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Recovery of oocytes from ovarian follicles by aspiration method in buffaloes

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Abstract
In the present study total of 155 ovaries were procured from a local slaughter house and follicular size categorization based on their diameter using vernier caliper as < 3 mm, 3-5 mm and > 5 mm size was carried out. Aspiration technique of oocytes retrival from buffalo ovarian follicles was done using an 18 gauge needle attached to a 5 ml syringe. Follicular contents were poured in a searching dish which had a square grid pattern and Cumulus Oocyte Complexes (COCs) were searched under a stereozoom microscope. The overall percentage of small, medium and large follicles in the ovaries were recorded as 16.29 ± 0.94%, 8.14±0.60%, 5.35 ± 0.76%, respectively. The overall recovery rate of COCs was 38%. The percentage of these oocytes were 16.74% (A), 15.25% (B), 25.26% (C), 18.33% (D) and 29.87% (E) respectively. Aspiration method is a quick method of oocytes recovery.

Keywords: aspiration method, COCs, follicle

1. Introduction
Interest in breeding bubaline species has tremendously increased worldwide, due to the fundamental role played by this amazing animal in several climatically disadvantaged agricultural systems. There is about 207 million buffalo populations in the world out of which roughly 97% are found in the Asian region [5]. Countries with the largest number of dairy buffaloes are India, Pakistan, China, Egypt followed by Nepal [5]. The total buffalo population in India is 109.85 million [16] ranking number one in the world with huge genetic diversity and showing an increase of about 1% over previous [15] where the population was 108.70 million.
Buffalo is the mainstay of livestock agriculture in Asia for many centuries primarily due to its acclimatization with the Indian scenario which furthermore enhances its working capacity and its milk yield. Also, there are no religious hindrances in slaughtering buffaloes in India. However, buffalo suffers from many reproductive issues like delayed puberty, postpartum ovarian inactivity, seasonality, low reproductive efficiency, low conception rate, high embryonic mortality and silent heat which can cause great economic loss to the farmers, and are the main hurdles in rearing them [19, 12, 13].
Buffalo females have few primordial *viz*. 12000 – 19000 follicles in the cortical region of ovaries and have a higher rate of follicular atresia [9]. Owing to the demand for technical expertise, the cost involved in the oocyte recovery from live animals is quite high compared to slaughterhouse derived oocytes which are a cheaper and easily available sources of COCs for *in vitro* production of embryos. Immature oocytes of dead animals are retrieved from the ovary using various methods such as aspiration, scoring, slicing, puncture or a combination of aspiration plus slicing methods [11, 17, 6]. Aspiration technique is a less time consuming method and a more practical method when a large number of ovaries are to be processed. This study aimed to recover oocytes from buffalo ovarian follicles by aspiration technique.

2. Materials and Methods
2.1 Study Area
For the present study, a total of 155 ovaries were processed at Departments of Animal Reproduction, Gynaecology and Obstetrics, Krantisinh Nana Patil College of Veterinary Science, Shirwal, Dist. Satara; and at Bull Mother Farm, Animal Husbandry (Government of Maharashtra) Tathawade, Pune.

2.2. Collection of Ovaries

For the present study, a total of 155 were procured from the local slaughter house at Kondhwa Abattoir, Pune, India. Immediately after collection the ovaries were placed in a stainless steel thermos flask containing 0.9% normal saline solution along with Inj. Gentamycin @ 50µg/ml and were transported to the laboratory within 2 hours of slaughter. In the laboratory, the perivaginal layer and stroma attached to the ovaries were trimmed off using scissors and ovaries were washed thrice under tap water and once with 0.9% Normal Saline Solution. These ovaries were then transferred to a beaker containing 0.9% Normal Saline Solution fortified with antibiotic gentamycin @ 50µg/ml [20]. Beaker was then placed on a stage warmer maintained at 37 ℃ temperature for 10 - 15 minutes for temperature equilibration.

2.3. Measurement of Follicles

Ovaries were then taken on an absorbant tissue paper one by one and ovarian surface follicles were measured with the help of a vernier caliper. Follicles based on their diameters were classified into three categories *viz*. small (<3mm), medium (3-5mm) and large (> 5mm) in diameter.

