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Effect of Hormones, Season and Lactation on Follicular Size, Oocyte Competency and Embryo Production in Sahiwal Cattle

K. Veera Bramhaiah[1#], N. Venkata Krishna[2#], G. Sandeep Kumar[2], K. Greeshma Reddy[2], Y. Nagendra Reddy[2], K.V.V. Tagore[2], D. Manjunath Reddy[2]

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ABSTRACT

Background: Transvaginal ultrasound-guided ovum pick-up (OPU) and *in vitro* embryo production (IVEP) techniques are widely used for enhancing reproductive efficiency. The number and quality of embryos have been reported to be influenced by various factors like source of hormones, season, lactation in cattle.

Methods: Twenty-one multiparous, normal cyclic Sahiwal cows (aged: 6-8 years; lactating: n-11 and non-lactating: n-10) were selected randomly and the experiment was conducted during hot (April to September) and cold (October to March) seasons. All the donor cows were administered 10 µg GnRH at random stage of oestrous cycle followed by FSH (Folltropin-V™ or Stimufol) at the rate of 100, 60 and 40 mg after 48, 60 and 72 h of GnRH administration. After 24 hours of coasting period, follicular aspiration was performed by stabilizing the targeted follicles with the help of ultrasonography. The procedures of COCs grading, *in vitro* maturation, fertilization, followed by culture were performed as per the standard protocols.

Results: The hormonal treatment was effective in improving the oocytes' developmental competency and *in vitro* embryo production; with no statistical differences between the two sources. The follicle size distribution revealed a lower (P<0.05) medium-sized and small-sized follicular proportion during the hot season with a higher average count of dominant (large-sized) follicles. While, good quality (A+B+C) oocytes, matured and fertilized oocytes were significantly higher (P<0.05) in non-lactating donors, especially among cool-season collections.

Key words: Embryo production, Heat stress, IVF, Lactation, Ovum pickup.

INTRODUCTION

The introduction of *Bos taurus* germplasm and cross-breeding programs in India aimed at augmenting milk production has resulted in a decline in the population and economic value of indigenous breeds, such as Sahiwal (Reddy *et al*., 2021). Assisted reproductive technologies have been used extensively to improve the reproductive efficiency and genetic makeup of indigenous cattle (Viana, 2021). Among these transvaginal ultrasound-guided ovum pick-up (OPU) and *in vitro* embryo production (IVEP) are being practiced to improve oocyte quantity and quality with FSH pre-treatment and to get good IVEP outcomes (Hayden *et al*., 2022).

At the same time, the ovarian response to OPU is dependent on several crucial factors, including the season and physiological condition of the animal (Khan *et al*., 2022) wherein hot season negatively influences the follicular growth, estrus expression, super ovulatory response, embryos quality and fertility (Khan *et al*., 2020). Further, in post-parturient or early lactating cows negative energy balance (NEB) is known to reduce the size of dominant follicle and changes in the blood glucose, IGF-1 (insulin growth factor-1), NEFA (non-esterified fatty acid), urea and total cholesterol (Reddy *et al*., 2018; 2019).

Considering the importance of the aforementioned factors, this study was planned to evaluate the influence of two commercial hormonal preparations, season and lactation on decisive parameters of OPU and IVEP techniques in sahiwal cows.

[1]YSR Administrative Building, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India.
[2]Department of Veterinary Gynaecology and Obstetrics, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India.
[#]These authors contributed equally to this work.

Corresponding Author: N. Venkata Krishna, Department of Veterinary Gynaecology and Obstetrics, Sri Venkateswara Veterinary University, Tirupati-517 502, Andhra Pradesh, India. Email: venkata26krishna@gmail.com

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MATERIALS AND METHODS

Study location and animals

The study was conducted at the Department of Veterinary Gynaecology, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India in 2022-23. Twenty-one multiparous, normal cyclic sahiwal cows (aged: 6-8 years; lactating: n=11 and non-lactating: n-10) were used as donors. Suckling was allowed and hand milking was practiced twice a day at 06:00 and 17:00. The animals were

treated during two different seasons; hot (April to September) and cold (October to March).

