# Effect of follicular wave emergence on OPU-IVEP attributes in *Bos indicus* breeds of cattle

B. Jagadheesh Kumar[1], K. Sunny Praveen[1], N.R. Srikanth[1], M. Mutha Rao[1]*, Ch. Srinivasa Prasad[2]

[1]Livestock Research Station, Lam, Guntur, [2]College of Veterinary Science, Proddatur, Sri Venkateswara Veterinary University, Andhra Pradesh, India.
*Corresponding author email: murakondamutharao@gmail.com

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## Abstract

A comparison of different Ovum Pick-up (OPU) and *invitro* Embryo Production (IVEP) attributes between two different OPU sessions each in Ongole and Gir cattle was attempted in the present study. Among the two OPU sessions, first OPU session was conducted on day 9 (luteal phase) of the induced estrous cycle and the second session was conducted on a random day of the cycle with a gap of 2-4 weeks apart between the OPU sessions. Study was conducted in two *Bos indicus* breeds *viz.*, Ongole cows (n=6) and Gir cows (n=6) with a total of 24 OPU sessions. Various OPU-IVEP attributes such as means of antral follicle count (AFC), follicles aspirated, COCs retrieved and embryos produced did not show any significant difference between different OPU sessions. Also, different attributes were similar between the two breeds. The results implied that in the present study OPU frequency did not have any impact on the studied attributes and also the different breeds *viz.*, Ongole and Gir (both *Bos indicus*) are similar in all the noted OPU-IVEP attributes.

**Keywords:** Ovum pick-up (OPU), *Invitro* Embryo Production (IVEP), Ongole, Gir and Cattle.

# Introduction

Ovum pick-up (OPU) and *invitro* embryo production (IVEP) in bovines is a third generation assisted reproductive technology (ART), first and second being Artificial Insemination (AI) and Multiple Ovulation and Embryo Transfer (MOET), respectively (Yusuf, 2024). IVEP used to be performed to obtain large number of embryos to conduct early embryonic studies on these embryos. Researchers then found in this technique a viable and more profound potential to be used in livestock breed improvement strategies (Looney *et.al.*,1994). Though previously for performing IVEP oocytes are sourced from ovaries of slaughtered animals, the latest technique to obtain oocytes in live animals through transvaginal ultrasound –guided follicular aspiration which is also referred to as ovum pick-up (OPU). This technique is minimally invasive and harmless to the elite bovine donor which enables repetitive harvests of oocytes from the antral follicles of the donors. OPU coupled with IVEP maximizes the reproductive output of genetically superior elite donors (Galli *et al.*, 2001). According to the global data for the year 2020, 79.7% of the total embryos transplanted were by *invitro* embryo production and 71.2% of those IVF embryos were developed from oocytes collected by OPU procedure (Dogan and Yenilmez, 2024). Scientific workers applied OPU successfully on bovines of different reproductive stages like pre-pubertal calves (Viana *et al*., 2024), primiparous and pluriparous cows (Du *et al*., 2024). OPU in general is carried out in bovines at random stage of estrous cycle (Pontes *et al.*, 2011). However, this type of non-synchronized OPU approach is often compromised by the presence of a dominant follicle, which induces follicular atresia in subordinate follicles (Hendriksen *et al.*, 2000). This phenomenon results in a diminished yield of viable oocytes due to the increased proportion of atretic follicles among the aspirated follicles. Through various studies it has been established that the first follicular wave typically emerges around ovulation or the day after the onset of the estrous (Ginther *et al.*, 1989), making it a viable target for super-stimulation. The second wave follicles respond to gonadotropin treatments just like first wave follicles (Mapletoft and Bó, 2011).

