo* culture of the fertilized oocytes in ligated rabbit (Totey *et al*., 1992) or sheep (Galli *et al*., 1998) oviduct. It was indicated that embryonic development depends on the proteins and mRNA stored within the matured oocytes. Once they are depleted, and the IVC conditions are not appropriate, embryonic development is arrested (Bagis *et al*., 2003). Therefore, the quality of the oocytes and the IVC environment dictates the developmental fate of the oocytes.

Results of the present study showed that re-selection of Grades A and B oocytes, the presence of hormone (FSH and E_2) and EGF in the IVM medium, and addition of energy substrates (pyruvate and lactate) in IVC medium were beneficial in the enhancement of blastocyst development of water buffalo. This demonstrated the uniformity of the oocytes and the components of the IVM and IVC medium as important in producing a developmentally competent oocyte and sustaining development to blastocyst.

It appeared that though the oocytes are classified as Grades A and B based on multi-layers of surrounding cumulus cells, the duration of IVM differed due to varying developmental speed which could be identified by the nature of the compactness of the

surrounding cumulus cells (compact or loosened) and the diameter of the ooplasm. Oocytes with compact cumulus and <100 μm ooplasm requires longer IVM period while those with loose cumulus and >120 μm ooplasm required shorter IVM period. The metabolic requirement of these oocytes, however, was not examined in the present experiment; hence, this is worth exploring in future investigations.

The addition of hormones and EGF in IVM medium showed that the interaction of gonadotrophins, steroid hormones and growth factors enhanced the development of the oocyte to MII. This may happen by altering the meiotic stage as described by Downs (1989). Improved blastocyst yield and quality were achieved due to stimulating of the pattern of protein neo-synthesized as indicated in bovine oocytes (Lonergan *et al*., 1996), accelerating the meiotic cell cycle possibly by increased H1 and MAP kinase activities as observed during the early stage of *in vivo* maturation in bovine (Sakaguchi *et al*., 2002), and regulating the paracrine and autocrine functions in the oocytes or initiated and activated signaling pathways necessary for acquisition of full developmental competence. This could have resulted in significantly higher embryo development rate and improved quality of the resultant embryos.

The presence of 10% FBS alone in TCM 199-base IVM medium may not be sufficient to support the maturation requirements in some of the oocytes resulting in lower cleavage and blastocyst development. However, the presence of the cumulus cells was found beneficial. The nature of products secreted by the somatic cells and their specific roles in embryonic development merit further investigations. Moreover, the culture medium used to culture the oocytes lacked some hormones (i.e. LH) and other growth factors (i.e. insulin) present in the *in vivo* system during follicular growth and development. This also merits further investigation.

Similarly, the IVC system to support the development of the embryos was not clearly defined in the present study. Though addition of pyruvate and lactate clearly demonstrated a beneficial effect on blastocyst development, the concentration and timing of administration were not investigated. The addition of glucose in the IVC medium merits a careful experimentation. Moreover, the beneficial effects of the increasing concentration of FBS in IVC medium (i.e. incidence of smaller size pre-implantation stage embryos) were not documented. Hence, this is another subject of future concerns.

On the other hand, chromosome abnormalities were found as major factor causing cellblock. Studies in cattle (Hare *et al*., 1980; Murray *et al*., 1985; Viuff *et al*., 2000, 2001) showed that chromosome abnormalities occur for up to 56 percent in the earlier stage embryos. Viuff *et al*. (2000) observed 22, 15, 16, and 42 percent mixoploidy in cattle embryos at Days 2, 3, 4, and 5 post inseminations, respectively.

In murine models, Santalo *et al*. (1992) found out a positive correlation between embryo IVP techniques and the incidence of different chromosomal abnormalities. They found out that aneuploidy was influenced by maternal age while polyspermy showed a positive correlation with IVF, immaturity and over-maturity of the oocytes employed and use of prepubertal females. On the other hand, Bongso *et al*. (1988) indicated that aneuploidy might have been brought about by errors in oogenesis (anaphase lagging or non-disjunction). This may offer one explanation for fertilization failure and overall low pregnancy rates after IVF. Though these issues were addressed in the present experiments, the incidence of the chromosome anomalies after each interventions were not examined; hence, it is worth addressing in future observations.