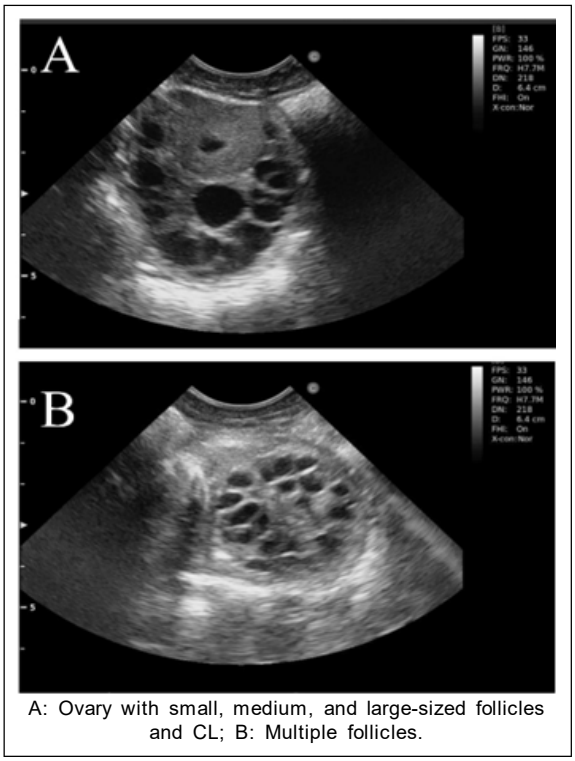
Super ovulation

The selected donor cows were randomly divided into two groups and one group received Folltropin and another group was administered Stimufol. Both the groups were administered with 10 µg GnRH (Receptal™ 2.5 ml i/m) at random stage of oestrous cycle. Then one group of animals received FSH (Folltropin-V™) and another group received (Stimufol™ at 200 mg i/m) at the rates of 100, 60 and 40 mg after 48, 60 and 72 h of GnRH administration.

Preparation of donor and ovum pick-up (OPU)

Following epidural anaesthesia, the lubricated transvaginal probe fitted in the plastic probe carrier (WTA, Sao Paulo, Cravinhos, Brazil) was advanced into the anterior vagina. Later, the ovary was positioned *via* transrectal manipulation together with the transducer to view and aspirate the follicles. The data relating to the number and diameter of follicles were recorded by freezing the image on the monitor and using an inbuilt calliper. Based on the diameter, the follicles were categorized as small (<4 mm), intermediate (4-<8 mm) and large (≥ 8 mm) as per the classification of Ginther *et al*. (1989). In addition, the presence of the corpus luteum on the ovary was visualized and its diameter was recorded.

After stabilizing the ovary and targeted follicle, the follicular aspiration was performed by using a real-time B mode ultrasound scanner (Chison Medical Imaging Co., China) equipped with a multi-frequency (4-9 MHz) micro convex probe (Fig 1). During the entire OPU session, the needle and aspiration line were thoroughly rinsed with pre-heated (37°C) OPU recovery medium (YVF Biotech Ltd, Brazil) to prevent blood from clotting or oocytes from sticking to the tubing. The negative pressure of 45 to 65 mm Hg was generated using an aspiration pump (WTA, So Paulo, Cravinhos, Brazil) and generate a fluid flow of 23-25 ml per minute.



A: Ovary with small, medium, and large-sized follicles and CL; B: Multiple follicles.

Fig 1: Transvaginal ultrasonographic images of FSH stimulated ovary.

Initial OPU was carried out with a coasting period of 24 h after the last FSH administration and the 2nd OPU at 96 h after 1st OPU. A total of 42 OPU sessions were performed on all the visible follicles and the oocyte yield and quality were analysed. The aspiration rate was calculated by dividing number of follicles aspirated with total number of follicles.

