The Recovery, *In Vitro* Maturation and Fertilization of Nili-Ravi Buffalo Follicular Oocytes

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ABSTRACT : Four types of serum supplements viz. estrus cow serum (ECS), estrus buffalo serum (EBS), pro-estrus buffalo serum (PrBS) and post-estrus buffalo serum (PtBS), added to TCM-199, were evaluated for *in vitro* maturation and fertilization of buffalo follicular oocytes. The oocytes were recovered from buffalo ovaries after slaughter, using either aspiration or scoring (multiple incisions) method. The recovered oocytes were categorized as A, B and C based on their cumulus investment and ooplasm homogeneity and cultured in four media. The *in vitro* matured oocytes were inseminated with 1×10^6 spermatozoa washed in 2.9% sodium citrate solution. The scoring method yielded greater number of morphologically good oocytes than the aspiration method (3.85 vs 1.76 per ovary, $p < 0.01$). The maturation rates of three categories of oocytes did not differ from one another. The maturation rates of 80.00, 82.08, 78.77 and 66.23%, while the fertilization rates of 54.54, 55.38, 52.80 and 36.76% were recorded for media containing ECS, EBS, PrBS, and PtBS, respectively. The medium containing PtBS gave lower maturation, as well as fertilization, rates than the other three media ($p < 0.05$). Thus, the scoring method was better than the aspiration method for the recovery of follicular oocytes. The oocytes categorized A, B and C had similar maturation capabilities. The TCM-199 containing buffalo/cow serum collected at pro-estrus or estrus appeared better for *in vitro* maturation and fertilization of buffalo follicular oocytes than that containing serum collected at post estrus.
(**Key Words**: Buffalo, Oocytes, *In Vitro* Maturation, Fertilization)

INTRODUCTION

In vitro maturation (IVM) and *in vitro* fertilization (IVF) appear to be useful techniques for the improvement of the reproductive efficiency of the buffalo. There are several reports on IVM-IVF and subsequent development of cattle oocytes (Lu et al., 1987a; Xu et al., 1987). However, very limited work has been done on the buffalo because of its being the native of developing countries. Previous reports concerning *in vitro* development of buffalo oocytes showed poor results. Among various reasons for this is the poor recovery rate of 0.46/ovary (Totey et al., 1992) of usable buffalo follicular oocytes as well as, lack of proper conditions to support *in vitro* maturation (Sirard, 1989). Therefore, this study was aimed to find out (i) a method that could enhance the usable follicular oocyte recovery rate per ovary (ii) to determine the *in vitro* maturation capabilities of buffalo follicular oocytes classified on the basis of their cumulus investment and (iii) to study the effect of various serum protein supplements (estrus cow serum, estrus buffalo serum, pro-estrus buffalo serum and post-estrus buffalo serum), on maturation and fertilization rates of buffalo follicular oocytes.

MATERIALS AND METHODS

Collection and preparation of ovaries

Buffalo ovaries were collected from a local abattoir within 1-2 hr after slaughter and transported immediately to the laboratory in a thermos containing sterile normal saline with added antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B). After cleaning in normal saline, the ovaries were rinsed in 70% ethanol followed by three rinses with sterile normal saline to remove the traces of ethanol. The recovery of oocytes from the ovaries was made by employing two different methods i.e. by the aspiration and by scoring the ovarian surface with a sharp blade.

Aspiration method

Ovarian follicles (2-6 mm in diameter) were aspirated

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with a 5 ml syringe and an 18-gauge needle filled with a modified tyrodelactate medium, TL-Hepes (Bavister, 1989), supplemented with 20% estrus buffalo serum, 0.2 mM sodium pyruvate and 0.1% gentamycin sulfate. The pH of the medium was adjusted to 7.4 and equilibrated at body temperature.

Scoring method (*multiple incisions*)

Follicular oocytes were recovered from 2-6 mm diameter follicles by scoring (making multiple incisions) the surface of ovary with a sterile surgical blade and instant rinsing and tapping the ovaries to release oocytes in a petri dish containing modified tyrodelactate medium, TL-Hepes (Bavister, 1989). The medium was supplemented with 20% estrus buffalo serum, 0.2 mM sodium pyruvate and 0.1% gentamycin sulphate.

The following criteria for classification of buffalo follicular oocytes, based on their cumulus investment and ooplasm homogeneity were followed for their categorization under stereomicroscope (De Loos et al., 1989; Lonergan et al., 1991).