5.3. Potential Application of the Present Findings to Ovum Pick Up and *in vitro* Production (OPU-IVP) System

Blastocyst development rate was improved significantly when oocytes used for IVM were re-selected based on the compactness of the surrounding cumulus cells and cultured for maturation in a desired IVM period. Although homogeneous population of oocytes was selected based on the surrounding cumulus cells, size of the donor follicle (Experiment 3-A) and the donor animal per se affected the developmental competence of the oocyte. Consequently, though the success rate of IVP of buffalo embryos reached the level achieved in cattle (Experiment 3-C), the ultimate efficiency from each ovary would be very low since buffalo ovary contains less number of follicles (Das *et al*., 1996; Kumar *et al*., 1997: Neglia *et al*., 2003). Number of follicles greater than $\geq$6mm in diameter per ovary in the buffalo is only about 0.5 in this study and 0.17 to 0.82 in other reports (Madan *et al*, 1996, Kumar *et al*, 1997). Hence, the results of the present

experiments could be of practical application in OPU-IVP system since follicles and donor animals could be screened.

Cleavage and blastocyst development was observed higher in OPU-derived oocytes than in oocytes from slaughterhouse-derived ovaries (Neglia *et al*., 2003). This means that the use of OPU-derived oocytes may also help minimize the variations caused by the oocyte population in conducting studies to improve the IVC systems.

In Experiment 3-A, the size of follicles was found to be linearly correlated with developmental competence of the oocytes. Since only Grades A and B oocytes from the different sizes of follicles were considered for IVM-IVF-IVC, the data showed that oocytes from <4mm follicles lacked developmental competence while oocytes from >4mm follicles showed higher developmental competence. Oocytes from ≥8 mm follicular group produced blastocysts *in vitro* at substantially greater rates than those from 2 to <8 mm follicles. Smaller follicles (<2 mm) yielded oocytes capable of fertilization but failed to cleave beyond the 8-cell stage while some oocytes from 2 to <4 mm follicular group acquired an intrinsic capacity to develop into an embryo but the proportion of competent oocytes was only 17.4 percent.

Since <2 mm follicles had the highest <100 μm oocytes, the failure to undergo cleavage could be supported by the results in the earlier study which indicated that oocytes with <100 μm lacked developmental competence. This means the oocytes from small follicles require an additional prematuration time to express their *in vitro* developmental competence.

Since maternal mRNA drives early stages of embryonic development until the embryonic genome takes over (Latham, 1999), oocytes from <4 mm follicles may have insufficient storage of maternal mRNA and proteins within the oocyte that led to developmental failure. Blondin *et al*. (1997) indicated that under normal conditions, oocytes acquire their developmental competence late in the follicular phase of the estrous cycle. Hence, the high blastocyst development potential in oocytes derived from follicles ≥8 mm may be due to the longer exposure of the oocytes within the follicles enabling them to accumulate and store proteins or stable mRNAs (Fair *et al*., 1996; Blondin and Sirard, 1995) necessary for development.

It is also possible that they have undergone capacitation described as the structural modifications that take place in the oocytes of dominant follicles before LH peak (Hyttel *et al*., 1997) necessary to achieve the oocytes' full development potential (Lonergan *et al*., 1994). The oocytes from the smaller follicular groups might have been depleted with this opportunity. If the percentage of morphologically healthy oocyte did not change over time during the follicular wave (Abdoon *et al*., 2001; Vassena *et al*., 2003), this observation has practical implication in an OPU–IVP system in water buffalo by applying a perfect timing of oocytes retrieval from the follicles.

