Journal of Agricultural Technology 2015 Vol. 11(8): 2343-2357
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

# A Protocol for the In Vitro Production of Bubaline Embryos: The Philippine Experience

**Marlon B. Ocampo[1,2]* and Lerma C. Ocampo[1]**

[1]Reproductive Biotechnology Unit, Philippine Carabao Center, Science City of Munoz, Nueva Ecija, Philippines 3120
[2]College of Veterinary Science and Medicine, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines 3120

Ocampo M.B. and Ocampo L.C. (2015). A Protocol for the In Vitro Production of Bubaline Embryos: The Philippine Experience. Journal of Agricultural Technology. 11(8): 2343-2357.

This study was designed to evaluate the effect of some factors in the in vitro production of bubaline embryos. In Experiment 1,aspiration, slicing and the combination of both methods had a mean oocyte recovery of 2.9, 3.2, 3.3 with rank A and B oocytes of 0.95, 1.1 and 1.2 per ovary, respectively. The fertilization rate of COCs recovered from either methods had no significant differences (52.6% - 58.3%). In Experiment 2, COCs cultured for 17-19 hr, 20-22 hr and 23-24 hr had a maturation rate of 57.0%, 80.3% and 86.7%, respectively. Cleavage rate post insemination have no difference but the blastocyst formation rate was higher in COCs matured for 20-24 hr (21.1 % - 23.3%) than 17-19 hr (12.7%). Total cell count was highest in blastocyst derived from COCs matured for 23-24 hr (102.2±3.5) than those matured for 20-22 hr (97.4±8.3) and 17-19 hr (89.9±6.4). In Experiment 3, COCs derived in vivo and in vitro had a maturation (53.0% and 77.1%), cleavage (39.6% and 57.5%) and blastocyst formation rate of 32.1% and 22.5%, respectively. In Experiment 4, COCs cultured in TCM-199 and mSOF medium had a maturation (81.3% and 78.3%), cleavage (55.4% and 70.7%), and blastocyst formation rate of 21.9% and 25.2%, respectively. The total cell count showed no difference.In summary, the factors considered and the techniques described in this study are consistent and reproducible in terms of achieving oocyte maturation, its fertilization and in the production of blastocyst stage embryos.

**Keywords**: Oocyte, maturation, fertilization, culture, embryos

## Introduction

In the Philippines, 1988 marked the beginning of the application of some reproductive biotechniques other than artificial insemination usingbubaline and caprine as a model (Ocampo *et al.*,1988a,b). Subsequently, the application of superovulation-embryo transfer (SOET) techniquehas resulted to the birth of a male buffalo calfin 1991(Venturina *et al.*, 1991). However, production of large number of embryos through SOET has been constrained by the low ovulation

***Corresponding author:** Marlon B. Ocampo; **Email:** ocampomarlon29@yahoo.com

response of buffaloes even after gonadotropin treatments. Thus, the development of an alternative approach using the oocytes of slaughtered femalehas become the most practical means of mass producing embryos both for accelerating calf production and/or for basic research.In our laboratory, production of embryos from oocytes derived in vivo, through ovum pick-up (Atabay *et al*., 2008; Aquino *et al.*, 2013) or in vitro, from slaughtered female in abattoirs (Ocampo *et al.*, 1998,2001c; Hufana-Duran *et al.*, 1998a) has enabled us to better understand the mechanisms surrounding the oocytes acquisition of developmental competence, sperm capacitation and acrosome reaction, sperm/egg attachment and penetration, and the eventual development of resulting zygotes to transferable stage. In fact, transfer of some of these embryos has resulted to live births (Ocampo *et al.*, 1996 a,b; Hufana-Duran *et al.*, 2004, 2005, 2008). However, the progress in elucidating details of these events has been slowed by technical problems inherent to the procedures and by difficulties in the interpretation of available data.Research efforts were mostly directed at accomplishing fertilization and it was only recently when significant insights into the mechanisms of sperm:egg interaction and embryo development have been made (Ocampo *et al.*, 2002,2003, 2015; Duran, 2004). Earlier attempts usually involved the incorporation of biological materials, eg., blood serum, follicular fluid, in the culture medium to maintain oocyte and sperm viability and/or to support its maturation and capacitation/acrosome reaction, respectively (Chuangsoongneon *et al.*, 1991; Totey *et al.*, 1993;Madan *et al.*, 1994a;Chauhan *et al.*, 1998;Samad *et al*., 1998; Aquino and Ocampo, 2014). These ill-defined conditions have been replaced with "defined" culture media in which the components are known, with the exceptions of factors associated with bovine serum albumin (BSA) preparations (Ocampo *et al.*, 2013). By using defined culture media, results become much more reproducible, data more meaningful and expertise is easier to acquire.