Handling and grading of oocytes

The washed and filtered follicular aspirate was transferred to a square grid petri dish (90 × 15 mm, Tarsons, Chennai) and examined under a zoom stereo microscope (SMZ - 1000, Nikon, Japan) at 20× magnification to identify the cumulus-oocyte complexes (COCs). The COCs were transferred to a 35 mm petri dish containing Vitrogen wash media (YVF Biotech Ltd, Brazil) and examined under a zoom stereomicroscope at 63× magnification and graded as per the classification provided by Looney *et al*. (1994) and Bungartz *et al*. (1995). The quality of aspirated oocytes was graded as A, B and C (good quality oocytes) and D, E grades were regarded as poor-quality oocytes. The oocyte recovery rate was calculated as number of oocytes recovered/ number of follicles aspirated.

***In vitro* maturation and staining**

All the good quality oocytes were subjected to *in-vitro* maturation and evaluated for its maturity based on the nuclear changes or stages of oocytes. Initially COCs were transferred to one of the wells of a 4-well dish (IVM dish) containing a 500 µl pre-equilibrated maturation medium (YVF Biotech Ltd, Brazil) and incubated for 24 hours. Later, the IVM dish containing oocytes was quickly examined for maturation. The cumulus cell expansion, mucification and extrusion of polar body were assessed under a zoom stereo microscope at 63 magnification (Nikon, Japan) and subjecting them to HOECHST 33342 fluorescent dye (1ug/ml) for 30 mins in dark at 38.5°C (Smith *et al*., 1993). The oocytes were manually denuded with the help of denudation pipette (Cooper surgicals, Malov, Denmark) and washed in maturation media thrice.

***In vitro* fertilization (IVF) and *in vitro* culture (IVC)**

The IVF and IVC were performed as per the standard protocol mentioned by Shahzad *et al*. (2020). A straw containing good quality frozen sexed semen of Sahiwal bull (Karim) was thawed at 37°C for 30 seconds and semen was mixed with percoll gradient consisting of 400 µl of percoll sexed and 200 µl of percoll diluted (200 µl sperm + 200 µl percoll sexed). Later, the vials were centrifuged at 600 × g for 6 minutes followed by discarding the supernatant leaving only the pellet. The residue was

transferred to a new Eppendorf tube with 400 µl of IVF medium. Second centrifugation was performed at 150× g for 3 minutes followed by supernatant removal. The pellet was evaluated for motility and concentration (Razza *et al*., 2019). The COCs and sperm were co-incubated for 16 h at 5% CO_2, 38.5°C temperature and 95% humidity for IVF. After 16 hours of insemination, the cumulus cells and excess sperm were removed from presumptive zygotes through gentle pipetting in pre-equilibrated culture (YVF Biotech Ltd, Brazil) medium. After three washings in culture medium, presumptive zygotes from each cow were placed separately in 100 µl drops of culture medium and incubated in bench top incubator (Planer BT 37, Cooper surgical, Denmark) for seven days under a controlled atmosphere (5% CO_2, 5% O_2 and 90% N_2) at 38.5°C. It would be beneficial to add "morula" on day 5, alongside the mention of "cleavage" on day 3 and "blastocyst" on day 8, to provide a more comprehensive overview of embryonic development.

Statistical analysis

Statistical analysis was performed using SAS (V 9.4). Data were tested for normal distribution using PROC UNIVARIATE statement and examining the p-value of Shapiro-Wilk test. The data were subjected to the independent t-test using the statement 'PROC t-test'. The *P* values less than 0.05 were assumed to be significant, while those between 0.05 and 0.01 were considered as a trend.

RESULTS AND DISCUSSION

The follicular size distribution and aspiration rates are presented in Table 1. Our earlier study on hormonal stimulation prior to OPU revealed a higher oocyte developmental competence and embryo production rates (Krishna *et al*., 2023). Accordingly, two hormones (Folltropin vs Stimufol) have been used and obtained equally effective responses in stimulating the follicular development but without any significant effect on the follicular diameter and oocyte quality including *in vitro* parameters.