Since follicles 4 to 5.9 mm contained highest (71%) good quality (Grades A and B) oocytes while <4 mm and ≥8 mm follicles contained poorer quality (Grades C, D and E) oocytes, retrieval of oocytes from 4 to 8 mm follicles for IVM in an OPU-IVP system would mean higher success rate of embryo IVP. This would also allow optimization of the reproduction potential of the donor animal since follicles <4 mm will be left to continue its growth and be used for the next OPU session.

This approach may have a potential impact not only in optimizing the embryo IVP success but also in managing time, resources, and welfare of the donor animal. It is therefore, important to establish the systems of ultrasound image characteristics of ovarian follicular status and follicular wave characteristics and schedule of each donor animal. This is useful in the retrieval of developmentally competent oocytes in optimizing production of embryos and the reproduction potential of preferred donors.

5.4. Challenges in the Field of Embryo Transfer in Water buffalo

The technology of ET was found effective in propagating river buffaloes with swamp buffaloes as recipient animals as shown by the birth of river calves both in the institutional and in the farmer/field level. Of the 16 calves born out of *in vitro*-derived-vitrified embryos, all males are now being used as donors of semen for nationwide artificial insemination programs and the females are used for breeding and eventually as dairy animals. Three of the first batch of females produced by this technology has delivered their own calves with an average age of 18 months at 1st calving and average calves of 2 heads within 6 years of age.

These results demonstrated that the technology is effective and this breakthrough guarantees the possibility of water buffalo genetic improvement in swamp buffalo dominated countries by international transport of embryos from one country to another. This can facilitate production of superior animals for milk and meat purposes and generate employment and increase income of rural farming communities, overcome malnutrition problems and address food security.

However, though improved success rates were achieved, the efficiency of ET in water buffalo is considerably low compared to cattle as discussed earlier. One major problem was observed in the use of synchronized recipients as shown by the tendency of higher success rate in recipients that received embryos following the natural estrus compared to recipients with synchronized estrus (Table 2-7 and 2-10). This resulted in a recipient-related factor that affected the accuracy of assessing the viability by fullterm development of the resultant *in vitro*-derived-vitrified embryos.

The embryo-recipient asynchrony was indicated as one major factor that contributed to low pregnancy rate in water buffalo (Misra and Joshi, 1991; Totey *et al*., 1996; Kajihara *et al*., 1992). In cattle, asynchrony was also recognized as one of the factors affecting the success of ET (Hasler *et al*., 1987; Albihn *et al*., 1991).

In the present study, a 16.1 and 40.0 percent calving rate was achieved in river and swamp buffalo recipients in natural estrus while only 4.2 and 5.7 percent in recipients in synchronized estrus. These results can be attributed to the variations in the individual response of animals to the synchronization agent, fixed-time ET, difficulty in discerning the actual standing estrus in water buffalo, and lack of concrete synchronization protocol that suits the physiology of water buffalo. Studies on estrus synchronization in water buffaloes have been limited. Protocols involving treatments developed in cattle and applied in buffaloes (Cruz *et al*., 1988) have been used. The synchronization scheme in water buffaloes requires intensive experimentation to develop a concrete protocol.

In the ET trials made in the present experiment, occurrence of estrus was monitored as basis for ET among synchronized recipients but lack of estrus manifestation meant fixed-time ET after the second injection of $PGF_{2\alpha}$. The failure to observe estrus occurrence might have resulted in the failure of perfect timing of ET.

Under village conditions, this factor was further enhanced by the less pronounce signs of estrus inherent in water buffaloes, making it difficult to perfectly time the transfer of embryo. The same problem was reported for artificial insemination (Seren *et al*., 1995; Ohashi, 1994) due to the relatively low expression of estrus behavior, variable duration of estrus from 4 to 64 h, and difficulty in predicting the time of ovulation (Baruselli, 2001).

Kasiraj *et al*. (1993) indicated that buffalo embryos produced *in vivo* and cryopreserved by slow freezing technique could only tolerate asynchrony of ±12 h which was confirmed by Misra *et al*. (1999b). They indicated that asynchrony beyond 12 h in water buffalo resulted conception failure. In cattle, asynchrony of ±24 h (Spell *et al*., 2001) to as long as ±36 h (Hasler *et al*., 1995) did not affect pregnancy rate.

