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Dr. Arul V
Department of Veterinary Science Agricultural College and Research Institute Vazhavachanur ThiruvannamalaI, Tamil Nadu, India

Correspondence
Dr. Arul V
Department of Veterinary Science Agricultural College and Research Institute Vazhavachanur ThiruvannamalaI, Tamil Nadu, India

Effect of oocyte retrieval techniques on yield and quality of buffalo (*Bubalus bubalus*) oocytes

Dr. Arul V

Abstract
The aim of this study was to investigate the recovery rate and quality of oocytes by slicing and aspiration methods. The cumulus oocytes complexes and denuded oocytes were collected from 2−8 mm follicles from local abattoir's ovaries. Although, the slicing method yielded a significantly (P<0.0001) large number (3.75 oocytes per ovary) of oocytes than the aspiration method (2.65 oocytes ovary). However, the slicing method resulted in providing large quantity of good quality oocytes compared to the aspiration techniques. Therefore it was concluded that slicing the ovarian surface is a better method to recover more number of good quality oocytes from ovaries for *in vitro* studies.

Keywords: *in vitro*, slicing, aspiration, ovaries, oocyte

Introduction
Buffalo is the mainstay of Indian dairy industry since it contributes over 55% of the milk production. Despite the importance of buffalo to the socioeconomic status, its population has been declining, partly due to poor reproductive performance. The low reproductive efficiency in female buffalo can be attributed to delayed puberty, higher age at calving, long postpartum anoestrus period, long calving interval, lack of overt sign of heat and low conception rate (Kumar and Anand, 2012) [14]. In addition, female buffaloes have a lower number of follicles in the ovary; poor superovulatory response and high percentage of atretic follicles (Haldar and Prakash, 2007; Hufana-Duran et *al*., 2007) [9, 10].
Oocyte maturation is the first and most critical step towards successful *in vitro* embryo production buffalo embryos has been gaining attention for its research and commercial application ever since the birth of the first buffalo calf through *in-vitro* fertilization of buffalo oocytes (Totey *et al*.,1992) [23]. Although the quality of *in vitro* matured oocytes (IVM) is less than *in vivo* matured oocytes (Moor and Dai, 2001) [18], it is a frequent technique carried out by *in vitro* fertilization (IVF) centres for augmenting more number of mature oocytes for IVF. The culture medium and selection of protein supplements and hormones for IVM plays an important role in the subsequent maturation rate and embryonic development following IVF (Bavister *et al*., 1992) [3].
In vitro handling and culture conditions causes oocytes and embryos to oxidative stress resulting from events such as exposure to light, elevated oxygen concentrations and unusual concentration of metabolites and substrates (Agawal *et al*., 2006) [1].
There is an increasing interest of *in vitro* maturation (IVM) of immature oocytes retrieved from unstimulated ovaries. However, this present study was designed to compare the efficiency of two methods for oocyte recovery in buffalo.

Materials and Method
The present study was carried out in the Centralized Embryo Biotechnology Unit at Madhavaram and Molecular Biology Lab at Department of Animal Biotechnology, Chennai

Collection of Ovary
The ovaries were collected from sexually matured buffaloes (*Bubalus bubalis*) irrespective of age, body condition, estrus cycle stage and season from Chennai Corporation abattoirs located at Perambur and Pallavaram. The ovaries were removed within 30 min of the slaughter and washed in phosphate buffer saline (PBS) supplemented with 50µg/ml gentamycin sulphate to remove blood and extraneous material. The ovaries were then transported to the laboratory, at 37 ^{0}C in a thermos flask containing the same media within 30 min of collection.

Preparation of Ovaries
The extra ovarian tissues were trimmed off and the ovaries were washed three times with normal saline to remove blood clots and superficial bacterial contamination. The washed ovaries were stored in a sterile beaker containing normal saline supplemented with 50µg/ml gentamycin till oocyte retrieval.

Retrieval of Oocytes
Cumulus oocyte complexes (COCs) were retrieved from buffalo ovaries by aspiration and slicing techniques.

