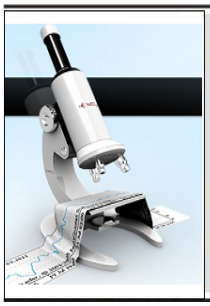

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Flocerfida P. Aquino
Philippine Carabao Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

Marlon B. Ocampo
Reproductive Biotechnology Laboratory, Philippine Carabao Center, Science City of Munoz, Nueva Ecija, Philippines.

Correspondence
Flocerfida P. Aquino
Philippine Carabao Center, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

In vitro maturation of bubaline oocytes using bubaline (Swamp Buffalo) follicular fluid

Flocerfida P. Aquino, Marlon B. Ocampo

Abstract
The mammalian ovaries are valuable source of oocytes for reproductive biotechnology. Oocytes attain developmental competence *in vivo* being in follicular fluid so using it as maturation medium could be an option. Swine, bovine, horse, ovine and even murine IVM studies using follicular fluid gave favorable effects.

Bubaline IVM experiment in CRD made use of 271 oocytes from buffalo ovaries. The oocytes were subjected to five maturation media: pure TCM 199 (T 1); 50% TCM 199 + 50% BuFF from 2-5 mm follicle size (FS) (T 2); pure BuFF from 2-5mm FS (T 3); 50% TCM 199 + 50% BuFF from 5-8mm FS (T 4) and pure BuFF from 5-8 mm FS (T 5). The oocytes were cultured for 22-24 hours at 38ºC, 5% CO_2 in air and high humidity inside the incubator. The oocytes were then fixed, stained, destained and evaluated their developmental competence.

In all the treatments, no significant differences were observed in the IVM stages of bubaline oocytes. The percentage values of matured oocytes ranged from 64.44% to 79.72%. The size of the follicles used in Treatments 2 and 3 showed no significant variations on oocyte maturation from Treatments 4 and 5. BuFF can be a substitute for TCM 199 in pure form or in combination with TCM 199 in bubaline IVM studies.

Keywords: Bubaline follicular fluid, buffalo, *In Vitro* Maturation, Oocytes

1. Introduction
Reproductive biotechnology has attracted the interests of researchers all over the world. With the advancement of micromanipulation, sexing and splitting of embryos, *in vitro* maturation/*in vitro* fertilization/*in vitro* culture/ embryo transfer (IVM/IVF/IVC/ET) technologies require the use of sophisticated equipment and technical capabilities to have tangible results. These technologies are breakthroughs that can be used as tools for accelerating the development of the livestock industry. In the Philippines, AI is still a practice in the upgrading of cattle and buffaloes. The success rate however, is only about 33% in synchronized buffaloes and 60% in natural estrus. In some private farms ET is used in improving the reproduction of buffaloes and cattle. It was in 1996 when the Philippine Carabao Center (PCC) demonstrated the application of IVM/IVF/IVC/ET in buffaloes that gave birth to two crossbred (PC x MB) calves.

At present the working protocol for IVM/IVF/IVC/ET in buffaloes is being refined. The refinement of the protocol considers the evaluation of different media in the maturation of bubaline oocytes. Incorporation of follicular fluid or total replacement of the IVM medium is used to evaluate the efficacy of bubaline follicular fluid. Follicular fluid supplementation is beneficial on *in vitro* maturation because of the presence of growth hormones, follicle stimulating hormone, luteinizing hormone and other nutrients found therein [1]. It is expected therefore that doing IVM using follicular fluid sourced from the same animal species may simulate the conditions of *in vivo* maturation which may achieve better *in vitro* maturation results. However, with the presence of hormones and nutrients in the follicular fluid, there is also the presence of oocyte maturation inhibitor factor [1]. Hence in this study, the efficacy of follicular fluid aspirated from different follicle sizes was evaluated and compared on the IVM of bubaline oocytes. Generally, the study aimed to test a suitable medium for the maturation of bubaline oocytes. Specifically, it aimed to 1) compare the extent of maturation of bubaline oocytes matured *in vitro* in bubaline follicular fluid (BuFF), Tissue Culture Medium (TCM) 199 and their combinations and 2) compare the effect of BFF aspirated from small size follicles (2-5 mm in diameter) and big size follicles (5-8 mm in diameter) in the *in vitro* maturation of bubaline oocytes)

2. Materials and Methods

From the ovaries of 90 newly slaughtered mature buffaloes, 271 oocytes were aspirated and were used in the study and were subjected to 5 culture media as follows:

Treatment I= TCM 199 + 10% calf serum and 50 ug/ml FSH
Treatment II= 50% TCM 199 + 50% BuFF aspirated from 2-5 mm follicle size (FS)
Treatment III= 100% BuFF aspirated from 2-5 mm FS
Treatment IV= 50% TCM 199+ 50% BuFF aspirated from 5-8 mm FS
Treatment V= 100% BuFF aspirated from 5-8 mm FS

The experimental design used was CRD with uneven replication. The statistical analysis of the data gathered was done using Chi Square Test.