The timing of ET after estrus synchronization may have affected and contributed to the inability to impregnate the estrus synchronized recipient animals. Estrus observation was conducted by visual observation from the herd and under village condition by the owner. However, the weak signs of estrus in water buffaloes proved to be a limiting factor to accurately determine the perfect timing for ET.

Another factor can be due to cases of short luteal phase among estrus-synchronized recipients as explained by the hypotheses on precocious luteolysis (Beal *et al*., 1980; Behrman *et al*., 1976; Henderson and McNatty, 1975; Ramirez-Godinez *et al*., 1981). Based on these results, there is a need to improve synchronization treatment and the determination of estrus in estrus-synchronized buffalo to achieve a perfect timing of ET. For better success rate, natural estrus buffaloes are best recipients for ET.

On the other hand, one case of stillbirth occurred in the use of swamp buffalo as recipient of river buffalo embryo. The case was due to failure of the farmer to summon technical assistance during parturition of the recipient animal. Close coordination between farmers and veterinarian is important in the implementation of ET program especially during the parturition of the recipient animal. The dead river buffalo calf was delivered out from the swamp buffalo surrogate by fetotomy without vaginal slicing.

This case demonstrated the problems associated with field conditions suggesting precautionary measures in using swamp buffaloes as recipients of river-type buffalo embryos. Jainudeen (1986) indicated that dystocia is higher in river than in swamp

buffaloes; however, with a river buffalo calf carried by a swamp buffalo, the problem of dystocia is inevitable. Furthermore, calves from the transfer of *in vitro*-derived embryos were reported to display cases of large calf syndrome (McEvoy *et al*., 2003) even if this problem was not observed in the present study. Improved management systems can help minimize stillbirths in swamp recipient/river embryo cases (Nanda *et al*., 2003).

5.5. General Conclusion and Recommendations

Based on the above studies and findings, it can be concluded that the techniques of embryo IVP developed in cattle yielded promising results and can be applied to water buffaloes. However, the success rate was sub-optimal suggesting a need to modify the culture systems to suit the water buffalo requirements. The speed of development of water buffalo embryos is a day faster than the cattle embryos; hence, extrapolating the systems in cattle may compromise the results in water buffaloes. Therefore, it is necessary to establish concrete biological requirements for IVC of the water buffalo oocytes and embryos.

The duration of ovary storage affects the developmental competence of the buffalo oocytes. Processing the donor ovaries stored in physiological saline at 30 to 33ºC should not be beyond 4 h for optimal development of the retrieved oocytes. Selection of homogeneous populations of oocytes for IVM is important in order to optimize the blastocyst development by implementing the desired culture system for each oocyte category.

The BCB test was effective for selecting homogeneous populations of oocytes and is recommended for use in selecting the developmentally competent oocytes for IVM. Follicle size ≥6 mm in diameter contained most of the developmentally competent oocytes; so, oocytes should be collected from these follicles to improve the efficiency of OPU-IVP system.

For the IVM of water buffalo oocytes, addition of hormones (FSH and E_2) and EGF in the IVM medium is necessary to improve the rate of blastocyst development and the quality of the resultant embryos. Success of IVF can be improved by the use of purely

motile sperm cells separated by density gradients of silica particles, and the reproduction potential of bulls with low sperm motility can also be optimized by this technique.

For the IVC for embryo development, supplementation of energy substrates in the IVC medium is important. Pyruvate and lactate are effective for enhancing blastocyst development rate. The resultant embryos following these techniques surpassed the stress brought about by cryopreservation procedures. The two-step ultra-rapid vitrification technique using open-poled straw with 10% ethylene glycol as pre-equilibration medium and EFS40 as cryoprotectant at an exposure time of 0.5 min was effective in the cryopreservation of water buffalo preimplantation stage embryos.