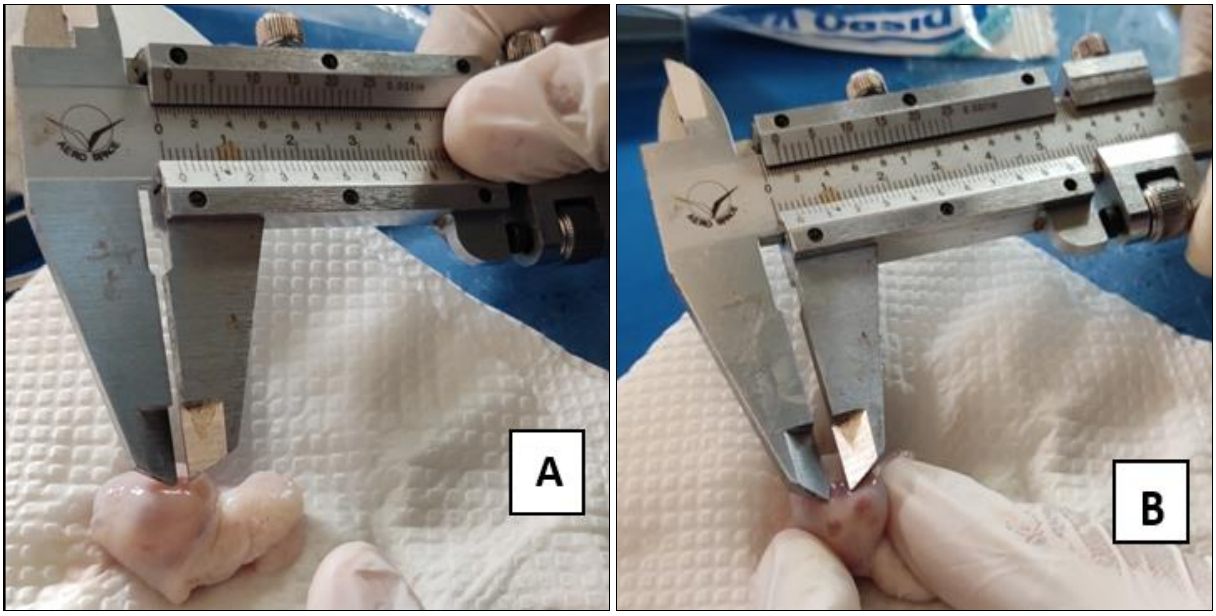

Plate 1: Measuring Follicles of Ovaries Using Vernier Caliper

2.4 Aspiration of Oocytes at Laboratory

Oocytes were collected by aspiration of follicles using an 18 G needle which was attached to a 5 ml disposable syringe. Aspirated contents were transferred to a centrifuge tube. This tube was placed at 37℃ in a tube warming unit for 10 minutes. The contents were then poured into a searching dish with a square grid pattern. The oocytes were searched and washed either in MOFA oocyte recovery media or DPBS fortified with 10% BSA @ 0.03 grams/ml followed by BO-Wash media. Grading was done after searching oocytes.

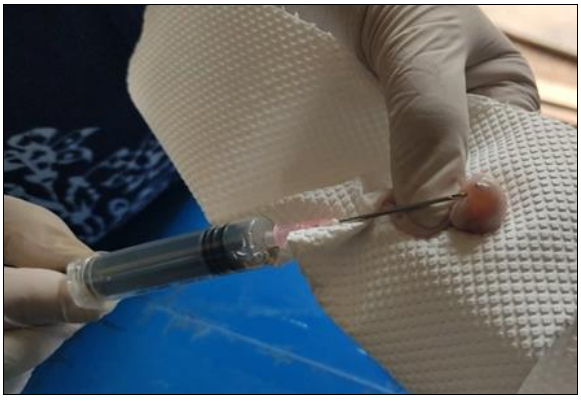

Plate 2: Aspiration of Ovarian Follicles Using 18 Gauge Needle Attached to 5 ml Syringe

Oocyte Evaluation and Grading Oocytes were examined under stereomicroscopy and classified based on their cumulus investment, compaction and ooplasm homogeneity as follows according to [3].

Table 1: Morphology of buffalo follicular oocytes

Category	Morphology of Follicular Oocytes
A	Dense and compact multilayered with (≥ 5) cumulus layers with homogenous ooplasm
B	Dense and compact multilayered with (3-4) cumulus layers with homogenous ooplasm
C	Less compact (1-2) cumulus layers with homogenous ooplasm
D	Denuded cumulus layer with homogenous ooplasm
E	Denuded cumulus layer with unevenly granulated ooplasm

Oocyte yield was counted and the oocyte recovery rate was determined as follows:

Oocyte recovery rate % = No. of oocytes recovered/ total no. of follicles x 100

3. Results

Buffalo ovarian follicles were graded using vernier caliper based on follicular diameter as small, medium and large (<3 mm, 3-5 mm, and >5 mm) respectively.