The use of bubaline gametes offers some advantages for studies on the in vitro production of embryos. The oocyte is dark in color with prominent zonapellucida permitting easy identification of the polar body during the completion of 1$^{st}$ meiosis. Similarly, the spermatozoa have prominent acrosome allowing assessment of acrosome reaction in living, motile samples. The tail and decondensation of penetrating sperm head in the eggcytoplasm is easily and clearly identifiable through phase contrast microscopy. The polar bodies do not degenerate soon following fertilization nor do they fragment, thus examination for evidence of fertilization is easier. The resulting zygotestarting from 30hrs post insemination and the subsequent developmental progression up to the blastocyst stage can easily observe by using a stereomicroscope (Hufana-Duran *et al.*, 1998b; Ocampo *et al.*, 2001a,b; 2015).

The techniques presented here represent the culmination of efforts to improve and simplify procedures for the in vitro production (IVP) of bubalineembryos which started as early as 20 yrs ago. One improvement is the ability to utilize a single based medium from the in vitro maturation of immature oocytes to the blastocyst stage following fertilization. At least 80% of immature oocytes are consistently completing the 1st meiotic division with more than 50% fertilization rate following insemination in vitro using frozen-thawed spermatozoa. The use of a modified synthetic oviductalfluid medium (mSOF) consistently support the development of cleaved embryos up to the blastocyst stage. For other researchers who wish to use the considerable advantagesof using bubaline IVP procedures for basic research and information gatherings, details of the techniques involved are hereby described.

## Materials and Methods

### *Reagents*

Unless specified, all chemicals, reagents and hormones were purchased from Sigma (St Louis, Mo, USA).The basic media for maturation of oocytes is a defined tissue culture medium with Earle's salts and L-glutamine (Krisher *et al.*, 1999), for fertilization is a Brackett-Oliphant medium (BO; Brackett and Oliphant, 1975) and for culture is the modified synthetic oviductal fluid medium (mSOF, Tervit *et al.*, 1972).

### *Water Quality*

Water purity is of utmost importance in the preparation of media to ensure repeatability of results between each treatment/trials. Satisfactory results have been obtained in using media with water from high-purity ion-exchange systems such as the Milli-Q (Serial No. 7LANO45 or F3MA86694B, Millipore Corporation, Bedford, MA) and/or Barnstead (Nalge Company, Rochester, NY) system.The presence of endotoxins in water/ or in chemicals may contribute to the variability in results which sometimes observed between batches of culture mediums.

### *Oil preparation*

Mineral oil (Squibb) is routinely used to overlay the media. Possible problems that maybe encountered with unsatisfactory brands or batches of mineral oil includes rapid death of sperm cells and fragmentation or flattening

of medium droplets as in the case when using paraffin oil. Oil is extracted with water (1:3 ratio) by carefully inverting for at least 3x without shaking, after separation for about 24 hr, the water is drained off and repeated one more time with water, then the last time with sterile saline solution (157 mMNaCL solution). This process equilibrates the oil with salt solution. The equilibrated oil is stored with equal amount of sterile saline solution for at least 24 hr in each respective incubator used for maturation, fertilization and culture before use. Sterilization of oil is unnecessary for short-term experiments. If it becomes necessary as for critical experiments, sterilization could be done by heating (110°C for several minutes) or by filtration before equilibration with saline solution. If oil is heated, it should be allowed to return to room temperature before equilibration with saline solution to prevent clouding.

***Oocyte collection and maturation***

Ovaries were obtained from swamp buffalo (SB)cows slaughtered at a local abattoir and subsequently transported to the laboratory in a thermos with 0.9% (w/v) saline solution at 30-35° C. Antral follicles (3-5 mm in diameter) were aspirated with an 18- gauge hypodermic needle attached to a 10- ml disposable syringe. The follicular aspirate was transferred in a conical tube maintained at 37°C water bath while aspiratingthe remaining follicles, allowing the oocytes to settle down the bottom. After 5 min the sediment from conical tubes were recovered using the transfer pipette, placed in a grid dish with phosphate buffered saline (PBS) medium and searched for cumulus-oocyte complexes (COCs) using a stereomicroscope. Oocytes enclosed by compact cumulus cells with more than three layers and having evenly granulated ooplasm (not pycnotic) were classified as normal oocytes and selected for in vitro maturation. A total of 10-15 COCs were placed in 100 ul drops of in vitro maturation mediumoverlaid with mineral oil in petri dishes (10-15 COCs/droplets; 35 mm x 10 mm, Falcon No. 1008, Becton Dickinson, Lincoln Park, NJ, USA). Oocytes were cultured for maturation in 5% $CO_2$ in air at 39° C.

After maturation, the oocyteswere denuded free of cumulus cells byvortexing, done by placing 100 µl of warmed PBS into a 1.5 ml microfuge tube plus 1 µl of 0.1% hyaluronidase (w/v) before placing the COCs. After vortexing for 30 sec the tube was rinsed with PBS (2x) and the fluid poured in a culture dish for denuded oocyte recovery and selection. The denuded and selected oocytes were washed (at least twice) to rinsed away hyaluronidase before putting them in the fertilization wells or drops. Without this precaution, prolonged exposure of oocytes to hyaluronidase may result to the loss of fertilizability.Or by pipetting,

done by aspirating the oocytes (in and out) using a finely drawn glass pipette with the diameter slightly bigger than that of the oocytes.

