Cleavage Rate of *in vitro* Matured Oocytes Fertilized with Conventional Semen in Buffaloes

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ABSTRACT

Background: *In vitro* embryo production in buffaloes has gained much importance in this current scenario due to ever increasing population and high demand of milk and meat. Slaughter house derived bubaline ovaries are a cheap and abundant source of cumulus oocyte complexes.

Methods: Oocytes from the buffalo ovarian follicles were recovered by aspiration technique as it facilitates quick recovery. Total 155 ovaries were used in the present study. Surface follicles were measured using vernier calliper and categorized into three groups *viz*. < 3 mm, 3-5 mm and > 5 mm based on follicular diameter and oocytes were processed for IVM, IVF and IVC using conventional non sorted semen.

Result: 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. 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. Maturation rate of oocytes were 81.96±2.70%. Fertilization rate was 74.98±3.87%, Cleavage rate % was 40.84±2.51% and Blastocyst percentage was 21.57±1.75% respectively. Application of *in vitro* embryo production technique using slaughter house ovaries can salvage the genetic potential of bubaline species.

Key words: Aspiration, Blastocyst, Cleavage, Conventional semen.

INTRODUCTION

There are about 207 million buffalo population in the world out of which roughly 97% are found in the Asian region (FAO, 2020). Countries with largest number of dairy buffaloes are India, Pakistan, China, Egypt followed by Nepal (FAO, 2020). The total buffalo population in India is 109.85 million (Livestock Census, 2019) ranking number one in the world with huge genetic diversity and showing an increase of about 1% over previous (Livestock Census, 2012) where the population was 108.70 million.

There is an imminent need to utilize recent advances in the field of reproduction to improve the outcome of bubaline industry. Laboratory production of embryos allows mass scale production of embryos for practical and scientific purposes as well as for the exploitation of oocytes from donors. Buffalo females have few primordial *viz*. 12000-19000 follicles in cortical region of ovaries and have higher rate of follicular atresia (Jamil *et al*., 2008). Owing to the demand of technical expertise, cost involved in the oocyte recovery from live animals is quite high compared to slaughterhouse derived oocytes which are cheaper and easily available source of COCs for *in vitro* production of embryos.

Fertilization process is considered as the most critical step for IVEP procedure in buffalo, as cleavage rates are lower in this species (Neglia, 2003). Furthermore, blastocyst rate of buffalo is poor (20%) when compared to cattle (35-48%) (Liang *et al*., 2008). These factors limits the applicability of IVEP in bubaline species (Sadeesh, 2015). It is therefore need to improve IVEP technology in buffaloes for improvement of genetic potential of ND breeds. For production of embryos *in vitro*, combinations of techniques are required *viz*. collection of immature oocytes, *In vitro* Maturation, *In vitro* fertilization and subsequent *In vitro* Culture.

In vitro maturation is a method in which 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 method (Khan *et al.,* 1997, Mehmood *et al.,* 2011, Hammad *et al*., 2014) and are allowed to mature in the laboratory which is a crutial step for fertilization and subsequent embryonic development. Maturation rate in cattle and buffaloes is almost similar (94 v/s 87% respectively) but a slightly lower cleavage rate is observed in buffalo as compared to cattle (84 v/s 65%) (Gasparrini, 2002, Neglia, 2003).

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In vitro fertilization has drawn interest of many researchers as it can salvage the genetic potential from infertile female and can also yield large number of embryos from slaughter house derived oocytes which may go waste otherwise (Kumar, 2012). Also, slaughter house ovaries are the potential source of immature oocytes. Therefore, large number of oocytes can be easily obtained for standardizing and optimizing various reproductive biotechnological techniques.

MATERIALS AND METHODS

The present study was conducted from the month of January 2020 to December 2020 by using 155 slaughter house derived ovaries for experiment at Department of Animal Reproduction, Gynaecology and Obstetrics, Krantisinh Nana Patil College of Veterinary Science, Shirwal, Dist. Satara; Maharashtra, India and Dr. Vijaypat Singhania, Centre of Excellence, Vadgaon, Rasai, Taluka-Shirur, Maharashtra, India.

