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Effect of FSH stimulation prior to ovum pick-up on follicular dynamics, oocyte competence, and *in vitro* embryo production in Ongole cows (*Bos indicus*)

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ABSTRACT. The current study evaluated the influence of follicle-stimulating hormone (FSH) administration on the stimulation of ovarian follicles, oocyte quality, and embryo production rate in Ongole cows with synchronized follicular wave emergence. Follicular waves were synchronized using 10 µg of gonadotropin-releasing hormone (GnRH), followed by FSH administration (250 µg) in three divided doses of 125, 75, and 50 µg at 48, 60, and 72 h post-GnRH administration, respectively. Although the total number of follicles was similar in both groups, the hormonal stimulation regimen increased ($P < 0.01$) the number of large follicles without affecting small and medium follicles. FSH stimulation had no effect on the mean count of aspirated follicles; however, the cows administered FSH showed a higher ($P < 0.001$) aspiration rate. An analysis of the proportion of cumulus oocyte complexes revealed that FSH stimulation did not affect their mean number. Nevertheless, FSH-stimulated cows had a lower recovery rate ($P < 0.001$) of cumulus oocyte complexes (COCs) compared to the control group. In addition, the proportion of good-quality COCs was higher ($P < 0.01$), with a simultaneous lower ($P < 0.01$) percentage of poor-quality COCs in the FSH group. *In vitro* fertilization of both good and poor quality COCs revealed a higher ($P < 0.01$) blastocyst formation rate within 7 to 8 days of fertilization. The study concluded that the FSH super-stimulation protocol improved follicular number, aspiration rate, oocyte competence and blastocyst percentage in cows synchronized for follicular wave emergence.

Introduction

Cattle, particularly indigenous breeds, play an indispensable role in alleviating rural poverty in developing countries. According to the 20th Livestock Census, India has a cattle population of 192.52 mln, with 73.76% being indigenous breeds (Livestock Census, 2019). However, the introduction and propagation of *Bos taurus* germplasm in the country over the past seven decades to increase milk production has put a severe strain on the very existence of the indigenous breeds such as Ongole. Indian farmers have increasingly resorted to crossbreeding programming, resulting in a loss of economic

importance of indigenous breeds. Consequently, the population of indigenous cattle has been steeply declining at alarming levels (Van Eenennaam, 2019). Among these breeds, Ongole (also known as Nellore) cattle is a dual-purpose indigenous breed known for its unique traits of disease resistance, heat tolerance, climate resilience, and performance under subsistent nutrition and management (Reddy et al., 2021).

The renewed interest in preserving purebred indigenous draught cattle breeds emphasizes the need to develop long-term conservation strategies. Although selective breeding through artificial insemination is a well-established procedure, it is constrained by the lack of genetically superior bulls and time requirements (Manmohan et al., 2021). Ovum pick-up and *in vitro* embryo production (OPU-IVEP) in combination with embryo transfer (ET) offers a promising alternative for preserving breeds and ensuring faster multiplication of superior germplasm (Baldassarerre et al., 2021).

Increasing the number of viable oocytes per aspiration is the key to producing more viable embryos. To this end, several techniques are being researched to increase the recovery of oocytes from a single aspiration. Among them, follicle-stimulating hormone (FSH) pre-treatment before ovum pick-up can be considered an easy, affordable, and promising strategy to increase oocyte retrieval efficiency (Hayden et al., 2022). Superstimulation protocols utilising FSH can improve the population of developmentally competent oocytes, thereby benefitting OPU-IVP programs (da Silva et al., 2017).

It is challenging to compare the results of previous studies on OPU and *in vitro* embryo production due to the use of different hormones and ovarian stimulation protocols, as well as variations in biological factors such as breed, age, stage of lactation, nutrition, and climate. Therefore, it is unrealistic to expect a single, simplified OPU protocol that would fulfil the requirements of every operation in the diverse cattle breeding industry. Currently, the OPU and *in vitro* embryo production technique is routinely used in *B. taurus* breeds in many parts of the world. Although initial work on OPU in Indian cattle has been described by Manik et al. (2003), information on the effect of hormonal pre-treatment on the number of follicles available for puncture, oocyte recovery rate, oocyte quality, and blastocyst development rate in Ongole cows is very scarce. Therefore, the purpose of this study was to investigate the effect of FSH pre-stimulation on the number and size of follicles, oocyte competence, and subsequent *in vitro* embryo production.