Category	Morphological features of follicular oocytes
A	Compact and dense multilayered (≥ 5) cumulus investment with homogeneous ooplasm.
B	Compact and dense multilayered (3-4) cumulus investment with homogeneous ooplasm.
C	Less compact cumulus cell layers (1-2) with less homogeneous ooplasm.
D	Denuded or naked oocytes with evenly granulated ooplasm.

Types A, B and C were considered good oocytes for *in vitro* maturation and fertilization. The category "D" oocytes, being poor candidates for *in vitro* maturation (De Loos et al., 1989), were excluded from the study. The selected oocytes were washed thrice with TL-Hepes at 37℃ before transfer to the muturation media.

In vitro maturation

For *in vitro* maturation of buffalo follicular oocytes, four media M_1, M_2, M_3, M_4 were prepared by adding serum supplements from four different sources i.e estrus cow serum (ECS), estrus buffalo serum (EBS), pro-estrus buffalo serum (PrBS) and post-estrus buffalo serum (PtBS) in TCM-199 supplemented with sodium pyruvate and gentamycin. The composition of media is shown in tabel 1. Drops of 100 μl size of each maturation medium were made in a polystyrene tissue culture dish covered with a layer of paraffin oil (Chian et al., 1995) and incubated for equilibration at 39℃ under 5% CO_2 in humidified air in the incubator for at least 4 hr prior to transfer of oocytes. A total of 20 oocytes were placed per drop. These dishes were cultured in an atmosphere of 5% CO_2 in air and high humidity at 39℃ for 24 hr in the incubator. Maturation of the oocytes was characterized by dispersion of cumulus cells surrounding the oocyte (figure 1) reaching at metaphase II with one polar body extruded. For staining, the oocytes were fixed in methanol acetic acid (3:1) and stained with 1% orecin in acetic acid. The stage of maturation was examined under inverted microscope (300-400x).

Table 1. Composition of the maturation media using various serum supplements

Ingredients	M1	M2	M3	M4
TCM-199 (ml)	7	7	7	7
Sodium pyruvate (ml)	1	1	1	1
Estrus cow serum* (ml)	2	–	–	–
Estrus buffalo serum* (ml)	–	2	–	–
Pro-estrus buffalo serum* (ml)	–	–	2	–
Post-estrus buffalo serum* (ml)	–	–	–	2
Gentamycin sulphate (U1)	50	50	50	50

* The serum was heated at 56℃ for 30 minutes.

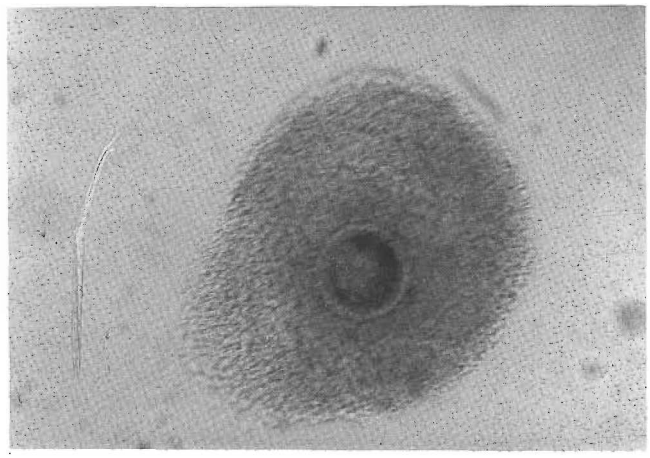

Figure 1. *In vitro* matured oocyte showing expansion of the cumulus cells.

Sperm preparation

Freshly collected buffallo bull semen was centrifuged at a rate of 3,000 rpm for 10-15 minutes and the seminal plasma was removed. The sediment was extended with an equal volume of sodium citrate (2.9%) and again

centrifuged for 5 minutes at a rate of 2,000 rpm. Again the supernatant was discarded and the sperm concentration in the pellet was determined by using hemocytometer. The pellet was diluted with equilibrated IVF-TL medium (Bavister and Yanagimachi, 1977) to obtain a sperm concentration of 25×10^6 sperm/ml. The method was fast, easy to perform, economical and yielded a high recovery of motile spermatozoa.