Table 2: (n=155): Grading of Buffalo Ovarian Follicle Oocytes Subjected to Fertilize with Conventional Non-Sorted Semen

Sr. No	No. of trails	Type of semen	No. of ovaries	No. of follicles			Total no. of follicles
				Small (<3mm)	Medium (3-5 mm)	Large (>5mm)	
1	1	CONVENTIONAL	10	14	9	3	26
2	2		20	19	7	6	32
3	3		10	15	6	1	22
4	4		10	13	9	5	27
5	5		10	21	7	5	33
6	6		10	22	8	4	34
7	7		10	13	4	2	19
8	8		10	19	11	9	39
9	9		10	15	12	9	36
10	10		10	13	10	10	33
11	11		10	17	5	4	26
12	12		10	18	9	4	31
13	13		10	10	9	9	28
14	14		15	19	8	4	31
Total			155	228	114	75	417
Mean ± SE			11.07 ± 0.774	16.286 ± 0.940	8.143 ± 0.592	5.357 ± 0.760	
SD			2.895	3.518	2.214	2.845	

Oocytes were subjected to fertilize with conventional non-sorted semen, out of 155 ovaries 228 small follicles, 114 medium follicles and 75 large follicles were observed. The overall percentage of small, medium and large follicles in the ovaries were recorded as 16.29 ± 0.94, 8.14 ± 0.60, 5.35 ± 0.76, respectively as depicted in table 2**.** A total, 417 surface follicles were measured using vernier caliper out of 155 ovaries by categorizing them based on follicular size diameter as small, medium and large respectively.

The grading of follicles based on the dimensions of buffalo ovarian follicles are depicted in table 2 and figure 1

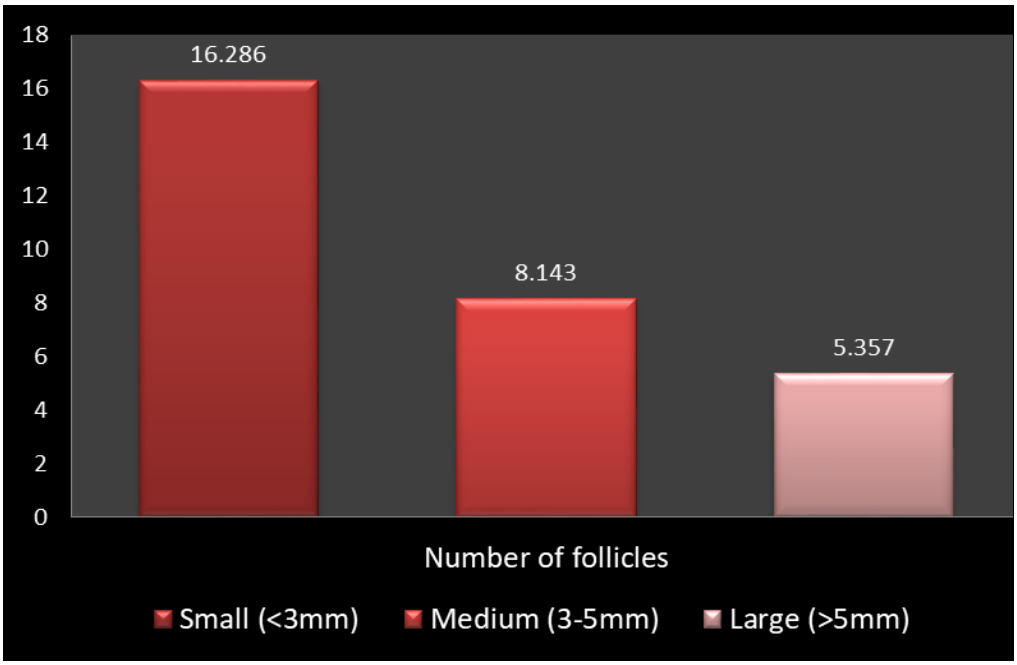

Fig 1: Grading of Follicles of Buffalo Ovaries Obtained From Abattoir. Oocytes Subjected to Fertilize in Conventional Non-Sorted Semen Trial

3.2 Aspiration of Buffalo Follicular Oocytes and Recovery Percentage

The technique used in the present study was aspiration of oocytes from the buffalo ovaries obtained from abattoir because it was a more practical method than others which enabled the quick collection of a significant quantity of oocytes within no time [26].

