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RESEARCH ARTICLE

Recombinant FSH (rFSH) superstimulation prior to ovum pick-up improved the rate of blastocyst production in zebu *(Bos indicus)* cattle

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Abstract : The present study was aimed to compare the efficacy of two differently sourced Follicle Stimulating Hormone viz., recombinant human FSH (rFSH) and conventional pituitary FSH (pFSH) on output of Cumulus Oocyte Complexes (COCs) retrieved through transvaginal ovum pick up followed by blastocyst production in Sahiwal cows. The selected donor cows were administered 10 µg of GnRH at random stage of estrous cycle and divided into two groups (1 and 2). Cows of Group 1were super-stimulated with rFSH and Group 2 were administered pFSH and subjected to OPU after 24h of last FSH injection. Retrieved COCs were subjected to *in vitro* maturation, *in vitro* fertilization and *in vitro* culture. The average number of follicles that were aspirated was 22.50± 4.47 in Group 1 and 29.33±5.14 in Group 2 respectively. The average number of COCs recovered along with their recovery rate in Groups 1 and 2 are 16.33±3.16 (72.50%) and 20.66±3.84 (70.45%). The average number of fertilized oocytes with rate of fertilisation was 7.83±1.88 (63.51%) in Group 1 and 10.83±3.28 (62.50%) in Group 2 respectively. The average number of blastocysts produced were 5.16±1.10 and 3.66±1.05 in Groups 1 and 2, respectively. The blastocyst conversion rates were 31.63% and 17.70%, in Groups 1 and 2, respectively. Group 1 stimulated with rFSH had a significantly ($p<0.05$) higher blastocyst conversion rate compared to Group 2stimulated with pFSH. Thus, rFSH can be used as a cost-effective alternative to pFSH for superstimulation prior to OPU for blastocyst production in Sahiwal cows.

Keywords: Blastocyst, Cumulus Oocyte Complexes, *In vitro* maturation, Ovum pick up, Recombinant FSH, Sahiwal cows.

Introduction

Domestic animals make major contribution to human requirements for food in the form of milk, meat, eggs, and fertilizer for crops as well as draught power**.** India, a country renowned for its rich livestock biodiversity, possesses 193.46 million cattle and 109.85 million buffaloes (DAHD Annual report, 2024). Of the total cattle population, about 142 million (74%) are indigenous and non-descript with low production potential. Enhancing the productivity of this huge chunk of animals will bring a quantum jump to the country's milk production. Reproductive technologies viz., artificial insemination, semen cryopreservation, estrous synchronization, embryo transfer (ET), embryo freezing and sexing are essential for conserving, propagating, and enhancing livestock productivity.

The transvaginal ultrasound-guided follicular aspiration (TVUFA) technique, initially developed for assisted reproduction in humans, was adapted for use in bovines (Pieterse et al. 1988). This facilitated ovum pick-up (OPU), which later became a key component of *in vitro* embryo production (IVEP) programs, offering a viable alternative to traditional methods of embryo production (Vennapureddy et al. 2022).

As the versatility and flexibility of OPU became evident, its application is extended to animals in various physiological states, including cyclic, non-cyclic, early pregnancy, and those unresponsive to hormonal stimulation. It was also successfully used in older cows with reproductive disorders (Galli et al. 2001; Vennapureddy et al. 2022), as well as juvenile calves and prepubertal heifers (Taneja et al. 2000). Today, OPU is recognized as the most flexible and repeatable technique for generating embryos from live and elite cows of Indigenous milch breeds like Sahiwal (Reddy et al. 2023; Farheen et al. 2024).

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The quantity and quality of oocytes collected during OPU are crucial factor that influence the yield of IVEP and their quality (Seneda et al. 2020; Vennapureddy et al. 2022; Krishna et al. 2023; Bramhaiah et al. 2024). These are affected by various factors including physiological status of the donor (Vieira et al. 2014), antral follicle count (AFC) (Baruselli et al. 2015), nutrition (Sales et al. 2015), breed (Sartori et al. 2016), age (Demetrio et al. 2021) and exogenous gonadotropins used for super-stimulation. The use of pituitary derived FSH (pFSH) in donor cows prior to OPU enhanced the consistency and size of the antral follicle population available for aspiration (Demissie et al. 2021; Farheen et al. 2024) and oocyte development competence during *in vitro* embryo production (Vieira et al. 2016). To avoid batch-to-batch variation in biological activity and LH contamination of the final pFSH product, recombinant FSH (rFSH) is used for stimulation in different dose rates (Carvalho et al. 2014). There are no studies on the use of rFSH super-stimulation prior to OPU in Zebu cattle. Hence, the study is undertaken to study the super-stimulatory effect of rFSH prior to OPU and its comparison with that of standard pFSH.