Material and methods

Animals, location, and feed management

The study was conducted at the Livestock Research Station (LRS), Lam Farm, Sri Venkateswara Veterinary University, Guntur, Andhra Pradesh. The region is located at 16.1800°N and 80.2900°E, an altitude of 31.5 m and an average rainfall of 925.7 mm. The guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA; IV, section 15(1)) for the prevention of cruelty to animals (PETA, 1960) were strictly followed. All procedures were approved by Sri Venkateswara Veterinary University Local Ethics Committee (2019-SVVU-VGO-01), and the animals were housed for further research after completing the trial.

A total of sixteen multiparous, clinically normal and cyclic Ongole cows (aged 12 ± 3 years; body weight 455 ± 42 kg) were selected from the Lam farm for oocyte aspiration and randomly divided into two equal groups.

The daily ration of each animal consisted of 2–4 kg of high protein feed containing 20% digestible crude protein (DCP) and 70% total digestible nutrients (TDN), 20–30 kg of chopped fodder and 7–8 kg of paddy straw. Water was available to the cows at all times. The animals were maintained under hygienic and optimal management conditions in a loose housing system and access to a large, open paddock for free movement. They were allowed to graze daily between 9:00 and 15:00 in the adjoining pasture lands. Calves were allowed to suckle the dams and hand milking was performed twice a day. Heat detection in the herd was carried out twice daily at 08:00 and 14:00 using a vasectomized bull (teaser). Health and vaccination protocols were followed as per the standard schedule.

Experimental design

The cows were randomly divided into two groups (control vs. stimulated), with eight replicates in each group. Animals from the control group (n = 8) were subjected to the OPU procedure at a random phase of the oestrous cycle, while cows in the stimulated group (n = 8) underwent follicular wave synchronization before the OPU procedure. Synchronization was initiated by administering 10 μg of GnRH (Receptol®, MSD Animal Health, India) on a random day of the oestrous cycle, followed by FSH stimulation (Stimufol®, Reprobiol,

Belgium) at a dose of 250 µg divided in three subdoses of 125, 75, and 50 µg administered 48, 60, and 72 h after GnRH administration, respectively. Ovum pick-up was performed 48 h (coasting period) after the last FSH injection. A total of 16 OPU sessions were conducted in 16 animals (one session per animal) with and without FSH pre-stimulation (Figure 1).

A lubricated transvaginal probe fitted in a plastic probe carrier (WTA, São Paulo, Cravinhos, Brazil) was inserted into the anterior vagina. Later, the ovary was positioned by transrectal manipulation along with a transducer to view and aspirate the follicles. Data on the number and diameter of follicles were recorded by freezing the image on a monitor and using a built-in calliper (Nagai et al., 2015).