***Sperm preparation and fertilization***

Locally processed frozen semen of water buffaloes from the Sperm Processing Unit of the Philippine Carabao Center stationed at Digdig, Carranglan, Nueva Ecijawere used for in vitro fertilization (IVF). For each trial, straw containing 0.5 ml semen was thawed in a water bath at 39°C for 15 sec and processed by the swim-up method. Briefly, the semen suspension was put in a test tube, added with 5 ml of fertilization medium + 10 % calf serum and centrifuged (460 g for 5 min) for washing (2x). The sperm pellet was then layered with 2 ml of fertilization medium and kept in the incubator for 1 hr.Afterwards, about 1.8 ml of the upper portion of the semen suspension was recovered and washed using the same fertilization medium. The sperm pellet was re-suspended in the same medium to give an initial sperm concentration of 10 x $10^6$ sperm/ml. Then, a 20 µl aliquot of the sperm suspension was introduced in droplets of 80 µl fertilization medium containing the pre-washed oocytes (10-15 oocytes/drop) to co-incubate. The final spermconcentration during fertilization consisted of 2 x $10^6$sperm/ml, 5 mM caffeine and 10 % calf serum. The motility rate of sperm during insemination was more than 50 %.

***Experimental***

***Experiment 1.*** Effect of different methods of oocyte recovery on the quality of COCs and its subsequent fertilization in vitro. The aspiration (using an 18- gauge needle and a syringe), slicing (the ovarian surface and tissue were cut lengthwise and crosswise with a sterile cutting device consisting of 5 razors arranged parallel to each other at 2 mm intervals) and a combination of both methods were compared in terms of obtainable good quality oocytes and its subsequent fertilizability.

***Experiment 2.*** Effect of maturation time on the completion of 1$^{st}$ meiosis and its subsequent fertilization in vitro. The selection of oocytes post-maturation in vitro is critical for successful fertilization. The presence of 2$^{nd}$ metaphase plate is important during sperm-oocyte coincubation. Reports on the time required for the completion of 1$^{st}$ meiosis in buffalo in different laboratories varies (Singh et al., 1992; Yadav et al., 1997; Neglia et al., 2001; Ocampo et al., 2001a). Thus, determination of the optimum time for maturation of the oocytes forfertilization studies is critical.

***Experiment 3.*** Effect of oocyte source on the acquisition of developmental competence of immature oocytes in vitro. COCs collected from the abattoir and by Transvaginal Ultrasound-guided Follicular Aspiration (TUFA; Model HCV 4710 MV, Japan, with the use of an Ultrasonic Scanner Model HS-2000, Honda Electronics Co., Ltd, Japan) were evaluated on their capacity to complete 1st meiosis, fertilization, cleavage and development to the blastocyst stage. It has been claimed that only a small proportion of IVM oocytes have full developmental potential compared to in vivo derived oocytes (Schroeder and Eppig, 1984) because they have not completed "oocyte capacitation", hence do not possess full molecular and cellular machinery required to support early embryogenesis (Gilchrist and Thompson, 2007). Thus, elucidation on the developmental competence of oocytes derived from in vitro or in vivo is important.

***Experiment 4.*** Culture medium effect on the acquisition of developmental competence of immature bubaline oocytes in vitro. TCM-199 medium (undefined) and mSOF medium (defined) were evaluated on their capacity to support the completion of 1st meiosis, cleavage and the subsequent developmental rate to the blastocyst stage. Chemically defined media are free of unknown enzymes, hormonal and other activities that may interfere with the responses of concern thus, giving a better/reproducible results than when using undefined culture conditions (Biggers, 1998). Besides, exposure of oocytes/embryos to changing culture environment may result in reduced developmental potential. Therefore, a reliable culture system that would provide a consistent and replicable result is important.

### *Assessment of meiotic maturation, fertilization and embryo cell number*

Oocytes or blastocysts at the time of examination were mounted on a glass slide and fixed for 10 min in 25 % (v/v) acetic acid in ethanol at room temperature. Fixation was done at 33° C on a warm plate for the past removal of lipids. They were stained with 1% (w/v) orcein in 45% (v/v) acetic acid solution, and examined under a phase-contrast microscope at a 400x magnification. The meiotic stage of the oocytes was assessed according to the method of Ocampo *et al.*, (1991, 2001a). Oocytes with 2nd metaphase plate (M-2 stage) and a 1st polar body were regarded as matured,whereas oocytes with male and female pronuclei (cytoplasmic maturation), 2nd polar body extrusion and a detached sperm tail in the ooplasm were classified as fertilized.The number of blastomeres in a blastocyst was countedon the 7th day using Thouas *et al.*, (2001) method.Briefly, blastocyst were washed in PBS-PVP, placed in 1 ml of Hoechst working solution (0.75 ml of 2.3 % Na citrate dehydrate

solution; 0.25 ml of ethanol; 10 µl of Hoechst 33342 stock solution of 1 mg/ml concentration dissolved in ethanol) in a microfuge tube, wrapped in aluminum foil and stored in the refrigerator for at least 24 hr. Subsequently, the blastocyst were recovered and washed in glycerol, mounted on a glass slide, flattened in glycerol by a cover slip to a level where all nuclei appeared at the same focal plane and examined by using a fluorescent microscope (Eclipse E-600; Nikon) under ultraviolet light. A digital image of each embryo was taken and the total cells (both inner cell mass and trophectoderm) counted.