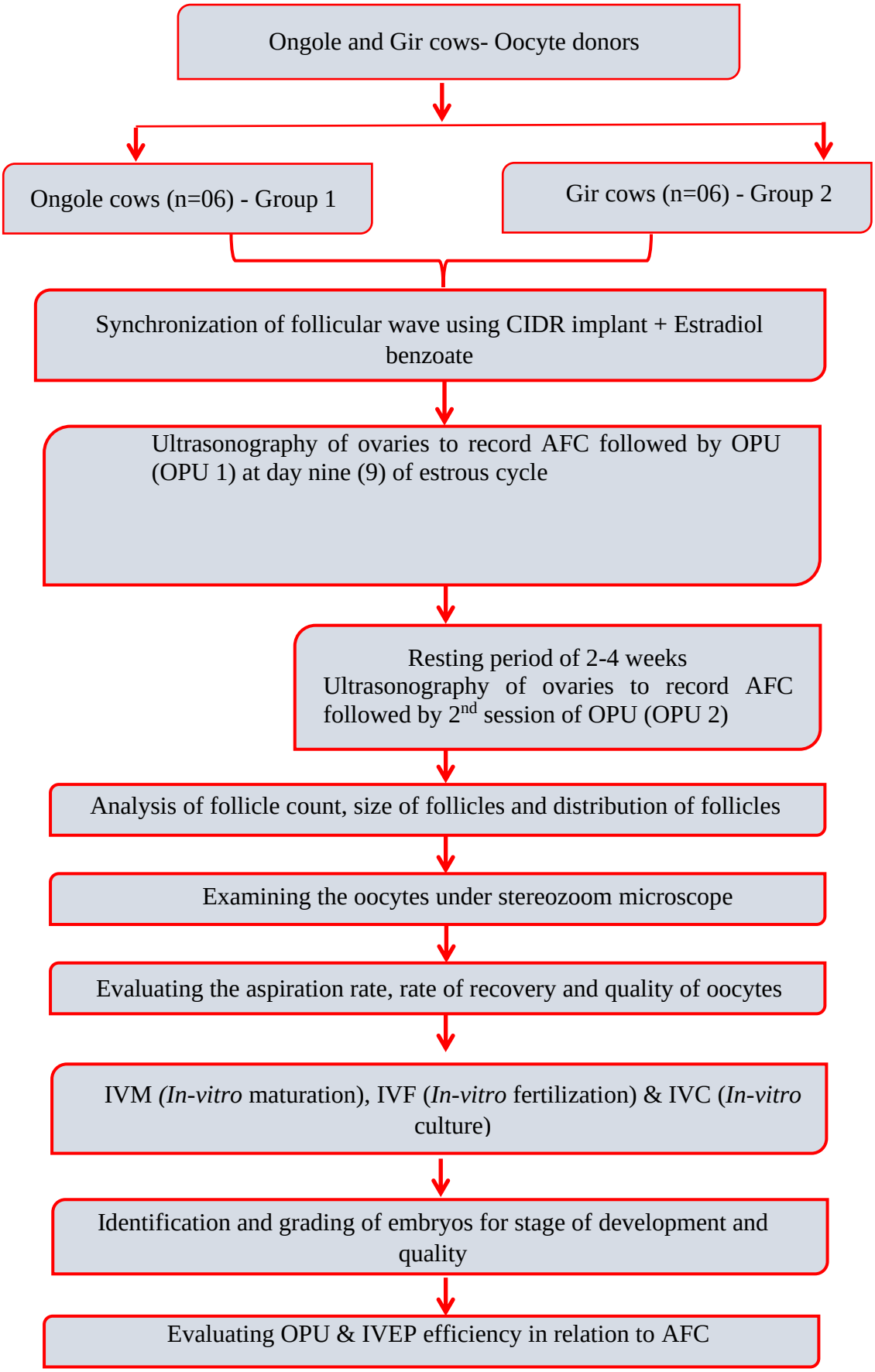
It is well documented that the response to super-stimulation is inferior when it is implemented after the selection of the dominant follicle and is much better if it is initiated during the wave emergence (Adams and Singh, 2021). To explore this further, the present investigation is designed to evaluate the impact of OPU timing on oocyte yield and subsequent embryo production in *Bos indicus* cows of Ongole and Gir breeds. Ongole is one of the best draught breeds among *Bos indicus* cattle and probably most widespread Indian cattle breed globally (Kamalakar *et al.*, 2015). Gir is very good milch breed among *Bos indicus* cattle (Mahesh *et al.*, 2022). These two *Bos indicus* breeds represent genetically distinct populations with recognized differences in reproductive physiology. Ongole is generally used breed in embryo transfer programs across South India while Gir is commonly used in Western India and globally for crossbreeding. Investigating IVEP parameters in these native elite breeds provides insight into optimizing ART applications for breed conservation and genetic advancement. Specifically, the OPU-IVEP attributes of OPU performed at the predicted day of follicular wave emergence (OPU1) was compared to OPU conducted at an undefined stage of follicular growth (OPU2) with a 2-4 weeks interval between the OPU sessions in both the breeds.

