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Ovum Pick Up and *in vitro* Embryo Production using Sex Sorted Semen in Cows

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ABSTRACT

Background: Ovum pick-up, a technique for extracting immature oocytes from antral follicles, with *in vitro* embryo production, is a way to produce large number of embryos from live donors.

Methods: By transvaginal ultrasound-guided ovum pick-up, oocytes were collected from large (9-19 mm), medium (4-8 mm) and small (2-3 mm) ovarian follicles of donor cows (n=8). The recovered oocytes were classified as A, B, C, D and E grades. Oocytes were subjected to *in vitro* maturation, fertilization (with sex sorted semen) and culture. Cleavage and blastocyst rates were recorded on day 4 and day 7 of culture respectively.

Result: Total of 278 follicles were aspirated. The follicles aspirated per cow per OPU session was 12.67±1.86. The oocyte recovery rate was 45.31±7.35. Mean percentage of grade A, B, C, D and E oocytes was 15.69±3.91, 24.97±3.02, 37.53±3.45, 19.44±3.76 and 2.38±2.38 respectively. *In vitro* maturation rate (IVM%) was 91.71±2.38% and fertilization rate was 90.18±1.52%. Cleavage and blastocyst rates observed was 55.1±5.90 and 24.81±2.95% respectively. The mean percentages of different grades of embryos observed was, code 1: 21.13±2.78, code 2: 14.41±3.25, code 3: 19.56±4.11 and code 4: 44.9±5.90%. Transvaginal ovum pick up can be performed repeatedly without hormonal stimulation. Sex sorted semen can be used for *in vitro* production of female embryos.

Key words: *In vitro*, Ovum pick-up, sex sorted semen, Ultrasound-guided.

INTRODUCTION

Ovum pick-up (OPU) is a technique for extracting immature oocytes from antral follicles that, when combined with *in vitro* embryo production (IVEP), is regarded the most efficient way to produce a large number of embryos from live donors, assuring the survival of endangered animals. Furthermore, OPU is a viable alternative to MOET (Multiple Ovulation and Embryo Transfer) for cattle embryo production as it can be carried out successfully regardless of the donor's reproductive status (*i.e*. acyclic and sub fertile cows, animals with patent tubes and those that are not responsive to MOET treatments) (Galli *et al.,* 2014). Success rate of OPU depends on the number and quality of follicles present on the ovaries during OPU. The number of follicles available for puncture depends upon the stage of the estrous cycle (Garcia and Salaheddine, 1998).

The use of sexed semen enables the selection of possibly superior females and the production of replacement heifers from these animals. In comparison to conventional semen, the genetic gain will be increased, along with the decrease in the cost of progeny testing, embryo transfer and genetic markers. For IVF only about 600-1500 sorted sperms are needed for fertilizing an oocyte (Boneya, 2021).

Considering the above things, the present study was conducted with the objectives to determine the efficiency of trans-vaginal ultrasound guided aspiration of ovarian follicles (Ovum Pickup) in cows, to study *in vitro* fertilization of oocytes using sex sorted semen, cleavage and blastocyst rates.

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MATERIALS AND METHODS

The present research work wascarried out on cows (n=8) with regular reproductive cyclicity in 6 OPU-IVEP trials at IVF Laboratory, Bull Mother Farm, Tathawade, Pune using IVF Bioscience media during the period of May to November 2021. The transvaginal ultrasound-guided follicular aspiration procedure was followed repeatedly once a week. Underan epidural anesthesia (3-5 ml of 2% lignocaine hydrochloride) at sacrococcygeal space, back raking of the

rectum was done and perineal region was cleaned thoroughly. Convex ultrasound 7.5-MHz trans-vaginal transducer with plastic handle was used to visualize the ovaries on the real time B mode ultrasound monitor (IMV, Model- Exago, France). After inserting the probe into the vaginal canal, all the visible follicles >2 mm were aspirated using an 18-gauge, 60 mm needle attached to needle holder, suction pump (maintained at 70-80 mmHg vacuum pressure) by means of silicone tubing (length-2 m, internal diameter-2 mm) passing into a 50-ml conical tube (maintained at 37°C temperature in tube warmer) for collection of the follicular fluid containing the oocytes. The follicles aspirated were categorized based on diameter as small (2-3 mm), medium (4-8 mm) and large (9-19 mm) follicles.