The *in vitro*-produced-vitrified water buffalo embryos are viable and swamp buffaloes were efficient as recipients and surrogate mothers of river embryos in spite of chromosome differences. Therefore, following the above interventions for IVC of water buffalo oocytes and embryos, the technology of IVP-vitrification-ET can become a potential tool for water buffalo genetic improvement, and for the propagation of river buffaloes in swamp buffalo dominated countries. However, more studies are recommended to further improve the current success rate and make the technology an efficient and effective tool for water buffalo breeding.

SUMMARY

A series of experiments was conducted to develop and improve the methods and efficiency of the techniques for *in vitro* production (IVP) and cryopreservation of water buffalo embryos and application of these techniques were examined for the production efficiency of viable embryos *in vitro*.

Experiment 1, divided into three sub-experiments, dealt on establishment of systems for water buffalo embryo IVP following systems develop on cattle, cryopreservation by vitrification, and tests of the viability of the *in vitro*-derived-vitrified embryos after embryo transfer (ET). In Experiment 1-A, oocytes surrounded by ≥3 multilayer of cumulus cells with granulated ooplasm (Grades A and B) were collected from abattoir-derived swamp buffalo ovaries. These were matured, fertilized, and cultured for development *in vitro* in tissue culture medium (TCM) 199 supplemented with 10% fetal bovine serum (FBS) and protected with antibiotics and co-cultured with cumulus cells for embryo development. *In vitro* fertilization (IVF) was carried out with $1x10^6$ sperm cells/mL, 5 mM caffeine, 2 units heparin/mL and 5 mg bovine serum albumin (BSA)/mL with Brackett and Oliphant (BO) as basal medium. Cleavage rate was 53.5 percent, and blastocyst development was 11.4 percent from oocytes and 21.4 percent from cleaved zygotes. The results suggested that the techniques developed for cattle may not be optimal for water buffalo. Therefore, studies were required to improve the IVC systems for efficient IVP of embryos in water buffalo. In Experiment 1-B, *in vitro*-derived embryos were subjected to cryopreservation following vitrification techniques. The in-straw vitrification method was examined using EFS40 (40% v/v ethylene glycol, 18% w/v ficoll, 0.3 M sucrose) as a cryoprotectant. The effect of exposure time (0.5 min or 1 min) and embryo stage (early blastocysts, mid blastocysts, expanded blastocysts) on post-warming hatching rate were assessed. The results showed that the optimum exposure time for embryos in EFS40 was 0.5 min with a post-warming hatching rate of 23.5 percent (8/34). Survival rate was low, affected by the developmental stage of embryo, and fracture damage was high. So, to avoid fracture damage, an ultra rapid vitrification technique with an open-poled straw was tried. The open-poled straw was prepared by removing the straw plug of a 0.25 mL French straw and shaping the opposite end with a

cutter to make an open-end where the embryo was loaded. Pre-implantation *in vitro*-produced embryos were pre-equilibrated with 10% ethylene glycol, exposed to EFS40 while loading in the open-poled straw within 0.5 min and immediately plunged in liquid nitrogen. Results showed a successful vitrification with a post-warming hatching rate of 82 to 88 percent for morula to blastocyst stage embryos indicating that the technique was effective for cryopreservation of preimplantation stage embryos. In Experiment 1-C, river buffalo embryos were produced and vitrified as described above and were transferred non-surgically in twos or threes to river and swamp buffalo recipients. The use of swamp buffaloes as recipients was done under field conditions. Results showed 6.3 percent (6/95) full-term development (10.9% calving rate, 6/55) in river buffalo recipients and 5.0 percent (4/80) full-term development (10.0% calving rate, 4/40) in swamp buffalo recipients. These results demonstrated viability of the resultant *in vitro*-derived-vitrified embryos and suggests that this technique is a promising tool for water buffalo genetic improvement particularly in propagating river buffaloes utilizing swamp buffaloes as surrogate mothers. However, the success rate was sub-optimal suggesting a need to improve the current embryo IVP system.