### *Statistical Analysis*

Data evaluation and statistical interpretation were done by using Student's t-test and Chi-square analysis. Differences were considered significant at $P<0.05$ level.

## Results

In Experiment 1, a total of 60 ovaries (divided equally into three methods compared) were collected from SB cows (age ranging from 1 – 7 yrs old) slaughtered from a local abattoir. The mean number of oocytes collectedthrough aspiration, slicing and a combination of both methods were 2.9, 3.2 and 3.3with rank A and B COCs of 0.95, 1.1 and 1.2 per ovary, respectively.Following the insemination of rank A and B COCs in vitro, a mean fertilization rate of 55.1% was obtained (Table 1).

**Table 1.** Oocyte quality and its fertilizability using different methods of recovery.

| Method of collection | Number of ovaries | Number of oocyte recovered (mean) | Oocyte rank A | B | C | D | No. of oocytes (%) inseminated | fertilized |
|---|---|---|---|---|---|---|---|---|
| Aspiration | 20 | 58 (2.9) | 8 | 11 | 21 | 18 | 19 | 10 (52.6) |
| Slicing | 20 | 63 (3.2) | 10 | 12 | 25 | 16 | 22 | 12 (54.5) |
| Aspiration + Slicing | 20 | 65 (3.3) | 9 | 15 | 32 | 9 | 24 | 14 (58.3) |

In Experiment 2, a total of 347 COCs were used (Table 2). Of these, 125, 117 and 105 were evaluated for the presence of 1$^{st}$ polar body post 17-19, 20-22 and 23-24 hr of culture, respectively. Significantly higher maturation rate was observed between 20-24 hr of culture than 17-19 hr of culture. Although no

significant differences were observed on the cleavage rate, the lowest cleavage rate was noticed on oocytes matured for 23-24 hr. The blastocyst formation rate was also higher significantly on oocytes matured between 20-24 hr (22.2%) versus 17-19 hr (12.7%). Total cell count was higher significantly on oocytes matured for 23-24 hr versus 17-19 hr.

**Table 2.** Maturation time effect on the developmental competence of COCs.

| Culture time | No. of COCs (%) cultured | matured | inseminated | cleaved | Blastocyst formation | Total cell count |
|---|---|---|---|---|---|---|
| 17-19 | 125 | 71 (57.0)[a] | 71 | 38 (53.5) | 9 (12.7)[a] | 89.9±6.4[a] |
| 20-22 | 117 | 94 (80.3)[b] | 90 | 52 (57.8) | 21 (23.3)[b] | 97.4±8.3[a,b] |
| 23-24 | 105 | 91 (86.7)[b] | 90 | 46 (51.1) | 19 (21.1)[b] | 102.2±3.5[b] |

[a,b]Values with different superscript differ significantly (P<0.05).

In Experiment 3, a total of 205 COCs were evaluated for maturation, fertilization and developmental competence (Table 3). In vivo derived COCs had a significantly lower maturation rate (53.0%) than in vitro derived COCs (77.1%). Similarly, significantly higher cleavage rate was observed on in vitro derived COCs (57.5%) than in vivo derived COCs (39.6%). However, the blastocyst formation and total cell count of embryos derived from oocytes in vivo was higher than oocytes derived in vitro.

**Table 3.** Effect of oocyte source on the maturation and developmental competence of COCs.

| Source of oocytes | No. of COCs (%) cultured | matured | inseminated | cleaved | Blastocyst formation | Total cell count |
|---|---|---|---|---|---|---|
| In vivo | 100 | 53 (53.0)[a] | 53 | 21 (39.6)[a] | 17 (32.1)[a] | 101.4±7.8 |
| In vitro | 105 | 81 (77.1)[b] | 80 | 46 (57.5)[b] | 18 (22.5)[b] | 96.8±9.2 |

[a,b] Values with different superscript differ significantly (P<0.05).

In Experiment 4,a total of 484 COCs were evaluated for their developmental competence in vitro (Table 4). The maturation rate of oocytes cultured in both culture medium had no difference but the cleavage rate of oocytes from mSOF medium (70.7%) was higher significantly than oocytes cultured in TCM-199 medium (55.4%). No significant difference was observed on the blastocyst formation of resulting zygotes and their total cell count.

**Table 4.** Culture medium effect on the developmental competence of COCs.

| Culture Medium | No. of COCs (%) cultured | matured | inseminated | cleaved | Blastocyst formation | Total cell count |
|---|---|---|---|---|---|---|
| TCM-199 | 327 | 266 (81.3) | 260 | 144 (55.4)[a] | 57 (21.9) | 98.8±7.0 |
| m-SOF | 157 | 123 (78.3) | 123 | 87 (70.7)[b] | 31 (25.2) | 97.3±8.1 |

[a,b]Values with different superscript differ significantly (P<0.05).

