INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2001/03–4–503–506
http://www.ijab.org

Studies on *In Vitro* Maturation and Fertilization of Nili-Ravi Buffalo Follicular Oocytes

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ABSTRACT

The present work was conducted to study the *in vitro* maturation and fertilization of Nili-Ravi buffalo follicular oocytes. Buffalo ovaries were collected from local abattoir within four hours of slaughter and transported immediately to the laboratory. Follicular oocytes were recovered by aspiration and scoring methods. It was found that the scoring method was an appropriate method for high recovery of good quality oocytes per ovary as it yielded 3.85 oocytes per ovary than aspiration method (1.76 oocytes per ovary). The effect of size of the ovary on oocyte harvest was non-significant while, the absence of corpus luteum (CL) from ovary had a significantly positive effect on oocyte harvest. The ovaries with CL yielded significantly lower ($P<0.05$) good quality and total oocytes (2.63 and 3.76 per ovary) than the ovary without CL (4.48 and 5.88 per ovary). The comparison of two experimental maturation media revealed that tissue culture medium-199 (TCM-199) resulted in significantly better maturation rate (73.33%) than Ham's F-10 (61.66%); whereas, these two media differ non-significantly for *in vitro* fertilization (42.66% and 36.00%).

Key Words: Buffalo; IVM; IVF; Ovarian size; Corpus luteum; Recovery method; Media

INTRODUCTION

Buffalo (*Bubalis bubalis*) play a prominent role in rural livestock production, particularly in Asia. Reproductive efficiency is the primary factor affecting productivity and is hampered in female buffalo by (i) inherent late maturity, (ii) poor estrous expression in summer, (iii) distinct seasonal reproductive patterns and (iv) prolonged intercalving intervals. Reproductive efficiency can be improved by introducing embryos produced *in vitro* using the fruits of now matured artificial insemination industry and many oocytes being wasted in the slaughterhouse. It is remarkable that viable embryos can be produced from ovarian oocytes collected hours after death of animals at the abattoir. Oocytes may be matured, fertilized and cultured *in vitro*, producing viable embryos with full developmental potential. Therefore, securing a plentiful and economical source of zygotes is central to capitalizing in buffalo on many new genetic technologies currently available in both research and commercial settings.

Very little work has been done previously on the development of an *in vitro* system for buffalo. These studies were undertaken to assess the two recovery methods (aspiration and scoring) for the yield of good quality oocytes; to study factors i.e., size of ovary and presence of corpus luteum (CL), affecting oocyte harvest from ovaries and to compare the two media i.e., tissue culture medium-199 (TCM-199) and Ham's F-10 for their effect on *in vitro* maturation (IVM) and subsequent fertilization (IVF) of follicular oocytes.

MATERIALS AND METHODS

Collection of ovaries. Buffalo ovaries (n=100) were collected from local abattoir within four hours of slaughtering and transported immediately to the laboratory in a thermos containing sterile normal saline with antibiotics (100 IU/mL penicillin G, 100 µg/mL streptomycin sulphate and 0.25 µg/mL amphotericin B) held at 30-35°C. Extraneous tissue was removed to clean the ovaries. Prior to oocyte collection the ovaries were rinsed in 70% ethanol to minimize the risk of contamination followed by three rinses with sterile normal saline to remove the traces of ethanol.

Recovery of oocytes. Follicular oocytes were recovered by following two methods:

Aspiration method. Ovarian follicles were aspirated from ovaries (n=50, divided in five groups, each comprising 10 ovaries) with an 18-guage needle fitted with a 10 mL syringe (Totey *et al*., 1992) filled with modified tyrode-lactate medium, Tl-Hepes (Bavister, 1989) supplemented with 20% estrus buffalo serum, 0.2 mM sodium pyruvate and 1mg gentamycin sulphate. The medium was adjusted to pH of 7.4 and then equilibrated at 37°C. Oocytes thus recovered were collected in a sterile 60 x 15 mm petri-dish containing modified tyrode-lactate medium.