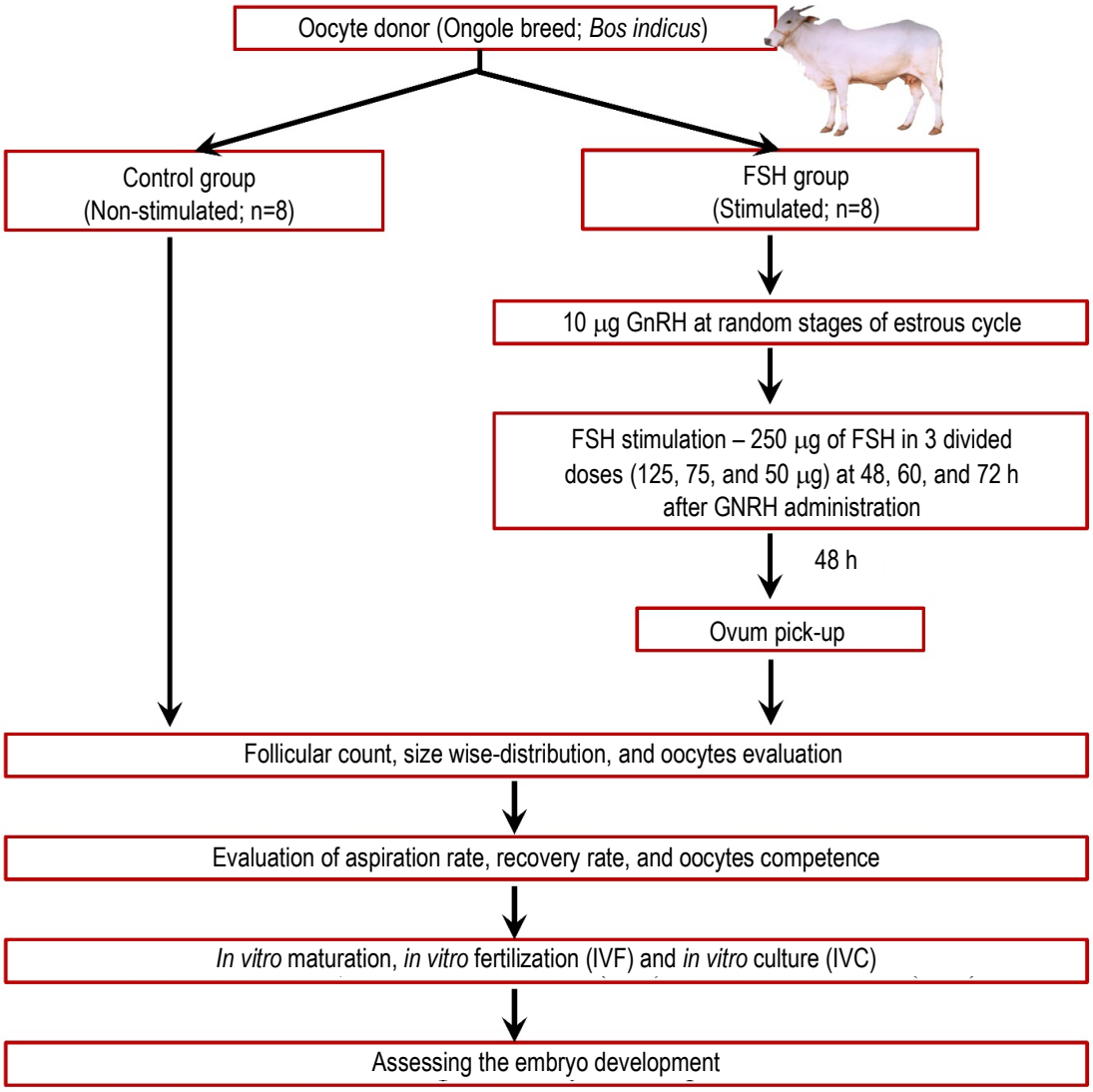

Figure 1. Experimental design

Preparation of donor and ovum pick-up (OPU)

Rectal evacuation was carried out with a gloved hand and the perineal region was cleaned with 70% ethanol. Before each OPU session, cows received 4 ml of Lox 2% (lignocaine hydrochloride, Neon, India) epidurally to prevent defecation and abdominal tension and to facilitate ovary handling.

Based on the diameter, the follicles were categorised as small (<4 mm), intermediate (4–<8 mm), and large (≥8 mm) according to the classification of Ginther et al. (1989). In addition, the presence of the corpus luteum on the ovary was visualised and its diameter was recorded.

Once the ovary and target follicle was stabilised, follicular aspiration was performed by

a single trained technician using a real-time B mode ultrasound scanner (My lab Gamma Vet, Esaote, Genova, Italy) equipped with a multi-frequency (4–9 MHz) microconvex probe (SC 3123, Esaote). During the entire OPU session, the needle and aspiration line were thoroughly rinsed with pre-warmed (37 °C) OPU recovery medium (IVF Bioscience, UK) to prevent blood clotting or oocytes sticking to the tubing. A negative pressure of 61 to 65 mm Hg was generated using an aspiration pump (V-MAR 5000, Cook Australia Pvt. Ltd., Australia) to create vacuum and generate a fluid flow of 23–25 ml per min for both groups. Transvaginal ultrasonographic images of an FSH-stimulated ovary are presented in Figure 2. Figures 2A and 2B depict multiple follicles of varying sizes and a dominant follicle with corpus luteum, respectively. The pre-aspiration follicle and the collapsed post-aspiration follicle are shown in Figures 2C and 2D, respectively. Aspiration rate (%) and oocyte recovery rate (%) were calculated as described by Goodhand et al. (2000):