## Discussion

In bubaline, the low number of primordial and antral follicles throughout the estrous cycle contributed significantly to a poor recovery rate of immature oocytes for use in the production of embryos in vitro (Kumar *et al.*, 1997). Moreover, the high incidence of follicular atresia limits the selection of quality oocytes for maturation(Ocampo *et al*., 1994; Palta *et al*., 1998). In bovine, it was suggested that the ability of oocytes to undergo maturation in vitro is not dependent on the size of the follicles and stage of estrous cycle (Leibfried and First,1979) hence, an efficient method to maximize oocyte recovery has become a necessity as in the case of buffalo. When using the aspiration method for oocyte recovery from all follicles that is visible on the surface of the ovary, a mean recovery rate of 2.9was obtained. This was further reduced to 0.95 oocyte per ovary after screening the oocytes into rank A or B. Others have reported an average oocyte recovery per ovary of 0.7 (Totey *et al*., 1992), 1.7 (Das *et al.*, 1996), 2.4 (Kumar *et al.*, 1997) and 4.3 (Gasparrini, 2002). The mean recovery rate of good quality oocytes was further reduced to 0.4 (Totey *et al*., 1992; Madan *et al*., 1994a), 0.9 (Das *et al*., 1996), 1.76 (Samad *et al.*, 1998) and 2.4 - 2.9 (Gasparrini *et al.*, 2000). When using the slicing method or a combination of both methods, a slightly higher mean recovery rate of 3.2 and 3.3 oocytes were obtained with rank A or B oocytes of 1.1 and 1.2 per ovary, respectively. Others have reported a mean oocyte recovery rate of 8.4 with good quality oocytes of 3.9 (Boni *et al*., 1994; Gasparrini, 2002) per ovary. Thesediscrepancies when using Italian buffalo cows have been attributed to their better nutritional status as the plane of nutrition affects the follicular dynamics (Smith, 1984; Cruz *et al*., 1992).In this study, the ovaries were collected from swamp buffaloes sold by local farmers which generally, are fed only with grasses and maybe legumes that are available in the pasture area without concentrate supplementation. Also, the oocyte recovery rate is further reduced with the presence of CL wherein follicular development is restricted

and small antral follicles becomes hardly accessible to aspiration method (Nandi *et al*., 2000).

Although slicing method in combination or not with the aspiration method can increase the number of recoverable oocytes, it is not practical to use when large number of ovaries is available due to extended length of time needed to collect the oocytes which may result to loss of viability.Nevertheless, the fertilizability of oocytes collected in either methods showed no significant difference. In contrast, oocytes recovered from ovaries by the cutting method was reported to have a better capacity to mature and be fertilized in bovine (Hamano and Kuwayama, 1993).

In vivo release of the oocytes from the follicles does not occur until maturation is accomplished. In in vitro condition, oocytes with visible 1st polar body is assumed to have completed 1st meiosis/nuclear maturation and therefore is ready for fertilization. The reported time required for bubaline oocytes to complete 1st meiosis varies in different laboratories (Singh, 1992; Jainudeen et al., 1993; Madan *et al.,* 1994a; Yadav *et al*., 1997; Neglia *et al*., 2001; Ocampo *et al*., 2001a; 2015), contributing to differences on the efficiency of IVF technology application.In this study, majority of the oocytes (more than 80.0%) have completed 1st meiosis at 20 hr of culture than between 17-19 hr(57.0%) of culture. Others have reported a much shorter time of culture at 15–16 hr and 19 hrs with more than 70.0% and 87.0% maturation rate, respectively (Neglia *et al.*, 2001; Gasparrini, 2002).The anatomical and physiological differences between the swamp and the river type buffaloes could possibly account for some differences on the timing of completion of oocyte meiosis in vitro (Drost and Elsden, 1985; Lohachit, 1987). Subsequent use of these oocytes for IVF in vitro showed no difference in cleavagerate but the resulting blastocyst formation was lower significantly in oocytes matured for 17-19 hr only. It has been suggested that oocytes that have not completed "oocyte capacitation" due to insufficient maturation time, do not possess the full molecular and cellular machinery required to support early embryogenesis (Gilchrist and Thompson, 2007), as in this case. Although morphological screening of the oocytes before maturation was done, the inherent limitation of IVM process dealing on mixed population of oocytes collected from follicles at varying degrees of development, dominance and atresia remains.