2.1 Collection of Ovaries: This was done from a local abattoir 120 km from the Embryo Technology Laboratory, Munoz, Nueva Ecija. Collection period was from 1:00 AM to 3:00 AM using normal saline solution (0.85% NaCl) supplemented with 100 µg streptomycin/ml and 100 IU penicillin/ml). Collected ovaries were transported not more than 6 hours to the laboratory to ensure the viability of oocytes.

2.2 Aspiration of Oocytes: The ovaries were thoroughly washed with normal saline thereafter, using an 18 g needle attached to a disposable syringe, 2-5 mm FS in each of the ovaries collected was aspirated and the aspirates were transferred to a sterile test tube for selection of viable oocytes. The same was done to 5-8 mm FS in each of the ovaries.

2.3 Selection of Oocytes: Aspirates were allowed to settle for 10 minutes after which the upper portions was decanted for use in the preparation of maturation medium and the sediment were used in searching for the cumulus oocyte complexes (COCs) for oocyte maturation. COCs were searched using a stereomicroscope, Nikon, SMZ-2T (Japan), 20-60 X magnification. Oocytes surrounded with compact multilayers of follicle cells and ooplasm with even granulation were selected and were used for the maturation study.

2.4 Collection of BuFF: The decanted fluid and the sediments used for searching the COCs obtained from the follicular aspirates of the collected ovaries were processed for use as medium for oocyte maturation. The BuFF was centrifuged at speed 1500xg for 20 minutes to remove any cell debris. After this process it was subjected to heat inactivation at 56 ^{o}C for 30 minutes in a water bath, and was sterilized through a 47 mm, 0.45 µm pore size (7404-004) using syringe filtering. Aliquots of BuFF were dispensed in 5 ml tubes and were stored in the freezer for subsequent use for IVM trials. Likewise, follicles having 5-8 mm diameter size were aspirated separately. The FF was collected following the procedures done in 2-5 mm FS.

2.5 Preparation of IVM Droplets: Maturation droplets were prepared 2-3 hours prior to maturation for equilibration purposes. Maturation droplets were prepared and labeled properly based on the treatments of the oocytes. All the droplets were covered with mineral oil. Oocytes were cultured for 22-24 hours at 38^{o}C, 5% CO_2 in air and high humidity inside the incubator

2.6 Fixing and Staining of Oocytes: After the appropriate maturation period, the oocytes were freed of cumulus cells by vortexing them for 5 minutes. The denuded oocytes were mounted on glass slides using a mixture of paraffin wax and vaseline (1:3) and were fixed with acetic acid and ethyl alcohol solution (1:3) Fixed oocytes were stained with 1% aceto-orcein dye and after 5 minutes, destaining followed using the solution composed of acetic acid, glycerol and distilled water (1:1:3).

2.7 Assessment of Nuclear Stages

1) Immature oocytes- oocytes that possessed an intact nuclear membrane or germinal vesicle (GV)
2) Oocyte with Germinal Vesicle Breakdown (GVBD)- distinguished by the disappearance of compact nucleolus, nuclear membrane and condensed chromatin material
3) Metaphase I- oocytes showing the complete formation of individual bivalents

Mature oocytes

4) Anaphase 1- oocytes characterized by the elongation and separation of two chromosome sets towards opposite poles
5) Telophase I- oocytes showing the complete separation of two sets of chromosomes

Metaphase 2- oocytes that emitted the first polar body.

3. Results and Discussion:

The highest number of immature oocytes was observed in Treatment 5 with a percentage value of 2.17% from 46 oocytes evaluated. No immature oocytes were observed in T3 which means that all oocytes resumed meiosis.

Statistical analysis showed no significant differences were observed among types of maturation media and the sizes of follicles where the BuFF was derived. The insignificant differences on the effect of TCM 199, BuFF and their combination on immature oocytes after 24 h IVM suggested that majority of the oocytes resumed meiosis.

There was no evident effect of so called oocyte maturation inhibitor (OMI) observed in BuFF. This confirmed the results obtained using bovine oocytes [2] with 18% of the oocytes reached the A-1 stage and 69% of the oocytes to be in M2 stage when matured *in vitro* using Bovine Follicular Fluid (BoFF). The same results were observed when oocytes were matured in TCM199, 79.72% or in combination with BuFF, 71.43% and 72.00% (2-5 mm FS and 5-8 mm FS) and even in 100% BuFF, 64.44% and 73.91% (2-5mm FS and 5-8mm FS). Studies conducted observed the same trend on the beneficial effects of BoFF on the maturation and developmental capacity of bovine oocytes [3, 4, 5] when added to the maturation medium. Bubaline oocytes that were matured in 100% BuFF from follicle size >5mm had a maturation rate comparable to TCM 199 [1]. Pure follicular fluid even from follicles 10-15 mm was able to increase in vitro maturation rates of equine oocytes [6].