Retrieval of Oocytes by Aspiration
COCs were aspirated from all the visible follicles measuring >2mm diameter using a sterile 18G hypodermic needle attached to a 10ml disposable syringe containing 0.5-1 ml of aspiration medium. The fluid thus obtained having the COCs was allowed to settle for 15-20 min in sterile 90 mm petridish at 37^0 C. After 15-20 min the supernatant was discarded and the pellet was resuspended in oocyte washing medium and screened for oocyte in 90 mm petridish. From a total of 150 ovaries 183 oocytes were recovered by aspiration method.

Retrieval of Oocytes by Slicing
Each ovary was held firmly with sterile artery forceps in a 90mm petridish containing oocyte collection medium (modified HEPES-buffered Tyrodes medium) and was sliced as per the standard technique described by Datta *et al*. (1998) [7]. The oocyte was screened under a stereozoom microscope and transferred to 35 mm petridish containing oocyte collection medium and then graded. From a total of 473 ovaries 2195 oocytes were recovered by slicing method.

Classification and Grading of Oocytes
The oocytes were screened under a stereozoom microscope at 10x magnification, then washed thrice in 35 mm petridishes and graded based on their cumulus mass investment and homogeneity of ooplasm as described by Nandi *et al*. (1998) [20]

Grade A: Compact COCs with unexpanded cumulus mass having >4 layers of cumulus cells and with homogenous evenly granular ooplasm.

Grade B: COCs not having much compaction, with 2-3 layers of cumulus cells and having a homogenous evenly granular ooplasm.

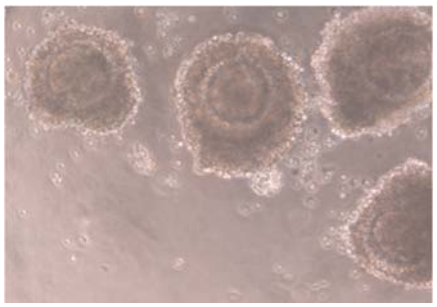
Grade C: COCs either partially denuded or with 1-2 layers of cumulus cells and with an irregular and dark ooplasm.

Grade D: Oocyte completely devoid of cumulus mass and having irregular and dark ooplasm.

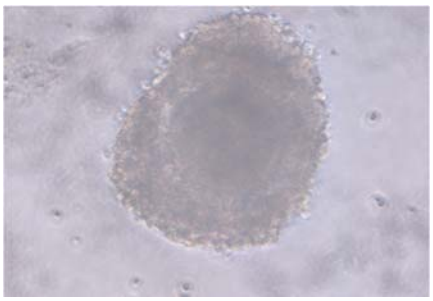
Grade E: COCs with highly expanded or scattered cumulus cells and with an irregular and dark ooplasm.

Statistical Analysis
The comparison of recovery of buffalo oocytes by aspiration and slicing methods the result were analyzed by percentage t-test. Difference was significant when $p< 0.05$.

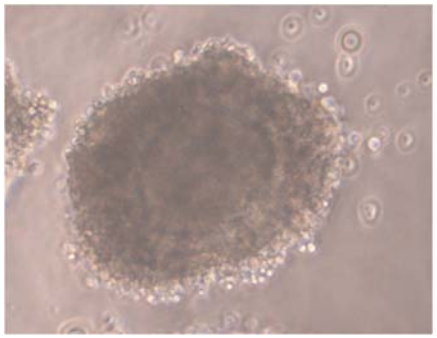
Results
Retrieval of Oocytes by Aspiration and Slicing Methods
From the total number of 623 ovaries collected from the abattoirs, oocytes were harvested from 150 ovaries by aspiration and 473 ovaries by slicing method and respectively a total of 183 and 2195 oocytes were retrieved.
The total number of oocytes retrieved per ovary by aspiration and slicing methods were 1.22 ± 0.06 and 4.64 ± 0.23 per cent for respectively. The number of oocytes retrieved per ovary by slicing method was highly significant ($p<0.001$) when compared with that of aspiration method.
The number and retrieval rate of oocytes harvested by aspiration and slicing methods are presented in Table 1, Plate 1(a-e) and Fig II.