In Experiment 3, the in vivoderived oocytes through TUFA had a lower maturation and cleavage rate post- insemination than in vitro derived oocytes. This could be attributed to the non-selection of TUFA derived oocytes prior to IVM, wherein all collected oocytes were immediately washed, placed in the maturation medium and used for IVF. This becomes necessary due to a lower proportion of rank A and B oocytes collected based on their morphological

characteristics. We assumed that a greater mechanical damage has been induced to the granulosa cells during TUFA procedure, the length of the needle (18 g; 50 cm long) which drew the samples under 50 mm Hg permanent negative pressure obtained with a vacuum pump and the line connected to the suction unit may have contributed to the loss of granulosa cells (Aquino et al., 2013). In contrast, in vitro derived oocytes were first evaluated morphologically before selecting for maturation and fertilization. Nonetheless, the blastocyst formation rate and the corresponding total cell count of in vivo derived oocytes was higher than in vitro derived oocytes. Apparently, acquisition of oocyte developmental competence occur in vivo during the course of folliculogenesis during which the oocyte acquires the cytoplasmic machinery necessary to fully support pre-implantation embryo development (Brevini-Gandolfi and Gandolfi, 2001; Sirard *et al.,* 2006).Whereas, spontaneous oocyte maturation in vitro occurs in the absence of certain crucial cytoplasmic events and components required for complete developmental competence of the oocytes (Gilchrist and Thompson, 2007).

Usually, sequential culture system is used in the in vitro production of buffalo embryos, from oocyte selection and culture, fertilization and in supporting embryonic development (Jainudeen *et al.,* 1993; Boni *et al*., 1994; Madan *et al*., 1994a; Chauhan *et al*., 1997; Aquino *et al*., 2014). Such system may expose the oocytes and embryos to changing osmolarity and/or pH which may negatively influence its developmental potential. Moreover, the supplementation of the medium with serumand/or feeder cells may contribute to the variabilities in response making the interpretation of results quite difficult (Madan et al., 1994b;Ocampo *et al.,* 2001c).In this study, the use of mSOF as a single IVM/IVF/IVC medium for buffalo supported the completion of 1$^{st}$ meiosis, its fertilization and the embryonic development of resulting zygotes to the blastocyst stagesimilar to, when using a sequential culture system. The choice of using a chemically defined medium (eg.,mSOF) offers some advantages over biological medium (eg., TCM-199). They are reproducible at different times, free of unknown enzyme, hormonal and other activities which may interfere with the responses being studied (Biggers, 1998).Also, it is capable of providing and maintainingthe proper environment for transcription or translation in overcoming the 8- to 16- cell block stage in this specie, resulting to blastocyst formation with a mean cell count of 97.3±8.1 on the 7$^{th}$ day of culture. It is hoped that with this culture system, a better hypotheses and experiments could be designed for identifying the missing link/requirements for culture of embryos that would support its developmental competence to produce live births.

## References


Aquino FP, Atabay EP, Atabay EC, Ocampo MB,Duran PG,Pedro PB,Duran DH, De Vera RV, Cruz LC.2013. In vitro embryo production and transfer of bubaline embryos using oocytes derived from TUFA. Buffalo Bull., 32:545-548.

Aquino FP,Ocampo MB.2014. In vitro maturation of bubaline oocytes using bubaline follicular fluid. European J Biotech BioSci., 2:35-38.

Atabay EP,Atabay EC,Duran PG,Flores EB,Abesamis AF,De Vera RV, Cruz LC. 2008. Production of embryos from prepubertal buffaloes using untrasound-guided ovum pick-up and in vitro embryo production techniques. Phil J Vet Anim Sci., 34:55-65.

Atabay EP,Atabay EC,De Vera RV, Aquino FP,Duran DH, Cruz LC.2010. Effect of holding bubaline and bovine ovaries at various temperature during transport and storage on in vitro embryo production. Phil J Vet Anim Sci., 36:81-93.

Biggers JD.1998. Reflections on the culture of preimplantation embryo.Int J Dev Biol., 42:879-884.

Boni R,Roviello S,Barbieri V, Zicarelli L.1994. In vitro embryo production in buffalo species.Atti XLVIII ConvegnoNazionale SISVET, 1:307-312.

Brackett BG, Oliphant G.1975.Capacitation of rabbit spermatozoa in vitro.BiolReprod., 12:260-274.

Brevini-Gandolfi T.A.L, Gandolfi F.2001.The maternal legacy to the embryo: cytoplasmic components and their effects on early development.Theriogenology, 55:1255-1276.

Chauhan MS, Palta P, Das SK,Katiyar P, Madan ML.1997. Replacement of serum and hormone additives with follicular fluid in the IVM medium; Effect on maturation, fertilization and subsequent development of buffalo oocytes in vitro.Theriogenology, 48: 461-469.

Chauhan MS,Singla SK,Palta P,Manik RS, Madan ML.1998. In vitro maturation, fertilization and subsequent development of buffalo embryos: Effects of oocyte quality and type of serum.ReprodFertil Dev., 10:173-177.

ChuangsoongneonU,Kamonpatana M. 1991. Oocyte maturation, in vitro fertilization and culture system for developing pre-implantation swamp buffalo embryos using frozen-thawed semen. Buffalo J., 7:189-198.