- Aspiration rate = number of follicles aspirated/ total number of follicles
- Oocyte recovery rate = number of oocytes recovered/number of follicles aspirated.

cumulus-oocyte complexes (COCs). COCs were transferred to a 35 mm Petri dish containing BO wash medium (IVF Bioscience, UK) and examined under a zoom stereomicroscope at 63x magnification and graded based on the cumulus investment as per the classification provided by Looney et al. (1994) and Bungartz et al. (1995). Oocyte quality deterioration is expressed using alphabetical coding in ascending order: A>B>C>D>E; The sum of A, B, and C is considered good quality oocytes, and the sum of D and E is regarded as poor-quality oocytes. All oocytes were used for *in vitro* fertilization (IVF) and embryo culture.

In vitro embryo production

After grading, COCs were initially transferred to one well of a 4-well dish (IVM dish) containing 500 µl of pre-equilibrated maturation medium (IVF Limited T/A IVF Bioscience, Falmouth, UK). After incubation in an atmosphere of 5% CO_2, a temperature of 38.5 °C, and a relative humidity of over 90% for 20–24 hours, the IVM dish containing oocytes was directly examined for cumulus cell expansion under a zoom stereo microscope at 63x magnification (Nikon, Tokyo, Japan).

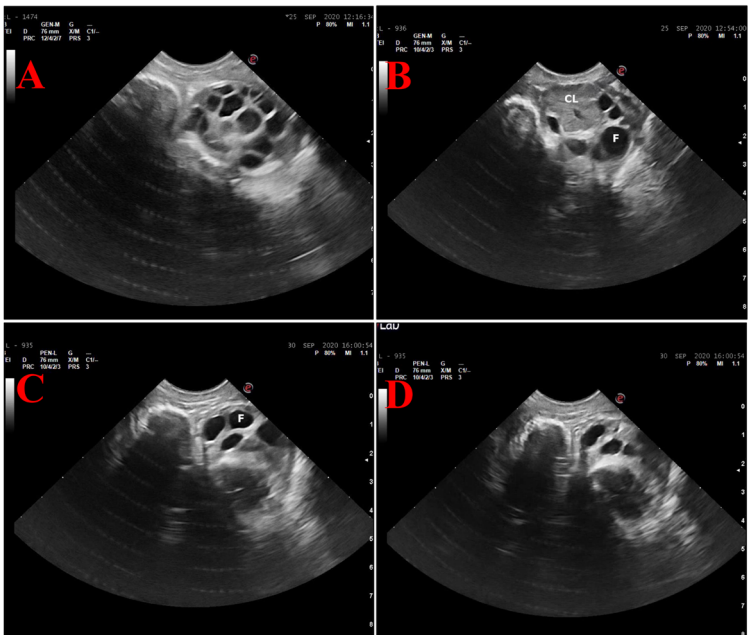

Figure 2. Transvaginal ultrasonographic images of FSH-stimulated ovary

(A) numerous follicles of different sizes; (B) dominant follicle with corpus luteum, CL – corpus luteum, F – follicile ; (C) follicle prior to aspiration; (D) collapsed follicle after aspiration

Washed and filtered follicular aspirate was transferred to a square grid Petri dish (90 × 15 mm, Tarsons, Chennai) and examined under a thermal stage-fitted zoom stereo microscope (SMZ 1000, Nikon, Japan) at 20x magnification to identify

Subsequently, approximately 10–40 µl of sperm pellet with a final sperm concentration of 2×10^6 ml^{-1} was placed in the IVF dish containing oocytes. Upon completion of the IVF procedure, the 4-well IVF dish was transferred to a CO_2 incubator (5% CO_2, 38.5°C,

>90% RH) for 16–20 h. Afterwards, the IVF dish containing presumptive zygotes was carefully removed from the CO_2 incubator and placed in an IVF workstation. Putative zygotes were then carefully stripped of loose cumulus cells and sperm using a denudation pipette (Origio®, Westborough, USA). On day 7, IVC plates containing embryos were examined under an inverted phase contrast microscope 400x (Carl Zeiss, Jena, Germany) to identify different stages and grades of embryos according to guidelines recommended by The International Embryo Transfer Society (IETS).