Medium

IVM media, IVF media, IVC media, Mineral oil, Washmedia, Percoll, Aliquot H, Aliquot PHE, Sperm(Dilution for percoll), Water for Humidification, these chemicals and media were obtained from Vitrogen (Av. Cel. José Nogueira Terra, 203 Cravinhos-SP, Brasil), MOFA Oocyte recovery Media (Global, Verona, WI, USA) and plasticware were procured from ThermoFisher SCIENTIFIC(Mumbai, India), SEWA MEDICALS LTD (Pune, India) and CORNING® (NY, USA).

Collection of ovaries

A total 155 ovaries were procured from the local slaughter house. Immediately after collection the ovaries were placed in a stainless steel thermos flask containing 0.9% normal saline solution along with Inj. Gentamycin @ 50 µg/ml and were transported to laboratory within 2 hours of slaughter. Oocytes were collected by aspiration of follicles using 18 G needle attached to a 5 ml disposable syringe. The oocytes were searched and washed in MOFA oocyte recovery media. Grading was done after searching of oocytes. Oocyte evaluation and graded oocytes were examined under stereomicroscopy and classified based on their cumulus investment, compaction and ooplasm homogeneity according to (de Loos *et al*., 1992) as A, B, C, D and E respectively.

In vitro maturation of buffalo oocytes using vitrogen media (Álvarez-Gallardo *et al*., 2020)

Aspiration of oocyte and transferring COCs to MOFA oocytes recovery media and later in a centrifuge tube. Pour the contents of centrifuge tube into searching dish.Then search the oocytes from searching dish, put and wash them into 3 droplets of 70 µl of Oocyte washing medium (Vitrogen) and a drop of 100 µl maturation mediain 60mm petri dish. Then finally put into maturation well and keep into Benchtop BT37 incubator at 5/6/90 gas (5% CO_2:6% O_2: 90% N_2 in Air) 38.5°C humidified atmosphere for 22-24 hrs.Within 20 minutes of aspiration place graded oocytes for IVM.

In vitro fertilization of buffalo oocytes using vitrogen media

Make IVF medium one day before. Make 3 droplets of 100 µl of IVF media (Vitrogen) into 60×15 mm Petridishes for oocytes washing. Add 70 µl of IVF medium + BO-Oil (~750 µl) *i.e.* (35 µl IVF media layered with 750 µl oil and then immerse the pipette tip and touch the 35 µl drop and release 2nd 35 µl droplet in order to increase the surface area for sperms to each well of Nunc 4 well plate (pipette 1ml sterile H_2O incenter well for humidification purpose) and equilibrate in bench top BT37 incubator having trigas (5% CO_2:6% O_2: 90% N2 in air) 38.5°C humidified atmosphere one day before of fertilization.

Semen setup

Thaw the semen straw in thaw unit water bath at 37°C for 30 seconds and check sperm motility. The volume of sperm for fertilization *i.e.* 2×10^6 sperms add to IVF dish containing oocytes. After maturation, on 22th hr start the washing of matured oocytes into 3 droplets of 100 µl of IVF media, wash well then remove and place into final 70 µl fertilization micro drop along with sperms in bench top BT37 incubator having trigas (5% CO_2 : 6% O_2 : 90% N_2 in Air) at 38.5°C. Humidified atmosphere for 18-20 hrs.

In vitro culture of buffalo embryos using vitrogen media

At 20th hr post IVF, remove fertilized potential zygotes and wash through 4 drops of 70 µl of warmed drop of IVC medium. In 1st drop, wash the potential zygotes then remove the cumulus in 2nd drop with help 5ul pipette (remaining hard cumulus of potential zygotes remove with help of stripper having pipettetip diameter of 135 µm). Wash denuded potential zygotes through remaining 2 drops of pre warmed IVC media. After washing, culture the potential zygotes in designated Nunc well containing 500 µl IVC medium + 400 µl oil in Bench top BT37 incubator having trigas (5% CO_2 : 6% O_2 : 90% N_2 in air) at 38.5°C humidified atmosphere for 7 day until the embryo development.