Materials and Methods

All the media viz., wash media, media for *in vitro* maturation of immature COCs, *in vitro* fertilization of mature oocytes, sperm and percoll media for sperm processing and *in vitro* culture media for culture of embryos till blastocyst were procured from Vitrogen, Brazil.

Ethical approval and Experimental animals

The experiment was carried out as per the mentioned guidelines of Institutional Animal Ethics Committee at Sri Venkateshwara Gosamrakshanashaala, Tirumala Tirupati Devasthanams, Tirupati and IVF laboratory, College of Veterinary Science, Tirupati betweenJune, 2024 to November, 2024 (Approval number: 281/ go/ ReBi/ S/ 2000/ CPCSEA/ CVSc/ TPTY/ 045/ Veterinary Gynaecology and Obstetrics/ 2024, Dated: 07-05-2024). Twelve (n=12) lactating / dry, pluriparous, high yielding, clinically normal, cyclic Sahiwal (*Bos indicus*) cows aged between 6-10 years weighing between 400 to 500 kg body weight were selected as donors after thorough clinical examination. The reproductive status was assessed by per rectal and ultrasonographic examination. All the selected animals were maintained with a daily ration of 2-4 kg with 20% DCP and 70% TDN along with 10-15 kg chopped fodder, 4-5 kg paddy straw and ad libitum drinking water. In general, animals were maintained under hygienic and optimum management conditions in loose housing system with a large, open paddock for free movement.

Superstimulation

All the selected donor cows were administered with 10 µg GnRH (Receptal @ 2.5 ml I/M) at random stage of estrous cycle. Half of the cows (n=6) were administered with recombinant human FSH (rFSH) @ 225 IU S/C in 2 divided doses (150 IU and 75 IU) at 48 and 72h after GnRH administration (1IU=17.0 µg). Other half (n=6) were administered with FSH (Follitropin-V (Vetoquinol) @ 200 mg I/M in 3 divided doses @ 100mg, 60mg and 40mg) at 48, 60 and 72 h after GnRH administration. Each vial of freeze dried Follitropin-V contains porcine follicle stimulating hormone (pFSH) 400 mg NIH that was reconstituted with 20 mL of bacteriostatic sodium chloride solution for injection USP so that final concentration was 20 mg of FSH/mL and OPU session was done. All the donors of Group 1 and 2 were subjected for OPU 24 h after the last FSH injection. The Cumulus Oocyte Complexes were collected and subjected to the process of in vitro maturation (IVM) followed by in vitro fertilization (IVF) and in vitro culture (IVC).

In vitro maturation

During each OPU session, all the visible follicles were aspirated in both groups of donor cows. Typically, the morphological evaluation and classification of cumulus oocyte complexes was based on the integrity of oocyte, homogeneity of the cytoplasm and the thickness of the cumulus cell layer around the oocyte. Basing on the evaluation, cumulus oocyte complexes were classified into four quality grades (A, B, C and D) and in turn in to viable (Grade A+B+C) and non-viable (Grade D) categories (Shanawaz et al. 2024).The recovered viable quality oocytes isolated from the colloidal solution were selected for maturation and washed in preequilibrated wash medium and maturation media by transferring them through 3-4 droplets of both wash medium and *in vitro* maturation medium, respectively. All of the COCs in the maturation media were examined under stereo zoom microscope after 20-24 h to observe the maturation changes such as cumulus cell expansion (Figure 1) or the extrusion of first polar body. COCs were classified as matured if they exhibited the cumulus cells expansion in a radiating pattern.