In Experiment 2, factors affecting blastocyst development were examined. Experiment 2-A assessed the effect the length of ovary storage. Ovaries were divided into two groups. Group 1 was stored for 3 to 4 h and Group 2 for 5 to 6 h upon retrieval from donor cow to oocyte aspiration. Collected Grades A and B oocytes from each group of ovaries were matured and fertilized *in vitro* and cleavage and blastocyst development were assessed. Results showed higher cleavage (58.6% versus 46.8%) and blastocyst development (21.4% versus 12.9%) in 3 to 4 h group than in 5 to 6 h group suggesting that duration of ovary storage affects the success of embryo IVP. In Experiment 2-B, the effect of the length of oocytes IVM was examined. Grades A and B oocytes were divided into five groups and cultured for IVM for 16, 19, 22, 25 and 28 h. In each time points, the oocytes were denuded from cumulus, fixed and stained to examine the nuclear maturation. Results showed varying nuclear status among oocytes with metaphase II (MII) observed at 16 h (15.0%) increased with the duration of IVM and reached peak (85.0%) at 25 h but decreased at 28 h. This shows heterogeneous population of oocytes suggesting a need to develop selection parameters to identify a homogeneous population of oocyte to optimize

developmental potentials. In Experiment 2-C, the effect of the culture condition during IVM was evaluated. Immature oocytes were divided into four groups and matured in IVM medium containing 10% FBS supplemented with either 1) hormones (0.02 units follicle stimulating hormone (FSH)/mL and 1 μg E_2/mL, 2) epidermal growth factor (EGF, 10 ng/mL), 3) a combination of hormones and growth factor, or 4) FBS alone. Meiotic competence after IVM and cleavage, blastocyst development and quality of embryos by the number of blastomeres were assessed after IVF. The results showed that addition of a combination of hormones and EGF to oocyte IVM medium significantly improved meiotic competence (81.9% MII) compared to the control group (65.0% MII). Furthermore, blastocyst development rate (27.1%) was significantly ($P<0.05$) improved compared to the control group (17.5%) suggesting that the nature of the IVM medium affects blastocyst yield. In Experiment 2-D, incidence of chromosomal anomalies among early stage embryos were carried out to determine factors causing development block to blastocyst. Early stage embryos (2 to 8 cells stage) derived from the best IVM medium in Experiment 2-C were treated with vinblastin sulfate, fixed with aceto-ethanol, stained with 2 % Giemsa and the incidence of chromosome anomalies examined. Results revealed high (47.7%) incidence of chromosome anomalies indicating this as major factor affecting blastocyst development of water buffaloes *in vitro*.

Based on the above findings, Experiment 3 was conducted to improve the culture systems for efficient production of viable embryos. Experiment 3-A was designed to establish selection parameters of developmentally competent and homogeneous population of oocytes. Oocytes graded as A and B were re-classified based on the density and compactness of the surrounding cumulus cells (compact versus loose), granulation (even versus uneven) and size of the ooplasm, and the size of the donor antral follicle. Brilliant cresyl blue staining technique was used to confirm heterogeneousity of the oocyte population and transmission electron microscopy (TEM) was used to describe ultrastructural differences. Results showed that density and compactness of the surrounding cumulus, diameter of the oocyte and the size of the donor follicles were important indicators of oocyte developmental competence. Oocytes with a compact cumulus required a longer (24 to 26 h) period of IVM and those with loose cumulus required a shorter (20 to 22 h) period of IVM for optimum blastocyst development.