In the present study, the ovaries were obtained from the slaughtered female buffaloes with an unknown history. After obtaining ovaries from abattoir, oocytes having good cumulus layers, intact zona pelluicida, granularity of ooplasm were selected for further *in vitro* embryo production. Furthermore, poor recovery of immature oocytes in buffaloes is a primary problem. Since, there is low number of follicles in buffalo ovaries, lower oocyte yield is obtained. The ovarian follicles from buffaloes were aspirated by using an 18 gauge needle attached to 5 ml syringe which was collected in a centrifuge tube kept at 37℃ in tube warmer. In aspiration technique of oocyte recovery, mainly large sized follicles are focused, leaving small embedded follicles inaccessible which could be the cause of poor oocyte yield. The aspirated contents were poured into searching dish with square grid pattern for easier search of COCs in a stepwise manner and searching was carried out.

Table 3: Group I: Total number of COCs recovered by aspiration of buffalo ovarian follicles and recovery rate% when oocytes were subjected to fertilize using conventional non-sorted semen

Sr. No	No. of trails	Type of semen	No. of ovaries	Total no. of follicles	No. of COCs recovered by aspiration method	Recovery rate %
1	1	CONVENTIONAL	10	26	20	2%
2	2		20	32	30	1.50%
3	3		10	22	22	2.20%
4	4		10	27	27	2.70%
5	5		10	33	33	3.30%
6	6		10	34	34	3.40%
7	7		10	19	19	1.90%
8	8		10	39	39	3.90%
9	9		10	36	36	3.60%
10	10		10	33	33	3.30%
11	11		10	26	26	2.60%
12	12		10	31	31	3.10%
13	13		10	28	28	2.80%
14	14		15	31	31	2.06%
Total			155	417	409	38%
Mean ± SE			11.07 ± 0.774		29.214± 1.587	2.74 ± 0.194
SD			2.895		5.938	0.726

Number of COCs recovered when oocytes were fertilized using conventional non-sorted semen was 409 and the mean oocytes recovered per trail were 29.21 ± 1.58. The overall recovery rate of COCs (cumulus oocytes complexes) in conventional non-sorted semen *in vitro* fertilization trail was 38% and the mean oocytes recovery rate per trial was 2.74 ± 0.19% as depicted in table 3

Table 4: Grading of Ovarian Oocytes Based on the Cumulus Layers Surrounding the Oocytes Subjected to Fertilize with Conventional Non Sorted Semen

Sr No.	No. of ovaries	No. of COCs recovered by aspiration method	Total oocyte recovered				
			A	B	C	D	E
1	10	26	4 (15.38%)	6(23.07%)	8(30.76%)	4(15.38%)	4(15.38%)
2	20	32	7(21.87%)	5(15.62%)	9(28.12%)	7(21.87%)	4(12.50%)
3	10	22	5(22.72%)	3(13.63%)	7(31.81%)	2(9.09%)	5(22.72%)
4	10	27	4(14.81%)	6(22.22%)	9(33.33%)	3(11.11%)	5(18.51%)
5	10	33	6(18.18%)	6(18.18%)	6(18.18%)	8(24.24%)	7(21.21%)
6	10	34	6(17.64%)	4(11.76%)	2(5.88%)	10(29.41%)	12(35.29%)
7	10	19	2(10.52%)	3(15.78%)	7(36.84%)	4(21.05%)	3(15.78%)
8	10	39	8(20.51)	6(15.38%)	6(15.38%)	8(20.51%)	11(28.20)
9	10	36	7(19.44%)	7(19.44%)	8(22.22%)	5(13.88%)	9(25%)
10	10	33	3(9.09%)	5(15.15%)	9(27.27%)	6(18.18%)	10(30.30%)
11	10	26	2(7.69%)	1(3.84%)	7(26.92%)	4(15.38%)	12(46.15%)
12	10	31	7(22.58)	8(25.80%)	5(16.12%)	3(9.67%)	8(25.80%)
13	10	28	5(17.85%)	2(7.14%)	8(28.57%)	5(17.85%)	8(28.57%)
14	15	31	5(16.12%)	2(6.45%)	10(32.25%)	9(29.03%)	5(16.12%)
Mean ± SE	11.07±0.77	29.79±1.46	5.07±0.51 (16.74%)	4.57±0.56 (15.25%)	7.21±0.55 (25.26%)	5.57±0.66 (18.33%)	7.36±0.83 (29.87%)
SD	2.89	5.45	1.9 (4.86%)	2.10 (6.42%)	2.04 (8.61%)	2.47 (6.48%)	3.01 (29.67%)