The analysis of follicle size distribution revealed a lower ($P<0.05$) medium-sized follicular proportion with a reduced tendency of large-sized follicular incidence and aspiration rate during the hot season. Similarly, a recent report focusing on the effect of season on growth of early antral (small-sized) follicles revealed a negative effect of heat stress on the major regulatory events of folliculogenesis and follicular diameter (Kawano *et al*., 2022). The follicular size decrease might be due to increasing temperature humidity index (THI) that suppresses the dominance size and in turn the follicular diameter (Schuller *et al*., 2017) and a high degree of follicular atresia and lower circulating concentrations of FSH and LH during summer months (Chu *et al*., 2018).

In the present study, the non-lactating cows exhibited a lower proportion of small follicles and a higher average count of dominant (large-sized) follicles than that of the lactating cows. The challenged metabolic responses in lactating cows might have altered the ovarian steroidogenesis leading to inadequate follicular development (Endo *et al*., 2012). As a positive energy balance is positively correlated with the count of large follicles and negatively correlated with small follicles, the higher count of small follicles in lactating cows could be attributed to negative energy balance during early lactation or stress (Reddy *et al*., 2018; 2019).

The quality of oocytes between the two regimens were non significantly different, however, the quantity and quality (A+B+C category) of oocytes was recorded to be higher during cold season. The lower tendency ($P=0.083$) of oocyte recovery per animal per session during hot season could be attributed to the lower number of follicles available for puncture. Further, the good quality oocytes (A+B+C grade) collected from non-lactating cows were observed to be higher ($P<0.05$) in count compared to those collected from

Table 1: Effect of hormonal source, season and lactation status on follicular size and quality of oocytes.

Size	Treatment		Season		Lactation status		SEM	*P* value		
	Folltropin (n=12)	Stimufol (n=09)	Cool (n=11)	Hot (n=10)	Lactating (n=10)	Non lactating (n=11)		T	C	LS
Small	31.17	26.44	29.73	28.50	31.60	26.91	2.25	0.105	0.970	0.036
Medium	34.83	37.33	37.64	34.00	33.80	37.82	2.43	0.180	0.041	0.515
Large	8.00	7.67	8.45	7.20	6.70	08.91	0.70	0.530	0.089	0.043
Total	74.00	71.44	75.82	69.70	72.10	73.64	3.76	0.151	0.118	0.987
No. of follicles aspirated	60.50	59.89	63.64	56.50	56.60	63.55	3.49	0.385	0.251	0.838
Aspiration rate	81.26	83.47	83.54	80.74	78.32	85.75	3.55	0.227	0.072	0.522
Quality of oocytes										
A	13.00	15.89	14.45	14.00	12.30	16.00	3.36	0.193	0.754	0.213
B	8.33	10.33	8.64	9.80	7.70	10.55	3.36	0.649	0.713	0.041
C	10.17	7.33	10.64	7.10	7.50	10.27	3.45	0.416	0.154	0.062
D	8.83	5.89	9.64	5.30	8.00	7.18	3.56	0.673	0.150	0.418
A+B+C	31.50	33.56	33.73	30.90	27.50	36.82	3.22	0.164	0.096	0.048
Total	40.33	39.44	43.36	36.20	35.50	44.00	3.37	0.345	0.083	0.322