Oocytes with a diameter of <100 μm lacked developmental competence evident by the failure to develop to MII after IVM while oocytes with a diameter ≥100 μm developed to MII and cleaved after IVF. Optimum cleavage (96.8%) and blastocyst development (27.0%) was observed in oocytes with ≥120 μm. The size of the donor follicle was linearly correlated with oocyte developmental competence with follicles ≥ 6 mm containing highly developmentally competent oocytes. BCB test showed a significantly higher BCB negative in oocytes with compact cumulus cells (39.1%) compared to the oocytes with loose cumuli (8.6%) indicating that some oocytes with very compact cumulus cells were still at the growing phase of development while most of the oocytes with loose cumulus were at the development phase. Transmission electron microscopy revealed structural differences in the nature of the cumulus cells, gap junctions and arrangement of organelles between oocyte classifications. Experiment 3-B studied the efficiency of density gradient sperm separation using silica particles in separating motile sperm cells to provide the IVF environment with good quality sperm cells and develop a tool to improve reproduction efficiency of bulls with low sperm post-thaw motilities. Three different suspensions of silica particles were combined to form discontinuous gradients: 45 and 95 percent (45/95), 65 and 95 percent (65/95), and 45, 65 and 95 percent (45/65/95). Frozen-thawed semen was layered at the top of the gradients and motile sperm were separated by centrifugation and used for IVF. Results showed that the combination of three discontinuous layers (45/65/95) effectively separated motile sperm cells and significantly ($P<0.01$) improved the sperm motility for IVF (from 23.9% to 96.4%) and the cleavage (from 28.9% to 69.1%) and blastocyst development (from 10.0% to 23.6%) rates. Significant differences ($P<0.01$) in sperm motility were observed among the bulls but density gradient sperm separation technique improved the sperm motility of bulls with low post-thaw motilities, and the subsequent cleavage and blastocyst development when the separated sperm cells were used for IVF. Experiment 3-C examined the effects of stage-dependent culture system and addition of energy substrates in IVC medium. *In vitro*-matured and fertilized oocytes were divided into four groups and subjected to *in vitro* co-culture with cumulus cells and the components of basic IVC medium modified as follows: 1) fixed dose of FBS (10%) without pyruvate and lactate; 2) fixed dose of FBS with pyruvate and lactate; 3) stage-dependent culture

system without pyruvate and lactate; and 4) stage-dependent culture system with pyruvate and lactate. Stage-dependent culture system involved the use of increasing concentration of FBS in the IVC medium (Day 0 to 3 of IVC: 1% FBS, Day 3 to 7 of IVC: 15% FBS). Results showed that the addition of pyruvate and lactate in the IVC medium improved embryo development to the pre-implantation stage. The increasing dose of FBS in the stage-dependent culture system had no beneficial effect in improving blastocyst development. Of the 52 vitrified embryos produced by the IVF system using sperm cells separated by density gradient and cultured for development in a stage dependent culture system with pyruvate and lactate, full term development of embryos after non-surgical transfer to recipient animals was 13.5 percent (7/52) with a 23.1 percent (6/26) calving rate including one twin indicating an improved efficiencies in the IVP of viable embryos.

Results of these studies demonstrated the methods of IVP for embryos in bovine functions in water buffaloes but success rate was sub-optimal (11.5%). Refinement and improvement of the systems to match the water buffalo requirements showed that the following points were useful: a) minimize long duration of ovary storage; b) selection of a homogeneous population of developmentally competent oocytes and adoption of a desired duration of IVM period; c) improvement in the culture medium by FSH, E_2 and EGF supplementation for IVM and lactate and pyruvate for IVC; and d) use of good quality motile sperm cells separated by density gradients with silica particles for IVF. These factors significantly improved the rate of blastocyst development (from 11.5 to 36.1%). For the cryopreservation of water buffalo embryos, a two-step ultra-rapid vitrification technique using open-poled straw with 10% ethylene glycol as pre-equilibration medium and EFS40 as cryoprotectant at an exposure time of 0.5 min was effective.

The *in vitro*-produced-vitrified embryos were viable and swamp buffaloes were efficient as recipients and surrogate mothers of river embryos in spite of chromosomal differences. This means, this technology is a potential tool for water buffalo genetic improvement and for the propagation of river buffaloes in swamp buffalo dominated countries. More studies, however, are recommended to further improve the current success rate and make the technology an efficient and effective tool for water buffalo breeding.

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