RESULTS AND DISCUSSION

The grading of follicles based on the dimensions of buffalo ovarian follicles in group I are depicted in Table 1. In group I where 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 1. 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.

In the present study, the number of small, medium and large follicles were far more than the findings of Bhajoni *et al.* (2018) who reported 4.36±0.31 small, 5.79±0.75 medium and 0.82±0.07 large follicles whereas Elbaz *et al.* (2019) reported 0.95 small, 0.57 medium and 0.24 large

follicles respectively when follicles of 155 ovaries were measured using vernier calliper. This could be because of the negative effect of CL which was surpassed by the presence of good number of follicles on ovary in present trial.

Aspiration of buffalo follicular oocytes and recovery percentage

The technique used in present study was aspiration of oocytes from the buffalo ovaries obtained from abattoir because it was more practical method than others which enabled quick collection of significant quantity of oocytes within no time (Katska, 1984; Totey *et al*., 1992; Mehmood *et al*., 2011; Rao and Uma Mahesh 2012; Shahid *et al*., 2014). No significant difference was observed in oocyte maturation, fertilization and development *in vitro* among the above mentioned methods of recovering oocytes (Pawshe *et al*., 1994). Thus in present study, comparatively practical method was used for recovery of COCs from ovaries.

Number of COCs recovered when oocytes were fertilized using conventional non sorted semen was 409 and mean oocytes recovered per trail were 29.21±1.58. Overall recovery rate of COCs (cumulus oocytes complexes) in conventional non sorted semen *in vitro* fertilization trail was 38% and mean oocytes recovery rate per trial was 2.74±0.19% as depicted in Table 2.

Khan *et al*. (1997) recovered 635 COCs from 192 ovaries; Nandi *et al*. (2000) recovered 425 COCs from 457 ovaries; Jamil *et al*. (2008) recovered 363 COCs from 298 ovaries of riverine buffaloes; Hammad *et al*. (2014) recovered 665 COCs from 206 ovaries of Egyptian buffaloes; and Elbaz *et al*., (2019) recovered 335 COCs from 187 ovaries.

In the present study, mean oocyte recovery rate per trail was 2.74±0.19%. These values were in accordance with Jamil *et al*. (2008) who reported oocytes recovery rate of 2.45±0.91% during peak breeding season and 1.93±0.68% during low breeding season respectively.

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

Usually buffaloes are slaughtered because of unproductiveness and poor fertility (Selvaraj *et al*., 2008). 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, Das *et al*., 1996). This is because follicular development is restricted when luteal cells occupy major part of the ovary (Kumar *et al*., 1997). Ovaries which 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). Furthermore, the stroma of buffalo ovaries are thick and follicles are deeply embedded in them and therefore aspiration of oocytes from follicles gets difficult (Selvaraj *et al*., 2008, Hammad *et al*., 2014). The ovaries which had CL yielded lower number of oocytes as compared to non CL bearing ovary as it was discussed by (Kumar *et al*. 1997). The lower number could be because of greater mechanical damage induced on the granulose cells during the procedure of recovery.

Evaluation of oocytes and subsequent grading of COCs

Oocytes were examined under stereomicroscopy and classified according to their compaction, number of cumulus

Table 1: Group 1(n=155): Grading of buffalo ovarian follicle oocytes subjected to fertilize with conventional Non-sorted semen.