Scoring method. Surface of ovaries (n=50, divided in five groups, each comprising 10 ovaries) was scored (Das *et al*., 1996) with a sterile surgical blade, with instant rinsing and tapping the ovary to release oocytes in a sterile 60 x 15 mm petridish containing modified tyrode-lactate medium, Tl-Hepes (Bavister, 1989) supplemented with 20 µg estrus buffalo serum, 0.2 mM sodium pyruvate and 1mg gentamycin sulphate. The medium was adjusted to pH of 7.4 and then equilibrated at 37°C.

Classification of oocytes. The following criteria for classification of oocytes, based on their cumulus investment and ooplasm homogeneity was followed for their categorization under stereomicroscope (De Loos *et al*., 1989; Lonergan *et al*., 1991). Only types A, B and C

oocytes were used for IVM and IVF studies. These were washed thrice with Tl-Hepes at 37°C before transfer to the maturation media.

Factors affecting the quality and quantity of oocytes. Two factors i.e., size of ovary and presence of CL in the ovary were studied (n=60, divided in five groups, each comprising 12 ovaries) for their effect on the quality and quantity of oocytes harvest for IVM. In laboratory, the dimensions of ovaries were measured by vernier calipers.

***In vitro* studies.** Two maturation media viz. TCM-199 and Ham's F-10 were compared for their effect on IVM and subsequent IVF of oocytes.

***In vitro* maturation.** Drops of 100 µL size of each maturation medium were made in a petri dish, covered with a layer of paraffin oil and incubated for equilibration at 39°C under 5% CO_2 in humidified air in the incubator for at least 4 h prior to transfer of oocytes.

A total of 120 oocytes were placed in drops of each maturation medium (12 per drop) and incubated for 24 h in an atmosphere of 5% CO_2 in humidified air at 39°C. Maturation of the oocytes was monitored through the dispersion of cumulus cells surrounding the oocytes.

***In vitro* fertilization.** For IVF, 200 µL IVF-Tl drops were made in petri dishes. The drops were covered with paraffin oil and equilibrated at 39°C under 5% CO_2 in humidified air in the incubator for at least 4 h prior to use. A total of 75 oocytes matured in each maturation medium were washed with Tl-Hepes and then transferred to these drops (15 oocytes per drop). Each drop was then inseminated with IVF-Tl diluted 1x106 sperm. The gametes were co-incubated for 24 h in 5% CO_2 with humidified air at 39°C. Fertilization was defined as the number of ova that cleaved to 2-cell stage.

RESULTS AND DISCUSSION

Recovery of buffalo follicular oocytes. The results revealed that scoring method yielded higher (5.74 per ovary) number and morphologically good quality oocytes (3.98 ovary) than aspiration method (3.20 and 1.68 ovary) (P<0.05; Table I). Das *et al.* (1996) also reported that better quality oocytes were recovered per ovary in buffalo by scoring (2.6) than by puncture (1.3) or aspiration (0.9) methods. In sheep, the number of oocytes recovered per ovary was higher by puncture and scoring than aspiration method. In the present study, the percentage of good quality oocytes was also higher in scoring method (4.28 per ovary) than aspiration method (1.69 per ovary). The recovery rates of buffalo follicular oocytes observed in the present study are higher than those reported by Totey *et al.* (1992), who obtained 0.46 usable oocytes per ovary using the aspiration method. The significantly (P<0.05) higher values of scoring method than aspiration method is attributed to the efficiency of this method. An average recovery of 11 oocytes per ovary has been reported in cows, about half of which were of good quality (Iwasaki *et al.*, 1987; Hamano & Kuwayma, 1993). Relatively low recovery of follicular oocytes in buffaloes than cows might be due to the lower number of primordial and graffian follicular population in the buffalo ovaries (Danell, 1987).