In the present study, in conventional method, grade A oocytes were 5.07 ± 0.51, Grade B were 4.57 ± 0.56, Grade C were 7.21 ± 0.55, Grade D were 5.57 ± 0.66 and Grade E were 7.36 ± 0.83 respectively as depicted in table 4 and Fig 2. The percentage of these oocytes were 16.74% (A), 15.25% (B), 25.26% (C), 18.33% (D) and 29.87% (E) respectively as depicted in table 4 and figure 2.

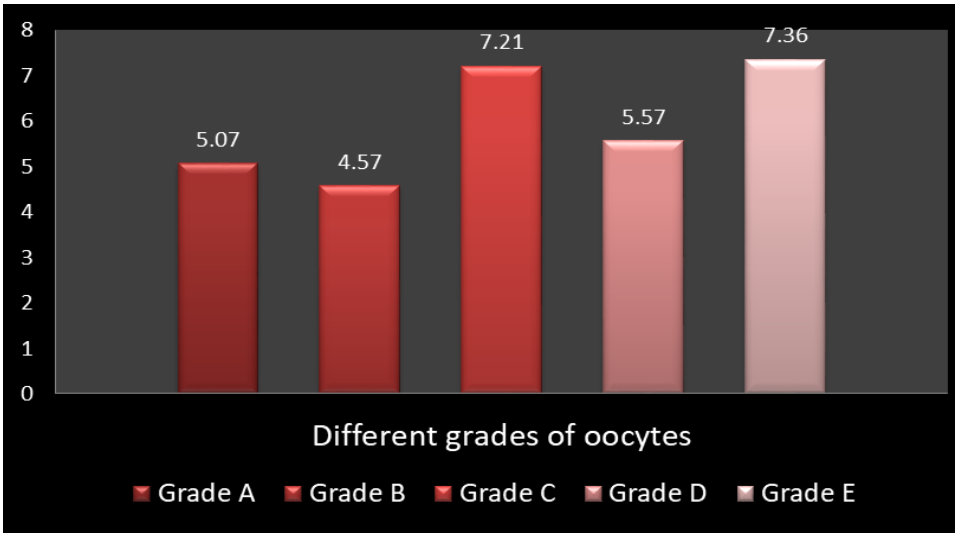

Fig 2: Grading of Ovarian Oocytes Based on the Compactness of Cumulus Layers. Oocytes Subjected to Fertilize in Conventional Non-Sorted Semen Trial

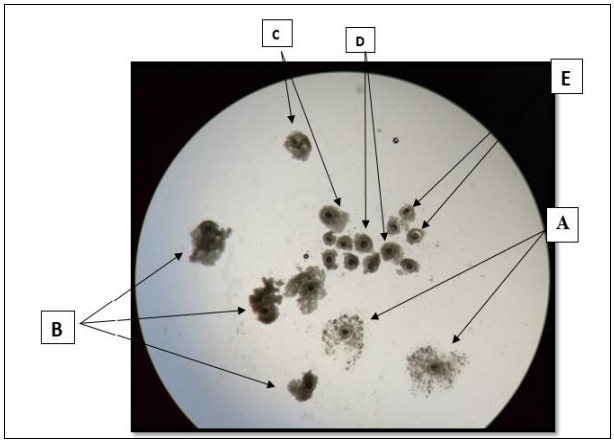

Plate 3: Grading of Oocytes (A, B, C, D and E) after Aspiration from Slaughtered Ovaries

4. Discussion

In the present study, the number of small, medium and large follicles were far more than the findings of Jamil *et al*., (2007) [8] who reported 4.36 ± 0.31 small, 5.79 ± 0.75 medium and 0.82 ± 0.07 large follicles whereas Elbaz *et al*., (2019) [4] reported 0.95 small, 0.57 medium and 0.24 large follicles respectively when follicles of 155 ovaries were measured using a vernier calliper. This could be because of the negative effect of CL which was surpassed by the presence of a good number of follicles on ovary in the present trial.