In vitro fertilization

The matured COCs were washed first in droplets of wash media, followed by IVF media. Finally, the matured COCs were transferred to IVF media containing 80 µl droplet @ 5 µl/cumulus oocyte complexes overlaid with mineral oil and placed in CO_2 incubator. The thawed sex sorted X sperm (Sexed Ultra XF 90 % DC SH 175 – SH- ULDB) was sourced through APLDA, a subsidiary unit of Animal Husbandry Department, Andhra Pradesh. The sperm is processed with Percoll for separation of motile fraction as per the instructions by Vitrogen. The sediment at the end of processing containing motile sperm was added @ 5-10 µL/ droplet of IVF media with matured oocytes of 10-15 in number. The matured oocytes are incubated with the frozen thawed X sperm for a period of 12 hrs for fertilization.

In vitro culture

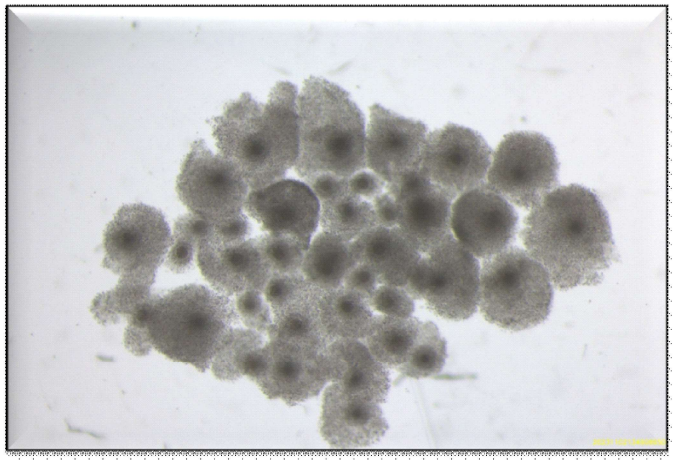

Fig. 1 Cumulus cell expansion of COCs after *in vitro* maturation

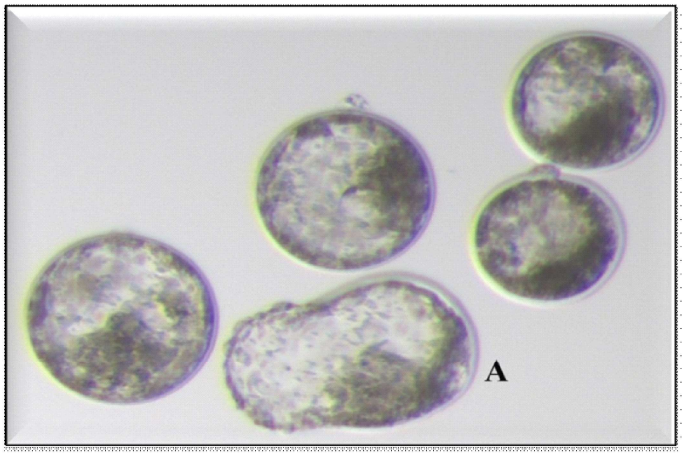

Fig. 2 Blastocyst (A) undergoing hatching

At the end of incubation period, the fertilized oocytes/ presumptive embryos are washed and denuded for removal of excess sperm and cumulus cells. They were then placed in 5 well petri dish containing pre- equilibrated IVC media (50-80 µl droplet) and overlaid with mineral oil, incubated in mixed gas incubator (5% CO_2, 5% $O_2$and 90% N_2) for 7-8 days till they reach the expanded blastocyst stage. The media was replaced with 60% fresh IVC media on 3rd and 5th day. After 72 h of IVF, the presumptive zygotes were examined for cleavage (Figure 2) as per the guidelines prescribed by International Embryo Transfer Society.

Statistical Analysis

Analysis of data was carried out by using independent t – test and Chi-square test. SPSS 15.0 software package for Windows was employed in the analysis. Graphical presentations were made using GraphPad Prism Software version 5.0 (San Diego, CA, USA). The level of significance of all the parameters was measured at $p<0.05$ (Snedecor and Cochran, 1994).

Results and Discussion

In FSH stimulated Sahiwal cows (recombinant FSH *vs.* pituitary FSH), information on follicular development and its availability for follicular aspiration, follicle aspiration rate, recovery of cumulus oocyte complexes (COCs), maturation rates of recovered COCs, fertilization rate and blastocyst conversion rates are provided in the Table 1.