In vitro fertilization

For IVF, 200 μl IVF-TL drops were taken in polystyrene culture dishes. IVF-TL consisted of IVF-TL stock (Bavister and Yanagimachi, 1977) supplemented with fatty acid free bovine serum albumin (BSA) fraction v, sodium pyruvate, heparin and gentamycin. The drops containing IVF-TL were covered with paraffin oil and equilibrated at 39℃ under 5% CO_2 in humidified air for at least 4 hr prior to use. After 24 hr, matured oocytes were washed twice in equilibrated TL-Hepes and 18-20 oocytes were transferred to each drop. Each drop was inseminated with 40 μl of IVF-TL diluted sperm having a final concentration of 1×10^6 motile sperms per drop. The drops were covered by paraffin oil. The gametes were co-incubated for 18-24 hr in 5% CO_2 and humidified air, at 39℃.

After 18-24 hr of gameter co-culture in IVF-TL, the oocytes were removed, washed with equilibrated TL-Hepes and transferred to CZB medium drops of 100 μl size (Chatot et al., 1989). The CZB medium was equilibrated at 39℃ under 5% CO_2 in humidified air for 4 hours prior to use. The oocytes were cultured in CZB medium for another 24 hr at 39℃ in 5% CO_2 in humidified air in an incubator for cleavage. The cultured oocytes were pipetted through a small bore glass tube to remove the cumulus cells and examined under stereomicroscope for counting 2 to 4 cell stage embryos (figure 2).

Statistical analysis

The mean values of oocytes recovered from two methods were computed using Chi square and the means were compared by Z test. The mean values of maturation and fertilization rates for the four culture media were computed. In order to see the magnitude of variation among four media, the data were subjected to analysis of variance using completely randomized design (Steel and Torrie, 1980). Duncan's multiple range test (Duncan, 1955) was applied for multiple mean comparison.

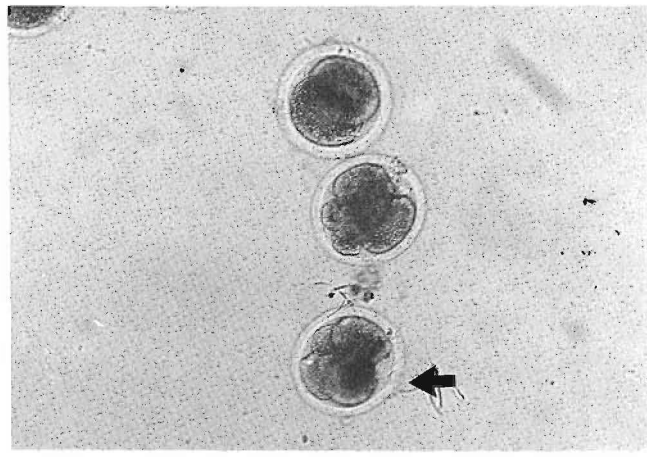

Figure 2. 2-4 cell *In vitro* embryos.

RESULTS

Recovery of oocytes

The recovery rate of buffalo follicular oocytes through aspiration and scoring methods is given in table 2. The study revealed that scoring method yielded significantly higher number of good quality oocytes (3.85 oocytes/ovary, type A, B & C) than the aspiration method (1.76 oocytes/ovary). There was a significant difference ($p < 0.01$) between the number and quality of oocytes recovered by the two methods (table 2).

In vitro maturation

Various categories of oocytes (A, B and C), classified according to their cumulus investment and ooplasm homogeneity, had similar capabilities to mature *in vitro*. Their maturation rates were 78.77, 75.59 and 75.75 percent for category A, B and C, respectively (table 3). Non significant difference was found between their maturation rates.

The results of this study revealed that the TCM-199 supplemented with sodium pyruvate and gentamycin containing ECS, EBS and PrBS had 80.00, 82.08 and 78.77 percent *in vitro* maturation rates of buffalo follicular oocytes, respectively. However, TCM-199 supplemented with sodium pyruvate and gentamycin containing PtBS had lower capability (66.23%) for *in vitro* maturation (table 3). There was non significant difference among culture media containing ECS, EBS and PrBs, however they differed significantly ($p < 0.05$) from culture medium containing PtBS.