Khan *et al*., (1997) [11] recovered 635 COCs from 192 ovaries; Nandi *et al*., (2000) [18] recovered 425 COCs from 457 ovaries; Jamil *et al*., (2008) [9] recovered 363 COCs from 298 ovaries of riverine buffaloes; Rajesh *et al*., (2018) [21] recovered 376 COCs from 234 ovaries; Rao & Uma (2012) [22] recovered 114 COCs from 48 ovaries; Hammad *et al*., (2014) [6] recovered 665 COCs from 206 ovaries of Egyptian buffaloes; Shahid *et al*., (2014) [25] recovered 409 COCs from 301 ovaries of Nili Ravi buffaloes; and Rajesh *et al*., (2018) [21] recovered 1909 COCS from 1010 ovaries; Elbaz *et al*., (2019) [4] recovered 335 COCs from 187 ovaries.

In the present study, mean oocyte recovery rate was 2.74±0.19% These values were in accordance with Jamil *et al*., (2008) [9] who reported oocytes recovery rate of 2.45±0.91% during peak breeding season and 1.93 ± 0.68% during low breeding season respectively. Also, Rajesh *et al*., (2018) [21] recorded 1.60% and 1.78% oocyte recovery rate.

Furthermore, Nandi *et al*., (2000) [18] recovered lower oocytes 0.12% whereas Rao & Uma (2012) [22] recorded higher oocytes 4.60 ± 0.33% than the present study.

The present study was conducted during climatic variation therefore the oocytes recovered in conventional non-sorted semen trails were more compared to sex sorted semen trials which were conducted during comparatively warmer climatic conditions. Usually buffaloes are slaughtered because of unproductiveness and poor fertility Selvaraju *et al*., (2008) [24]. The lower oocyte recovery rate in buffaloes may be due to anestrous condition as well as acyclicity and also because of presence of CL Totey *et al*., (1992) [27], Das *et al*., (1996) [2]. This is because follicular development is restricted when luteal cells occupy the major part of the ovary Kumar *et al*., (1997) [14]. Ovaries that do not possess CL have higher number of follicles being recovered and also higher grade COCs than those non CL bearing ovaries Khandoker *et al*., (2011) [12]. Furthermore, the stroma of buffalo ovaries is thick and follicles are deeply embedded in them and therefore aspiration of oocytes from follicles gets difficult Selvaraju *et al*., (2008) [24], Hammad *et al*., (2014) [6]. The ovaries which had CL yielded a lower number of oocytes as compared to non CL bearing ovary as was discussed by Kumar *et al*., (1997) [14]. The lower number could be because of greater mechanical damage induced on the granulose cells during the procedure of recovery Jainudeen *et al*., (1993) [7].

After searching the oocytes, they were washed in an oocytes recovery medium containing DPBS fortified with 10% BSA @ 0.03 grams/ml and were subsequently graded Kataska (1984), Nandi *et al*., (2000) [18], Elbaz *et al*., (2019) [4].

The oocyte quality plays a major role in assessment of oocyte development therefore it is essential to grade the oocytes which in this study were correlated with Elbaz *et al*., (2019) [4] who got similar results, grade A oocytes were 5 ± 0.68, Grade B was 6.1 ± 1.1, Grade C was 4.8 ± 0.84 and Grade D were 4.8 ± 0.55 respectively.

Percentage of graded oocytes based on the compactness of cumulus layers were in accordance with Khan *et al*., (1997) [11] who recorded A (18.42%), B (15.43%), C (19.37%) and D (46.77%) whereas Samad *et al*., (1998) recoded A (18.42%),

B (15.43%), C (19.57%) and D (46.77%) respectively. Bhajoni *et al*., (2018) [1] recorded A (47.58%), B (37.42%), C (8.82%) and D (6.12%) oocytes which were higher than the present study and Nandi *et al*., (2000) [18] recorded A (0.10%), B (0.13%) and C (0.11%) oocytes which were lower than the present study.

5. Conclusion

The overall percentage of small, medium and large follicles in the ovaries were recorded as 16.29 ± 0.94%, 8.14±0.60%, 5.35 ± 0.76% and 11.5 ± 0.71%, 7.14±0.48%, 4.78 ± 0.49% respectively in conventional non-sorted semen trail and sex sorted semen trial respectively. The overall recovery rate of cumulus oocyte complexes was 38%. The percentage of recovered oocytes were 16.74% (A), 15.25% (B), 25.26% (C), 18.33% (D) and 29.87% (E) and 20.82%. Aspiration method of oocyte recovery is practical and less time consuming method.

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