Group 1 (rFSH stimulated) and Group 2 (pFSH stimulated) cows had 159 and 206 available follicles with an average of 26.50±5.38 and 34.33±6.02 follicles in respective groups 1 & 2 respectively. The mean number of follicles aspirated per cow per session with aspiration rates was 22.50±4.47 (84.90%) and 29.33±5.14 (85.43%) in Group 1 (rFSH stimulated) and Group 2 (pFSH stimulated), respectively. The total COCs recovered were 98 and 124 COCs in Group 1 (rFSH stimulated) and Group 2 (pFSH stimulated) respectively with recovery rates of 72.59% and 70.45%(Figure 3). A total of 83 and 112 viable COCs (grade A+B+C) were recovered in Group 1 and Group 2, respectively. The rates of recovery of viable COCs observed were 84.69 % in Group 1and 90.32 % in Group 2 (Figure 3). For all the parameters studied and compared, there was no significant difference between the groups

After exposing the COCs recovered to the IVM process for 24 hours, the mean number of matured oocytes and rate of maturation was 12.33±2.29 (75.51%) and 17.33± 2.44 (83.87%) in Group 1 and Group 2, respectively. The mean number of mature oocytes fertilized with rate of fertilisation after IVF procedures was 7.83±1.88 (63.51%) for Group 1 and 10.83±3.28 (62.50%) for Group 2 respectively (Figure 4). The mean number of blastocysts produced in Group 1 being 5.16±1.10 and Group 2 being 3.66±1.05. There was no significant difference between the two groups for all the parameters studied and compared till now. However, the blastocyst conversion rates were 31.63% and 17.74 % for the Groups 1 and 2, respectively that differs significantly ($p<0.05$) (Figure 4).

This study compared the super-stimulatory effects of recombinant human FSH (rFSH) Group 1 and pituitary-derived FSH (pFSH) Group 2 on ovarian stimulation in Zebu (Sahiwal breed) cattle.

The similarity in the mean number of follicles available for aspiration in Groups 1 and 2 are in agreement with that of Carvalho *et al.* (2014), and Viana *et al.* (2024). The number of follicles aspirated per session per cow per session are similar in group 1 stimulated with a lower dose (17 µg) of r FSH, exhibiting a comparable efficacy to that in group 2, stimulated with a higher dose of pFSH (200 mg). This outcome is attributed to the higher purity, homogeneity, bioactivity (Carvalho et al. 2014), and longer half-life of rFSH (Song et al. 2024) that probably enhanced the antral follicular recruitment during folliculogenesis comparable to pFSH. Further, the heterogenous nature and lesser purity of

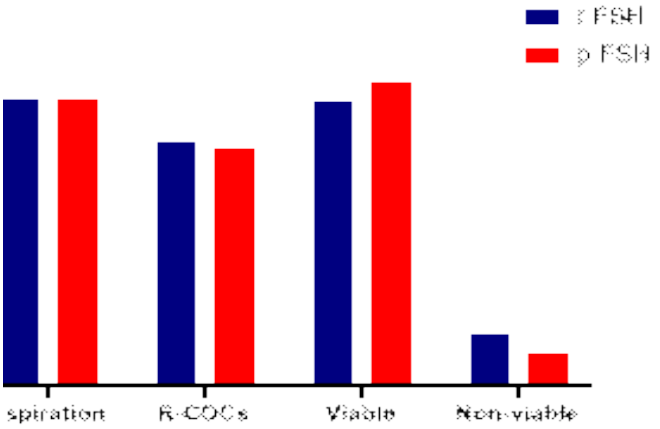

Fig. 3 Effect of rFSH and pFSH on rates (%) of follicles aspirated, viable and non-viable Cumulus oocyte complexes (COCs) recovered through OPU in Sahiwal donor cows.