# Materials and methods

The present study conducted at the Livestock Research Station (LRS), Lam, Guntur, Andhra Pradesh, an affiliate of Sri Venkateswara Veterinary University, from August to December 2024, utilized randomly selected, reproductively sound *Bos indicus* cows of Ongole and Gir breeds, aged 5-10 years. This station has an average elevation of 108 ft above mean sea level and is situated on the plains, with 16°.3 N latitude and 80°.4 E longitude. The selected elite oocyte donor cows were maintained under a loose housing system with access to an open paddock. Nutritional management consisted of a daily ration of 3-5 kg high-protein concentrate (20% DCP, 70% TDN), 20-30 kg chopped fodder, and 6-7 kg paddy straw. Oocyte donors were grouped under Groups I and II with Ongole (n=6) and Gir (n=6) cows, respectively.

On a random day of the estrous cycle, synchronization was initiated in all the donors of both the groups, by intravaginal insertion of a 1.38 g progesterone-releasing, controlled internal drug release dispenser - CIDR (EAZI-Breed, Pfizer Animal Health) with a simultaneous administration of 2 mg oestradiol benzoate (Inj. Pregheat®- 2ml, VHB Medi Science Limited), intramuscularly. On day 7$^{th}$ post-CIDR insertion, the intravaginal implant was removed and 500 µg cloprostenol (Inj.Vetmate - 2 mL; Provimi Animal Nutrition India Pvt. Ltd.) was administered intramuscularly. One mg of oestradiol benzoate was injected intramuscularly on day eight and the next day which is considered the day of estrus was used to detect oestrus (Kim *et al.*, 2007). Transvaginal ultrasonography (B-mode, 7.5 MHz micro convex) was performed on both ovaries of the oocyte donors in Groups 1 and 2 on the ninth day of the synchronised oestrous cycle using the day of oestrus as the baseline. Antral follicle count (AFC ≥3 mm) was determined, and oocyte pick-up (OPU1) was subsequently performed. After a 2-4 weeks interval, cows were subjected to a subsequent transvaginal ultrasound examination for AFC determination and oocyte retrieval (OPU2), performed at an undetermined phase of the estrous cycle. For oocyte retrieval, MyLab Gamma Vet ultrasound scanner (Esaote, Genova, Italy) equipped with a 4-9 MHz microconvex probe (Model: SC 3123, Esaote) was employed. The probe was mounted within a plastic vaginal probe carrier, which also housed

**Fig 1**: Experimental design



the needle, needle guide, and aspiration line (WTA, Brazil). A vacuum pump (Cook, USA), maintaining a negative pressure of 70-75 mm Hg and controlled *via* a foot-operated switch, was connected to the aspiration system. The ovary was gently manipulated to appose the probe head, facilitating clear visualization of follicles on the ultrasound monitor. Subsequently, follicles with a diameter of ≥3 mm were enumerated on both ovaries, yielding the antral follicle count (AFC). The aspiration needle was inserted into the antral cavity of the selected follicle, and the follicular fluid was aspirated into a pre-warmed conical tube containing OPU recovery medium (IMV, France). Successful aspiration was confirmed by the real-time disappearance of the anechoic follicular image from the ultrasound display.

During each oocyte pick-up (OPU) session all visually identified follicles were aspirated and the collected cumulus-oocyte complexes (COCs) were subjected to *invitro* maturation (IVM), *invitro* fertilization (IVF) and *invitro* culture (IVC) following standardized procedures (Vieira *et al.,* 2014, Krishna *et al*., 2023 and Sreemannarayana *et al*., 2024).