Cruz LC,Smith OF, Adriano FA,Venturina HV, Lorenzo N,Mamuad FV,Atabay EC, Duran PG. 1992. Ovarian follicular dynamics and responses to superovulatory treatments in water buffaloes. Phil J Vet Anim Sci., 18:19-32.

Das GK, Jain GC,Solanski VS, Tripathi VN.1996. Efficacy of various collection methods for oocyte retrieval in buffalo.Theriogenology, 46:1403-1411.

DrostM, Elsden RP.1985. Blastocyst development in the water buffalo.Theriogenology, 23:191.

Duran DH. 2004. In vitro systems for oocyte maturation, fertilization and culture inwater buffalo. 7th World BuffaloCong.,Oct. 4-19,Mla, Phil., pp. 136-150.

Gasparrini B,Neglia G,Di Palo R,Campanile G, Zicarelli L. 2000. Effect of cysteamine during in vitro maturation on buffalo embryo development. Theriogenology, 54:1537-1542.

Gasparrini B. 2002. In vitro embryo production in buffalo species:The state of the art. Theriogenology, 57:237-256.

Gilchrist RB, Thompson JG. 2007. Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro. Theriogenology, 67:6-15.

Hamano S, Kuwayama M. 1993. In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: A comparison between the cutting and aspiration method. Theriogenology, 39:703-712.

Hufana-Duran D, Aquino FP, De Vera RV, Pedro PB, Cruz LC. 1998a. Some physical factors affecting development of buffalo oocytes matured and fertilized in vitro. Korean J AnimReprod., 21:265-272.

Hufana-Duran D,Pedro PB, Aquino FP, De Vera RV, Duran PG, Cruz LC. 1998b. Nuclear progression of immature swamp buffalo oocytes in minimum essential medium after cryopreservation by vitrification method. Proc 4th Symposium on Animal Biotechnology, October 7-9, 1998, Kamina, Japan, pp.84-89.

Hufana-Duran D,Pedro PB,Venturina HV,Hufana RD,Salazar AL,Duran PG, Cruz LC. 2004. Post-warming hatching and birth of live calves following transfer of in vitro-derived vitrified water buffalo embryos. Theriogenology, 61:1429-1439.

Hufana-Duran D,Pedro PB,Venturina HV,Duran PG, Cruz LC. 2005. Full term development of in vitro produced-vitrified water buffalo embryos. Phil J Sci., 134:89-94.

Hufana-Duran D,Pedro PB, Salazar AL,Venturina HV, Duran PG, Cruz LC. 2008. Twin calf production in water buffaloes following non-surgical transfer of in vitro produced-vitrified embryos. Phil J Sci., 137:99-104.

Jainudeen MR,Takahashi Y,Nihayah M, Kanagawa H. 1993. In vitro maturation and fertilization of swamp buffalo oocytes. AnimReprod Sci., 31:205-212.

KrisherRL, Lane M, Bavister BD. 1999. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. BiolReprod., 60:1345-1352.

Kumar A,Solanski VS, Jindal SK,Tripathi VN, Jain GC. 1997. Oocyte retrieval and histological studies of follicular population in buffalo ovaries. AnimReprod Sci., 47:189-195.

Leibfried L, First NL. 1979. Characterization of bovine follicular oocytes and their ability to mature in vitro. J Anim Sci., 48:76-86.

Lohachit C. 1987. Anatomy and clinical examination of female reproductive organ of swamp buffalo. In: Chantaraprateep P, Virakul P, Lohachit C and Kunavongkrit A (eds). Swamp Buffalo Reproduction. Chulalongkorn University Press: Bangkok, Thailand. Pp. 93-115.

Madan ML,Singla SK,Chauhan MS, Manik RS.1994a. In vitro production and transfer of embryos in buffaloes. Theriogenology, 41:139-143.

Madan ML,Chauhan MS,Singla SK, Manik RS. 1984b. Pregnancies established from water buffalo blastocysts derived from IVM/IVF oocytes and co-cultured with cumulus and oviductal cells. Theriogenology, 42:591-600.

Nandi S,Chauhan MS, Palta P. 2000. Effect of corpus luteum on the recovery and developmental potential of buffalo oocytes. Vet Rec., 147:580-581.

Neglia G,Marino M,Di Palo R,Wilding M,Caracioolo Di Brienza V, Dale B,Gasparrini B. 2001. A comparison of in vitro maturation in buffalo and bovine oocytes using confocal microscopy. Theriogenology, 55:488.

Ocampo MB,Uenishi RS, Valdez CA,Muralla Z,Hishinuma M, Kanagawa H. 1988a. Superovulation response of upgraded Philippine goat. Jpn J Vet Res., 36:249-255.

Ocampo MB, R.S.Uenishi, C.A. Valdez, J. Pastor, L.C. Cruz and H. Kanagawa. 1988b. Non-surgical embryo recovery in the water buffalo. Jpn J Vet Res., 36:257-263.

Ocampo MB,Ocampo LC, Kanagawa H. 1991. Timing of sequential changes in chromosome configurations during the 1st meiotic division of pig oocytes cultured in vitro. Jpn J Vet Res 38:127-137.