Statistical analysis

All statistical analyses were performed using SAS (Statistica, ver. 9.4). Data were tested for normal distribution using the PROC UNIVARIATE statement and *P*-values from the Shapiro-Wilk test. Non-categorical or descriptive data lacking a normal distribution and homoscedasticity were analysed by the Mann-Whitney test. Data considered normal with homoscedasticity were subjected to an independent t-test using the 'PROC t-test' statement. When conducting the independent t-test, the assumption of equal variances was tested by the 'Folded F' method. The pvalues of equal variances were taken from the pooled method, while those of unequal variances were obtained using Satterthwaite's approximation. Categorical variables represent two outcomes (Yes/No); percentages were calculated using the 'PROC FREQ' statement. Initially, the percentages and categorical variables were subjected to a chi-squared test by cross-tabulation. However, a 2×2 contingency table revealed that more than 90% of the cells had an expected number less than 5; hence, the percentages were tested for significance using Fisher's exact test. Graphs were generated using Graph pad Prism Version 7.0. *P*-values less than 0.05 were considered significant, while those between 0.05 and 0.01 were considered a trend.

Results

The number of follicles and distribution of follicle size in Ongole cows subjected to transvaginal ovum pick-up are presented in Table 1. The count of follicles in the right and left ovary was equal. No significant differences were observed for any of the parameters, except for large-sized follicles, which were more common ($P < 0.01$) in synchronized cows. The variation in the number of follicles between individual animals was higher than between the groups (control vs. stimulated), ranging from 13 to 59, which could be due to the high age difference of the animals tested. Iwata (2017) reported an age-associated decrease in follicular fluid, which impaired oocyte development.

Table 1. Follicle count and size distribution in Ongole cows subjected to transvaginal ovum pick-up

Item	Control	FSH	SEM	*P*-value
Number of follicles				
right ovary	136	110	1.90	0.272
left ovary	116	110	1.59	0.446
total number of follicles	252	220	3.31	0.374
mean number of follicles/cow	31.50 (13–59)*	27.50 (19–44)*	3.31	0.564
Mean number of follicles by size				
small	19.50	11.00	2.97	0.176
medium	10.25	11.00	2.11	0.865
large	1.75	5.50	0.68	0.002

FSH – follicle-stimulating hormone, SEM – standard error of the mean; $P < 0.05$ indicates significant difference; * numbers in parenthesis indicate range

The results of transvaginal ovum pick-up in Ongole cows is presented in Table 2. A wide range in the number of aspirated follicles was observed among individual animals, ranging from 7 to 36. Similarly, the number of obtained oocytes varied from 2 to 26 among individual cows. The chi-square test revealed a higher ($P < 0.001$) aspiration rate and a lower ($P < 0.001$) recovery rate among synchronized cows. Nevertheless, the average number of COCs recovered per cow per session was the same for both groups. The percentage of good-quality COCs was higher ($P < 0.01$) in FSH-stimulated cows, while the percentage of poor-quality COCs was higher in the control group.

Table 2. Efficiency of transvaginal ovum pick-up in Ongole cows

Item	Control	FSH	SEM	*P*-value
Number of follicles aspirated	143	160	2.32	0.501
Mean number of aspirated follicles/cow	17.88 (7–34)*	20.00 (11–36)*	2.32	0.663
Aspiration rate, %	56.75	72.73	4.30	0.001
Number of COCs recovered	88	88	2.04	1.000
Recovery rate, %	61.54	55.00	0.05	0.001
Mean number of oocytes/cow	11 (2–23)*	11 (3–26)*	2.04	1.000
Quality of COCs				
good-quality COCs, %	33.36	72.73	9.70	0.002
poor-quality COCs, %	63.64	27.27	9.70	0.002

FSH – follicle-stimulating hormone, COCs – cumulus-oocyte complexes, SEM – standard error of the mean; $P < 0.05$ indicates significant difference; * numbers in parenthesis indicate range

The results of the analysis concerning the proportion of A, B, C, D and E oocytes are illustrated in Figure 3. FSH stimulation increased ($P < 0.05$) the mean count of grade C oocytes and decreased ($P < 0.05$) the number of grade D oocytes without affecting the proportions of grade A, B, and E oocytes. The effect of FSH stimulation on the average number of good-quality (A+B+C) and poor-quality oocytes (D+E) is presented in Figure 4. Stimulation resulted in a marked reduction ($P < 0.05$) in the number of poor-quality oocytes (Figure 5A), while increasing ($P < 0.05$) the count of good-quality oocytes (Figure 5B).