Follicular aspirate that was collected by OPU procedure was initially filtered using a mini oocyte filter (WTA, Brazil) to remove unwanted debris. The filtrate was then repeatedly washed with OPU recovery media until the sample look clear from debris and blood tinge. Subsequently, the washed aspirate was transferred to a bottom gridded petri dish and visualized under a Nikon SMZ 1000 stereo microscope at 20x magnification to screen cumulus-oocyte complexes (COCs). COCs were evaluated at 80x magnification and graded from A to E based on oocyte integrity, cytoplasmic homogeneity and cumulus cell layers. Further, COCs were then classified as viable (grades A, B, and C) or non-viable (grades D and E) (Looney et al., 1994).

Following the discarding of non-viable COCs, Viable COCs were placed in a four-well IVM plate (NUNC© Thermo Scientific) with maturation medium and layered with oil and incubated for 20-24 hours under controlled conditions (6% CO2, 5% O2, 89% N2, 38.8°C and >90% RH), after which cumulus expansion was evaluated at 80x magnification. After the maturation of the COCs, they were transferred to a four-well IVF dish with pre-equilibrated fertilization medium (Vitrogen – YVF Biotech, Brazil). A proven semen for IVF was processed by centrifugation method using semen processing media (Vitrogen – YVF Biotech, Brazil) and the COCs were fertilized with 10 µL of processed sperm pellet with a final concentration of about $2x10^6$ sperm/ml. After 16-20 hours of incubation, presumptive zygotes were denuded using a denudation pipette (Origio®, Denmark) and transferred to a four-well IVC plate containing 500 µL IVC medium overlaid with 300 µL sterile oil (Vitrogen – YVF Biotech, Brazil). On day 7, embryo development was assessed at 80x magnification (Nikon, Japan) and graded according to IETS guidelines. The experimental design of the present study is illustrated in figure 1.

The data regarding OPU-IVEP attributes was then analysed using student's t-test and ANOVA as per Snedecor and Cochran (1994).

# Results and Discussion

The results of OPU-IVEP attributes for the two OPU sessions involving the Ongole and Gir cow donors were presented in Table 1.

The mean number of available follicles, also referred to as antral follicle count (AFC) did not show any significant difference (P>0.05) between the two OPU sessions in either of the breeds and in total as well. Also, the mean AFC recorded similar values in both the breeds showing that there was no difference in follicular pool between the breeds. However, the AFC ranged widely between individual animals in both Ongole (10 to 37) and Gir (19 to 48) cows. Burns *et al.* (2005) published that the peak number of antral follicles ≥ 3 mm in diameter recruited per follicular wave was highly variable (range 8-54) among individuals. The same kind of results were obtained in the present study and the values were highly repeatable with in an animal under the study. Gobikrushanth *et al.* (2017) found 10-53 at unknown stage of follicular growth and 6-45 on the expected day of follicular wave emergence, which was in line with the present finding in the Group-I of Ongole donors with an AFC of 12-37 at unknown stage of follicular growth and 10-18 on the expected day of follicular wave emergence. While the Ongole breed donor cows shown a lower value in AFC on the day of expected wave emergence compared to that in unknown stage of follicular growth, Gir cow donors exhibited higher AFC with a range of 24-48 on the expected day of follicular wave emergence compared to the unknown stage with a range of 19-31. Baldrighi *et al.* (2014) reported an AFC range of 2-125 in Gir cows. Krishna *et al.* (2023) discovered an AFC of 13-59 irrespective of FSH pre-treatment in Ongole cows. In all these studies AFC varied from study to study and established individual variation in AFC with highly repetitive values with in a given animal.