No. of trails	Type of semen	No. of ovaries	No. of follicles			Total no. of follicles
			Small (<3mm)	Medium (3-5 mm)	Large (>5 mm)	
1		10	14	9	3	26
2		20	19	7	6	32
3		10	15	6	1	22
4		10	13	9	5	27
5		10	21	7	5	33
6		10	22	8	4	34
7	Conventional	10	13	4	2	19
8		10	19	11	9	39
9		10	15	12	9	36
10		10	13	10	10	33
11		10	17	5	4	26
12		10	18	9	4	31
13		10	10	9	9	28
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	

cell layers and homogeneity of ooplasm (Ravindranatha *et al*., 2003) into 5 categories as:

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.

Accordance to above criteria the oocytes were classified as A, B, C, D and E (De Loos *et al*., 1992, Khan *et al*., 1997, Jamil *et al*., 2008).

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 3. The percentage of these oocytes were 16.74% (A), 15.25% (B), 25.26% (C), 18.33% (D) and 29.87% (E) respectively.

The oocyte quality plays 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) who got similar results, grade A oocytes were 5±0.68, Grade B were 6.1±1.1, Grade C were 4.8±0.84 and Grade D were 4.8±0.55 respectively.

Percentage of graded oocytes based on the compactness of cumulus layers in both the groups were in

Table 2: 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.

No. of trails	Type of semen	No. of ovaries	Total no. of follicles	No. of COCs recovered by aspiration method	Recovery rate %
1		10	26	20	2%
2		20	32	30	1.50%
3		10	22	22	2.20%
4		10	27	27	2.70%
5		10	33	33	3.30%
6		10	34	34	3.40%
7	Conventional	10	19	19	1.90%
8		10	39	39	3.90%
9		10	36	36	3.60%
10		10	33	33	3.30%
11		10	26	26	2.60%
12		10	31	31	3.10%
13		10	28	28	2.80%
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



Plate 1: Cumulus expansion of oocytes post IVM in conventional non sorted semen trial.

accordance with Khan *et al*. (1997) 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) 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) recorded A (0.10%), B (0.13%) and C (0.11%) oocytes which were lower than the present study.

Maturation rate of COC's subjected for maturation using conventional non sorted semen

In the present study, average number of COCs used for IVM in 14 trials of non sorted group was 29.21±1.58. Maturation rate of oocytes were 81.96±2.70% in conventional non sorted trail as depicted in Table 4. This maturation rate was in accordance with (Arul *et al*., 2017, Totey *et al*., 1992) who got maturation rate as 81.7 1±1.81% and 81.7±14.5% respectively as depicted in Plate 1.

Table 3: 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%)	2.1 (6.42%)	2.04 (8.61%)	2.47 (6.48%)	3.01 (29.67%)

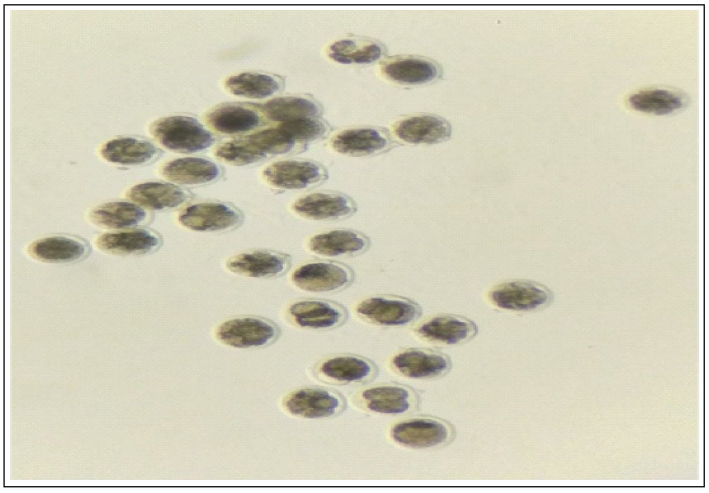

Plate 2: Cleavage of potential zygotes observed on day 4 post *in vitro* fertilization in conventional non sorted semen trial.