Recovery of oocytes

Immediately after OPU the follicular fluid containing COCs was strained by using a 100 µm cell strainer. Then COCs were washed into a 60 mm searching dish using flush media. After searching through the dish systematically under stereo zoom microscope, the cumulus-oocyte-complexes (COCs) were identified and graded morphologically as A, B, C, D and E grades (Khan *et al.*, 1997).

***In vitro* maturation**

All the recovered COCs were washed in 5 drops of 70 µl wash media and then rinsed once in 100 µl BO-IVM drop. Then COCs were transferred to a 4 Well Plate containing 100 µl of BO-IVM medium with 800 µl oil overlay (25 COC sper well of 100 µl BO-IVM) and incubated at 38.5°C at 6% CO_2, 5% O_2 and 89% N_2 in the bench top incubator for 20 to 22 hours.

***In vitro* fertilization**

Matured COCs were removed from the incubator, evaluated for cumulus expansion, washed in 5 drops (70 µl each) of wash media and then rinsed in 100 µl BO-IVF media. Then COCs were transferred to a well of BO-IVF 4 WP containing 100 µl IVF drop with oil overlay and incubated at 38.5°C, 6% CO_2, 5% O_2 and 89% N_2 in humidified atmospheric whilst the semen is prepared.

Semen preparation

2 ml of BO-Semen Prep medium was added into two 15 ml centrifuge tubes and labelled them as Tube A and Tube B. Frozen thawed (at 37°C for 30 seconds) semen was added to tube A for centrifugation. Some sperm from the straw was added to a warmed glass slide to evaluate the motility under a microscope. Tube A was centrifuged for 5 minutes at 328 × g. Supernatant was removed from tube A and then 2 ml of BO-Semen Prep from tube B was added to tube A, mixed gently and centrifuged again for 5 minutes at 3 28 × g. Again the supernatant was removed. To achieve a recommended final concentration of 2.0×10^6 sperm/ml in the IVF medium Neubauer's counting chamber was used. Calculated volume of sperms us pension was added to IVF well containing *in vitro* matured oocytes and incubated at 38.5°C, 6% CO_2, 5% O_2 and 89% N_2 in humidified atmosphere in benchtop incubator for 22 h.

***In vitro* culture**

Inseminated oocytes were removed from the 4 well plate, washed and denuded by gentle pipetting in 5 drops (70 µl each) of BO-Wash medium. Then the oocytes were rinsed in a 100 µl drop of BO-IVC medium and transferred to 4WP with a 100 µl drop of BO-IVC medium with 800 µl oil overlay and cultured at 38.5°C, 6% CO_2, 5% O_2 and 89% N_2 humidified atmosphere in benchtop incubator. On day 4 of culture, cleavage rate was observed and all the embryos were transferred to a new 4WP with BO IVC media with oil overlay and incubated again. On day 7 blastocyst rate was observed.

Grading of embryos

Embryo quality codes were given based on morphological integrity of embryos. The codes range from "1" to "4" (Bó and Mapletoft 2018): Code 1- Excellent or Good, Code 2- Fair, Code 3- Poor and Code 4- Dead or degenerating.

Statistical analysis

Collected data was analyzed statistically as per the methods described by Snedecor and Cochran (1994) and Student's test was applied using IBM SPSS Software version 20.

RESULTS AND DISCUSSION

In the present study, 19 OPU (Ovum Pickup) sessions were conducted for which 8 donor cows (n=8) were subjected to ultrasound guided follicular aspiration. 278 follicles were aspirate din total. The mean number of OPU sessions conducted per donor cow was 2.38±0.60. The number of large, medium and small follicles aspirated per cow was 3.13±1.19, 14.63±5.89 and 17.0 ±7.01 respectively. The total follicles aspirated per cow and follicles aspirated per cow per OPU session were 34.75±12.83 and 12.67±1.86 respectively (Table 1).

Table 1: Grading of Aspirated Follicles, follicles aspirated per session per cow and oocyte recovery rate.