The potential for *in vitro* embryo production in both groups is shown in Table 3. During *in vitro* fertilization, an unfertilized embryo, embryos with 2–16 cells, early morula, morula, early blastocyst, blastocyst, and expanded blastocyst during days 1, 2 to 5, 5 to 6, 6, 7, and 7 to 8 were observed, respectively. The percentage of blastocysts from good-quality oocytes and the overall blastocyst formation rate were higher ($P < 0.01$) in the stimulated group. Figure 5C depicts the expansion of cumulus cells after *in vitro* maturation, while Figure 5D shows grade a and grade B embryos.

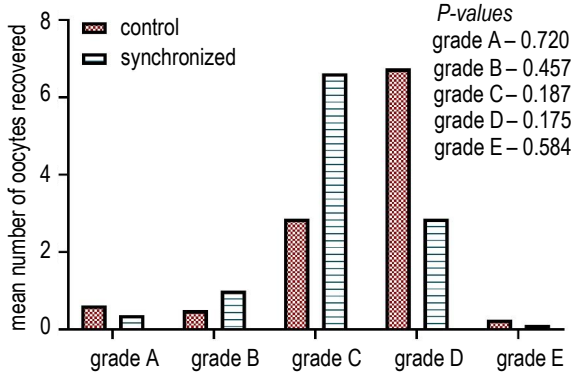

Figure 3. Five quality grades of oocytes retrieved by ovum pick-up

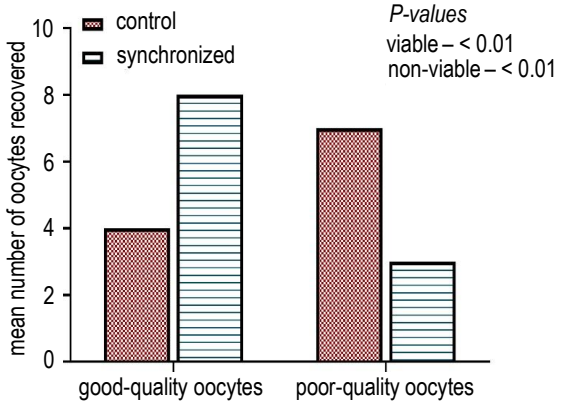

Figure 4. Quality range of oocytes retrieved by ovum pick-up

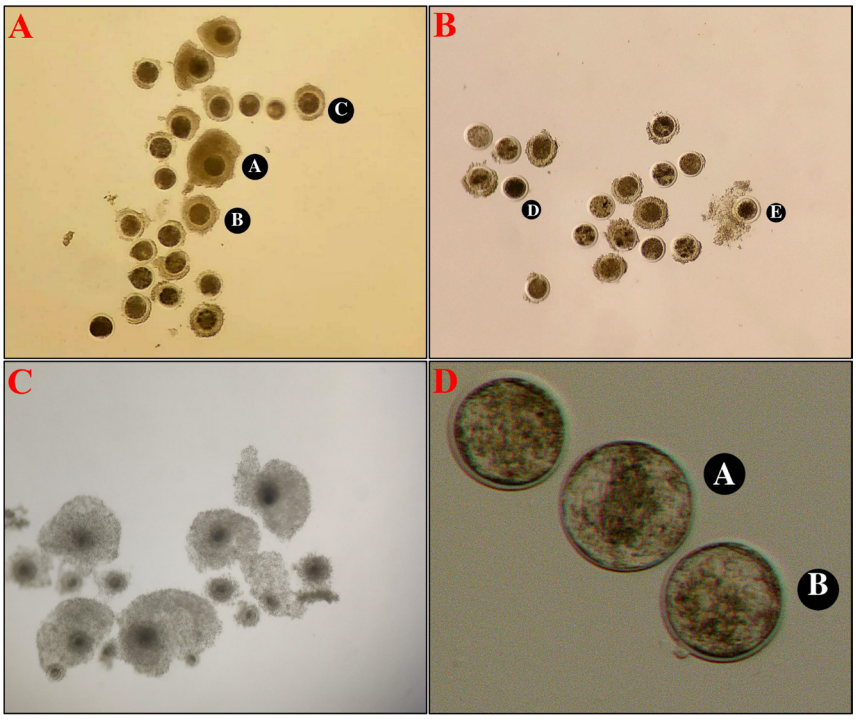

Figure 5. Different grades of oocytes, oocytes during *in vitro* maturation, and different embryo grades

(A) good-quality oocytes (A, B, and C); (B) poor-quality oocytes (D and E); (C) cumulus cell expansion after *in vitro* maturation; (D) good grade blastocysts (A and B)

Table 3. *In vitro* embryo production through ovum pick-up (OPU) in Ongole cows

Attribute	Control	FSH	SEM	*P*-value
Embryos produced	8	22	0.72	0.612
Mean number of embryos/cow	1	2.75	0.72	0.250
Blastocyst rate				
from good-quality oocytes, %	18.75	34.38	4.96	0.009
from poor-quality oocytes, %	3.57	0.00	0.54	-
overall rate, %	9.09	25.00	3.60	0.005

FSH – follicle-stimulating hormone; SEM – standard error of the mean; $P < 0.05$ indicates significant difference

Discussion

The present study was conducted to test the efficacy of FSH superstimulation in promoting the growth of antral follicles and improving oocyte competence, ultimately leading to a higher number of viable embryos. FSH pre-stimulation did not affect the total number of follicles available for aspiration. On the contrary, few authors have reported a higher number of follicles available for puncture in cows subjected to FSH stimulation (Vennapureddy et al., 2022), highlighting the importance of FSH action in antral follicle development and growth. However, the follicular count is highly variable due to various intrinsic (follicle number in the reserve pool, follicle recruitment, development, and atresia) and extrinsic (breed, age, cyclicity, nutritional