Maturation rates obtained by (Kumar and Maurya 2000, Adlak *et al*., 2007, Leal *et al*., 2007 and Elbaz *et al*., 2019) was 53.35%, 71.05%, 36.68% and 28.82% respectively were lower than the present study. Furthermore maturation rates observed were higher in studies of (De Matos *et al*., 1995, Umadevi *et al*., 1998 and Habeeb *et al*., 2018) was 90±2%, 83.33% and 86.5% respectively. This maturation rate was in accordance with (Suresh *et al*., 2009) who got maturation rate as 78.36±2.16% respectively.

In conclusion, such variations in the maturation rates could be due to variations in the number of COCs used for IVM, variation in the choice of media used must have influenced maturation rates.

The average number of oocytes used for fertilization in conventional trial was 23.78±1.33 and fertilization rate was 74.98±3.87% as depicted in Table 5.

Fertilization rates were in accordance with (Nain *et al*., 2006, Barkawi *et al*., 2007) who recorded 60.70% and 80.2% of fertilization rates. Furthermore, Bavister *et al*. (1992) recoded higher fertilization rates as 85±5% and (Jamil *et al*. (2008); Palta and Chauhan (1998) and Baczkowski *et al*. (2004) recorded 63.75±2.81%, 40-55% and 57.1% respectively which were lower than present study.

Table 4: Maturation rate of COC's subjected for maturation using conventional non sorted semen.

No. of trails	Type of semen	No. of ovaries	No. of COCs recovered by aspiration method	IVM	Maturation rate%
1		10	20	20	100%
2		20	30	30	90%
3		10	22	22	86.36%
4		10	27	27	85.18%
5		10	33	33	75.75%
6		10	34	34	64.70%
7	Conventional	10	19	19	78.94%
8		10	39	39	71.79%
9		10	36	36	94.44%
10		10	33	33	81.81%
11		10	26	26	65.38%
12		10	31	31	87.09%
13		10	28	28	82.14%
14		15	31	31	83.87%
Mean±SE		11.071±0.774	29.214±1.587	29.214±1.587	81.961±2.703
SD		2.895	5.938	5.938	10.113

Table 5: Fertilization rate of COC's subjected for fertilization using conventional non sorted semen.

Trial no.	Type of semen	No. of ovaries	No. of COCs recovered by aspiration method	IVF	Fertilization rate%
1		10	21	20	85%
2		10	20	27	62.96%
3		15	21	19	78.94%
4		10	21	23	82.60%
5		10	17	25	40%
6		15	26	22	72.72%
7	Conventional	10	21	15	73.33%
8		10	23	28	78.57%
9		10	14	34	76.47%
10		15	13	27	77.78%
11		10	27	17	52.94%
12		10	22	27	92.59%
13		10	16	23	91.30%
14		10	20	26	84.61%
Mean±SE		11.071±0.774	20.143±1.079	23.786±1.335	74.986±3.872
SD		2.895	4.036	4.995	14.49%

Cleavage rate of potential zygotes in conventional unsorted group

In the present study, average number of potential zygotes used in IVC are 17.92±1.43 and the number of average cleaved embryos obtained were 7.35±0.76 in conventional trial whereas cleavage rate % was 40.84±2.51% as depicted in Table 6 which was in accordance with Chauhan *et al*., (1998) and Nandi *et al*. (2000) who observed cleavage rate of 40%

Cleavage rate was in accordance with (Nain *et al*., 2006 and Aquino *et al*., 2013) was 40-50% and 40% respectively. Cleavage rates observed (Madan *et al*., 1994, Mehmood *et al*., 2007, Suresh *et al*., 2009 and Gonclaves *et al*., 2013) were higher 54.4%, 75.9%, 52.14±1.15% and 71.8% respectively. Furthermore, cleavage rates observed (Ocampo *et al*., 2015, Sadeesh *et al*., 2016) were lower 39.6%, 22.5±4.2% respectively than present study as depicted in Plate 2.