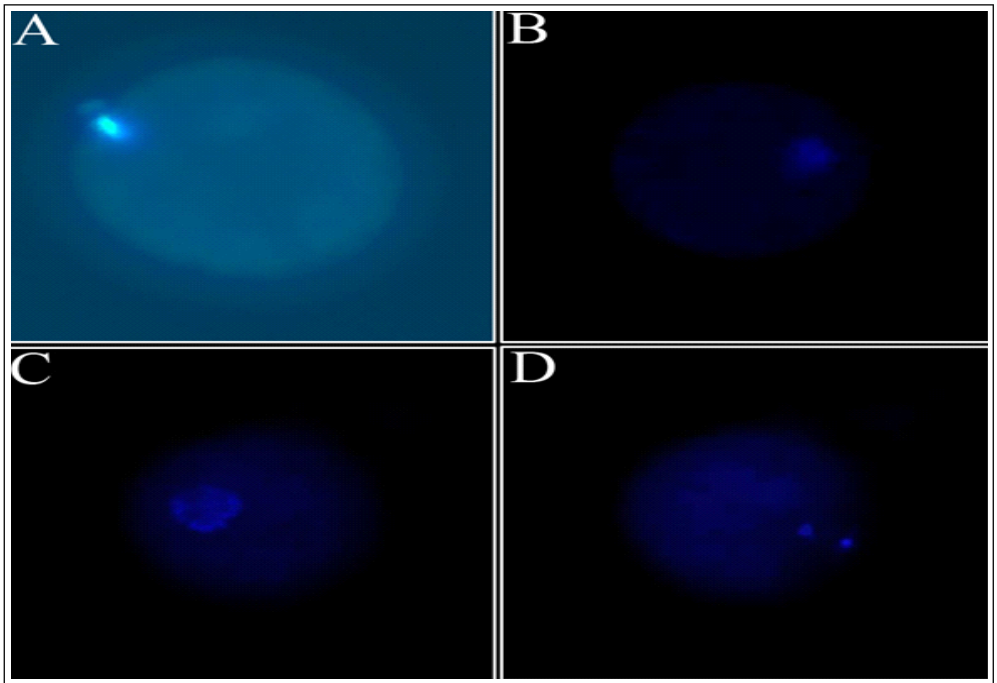
lactating cows. The metabolic profile of lactating cows is characterized by lower concentrations of progesterone and higher concentrations of NEFA and BHBA and this negative energy balance-associated metabolic profile has been proved to be associated with a suboptimal follicle microenvironment, thereby compromising oocyte quality (Missio *et al*., 2022). In addition, the imbalance in the metabolism of NEFA and pyruvate during lactation and nutrition stress is further known to reduce oocyte quality and developmental competence (Shri and Sirard, 2022).

The *in vitro* fertilization and maturation rates are presented in Table 2. The oocyte was stained with Hoechst 33342 during maturation and the nuclear status of oocyte is exhibited in Fig 2. A spherical and uniformly brilliant disc representing germinal vesicle with germinal vesicle break down (GVBD) showing more condensed nuclear material and disintegration of the nuclear membrane was observed. The sequence of oocyte retrieval and embryo culture from oocytes' aspiration to blastocyst formation was depicted in Fig 3.

The prolonged higher temperatures of summer season during oocyte collection might have compromised maturation and fertilization of COCs by disrupting the oocyte physiology and causing hormonal imbalances on the hypothalamic-pituitary axis (Mietkiewska *et al*., 2022). Furthermore, the compromised oocyte quality due to altered

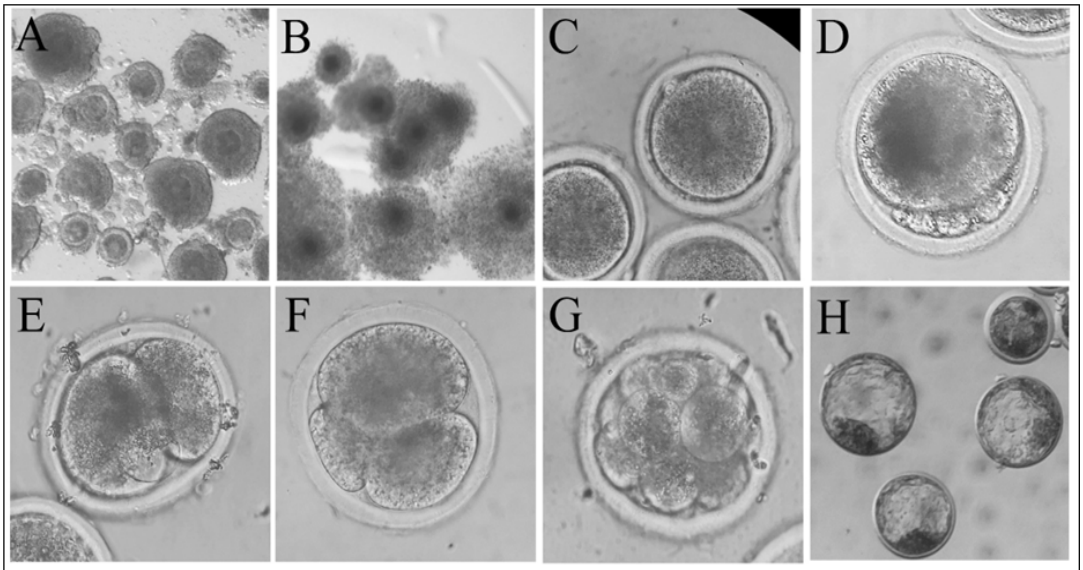
Table 2: Effect of hormonal source, season and lactation status on *in vitro* maturation rate, fertilization rate and embryo production.