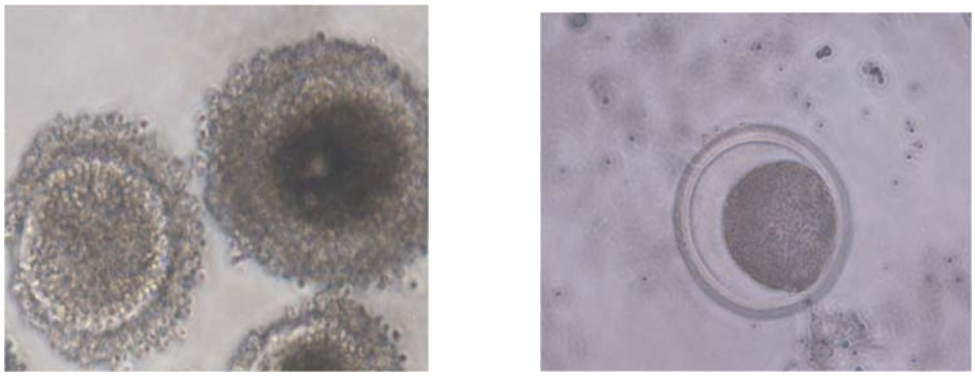
1a: grade a : cocs with more than 4 layers of cumulus cells



1b: grade b : cocs with 2 - 4 layers of cumulus cells



1c: Grade C: COCs with one layer of cumulus cells



1d: Grade D: COCs with denuded layers of scattered cumulus cells 1E: Grade E: Denuded oocytes

Plate 1: Different Grades of Buffalo Oocytes (200x)

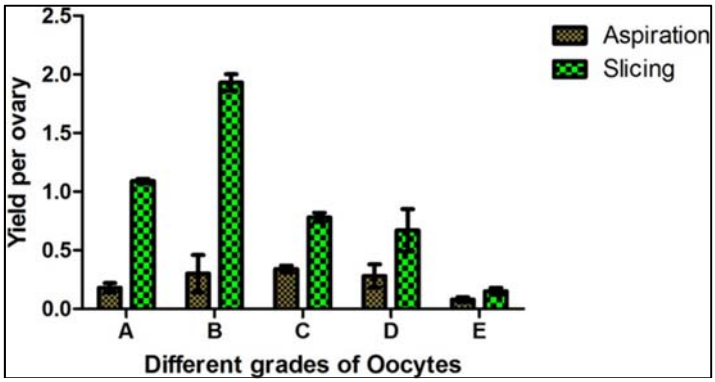


Fig 2: Number Quality and Retrieval Rate of Oocytes Harvested From Buffalo Ovaries by Aspiration and Slicing Methods

Quality of Oocytes Retrieved by Aspiration and Slicing Methods

The quality of oocytes retrieved from both the methods were graded into five categories A,B,C,D and E based on their diameter, compactness, the extent of surrounding investment, homogeneity and colour of the ooplasm.

The percentage yield of oocytes by aspiration and slicing methods were respectively 15.30 / 23.69 in Grade A, 26.23 / 41.59 in Grade B, 28.41/ 16.90 in Grade C, 23.49 / 14,53 in Grade D and 6.55 / 3.28 in Grade E.

It was found that in aspiration method percentage yield of C Grade oocytes were higher (28.41) and in slicing method B Grade oocytes were higher (41.59) whereas the percentage yield of C Grade oocytes were 16.90 in slicing method and the percentage yield of B Grade oocytes were 26.23 in aspiration method. The differences were highly significant ($p<0.001$).

The rate of oocyte yield per ovary by aspiration and slicing methods were respectively 0.18 ± 0.04 / 1.09 ± 0.02 in Grade A, 0.30 ± 0.16 / 1.93 ± 0.07 in Grade B, 0.34 ± 0.03 / 0.78 ± 0.04 in Grade C, 0.28 ± 0.10 / 0.67 ± 0.18 in Grade D and 0.08 ± 0.02 / 0.15 ± 0.03 in Grade E.