The current study and the observation of Mossa *et al.* (2012) make it clear that both ovarian reserve (the total number of healthy follicles and oocytes) and oocyte recovery are dependent on AFC, which in turn determines the other OPU and IVEP characteristics. The current study revealed that all the OPU and IVEP attributes had mean values that were comparable to the AFC and did not differ significantly. This suggests that the AFC is the primary determinant of attributes like oocyte recovery, the number of viable oocytes that follow and therefore the formation of embryos. It was clearly evident from our study that the wave of follicular emergence could not able to place any emphasis on these characteristics neither the breed. Cavalieri *et al.* (2018) observed

**Table 1**: OPU-IVEP attributes for the two OPU sessions involving the Ongole and Gir cow donors

| Attribute | Treatment type | Group 1 (Ongole n=6) | Group 2 (Gir n=6) | Total (n=12) |
|---|---|---|---|---|
| No. of follicles available | OPU 1 | 129 | 174 | 303 |
| | OPU 2 | 128 | 143 | 271 |
| Mean no. of available follicles | OPU 1 | 21.50±1.70 | 29.00±3.54 | 25.25±3.14 |
| | | (10-18) | (24-48) | (10-48) |
| | OPU 2 | 21.33±3.61 | 23.83±1.64 | 22.58±2.65 |
| | | (12-37) | (19-31) | (12-37) |
| No. of follicles aspirated | OPU 1 | 87 | 120 | 207 |
| | OPU 2 | 92 | 96 | 188 |
| Mean no. of follicles aspirated | OPU 1 | 14.50±1.23 | 20.00±2.99 | 17.25±2.52 |
| | | (16-27) | (15-36) | (15-36) |
| | OPU 2 | 15.33±2.69 | 16.00±1.52 | 15.67±2.05 |
| | | (8-32) | (11-23) | (8-32) |
| No. of COCs recovered | OPU 1 | 47 | 75 | 122 |
| | OPU 2 | 68 | 54 | 122 |
| Mean No. of COCs recovered | OPU 1 | 7.83±1.04 | 12.50±3.36 | 10.17±2.84 |
| | | (5-11) | (7-32) | (5-32) |
| | OPU 2 | 11.33±2.81 | 9.00±1.59 | 10.17±2.19 |
| | | (3-21) | (4-14) | (3-21) |
| Viable COCs | OPU 1 | 42 | 65 | 107 |
| | OPU 2 | 62 | 53 | 115 |
| Mean viable COCs recovered | OPU 1 | 7.00±1.02 | 10.83±3.17 | 8.92±2.49 |
| | | (4-11) | (6-28) | (4-28) |
| | OPU 2 | 10.33±2.65 | 8.83±1.51 | 9.58±2.04 |
| | | (3-21) | (4-13) | (3-21) |
| Embryos produced | OPU 1 | 19 | 29 | 48 |
| | OPU 2 | 24 | 24 | 48 |
| Mean no. of embryos produced | OPU 1 | 3.16±1.07 | 4.83±1.69 | 4.00±1.42 |
| | | (1-8) | (2-14) | (1-14) |
| | OPU 2 | 4.00±1.23 | 4.00±1.26 | 4.00±1.20 |
| | | (1-9) | (1-10) | (1-10) |

that synchronizing the estrous cycle and performing OPU on day 5 enhanced embryonic production in Nelore (Ongole) cows, a result that stands in contrast to current study. However, Gobikrushanth *et al.* (2018) noted no significant variation in AFC that was noted at an unknown stage of follicular growth compared to that was noted at expected day of wave emergence. Established research consistently indicates superior oocyte yield and embryo developmental percent in *Bos indicus* breeds compared to *Bos taurus* breeds (Pontes *et al.,* 2010; Sales *et al.,* 2015). This study revealed no significant differences in AFC, oocyte recovery, or embryo development between the two *Bos indicus* breeds of the study *i.e.,* Ongole and Gir.

**Conclusion**

In conclusion, the present data revealed no statistically significant differences in assessed OPU-IVEP parameters across various stages of the estrous cycle in either of the *Bos indicus* breeds of the study. Further to state, these findings indicate that, within the tested population of Ongole and Gir cows, the inherent variability in individual animal response may outweigh the potential benefits of precisely timed OPU based on synchronized follicular waves. Furthermore, this suggests that for these breeds, routine OPU procedures may be performed independent of estrous cycle synchronizations without compromising on oocyte quantity or quality for IVEP purposes.

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