	Donors	No. of OPU sessions	No. of follicles aspirated			Total no of follicles	Follicles/ session/ cow	COCs recovered	Oocyte recovery %
			Large 9-19 mm	Medium 4-8 mm	Small 2-3 mm				
Total	8	19	25	117	136	278		145	
Mean±Sem		2.38±0.6 0	3.13±1.1 9	14.63±5. 89	17.0 ±7.01	34.75±12.83	12.67±1.8 6	18.13±6.66	45.31±7.35
SD		1.69	3.36	16.67	19.81	36.3	5.25	18.85	20.79

In total, 145 COCs were recovered (Fig 1). The mean number of COCs recovered from each donor cow was 18.13±6.66 and the mean oocyte recovery rate was 45.31±7.35 (Table 1). Percentage of grade A, B, C, D and E oocytes was 15.69±3.91, 24.97±3.02, 37.53±3.45, 19.44±3.76 and 2.38±2.38respectively (Table 2). More number of grade C oocytes were recovered followed by grades B, D, A and E oocytes.

Oocytes with grades A, B, C and D were selected for *in vitro* maturation. Visualization of cumulus expansion after *in vitro* maturation was considered for calculation of maturation rates. Out of 125 oocytes subjected for *in vitro* maturation, 114 oocytes achieved maturation. The mean *in vitro* maturation rate (IVM%) was 91.71±2.38%. Total of 106 matured oocytes were subjected for *in vitro* fertilization using sex sorted bovine semen among which 96 oocytes were fertilized. The mean number of oocytes fertilized was 16±1.61 with the total mean percentage of 90.18±1.52% (Table 2).

The mean number of oocytes cultured was 16.33±1.41, mean number of embryos cleaved was 9.17±1.45 (Fig 2) and the mean cleavage rates observed was 55.1±5.90%. The mean number of blastocysts formed was 4.17±0.75 (Fig 3) and the mean blastocyst rate was 24.81±2.95% (Table 3).

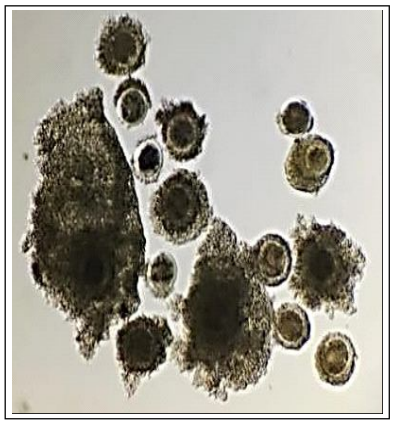

Fig 1: Recovered oocytes.

Fig 2: Cleavage on day 4.

Fig 3: Blastocysts on day 7.

Grading of embryos

The mean percentages of different grades of embryos observed were, code 1: 21.13±2.78, code 2: 14.41±3.25, code 3: 19.56±4.11 and code 4: 44.9±5.90 (Table 3). Highest number of code 4 embryos was produced in the present study.

In the present study small follicles were aspirated in greater numbers than medium and large follicles Similar to the results of Manik *et al.* (2003), Goodhand *et al.* (1999), Presicce *et al.* (2020) Sakhong *et al.* (2012), da Silva *et al.* (2017) and Egashira *et al.* (2019). This suggests that breed, season, environment, nutrition and stimulation protocols have an impact on follicular populations of various sizes.

The oocyte recovery rate achieved in the current study (45.31±7.35%) is in consistent with the results obtained by Gibbons *et al.* (1994) and Garcia and Salaheddine, (1998) who achieved43.9% and 44.2% oocytes recovery rates, respectively. They found that performing OPU twice a week instead of once a week resulted in a higher number of harvestable follicles and higher recovery rates. The current findings are higher than Sisodiya (2008), Pieterse *et al.*

Table 2: Grading of recovered oocytes, *in vitro* maturation and fertilization rates.

	Trials	Oocytes recovered	Grading of recovered oocytes (%)					IVM%	IVF%
			A	B	C	D	E		
Total	6	145	25	36	52	29	3		
Mean±sem			15.69±3.91	24.97±3.02	37.53±3.45	19.44±3.76	2.38±2.38	91.71±2.38	90.18±1.52
SD			9.57	7.4	8.46	9.20	5.83	5.84	3.73

Table 3: Cleavage rate, blastocyst rates and grading of embryos.