The results revealed that the effect of OMI was overcome by the substance called meiotic promoting substance (MPS) that caused the bubaline oocytes to resume meiotic changes.

Data gathered in this study are in agreement with those obtained in previous studies on the effects of FF supplementation of IVM medium in other species. In pigs, FF induced oocyte maturation *in vitro* and improved the rate of male and female pronuclei formation [7, 8, 9] and subsequent developmental capacity [10, 11].In murine IVF [12] media supplemented with concentration of 20% BoFF and estrous cow serum support murine 2-cell embryo development to blastocyst stage. In sheep [13], supplementing IVM medium with FF showed favorable effects in the maturation of sheep oocytes and also in horse IVM [14]. Another research in equine IVM-ICSI [15] which made use of pure follicular fluid from subordinate follicles and pure follicular fluid collected from mares administered crude equine

gonadotropin gave high proportions of normally fertilized oocytes after ICSI with 40 % and 38% for both media used. A direct comparison on the effects of FF and oestrous cow serum [16], revealed that the presence of FF in culture medium during IVM-IVF of bovine oocytes increased the fertilization rate and percentage of morulae/blastocysts obtained. Results in the studies using follicular fluid derived from small, medium and pre-ovulatory follicles supplemented to the maturation medium at the range of 10% [17, 18, 19] 20% [20] and 100% [21] improved the developmental capacity of the bovine oocytes.

Statistical results showed that T1 when compared to Treatments 2, 3 4 and 5 had comparable effect as regards the percentage values obtained during A-1 and T-1 stages of meiosis. Similarly, the sizes of the follicles (2-5 mm vs 5-8 mm) where the BFF was prepared had no significant effect on oocyte maturation.

Table 1: Research results in bubaline oocyte maturation using TCM 199, follicular fluid or its combination, (%)

Treatment	No. of Oocytes Examined	GV	GVBD	M-1	A-1	T-1	M-2
TI TCM 199	74	1.35 (1)	4.05 (3)	9.46 (7)	0	0	79.72 (59)
T2 50%TCM+50% BuFF from 2-5 mm FS	56	1.79 (1)	3.57 (2)	5.37 (3)	3.57 (2)	5.36 (3)	71.43 (40)
T3 100% BuFF from 2-5mm FS	45	0	11.11 (5)	2.22 (1)	4.44 (2)	6.67 (3)	64.44 (29)
T4 50% TCM +50% BuFF from 5-8mm FS	50	2.00 (1)	4.00 (2)	8.00 (4)	2.00 (1)	2.00 (1)	72.00 (36)
T5 100% BuFF from 5-8mm FS	46	2.17 (1)	4.34 (2)	6.52 (3)	6.52 (3)	0	73.91 (34)

Table 2: Effect of TCM 199, BFF and Their Combinations on Degenerated Bubaline Oocytes

Maturation Media	No. of Trials	No. of Oocytes Examined	Degenerated/ Parthenogenetically Activated n	%
TCM199	3	74	3	4.05 ns
50%TCM 199 +50% BuFF from 2-5 mm FS	9	56	5	8.93 ns
100% BuFF from 2-5mm FS	7	45	5	11.11 ns
50% TCM 199 +50% BuFF from 5-8mm FS	8	50	5	10.00 ns
100% BuFF from 5-8mm FS	7	46	3	6.52 ns

ns- not significant

The number of degenerated oocytes was highest (11.11%) in T3 followed by oocytes in T4, (10.00%), those oocytes from T2 (8.93%) and T5 with 6.52%. The lowest incidence of degenerated oocytes was observed in T1 with only 4.05%. Statistical analysis showed insignificant differences among the maturation media used in terms of percent degenerated oocytes. The values observed, however, could not be attributed only to the effect of the media used but also to the handling of the oocytes during aspiration and during the fixing of matured oocytes.

4. Conclusion

BuFF can be used as a substitute for TCM 199 without any significant difference in the IVM of bubaline oocytes. BuFF can be used as economic and easily prepared *in vitro* maturation medium without any supplementation.

The oocyte maturation inhibitor (OMI) present in some follicular fluid causing meiotic arrest was not observed in the IVM of bubaline oocytes.

Follicle sizes (2-5 mm and 5-8 mm) in which the BuFF was sourced and used as IVM medium had no significant effect on the IVM of bubaline oocytes.

The degenerated bubaline oocytes were not only affected by the maturation medium used but also by the handling of the oocytes during the aspiration or fixing of the oocytes.

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