In vitro fertilization

In vitro fertilization rate of *In vitro* matured oocytes after 48 hours is given in table 4. Oocytes matured in

Table 2. Influence of recovery method on quantity and quality of buffalo follicular oocyte types obtained for *in vitro* maturation

	Method	
	Aspiration	Scoring (multiple incisions)
No. of Ovaries	192	185
No. of follicles aspirated/scored	1,152 (6/ovary)[a]	1,221 (6.6/ovary)[a]
Good quality (A, B & C) oocytes recovered	1.76/ovary[b]	3.85/ovary[a]
Oocytes recovered:		
Total	635 (55.12%)[b]	954 (78.13%)[a]
A	117 (18.42%)[b]	334 (35.01%)[a]
B	98 (15.43%)[b]	268 (28.09%)[a]
C	123 (19.57%)[b]	111 (11.63%)[a]
D	297 (46.77%)[b]	241 (25.26%)[a]

Values with different letters within a row differ significantly ($p < 0.01$).

Table 3. Effect of four culture media on maturation rates of various categories of buffalo follicular oocytes classified on the basis of their cumulus investment and ooplasm homogeneity

Media	Categories of oocytes			Overall mean
	A	B	C	
M_1	84/103 (81.55%)	79/ 99 (79.79%)	73/ 93 (78.49%)	80.00% [a]
M_2	79/ 96 (82.28%)	86/107 (80.37%)	87/104 (83.65%)	82.08% [a]
M_3	69/ 86 (80.23%)	80/101 (79.20%)	70/ 91 (76.92%)	78.77% [a]
M_4	65/ 92 (70.65%)	71/111 (63.96%)	70/108 (64.81%)	66.23% [b]
Overall mean	78.77% [a]	75.59% [a]	75.75% [a]	

Values with different letters within a column or within a row differ significantly ($p < 0.05$).

Table 4. Fertilization rates of buffalo follicular oocytes matured *in vitro* using four culture media

Culture media	Oocytes inseminated	Oocytes reaching 2 cell stage	Oocytes reaching 4 cell stage	Overall fertilization rate (%)
M_1	121	18 (14.87%)	48 (39.66%)	54.54 [a]
M_2	130	20 (15.38%)	52 (40.00%)	55.38 [a]
M_3	125	18 (14.40%)	48 (38.40%)	52.80 [a]
M_4	136	20 (14.70%)	30 (22.05%)	36.76 [b]

Values with different letters in a column differ significantly ($p < 0.05$).

TCM-199 containing ECS, EBS and PrBS gave the fertilization rates of 54.54, 55.38 and 52.80 percent respectively, the difference was not significant. However, the fertilization rate was significantly lower ($p < 0.05$) for the oocytes matured in the medium containing PtBS (36.76%) than the other three media.

DISCUSSION

For *in vitro* maturation, fertilization and development of follicular oocytes, abundant recovery of good quality oocytes is one of the prerequisite. Unfortunately, poor recovery of immature oocytes in buffalo is a primary problem for the development of an *in vitro* system. In the present study, the scoring method gave higher recovery of good quality oocytes (3.85/ovary) than aspiration method (1.76/ovary). These values are higher than those reported by Totey et al. (1992), who obtained 0.46 of usable oocytes per ovary, by using aspiration method. Similarly, Madan, et al. (1994) reported that aspiration of more than 4,600 buffalo ovaries yielded an average of 0.42 good oocytes per ovary. The significantly ($p < 0.01$) higher values observed in this study can be attributed to the efficiency of the method applied for oocyte recovery. An average collection of about 11 oocytes/ovary has been reported in cows, about half of which were of good quality (Iwasaki et al., 1987; Hamano and Kuwayama, 1993). Relatively low recovery of follicular oocytes in buffaloes than cows might be due to the lower number of primordial and Graafian follicular population in the buffalo ovaries (Danell, 1987). This lower number of follicles in the buffalo ovaries results in lower oocyte yield per ovary, as well as low number of good quality oocytes (9.5%) with the majority of oocytes (55%) being degenerated (Suzuki, et al., 1991). In the present study, the overall recovery rates for ovaries from adult Nili-Ravi buffaloes were 55.12 and 78.13% with aspiration and scoring methods, respectively. Jainudeen et al. (1993) recorded 83% recovery rate for adult and 74% for prepubertal swamp buffaloes treated with equine chorionic gonadotrophin hormone 72 hr before slaughter.

The existence of a healthy population of somatic cells surrounding the oocyte is mandatory to facilitate the transport of nutrients and signals into, and out of, the oocyte. The cumulus cells play a supportive role by facilitating the entry of essential products and sending instructive signals to the oocyte for maturation through the gap junction (Osborn and Moor, 1982; Moor and Seamark, 1986). Oocytes surrounded by a tight and complete multilayered cumulus investment and containing ooplasm with a sandy appearance are developmentally more competent than oocytes that do not possess these characters (De Loos et al., 1989). The present study revealed that the buffalo follicular oocytes of category A, B and C had similar capabilities to mature *in vitro*. These observations confirm the studies of De Loos et al. (1989) who reported similar observations in cattle.