It was found that in aspiration method oocyte yield per ovary of C Grade were higher (0.34 ± 0.03) and in slicing method B Grade oocytes were higher (1.93 ± 0.07) whereas the yield per ovary of C Grade oocytes were 0.78 ± 0.04 in slicing method and B Grade oocytes were 0.30 ± 0.16 in aspiration method. The differences were highly significant ($p<0.001$).

Discussion

Retrieval Rate of Oocytes by Aspiration and Slicing Methods

In the present study, from the total number of 623 ovaries collected from the abattoirs, 183 oocytes were harvested from 150 ovaries by aspiration and 2195 oocytes from 473 ovaries by slicing method. The observed retrieval rate of oocytes per ovary by aspiration and slicing methods were 1.22 ± 0.06 and 4.64 ± 0.23 respectively (Table 1).

Similar results were observed by Nandi *et al*. (2000a) [22] with a retrieval rate of 1.06 from ovaries with corpus luteum and 1.25 in ovaries without corpus luteum.

The retrieval rate of buffalo oocytes per ovary by aspiration method in this study was also in accordance with the findings of Das *et al*. (1996) [6] but the same was higher than the value (0.73-1.3) reported by Totey *et al*. (1992) [23], Ravindranatha *et al*. (2002b) and Loganathasamy, (2004) and lower than 2.8 reported by Mishra *et al*. (2008).

On the contrary, Khan *et al.* (1997) [14] reported a higher oocyte retrieval rate of 5.15 per ovary by aspiration method, out of which retrieval rate of good quality oocytes was 3.30 per ovary.

Using slicing technique, from 473 ovaries, 2195 oocytes were retrieved with an average yield of 4.64 ± 0.23 oocytes per ovary. The retrieval rate observed in this study was lower than the value (5.7-7.02) reported by Jain *et al*. (1995) [13] and Das *et al.* (1996) [6].

In the present study, slicing of ovaries yielded higher retrieval of oocytes per ovary than by aspiration method. The low retrieval rate of oocytes per ovary by aspiration method could be attributed to the reason that only surface follicles are accessible and deep set follicles cannot be reached. Whereas in slicing method the oocytes from both superficial follicles and those in the deeper cortical stroma can be accessed (Das *et al.*, 1996) [6].

Das *et al.* (1996) [6] compared the oocyte retrieval methods in buffaloes and found that slicing yielded significantly more (5.7) oocytes per ovary than follicular puncture (2.6) or

aspiration (1.7) method and conferred that more of better quality oocytes (good or fair) were retrieved per ovary by slicing (2.6) than by follicular puncture (1.3) or aspiration (1.9) method. They further reported that slicing the ovaries released oocytes from two sources: surface follicles and those in deeper cortical stroma and this factor accounted for increased yield of oocytes after slicing. They concluded that slicing was the best oocyte collection method.

Also those oocytes which are not free floating and remain firmly attached (in small or medium sized follicles) could not be aspirated before the onset of cumulus expansion but could easily be retrieved, by slicing (Ball *et al*., 1983). It was also reported that the efficient method for retrieving higher number of cumulus oocyte complexes was by slicing of ovaries (Pawshe *et al*., 1994) [21].

Mutha Rao and Uma Mahesh (2012) concluded that slicing method was superior in terms of both total retrieval and higher number of culture grade oocytes, asslicing method yielded higher number of oocytes (7.98 ± 0.70) compared to aspiration method (2.38 ± 0.19) per ovary.

This was in corroboration with the findings (Gupta, 2003) [8] that, slicing of ovaries yielded high retrieval rate of total number of oocytes in comparison to aspiration method and added that poor rate of retrieval of oocytes by aspiration might be due to inaccessibility of deep set follicles located in the deeper cortical stroma.

However, the variation in oocytes retrieval by the same technique in different studies reported by different authors as mentioned above might be attributed to other variables like breed characters, nutritional status of the animals and agro climatic conditions. Furthermore, the expertise of the individual in processing the ovaries and the stress of handling large number of ovaries per day could also have affected the efficiency of oocyte retrieval (Mishra *et al*., 2008).