	Oocytes cultured	Cleavage %	Blastocyst %	Grading of embryos (%)			
				Code 1	Code 2	Code 3	Code 4
Total	98			21	15	19	43
Mean±Sem	16.33±1.41	55.1±5.90	24.81±2.95	21.13±2.78	14.41±3.25	19.56±4.11	44.9±5.90
SD	3.44	14.45	7.22	6.82	7.96	10.07	14.45

(1988) and Brogliatti and Adams (1996) who achieved 14.2%, 18% and 25% of recovery rates respectively. Bungartz *et al.* (1995) reported 65.4% recovery rates; Manik *et al.* (2003) 59%; Verma (2005) 53.37%; Li *et al.* (2007) 49.2±6.9%; Egashira *et al.* (2019) 69.8±5.6%; Baruselli *et al.* (2012) 91.2% and 61.1% in Gir and Holstein cows respectively, indicating that they achieved higher recovery rates than the present findings. When the OPU was performed close to the emergence of the follicular wave, higher recovery rate can be achieved. Harkal (2019) found a greater oocyte recovery when follicular aspiration was done at 100 mm Hg vacuum pressure than when it was done at 80 mm Hg or 90 mmHg vacuum pressure highlighting that recovery rate is also influenced by the vacuum pressure used during follicular aspiration.

The recovery of grade C oocytes was higher than grades A, B, D and E oocytes in the present study. Results reported by Looney *et al.* (1994), Manik *et al.* (2003), Chaubal *et al.* (2006), Sakhong *et al.* (2012) are in agreement with the present study who recovered highest percentage of grade C oocytes than grade A, B, D and E oocytes. Sanjeeva Kumar *et al.* (2020) recovered more number of grade A oocytes followed by grade B, C and D oocytes. Oocyte quality is influenced by stage of oestrus cycle at the time of OPU session, nutritional deficiencies (Santos *et al.* 2008), type of needle used, diameter of silicone tubing, experience as well as expertize of OPU technician and, Summer season negatively affects oocyte quality as compared to winter (Guerrero-Gallego *et al*. 2021).

The mean *in vitro* maturation rate achieved in the present study was 91.71±2.38% which is in accordance with Sanjeeva Kumar *et al.* (2020) who reported 91.30±1.27%. Lower maturation rates were reported by Verma (2005) 81.54%, Lonergon *et al. (*1994) 70.20%, Pontes *et al., (*2010) 75.53% and Cabrera-Ramos *et al.* (2017) 70.34% where as higher maturation rates were achieved by Chohan and Hunter (2004) who reported 92% maturation rate. According to various studies, IVM can be improved by supple metation of various compounds into maturation medium like amino acids, cysteine, insulin, transferrin, selenium, triiodothyronine and resveratrol.

The overall fertilization rates achieved in the present study was 90.18±1.52% which is higher than Carvalho *et al.* (2010) who reported 67.2±5.7% fertilization rates. Their study suggested that sex-sorting procedure by flow cytometry did not reduce sperm's capacity to produce embryos *in vitro*.

The cleavage rates observed in the present study was 55.1±5.90%. Lower cleavage rates were reported by Liang *et al.* (2008) 50.5±13.0% and Presicce *et al.* (2011) 41.2±6.1%; and higher cleavage rates reported by de Oliveira Bezerra *et al.* (2019) 63.80%; Matoba *et al.* (2014) 75.4±5.2%; Underwood *et al.* (2010) 78.8±4.6% and Nogueira *et al.* (2021) 81.61%.

The present report of blastocyst percentage (24.81±2.95%) is higher than Cebrian-Serrano *et al.* (2013) 5.56%; Liang *et al.* (2008) 15.3±7.4%; Presicce *et al.* (2011) 12.8±2.3% and Pontes *et al.* (2010) 18.8%; and lower than those reported by de Oliveira Bezerra *et al.* (2019) 30.47%; Morotti *et al.* (2014) 29%; Matoba *et al.* (2014) 31.8±8.2%; Underwood *et al.* (2010) 35.9±4.8% and Nogueira *et al.* (2021) 30.08%. According to Nogueira *et al.* (2021), breed, blood group and frequency of aspirations have a strong influence on embryonic production.

Wilson *et al.* (2006) produced more number of grade 2 embryos followed by grade 1 and grade 3 respectively in contrast to the present study. In the present study, higher number of code 4 embryos were produced possibly due to effect of quality of oocytes, once weekly of aspiration, media used and the use of sex sorted sperm.

CONCLUSION

In conclusion, ultrasound guided transvaginal ovum pick up can be performed repeatedly without hormonal stimulation of the ovaries. Sex sorted semen can be used efficiently for *in vitro* production of embryos to increase the female population for higher milk production.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data organization and analysis were performed by M.R. Hadimani. The first draft of the manuscript was written by the first author and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics approval

Not applicable

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Informed consent

Not applicable.

Availability of data and material

All data are available via the corresponding author.

Code availability

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

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