Table I. Effect of recovery method on oocytes harvest

Method	Aspiration	Scoring
No. of ovaries	50	50
Oocytes recovered	160b	287a
Oocyte recovered per ovary	3.20	5.74
Oocytes recovered		
A	33b (20.62%)	87a (30.31%)
B	25b (15.62%)	79a (27.52%)
C	26a (16.25%)	33a (11.49%)
D	76a (47.50%)	82a (28.57%)
Good quality (Category A, B and C) oocytes recovered per ovary	1.68b	3.98a

Values with different letters within a row differ significantly (P<0.05).

The existence of healthy population somatic cells surrounding the oocytes is mandatory to facilitate the transport of nutrients and signals into, and out of, the oocyte. The cumulus cells surrounding the oocytes play a supportive role by facilitating the entry of essential products and sending instructive signals to the oocyte through the gap junction for maturation (Osborn & Moor, 1982; Moor & Seamark, 1986). Oocytes surrounded by tight and multi-layered cumulus investment containing ooplasm with homogeneous appearance are most likely to be developmentally competent. Oocytes that do not possess these characters complete meiosis at a lower frequency *in vitro* (De Loos *et al.* 1989).

Thus, it can be concluded that scoring the ovarian surface with a sharp blade is a better method for oocytes recovery in buffalo than aspiration method. Moreover oocytes of categories A, B and C according to their cumulus investment and ooplasm homogeneity have similar maturation capabilities.

Effect of size of ovary on oocyte harvest. The effect of size of ovary on the total and usable oocytes recovered per ovary is given in Table II. The ovaries <2.25x1.75x1.25 cm yielded 3.80 and 5.68 usable and total oocytes per ovary while the ovaries >2.25x1.75x1.25cm yielded 3.66 and 5.30 usable and total oocytes, respectively per ovary. However, the difference was non-significant (P<0.05). Wani (1995) reported that in the sheep larger ovaries yielded significantly more number of oocytes than smaller ovaries by aspiration technique, while the difference was non-significant with the scoring method. Since no work has been reported about the size of ovary on the number and the quality of oocytes in buffaloes, the results can't be compared.

Effect of CL on oocyte harvest. Data regarding the effect of CL presence in the ovary on the oocyte recovery is given in Table II. The ovaries with CL yielded significantly less (P<0.05) number of usable (2.63) and total oocytes (3.76) per ovary than the ovaries without CL (4.48 and 5.88). The cause of lower yield of oocytes from the ovaries with CL may be attributed to the fact that CL reduces the growth of

Table II. Effect of ovarian size and corpus luteum on oocyte harvest

Ovarian Size	<2.25x1.75 x 1.25 cm	>2.25x1.75 x 1.25 cm	CL Present	CL Absent
No. of ovaries	60	60	60	60
Oocytes recovered	341a	318a	226 a	353 b
Oocyte recovered per ovary	5.68	5.30	3.76 a	5.88 b
Oocytes recovered				
A	76 a (22.28%)	81 a (25.47%)	61 a (26.99%)	94 b (26.62%)
B	56 a (16.42%)	77 a (24.21%)	50 a (22.12%)	108 b (30.59%)
C	96 a (28.15%)	62 b (19.49%)	47 a (20.79%)	67 b (18.98%)
D	113 a (33.13%)	98 a (30.81%)	68 a (30.08%)	84 b (23.79%)
Good quality (Category A, B and C) oocytes recovered per ovary	3.80	3.66	2.63 a	4.48 b

Values with different letters within a row differ significantly (P<0.05)

follicles and increases the atresia of follicles (Hafez, 1993).

Effect of media on IVM of oocytes. The IVM rates (Table III) of oocytes were 73.33 and 61.66% for TCM-199 and Ham's F-10, respectively. These media differed significantly (P<0.05) for IVM. Culture environment and media are important factors for oocytes to attain maturation *in vitro*. In addition to nuclear maturation, cytoplasmic and membrane maturation are critical components of the maturation process for continued viability of oocytes following fertilization (Thibault & Gerard, 1973). Cytoplasmic maturation remains ill defined, but involves both reorganization of cytoplasmic components (Hyttel *et al.*, 1986; Ducibilla *et al.*, 1990) and synthesis of ooplasmic proteins (Moor *et al.*, 1980, 1983; Kastrop *et al.*, 1990), in preparation for fertilization and embryonic development.