Oocyte maturation involves changes in the plasma membrane, nuclear and cytoplasmic maturation. A wide variety of media, ranging from simple salt solutions to complex culture media, have been used for *in vitro* maturation of bovine follicular oocytes. TCM-199 has emerged as the most commonly used medium for this purpose (Staigmiller, 1988). To achieve optimum maturation of oocytes, supplementation with serum has been found beneficial (Sirard, 1989). None of the hormones, growth factors or glycosaminoglycan could be substituted for serum (Eppig and Allen, 1986). The results of this study revealed that the effect of various serum supplements on maturation rates was significant ($p < 0.05$). This might be due to the fact that ECS, EBS and PrBS contain optimum level of various hormones (FSH, LH and E_2) that might be helpful in maturation and fertilization of buffalo follicular oocytes. However, the serum collected 24 hr after ovulation may not have enough concentrations of these hormones to meet the requirements of maturation and thereafter fertilization. In the buffalo serum concentrations of FSH were 5.16 ± 0.18, 5.75 ± 0.24 and 5.05 ± 0.03 m IU/ml during proestrus, estrus and post estrus phases of the cycle, respectively. The corresponding values were 6.31 ± 0.29, 8.38 ± 0.35 and 5.05 ± 0.31 m IU/ml for serum LH and 17.80 ± 9.09, 20.75 ± 1.09 and 9.93 ± 0.54 pg/ml for estradiol 17-β (Jindal et al., 1988; Sharma et al., 1990). However, Sanbuissho and Thralfall (1986) reported that ECS had non significant effect on maturation but significantly ($p < 0.05$) improved the fertilization and development of bovine oocytes over the serum collected 24 hr post ovulation.

According to Madan et al. (1994), addition of 10% buffalo estrus serum (BES) to TCM-199 significantly affected the maturation process over 5% BES with a higher percentage of matured oocytes at the metaphase II stage 22 hr after incubation. TCM-199 plus 20% BES supplemented with FSH, LH and estradiol has also been reported to result in a maturation rate of 81.7% while the addition of 20% BES alone resulted in only 47.4% oocyte maturation rate in buffaloes (Totey et al., 1992). In swamp buffaloes, Jainudeen et al. (1993) recorded a maturation rate of 47% using TCM-199 supplemented with 10% fetal calf serum, FSH and estradiol 17 β. A similar maturation medium resulted in a maturation rate

of 40% in river buffalo (Totey et al., 1991).

In vitro fertilization rate of the oocytes matured in media containing ECS (54.54%), EBS (55.38%) and PrBS (52.80%) did not differ from one another but the fertilization rates observed with medium containing PtBS were significantly lower ($p < 0.05$) than the other three media. Kajihara et al. (1991) recorded a cleavage rate of 48.3% in oocytes taken from ovaries of heifer calves. However, these values are higher than cleavage rates of 10 -28% reported in earlier studies on buffaloes (Suzuki et al., 1991; Totey et al., 1991; Jainudeen et al., 1993). The values of the present studies are comparable to 54.4% cleavage rate for buffalo oocytes in TCM-199 medium containing 10% buffalo estrus serum with cumulus cells (Madan et al., 1994). These workers recorded higher cleavage rate (77.2%) in TCM-199 medium containing 10% buffalo estrus serum with cumulus and oviductal epithelial cells. According to Keefer et al. (1991), the addition of ECS in TCM-199 enhances maturation and fertilization of bovine oocytes. LU et al. (1987b) have also suggested that serum of estrus animals enhances the fertilizability and developmental ability of cattle oocytes matured *in vitro*. The findings of this study do not agree with those reported by Larocca et al. (1993) who observed non significant effect of ECS on maturation, fertilization and culture of bovine oocytes.

Based on the results of the present study it can be concluded that scoring the ovarian surface is a better method for oocyte recovery in buffalo and the oocytes categorized A, B and C according to their cumulus investment and ooplasm homogeneity have similar maturation capabilities. TCM-199 containing buffalo or cow serum collected either at pro-estrus or estrus are better for *in vitro* maturation and fertilization of buffalo follicular oocytes.

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