status etc.) factors (Cox and Takov, 2022). Although no significant differences were observed for small and medium-sized follicles, the frequency distribution revealed a threefold higher mean count of large-sized follicles in the FSH-stimulated group. Likewise, Goodhand et al. (2000) observed a significant increase in the number of larger follicles in FSH-stimulated cows. A recent study conducted to conserve indigenous breeds obtained a similar result regarding a higher count of large-sized follicles in response to FSH stimulation (Vennapureddy et al., 2022). According to the most recent literature, the number of large follicles is the most reliable parameter for comparing superstimulation protocols as oocytes originating from them are more competent for maturation (Cox and Takov, 2022; Helmer et al., 2022). However, there was considerable variation in the follicular count at the individual animal level (range of 13 to 59).

There were no significant differences in the mean number of aspirated follicles and recovered oocytes between the two groups; however, FSH stimulation increased the rate of follicle aspiration. Goodhand et al. (2000) also showed an increased aspiration rate with no change in the average number of follicles and oocytes with FSH pre-stimulation. In another study, Seneda et al. (2001) demonstrated a profound effect of follicular wave stage on the number and quality of recovered COCs during OPU sessions, thereby masking the beneficial effects of FSH stimulation. Hence, in this study, GnRH was administered to the stimulated group to synchronize the follicular wave and prevent its influence. The cows subjected to FSH stimulation had a higher recovery rate, presumably due to a higher proportion of large-sized follicles (Majeed et al., 2019). Contrary to expectation, the oocyte recovery rate was higher in the control group. These results were consistent with reports by Pieterse et al. (1991) and Walton et al. (1993), while De Roover et al. (2008) found better recovery rates in FSH-stimulated cows. As shown by the equation (Oocyte recovery rate = number of oocytes re-covered/number of follicles aspirated), the higher recovery rate in non-stimulated cows could be attributed to the relatively lower count of aspirated follicles in the control group compared to the FSH-stimulated group (143 vs. 160).