Hence, from the present study it was inferred that slicing technique is more advantageous than aspiration for retrieval of more number of oocytes.

Table 1: Number Qualityand Recovery Rate of Oocytes Harvested From Buffalo Ovaries by Aspiration and Slicing Methods

Methods	No. of ovaries	Oocytes			
		Grades	Retrieved	Per cent	Yield per ovary
Aspiration	150	A	28	15.30	0.18 ± 0.04^{b}
		B	48	26.23	0.30 ± 0.16^{b}
		C	52	28.41	0.34 ± 0.03^{b}
		D	43	23.49	0.28 ± 0.10^{a}
		E	12	6.55	0.08 ± 0.02^{a}
		Total oocytes	183	100	1.22 ± 0.06^{b}
Slicing	473	A	520	23.69	1.09 ± 0.02^{a}
		B	913	41.59	1.93 ± 0.07^{a}
		C	371	16.90	0.78 ± 0.04^{a}
		D	319	14.53	0.67 ± 0.18^{a}
		E	72	3.28	0.15 ± 0.03^{a}
		Total oocytes	2195	100	4.64 ± 0.23^{a}

Values of yield per ovary are given as Mean ± S.E.

Values with different superscript within column differ highly significant ($P < 0.001$)

Quality of Oocytes Retrieved by Aspiration and Slicing Methods

In the present study all the oocytes retrieved by both the methods were graded based on their morphology i.e number of layers of cumulus cells and ooplasm character, and then classified as Grade A,B,C,D and E oocytes with specific features as described by Nandi *et al*. (1998) [20].

Several studies in buffaloes have equivocally demonstrated that the visual assessment of the compactness and extent of cumulus investment surrounding the oocyte served as one of the best guide for its developmental ability (Chuangsoongueon and Kamonpatana, 1991) [4] and therefore oocytes are graded based on those characters.

The percentage yield of oocytes in the current experiment by aspiration and slicing methods were respectively 15.30 / 23.69 in Grade A, 26.23 /41.59 in Grade B, 28.41/ 16.90 in Grade C, 23.49 / 14,53 in Grade D and 6.55 / 3.28 in Grade E.

It could be seen from the above values that more of better quality oocytes were obtained by the slicing method than by the aspiration as the highest number of Grade A (23.69) and Grade B (41.59) oocytes were retrieved by slicing method. Also the least number of Grade E (3.28) oocytes was obtained by slicing method.

The rate of oocyte yield per ovary by aspiration and slicing methods were respectively 0.18 ± 0.04 / 1.09 ± 0.02 in Grade A, 0.30 ± 0.16 / 1.93 ± 0.07 in Grade B, 0.34 ± 0.03/ 0.78 ± 0.04 in Grade C, 0.28 ± 0.10 / 0.67 ± 0.18 in Grade D and 0.08 ± 0.02 / 0.15 ± 0.03 in Grade E.

It could be seen from the above values that more of better quality oocytes were obtained by the slicing method than by the aspiration as the highest oocyte yield per ovary of Grade A (1.09 ± 0.02) and Grade B (1.93 ± 0.07) oocytes were retrieved by slicing method. Also the least oocyte yield per ovary of Grade E (0.15 ± 0.03) were obtained by slicing method.

The reported yield of cultivable oocytes (A and B grade) per ovary by aspiration technique by different authors were 0.43 (Totey *et al*., 1992) [23], 0.42 (Madan *et al*., 1994) [17], 0.9 (Das *et al*., 1996) [6], 0.77 (Loganathasamy, 2004) [16] and 1.9 (Mishra *et al*., 2008).

The pooled yield per ovary of A and B grade (0.48) by aspiration method in this experiment corresponds with that of the findings of Totey *et al.* 1992 Totey [23], (0.43) and Madan *et al*. 1994 [17], (0.42) by aspiration method.

Das *et al*. (1996) [6] also reported that the retrieval rate of good quality or cultivable grade (A and B grade) oocytes was higher by slicing than by aspiration method as observed in this research.

Therefore it is inferred that slicing method is more advantageous than aspiration method to retrieve more number of good quality cultivable buffalo oocyte.

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