Table III. Effect of media on *in vitro* maturation

Maturation media	Oocytes incubated	Oocytes matured	Percentage (%)
TCM-199	120	88 b	73.33 b
Ham's F-10	120	74 a	61.66 a

Values with different letters within a column differ significantly (P<0.05).

A wide variety of media have been used for *in vitro* maturation in domestic animals, vitamins, purine and other compounds regarded as essential for general cell culture. Complex culture medium TCM-199 buffered with bicarbonate or HEPES and supplemented with various sera, and/or gonadotropic (FSH and LH) and/or steroid (Estradiol-17b) hormones have been most widely used to study bovine oocytes IVM. Although acceptable rates of maturation have been obtained with most of media but TCM-199 has emerged as the most commonly used media for bovine oocytes (Staigmiller, 1988). The medium used for oocyte maturation can strongly affect the developmental capacity of *in vitro* produced bovine embryos. It is well established that the culture conditions employed for IVM of mammalian oocytes can significantly influence IVF rates and subsequent embryonic development. A recent comparison of several commercially available chemically defined cell culture media revealed that IVM in either Ham's F-12 or Waymouth's medium MB 752/1 resulted in significantly reduced fertilization and embryonic development of bovine oocytes compared to IVM in either TCM-199 or MEM (Bavister *et al.*, 1992).

The present findings indicate that the buffalo oocytes can be successfully matured in TCM-199 and Ham's F-10 and these oocytes can cleave after getting matured in these media. These results are in agreement to those of Sirard and Coeman (1993), who reported non-significant difference in maturation and cleavage rates of cattle oocytes matured either in TCM-199 and Ham's F-10. Our results are not in accordance with those of Totey *et al.* (1991) who achieved higher maturation rates in Ham's F-10 than TCM-199 medium.

According to Madan *et al*. (1994), addition of 10% estrous buffalo serum (EBS) to TCM-199 significantly affect the maturation process over 5% EBS with a higher percentage of matured oocytes at metaphase II stage 22h after incubation. TCM-199+20% EBS supplemented with FSH, LH and estradiol has also been reported to result in a maturation of 81.7% while the addition of 20% EBS alone resulted in only 47.4% oocytes maturation rate in buffaloes. In swamp buffaloes, Jainudeen *et al*. (1993) recorded a maturation of 47.0% using TCM-199 supplemented with 10% fetal calf serum, FSH and estradiol-17b. A similar maturation medium resulted in maturation rate of 40% in river buffalo (Totey *et al.,* 1991). Bavister *et al.* (1992) stated that the medium used for oocyte maturation could strongly affect the developmental capacity of *in vitro* produced embryos. Furthermore the effects of media on oocyte maturation are manifested both before and after the first cleavage.

***In vitro* fertilization of oocytes.** The IVF rates (Table IV) of oocytes matured in TCM-199 and Ham's F-10 were 42.66 and 36.00%, respectively. The fertilization rates of oocytes matured in TCM-199 and Ham's F-10 differed

Table IV. Effect of maturation media on subsequent *in vitro* fertilization

Media	Oocytes inseminated	Oocytes fertilized	Percentage (%)
TCM-199	75	32 a	42.66 a
Ham's F-10	75	27 a	36.00 a

Values with different letters within a column differ significantly (P<0.05).

significantly (P<0.05) for IVF. When the oocytes matured in these media were co-incubated with spermatozoa, it was observed that TCM-199 and Ham's F-10 differ non-significantly (P<0.05) for the fertilization of these oocytes as their fertilization rates were 42.66 and 36.00% for TCM-199 and Ham's F-10. Differential effects of maturation media were previously demonstrated in mouse (Van de Sandi *et al*., 1990).

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(Received 13 August 2001; Accepted 10 September 2001)