The number and quality of oocytes recovered from donor animals directly reflect the commercial application of *in vitro* embryos (Ferre et al., 2020). Although the number of oocytes did not differ between the groups, the stimulated group had a higher proportion of good-quality and lower proportion of poor-quality oocytes. This highlights the importance of synchronizing follicular wave emergence and using FSH superstimulation prior to OPU as a strategy to improve the embryo production rate.

Based on the classification of Chaubal et al. (2006), the oocytes shown in Figure 5A were graded as A, B, and C, which corresponded to compact cumulus (>4 layers), 3 to 4 cumulus layers, and 1 to 2 cumulus layers, respectively. Similarly, Figure 5B shows grade D and E oocytes, which are denuded and have expanded cumulus, respectively. FSH stimulation prior to OPU increased the mean count of grade C oocytes and reduced the number of grade D oocytes. In another study, Jeyakumar (2004) classified oocytes using a similar procedure and recorded a higher proportion of grades B and C in cows injected with FSH. Although there were no significant differences observed in individual grades A, B, and E, FSH pre-treatment improved the overall recovery of good-quality COCs (A+B+C,) while decreasing the recovery of poor-quality COCs (D+E). Petrovas et al. (2020) also showed a higher recovery rate of good-quality COCs in Italian Mediterranean buffaloes subjected to FSH stimulation. The increased competency of oocytes could be due to FSH's properties in controlling steroidogenesis and gene expression

(de Souza et al., 2018). Moreover, the FSH stimulation group had a higher proportion of large-sized follicles, which may result in a greater extent of follicular aspiration and more cumulus investment, ultimately affecting the quality of oocytes (Vennapureddy et al., 2022). The mean number of oocytes per cow (2–26) was higher compared to an earlier study by Hendriksen et al. (2004); however, a wide range as high as 0-26 is possible due to individual variability in donor animals (Dorice et al., 2019). Various environmental factors such as genetics, donor age, oestrous cycle stage, proportion of FSH-responsive follicles in the ovary, as well as the size of the primary and tertiary follicle pools contribute to the differences between individual donor animals (Boni, 2012).

The overall blastocyst rate formation was higher in stimulated cows due to the higher proportion of good-quality oocytes. Saini et al. (2015) demonstrated that the cleavage rate was strongly influenced by the quality of COCs and that good-quality COCs showed a better developmental potential. The cleavage in good-quality COCs progresses to the morula and blastocyst stages, where the development of poor-quality COCs is arrested at 8 to 16 cell stages. This phenomenon indicated a better developmental potential of good-quality COCs, whose percentage was higher in the FSH-stimulated group. The positive correlation between the ability of oocytes to develop to the blastocyst stage and the presence of large-sized follicles is well established (Kahraman et al., 2018). According to Baldassarre (2021), the acquisition of developmental competence by an oocyte requires the accumulation of critical molecules produced by cumulus granulosa cells of large follicles at the pre-ovulatory stage. In addition, a 48-hour coasting period, i.e., the time gap between the last FSH administration and OPU session, may have contributed to the observed positive rate of blastocyst formation (Jeyakumar, 2004). Coasting period has been projected as an efficient technique to address reduced developmental rates caused by FSH-induced asynchrony between the maturation of the oocyte and its surrounding follicle or cytoplasmic and nuclear maturation (da Silva et al., 2017).

The present findings also support the hypothesis that FSH treatment in three divided doses before OPU increases the size of aspirated follicles, oocyte competency, and blastocyst formation rate. It should be noted that the evaluation of embryo stage and grade is always subjective and may vary between embryologists. In the current study, the sample size was limited to n = 16, and it is likely that the low sample size and large variations within the study subjects (individual cows) may have influenced the results. Therefore, we recommend future studies on Ongole cows (*Bos indicus*) using a larger sample size.

Conclusions

The study findings suggest that follicle-stimulating hormone (FSH) is beneficial in stimulating the growth of medium-sized antral follicles prior to ovum pick-up. In addition, FSH administration improves *in vitro* oocyte competence and overall blastocyst formation rate in lactating Ongole cows, thereby enhancing the effectiveness of ovum pick-up and *in vitro* embryo production programs.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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