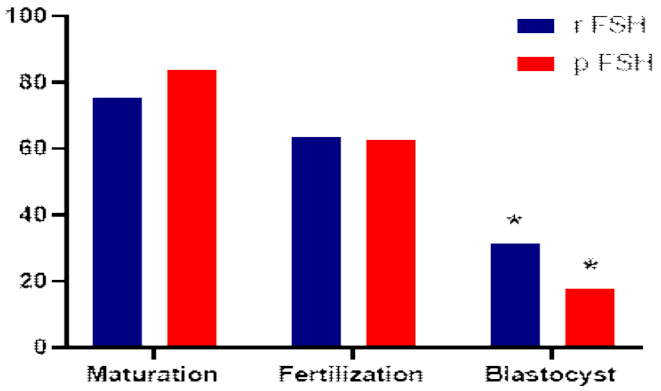

Fig. 4 Effect of rFSH and pFSH on the rate (%) of COCs undergone maturation, fertilization and converted into blastocysts after retrieval through OPU in Sahiwal donor cows. Rates with a significant difference at $p<0.05$ are indicated by an asterisk (*).

Table 1: Performance of Sahiwal cows with super stimulation using r FSH and p FSH during transvaginal ovum pick-up on various parameters set for the study

Attribute	Group 1 (rFSH stimulated)	Group 2 (pFSH stimulated)
No. of animals	6	6
Total no. of follicles available for aspiration	159	206
No. of follicles aspirated	135	176
Mean No. of follicles aspirated / cow / session	22.50±4.47[a]	29.33±5.14[a]
Aspiration rate (%)	84.90[a]	85.43 [a]
Total No. of COCs recovered and subjected to IVM for 24 h	98	124
Recovery rate (COCs / aspirated follicles) (%)	72.59[a]	70.45[a]
Number of oocytes matured after 24 h of IVM & incubated with sperm for 12 h	74	104
Mean number of matured oocytes	12.33±2.29[a]	17.33±2.44[a]
Rate of maturation (%)	75.51[a]	83.87[a]
Number of oocytes fertilized	47	65
Mean number of oocytes fertilized	7.83±1.88[a]	10.83±3.28[a]
Fertilization rate w.r.t number of matured oocytes (No. of oocytes fertilized / No. of oocytes matured) %	63.51[a]	62.50[a]
Blastocyst produced	31	22
Mean no. of blastocysts produced / cow / session	5.16±1.10	3.66±1.05
Blastocyst production w.r.t no of oocytes subjected to IVM (No. of embryos produced / no. of oocytes recovered) %	31.63[a]	17.74[b]

Values bearing different superscripts with in a row differ significantly ($p<0.05$)

pFSH (contaminated with LH, the ratio of FSH is 5.25:1) (Rodrigues et al. 2023) would have necessitated a higher dosage for stimulatory effect on follicles.

The mean numbers of total as well as viable cumulus-oocyte complexes (COCs) recovered along with their recovery rates are similar in both the groups. This is probably due to the prolonged half-life of the rFSH as reported by Song *et al.* (2024) in sheep. Nevertheless, the higher purity and homogenous nature combined with higher bioactivity of the rFSH might have exerted a positive effect on the retrieval and recovery rate of COCs even when used in lower doses that was comparable to that of higher doses of pFSH (Carvalho et al.2014).

The mean number of blastocysts produced per cow exhibited a non-significant higher value (5.16±1.10) and significant conversion rate in group 1 stimulated using rFSH compared to that of group 2 stimulated by p FSH with a mean yield of (3.66±1.05) and conversion rate of 17.7%. The higher conversion rates of blastocysts with rFSH stimulated group 1 might be linked to the enhanced developmental competence of COCs and better receptor-level activity of rFSH, which is similar to the findings of Gutierrez-Reinoso *et al.* (2025). While previous studies (Arreseigor et al. 2022; Gutierrez-Reinoso et al. 2022; Khodadadi et al. 2022) corroborate the advantages of rFSH, the lack of direct comparisons in cattle limits broader conclusions. The findings highlight rFSH as a potent alternative to pFSH for OPU and *in vitro* embryo production, offering comparable or superior outcomes with lower doses and lower frequency of administration.

Conclusion

The mean number of follicles available for aspiration, the mean number of follicles aspirated, the COCs recovered, the maturation rate, and the fertilization rate were all found to be statistically similar in donor cows stimulated with rFSH and pFSH. Interestingly, however, the blastocyst conversion rate was significantly higher in rFSH-treated Sahiwal cows than in pFSH donor cows. It can be concluded that recombinant follicle stimulating hormone is a viable alternative to conventional pituitary follicle stimulating hormone for super stimulation. Additionally, rFSH offers beneficial effects in being cost effective and long-acting apart from reducing the need for animal handling.

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