	Treatment		Season		Lactation status		SEM	*P* value		
	Folltropin	Stimufol	Cool	Hot	Lactating	Non lactating		T	C	LS
No. of matured oocytes	33.42	32.22	35.64	29.90	27.80	37.55	2.53	0.101	0.033	0.030
Maturation rate	81.26	82.65	81.49	82.27	78.87	84.58	2.82	0.579	0.646	0.047
No. of fertilized oocytes	26.42	27.22	29.09	24.20	22.50	30.64	2.03	0.133	0.049	0.042
Fertilization rate	79.65	84.15	82.50	80.56	81.05	82.06	1.60	0.290	0.163	0.681
2-celled	22.42	21.33	23.36	20.40	21.20	22.64	1.53	0.187	0.761	0.892
4-celled	21.33	19.89	22.18	19.10	20.10	21.27	1.37	0.204	0.980	0.856
8-celled	18.83	17.11	19.55	16.50	18.20	18.00	1.14	0.548	0.791	0.311
16-celled	17.08	15.11	18.09	14.20	16.50	16.00	1.11	0.468	0.615	0.382
Morula	15.42	13.11	16.27	12.40	14.60	14.27	1.04	0.723	0.690	0.959
No. of embryos	10.50	10.11	10.91	9.70	10.90	9.82	3.47	0.695	0.686	0.143



Stained with Hoechst 33342. 2A: Oocyte showing polar body; 2B: Oocyte showing nucleolus; 2C: Germinal vesicle membrane disintegration; 2D: Oocyte showing germinal vesicle stage.

Fig 2: Nuclear status of Sahiwal cow oocyte during maturation.



3A: Aspirated COCs; 3B: Oocytes post *in vitro* maturation; 3C: Oocyte in Metaphase II with a pro nucleus; 3D: Post fertilized oocyte with 3 pro nuclei; 3E: 2-celled stage embryo (First cleavage); 3F: 4-celled stage embryo; 3G: Morula stage embryo; 3H: Blastocyst stage embryo.

Fig 3: Sequence of oocyte retrieval and embryo culture.

metabolic profile of lactating cows may explain the lower *in vitro* maturation and fertilization per cent of COCs collected from lactating cow donors. However, it is noteworthy that the *in vitro* embryo culture was conducted under controlled conditions. Despite the higher ($P<0.05$) mean number of matured and fertilized oocytes in cool climate and non-lactating cows, no effects were seen on cleavage rate or the proportion of embryos reaching the 2-cell, 4-8 cell or >8-cell stages and embryo production.

The results of the present study revealed that the cool season and non-lactating donors are the preferred conditions for enrolling in OPU-IVP programs. Finally, shortcomings should be considered in order to transfer the results to field level *viz*. the variations in the aspiration of follicles, variation in the exogenous gonadotropin and FSH regimen, oocyte collection criteria, intervals between subsequent OPU sessions and pressure and needle diameter.

CONCLUSION

There was no effect of the source of hormones on the superovulatory response for OPU protocols. The study concludes that the oocyte quality reduces with summer stress and non-lactating cows are good donors for Ovum Pick Up *In-vitro* embryo production (OPU-IVP) programs. These results create a paradigm for future *in vitro* embryo production program-based studies in Sahiwal cows under different climatic and physiological conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

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