In the present study, average number of blastocyst formed were 3.92±0.46 and blastocyst percentage was 21.57±1.75% as depicted in Table 7 which was in accordance with (Neglia *et al*., 2003) who observed blastocyst % of 19.9±4.2%. Blastocyst % observed was in accordance with (Ocampo *et al*., 2016 and Sadeesh *et al*., 2016) as 19.6%, 20.4±2.5% respectively. Blastocyst percentage was higher than present study (Aquino *et al*., 2013, Ocampo *et al*., 2015 and Shabankareh *et al*., 2015) as 32.50%, 22.5% and 38.7% respectively and lower than present study (Palta and Chauhan *et al*., 1998, Boni *et al*., 1999, Prasad *et al*., 2013) as 10-15%, 13.5%, 10.65±0.45% respectively in Plate 3.

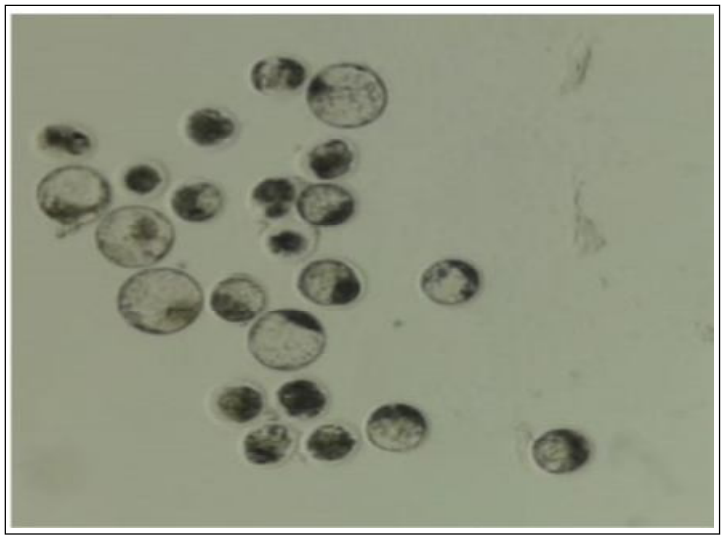

Plate 3: Blastocyst formed on day 7 post *in vitro* culture in conventional non sorted semen trial.

Table 6: Number of COC's subjected for IVC and their subsequent cleavage rate in conventional non sorted semen trial.

Trial no.	Type of semen	IVC	no. of embryos cleaved	Cleavage rate %	Significance
1		17	4	23.52%	
2		17	5	29.41%	
3		15	8	53.33%	
4		19	7	36.84%	
5		10	4	40%	
6		16	6	37.50%	
7	Conventional	11	6	54.54%	NS
8		22	9	40.90%	
9		26	12	46.15%	
10		21	8	38.09%	
11		9	3	33.33%	
12		25	9	36%	
13		21	10	47.61%	
14		22	12	54.54%	
Mean±SE		17.929±1.431	7.357±0.768	40.84±2.515	
SD		5.356	2.872	9.41%	

P<0.01.

Table 7: Number of blastocyst formed and blastocyst percentage of embryos in conventional unsorted group.

Trial no.	Type of semen	IVC	Blastocyst formed	Blastocyst %	Significance
1		17	2	12%	
2		17	3	18%	
3		15	5	33%	
4		19	4	21%	
5		10	2	20%	
6		16	4	25%	
7		11	3	27%	NS
8	Conventional	22	4	18%	
9		26	7	27%	
10		21	4	19%	
11		9	1	11%	
12		25	5	20%	
13		21	4	19%	
14		22	7	32%	
Mean±SE		17.929±1.431	3.929±0.462	21.571±1.750	
SD		5.356	1.73	6.55%	

P<0.01.

CONCLUSION

In vitro fertilization of oocytes recovered from slaughter house ovaries is the cheapest method of production of large number of embryos using conventional semen for *in vitro* embryo production in buffaloes.

Conflict of interest

All authors declare that they have no conflict of interest.

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