Ocampo MB,DeAsis AT, Ocampo LC, Kanagawa H. 1994. Histological observation of follicular atresia in swamp buffalo. Buffalo Bull., 13:51-55.

Ocampo LC,Ocampo MB, Aquino FP, De Vera RV, Cruz LC. 1996a. Blastocyst formation of swamp buffalo embryos in co-culture systems. Proc 2nd ABA Cong., Manila, Philippines, pp. 412-416

Ocampo MB, Ocampo LC, Lorenzo ND, De Vera RV, Aquino FP, Cruz LC. 1996b. Pregnancies resulting from swamp buffalo oocytes matured and fertilized in vitro. Proc 2nd ABA Cong., Oct. 9-12, Mla, Phil., pp. 210-214.

Ocampo MB, Ocampo LC, Lorenzo ND,Mamuad FV, Aquino FP, Mori T,Shimizu H, Cruz LC. 2000. Live births resulting from swamp buffalo oocytes matured, fertilized and cultured in vitro. Austr J Anim Sci., 13:279-281

Ocampo LC, Ocampo MB,Mamuad FV,Venturina EV, Mori T, Shimizu H. 2001a. Timing of sequential changes on chromosome configurations during the 1st meiosis and cytoplasmic events swamp buffalo oocytes matured and fertilized in vitro. Phil J Vet Anim Sci.,27:1-11.

Ocampo LC, Mori T,Mamuad FV, Ocampo MB, Shimizu H. 2001b. Maturation and fertilization of swamp buffalo oocytes in vitro; effect of length of ovarian transport. *Bubalusbubalis*: J Buff Sci Tech., 11:68-77.

Ocampo LC,Mamuad FV,Mori T,Shimizu H, Ocampo MB. 2001c. In vitro production of pre-implantation buffalo embryos. Buffalo J.,1:145-154.

Ocampo LC,Ocampo MB,Mamuad FV,Venturina EV, Mori T, Shimizu H. 2002. Intracytoplasmic GSH concentration of swamp buffalo oocytes cultured in vitro. Buffalo Bull., 19:27-29

Ocampo LC,Cruz LC, Ocampo MB. 2003. Some factors influencing the maturation, fertilization and development of buffalo oocytes in vitro. Proc 25th VPAP SciConf., June 20-21, Mla. Phil.,pp. 75-81.

Ocampo LC, Duran DH, Pedro PB, Aquino FP,Atabay EP, Ocampo MB,Maylem ER, Cruz LC. 2013. Developmental competence of water buffalo oocytes in vitro in single-based medium SOF. 10th World Buffalo Congress, Phuket, Thailand, May 6-8, 2013, pp. 67.

Ocampo MB, Manuel JA, Soriano RP, Ocampo LC. 2015. Chromosome complement and developmental competence of swamp buffalo oocytes matured and fertilized in vitro. J Agric Tech., 11:57-68.

Ocampo LC, Ocampo MB. 2015. Improved developmental competence of swamp buffalo oocytes matured in the presence of cysteamine. J Agric Tech., 11:31-40.

PaltaP,Banzal N,Prakash BS,Manik RS, Madan ML. 1998. Endocrinological observation of atresia in individual buffalo ovarian follicles. Ind J Anim Sci., 68:444-447.

Samad HA,Khan IQ,Rehman NU, Ahmad N. 1998. In vitro maturation and fertilization of Nili-Ravi buffalo follicular oocytes. Asian Aust J Anim Sci., 11:491-497.

Schroeder AC, Eppig JJ. 1984. The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. Dev Biol., 102:493-497.

Singh R, Majundar AC. 1992. Chronological changes of buffalo follicular oocytematuration in vitro. Ind J Anim Sci., 62:205-209.

Sirard MA, Richard F,Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. Theriogenology, 65:126-136.

Smith JF. 1984. Protein, energy and ovulation rate. In: Land RB, Robinson DW (eds). Genetics of Reproduction in sheep. London, Butterworth, England, pp. 349-359.

Tervit HR,WhittinghamDG, Rowson LE. 1972. Successful culture *in vitro* of sheep and cattle ova. J ReprodFertil., 30:493-497.

Thouas G.A. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod Biomed Online 3:25-29.

Totey SM, Singh G,Taneja M,Pawshe CH, Talwar GP. 1992. In vitro maturation, fertilization and development of follicular oocytes from buffalo. J ReprodFertil., 95:597-607.

Totey SM,Pawshe CH, Singh GP. 1993. In vitro maturation and fertilization of buffalo oocytes:Effects of media, hormones and sera. Theriogenology, 39:1153-1171.

Venturina HV,Adriano F, Duran P, Lorenzo ND, Smith OF, Cruz LC. 1991. Successful pregnancy of purebred buffalo embryo in Philippine carabao as recipient. Phil J Anim Sci., 17:55.

Yadav BR,Katiyar PK,Chauhan MS, Madan ML. 1997. Chromosome configuration during in vitro maturation of goat, sheep and buffalo oocytes. Theriogenology, 47:943-951.