RESEARCH ARTICLE

Ovum-Pick Up and *In Vitro* Embryo Production Responses in Stimulated and Non-Stimulated Sahiwal Cows

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Abstract

The aim of the present study was to evaluate follicular population, oocyte recovery, quality, and *in vitro* embryo developmental competence in non-stimulated and FSH stimulated Sahiwal cows subjected to ovum pick-up (OPU) and *in vitro* embryo production (IVEP). The study was conducted on total 16 cows divided into two groups; group 1 FSH stimulated (n=08) and group 2 non-stimulated (n=08) Sahiwal cows. A total of 16 OPU sessions were performed in early luteal phase of estrous cycles. The mean number of follicles aspirated (17.13±1.87 Vs. 12.38±0.85) and the mean number of oocytes recovered (10.25±0.98 Vs. 6.75±0.60) per OPU per animal were significantly ($p<0.05$) higher in FSH-stimulated as compared to the non-stimulated cows. In stimulated group, a significantly ($p<0.05$) high Grade-A oocytes were recovered as compared to the non-stimulated group (60.98±0.49% Vs. 24.07±0.40%). However, maturation rate (84.41 Vs. 81.10%) and fertilization rate (78.46 Vs. 72.08) did not differ significantly between stimulated and non-stimulated groups. Further when fertilized oocytes were cultured *in vitro* for 7 days higher ($p<0.05$) cleavage rate (73.44% Vs. 61.25%) and embryo production (4.50±0.50 Vs. 2.38±0.43 per OPU session) were recorded in stimulated cows as compared to the non-stimulated cows. Further all the zygotes cleaved into 2-cell stage in both the groups, but subsequently significantly ($p<0.05$) more 4-cell stage, 8-cell stage and numerically higher 16-cell stage and compact morula (13.89% Vs. 5.26%) were recorded in stimulated than in non-stimulated Sahiwal cows. In conclusion, the FSH pre-stimulation prior to OPU-IVF increased the number of medium and large follicles, oocyte recovery and embryo quality in Sahiwal cows.

Key words: Embryo, FSH, OPU-IVF, Quality, Sahiwal cows.

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Introduction

The global demand for *in-vitro* produced embryos (IVP) has surpassed the multiple ovulation and embryo transfer (MOET) for the first time in recorded history (Viana, 2018). In mid 1980s, the new reproductive technology, Ovum pick-up (OPU) was developed to harvest oocytes from live cattle. The results of an *in-vitro* embryo production (IVEP) program are largely dependent on the antral follicular population and their sizes available during OPU procedure (Chasombat *et al.,* 2013). The acquisition of developmental competence of oocytes is related to follicular size as the oocytes retrieved from medium-size follicles had better developmental competence than the small-size follicles (Sarwar *et al.,* 2020). Therefore, a homogenous population of medium size (6-10 mm) follicles is aimed for improving the efficiency of *in-vitro* embryo production. In this context, gonadotropin pre-stimulation was used to increase *in-vitro* embryo production efficiency (Bo and Mapletoft, 2020).

The hormonal stimulation using gonadotropins is preferred method to increase the number of follicles available for aspiration, the oocyte yield, quality and thus the subsequent embryo production (Sathanawongs *et al*., 2004). Ovarian response, follicle number in ovaries and oocyte quality are affected by the type of gonadotropin used and FSH is better alternative than eCG for OPU treatment (Sendag *et al*., 2008). To the best of our knowledge, very meagre reports are available on effect of gonadotropin stimulation prior to OPU and oocyte recovery, *in-vitro* maturation, *in-vitro* fertilization, and IVEP in Sahiwal (*Bos Indicus*) cows under

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tropical climatic conditions. Hence, this study was designed to know the follicular population, oocyte recovery, quality, and embryo developmental competence in FSH-stimulated and non-stimulated Sahiwal cows during ovum pick-up and *in-vitro* embryo production.

Materials and Methods

Experimental Design and Ovum Pick-Up

The study was conducted on total 16 Sahiwal cows of 1st to 4th parity, maintained under iso-managerial conditions at Cattle and Buffalo Farm, LPM Section, ICAR-Indian Veterinary Research Institute, Izatnagar (UP, India). The average body weight of cows taken in the study was 350±25 kg. The experiment was conducted after permission from Ethical Committee of IVRI, Izatnagar, UP, India, vide Ref No. 26-3/2020-21/JD(R)/IAEC.

The experimental cows were divided into two groups (n=08 each). In stimulated group, cows were pre-stimulated with FSH (total dose 130 µg (40, 40, 25, 25 µg; morning & evening, i/m) on day-2 and 3 post-estrus followed by coasting period of 36-48 h. Trans-rectal ultrasonography (Exago-ECM, IMV) at 7.5 MHz frequency was performed on day-1 and 2 of FSH and on the day of OPU to access the effect of gonadotropin stimulation on different follicular categories, *i.e.,* large follicle (LF), medium follicle (MF) and small follicle (SF) (Table 1). On 4th day post-estrus (44 h after last FSH injection), OPU was performed by aspirating all visible follicles of 3-10 mm size as described by Vennapureddy *et al.* (2024). The collected oocytes were graded under stereozoom microscope.

In-Vitro Embryo Production

The cumulus oocyte complexes (COCs) recovered after OPU were washed with BO-wash media and subsequently with BO-HEPES media. After *in-vitro* maturation for 24 h at 5% CO_2, 90-95% RH at 37°C, the maturation status of oocytes was assessed based on expansion of cumulus layers and presence of polar body under stereozoom microscope (Olympus SZX12). The COCs were washed thrice in BO-IVF medium and then transferred to 100 µL fertilization droplet of BO-IVF medium. Each IVF drop contained 10-20 oocytes received and 10-15 µL of frozen-thawed Sahiwal semen with a final concentration of 2×10^6 live sperm/mL. Then the COCs and sperm were co-incubated for 16 h at 5% CO_2, 38.5°C temperature, and 95% humidity for IVF. After 16-18 h of IVF, the presumptive zygotes were visualized for presence of sperm in the ooplasm and two pronuclei. Before keeping presumptive zygotes to IVC media, the cumulus cells and excess sperm were removed by washing (3 times) in 100 µL droplets of prewarmed IVC media. After three times washing in BO-IVC media, 10-20 presumptive zygotes were placed separately in 100 µL drops of prewarmed BO-IVC and further incubated for 7-days. The cleavage and embryo development rates were assessed on 3rd, 5th, and 7th day of embryo culture. The developed embryo was evaluated as per guidelines of IETS (International Embryo Technology Society).

Statistical Analysis

The data related to comparative oocyte recovery, quality, *in vitro* maturation, fertilization, and culture rates between non-stimulated and stimulated groups were statistically analyzed using SPSS (20.0). Data were presented as mean ±SEM. OPU-IVEP parameters were compared using unpaired 't'-test. Difference between the mean values was considered significant when the level of significance was $p<0.05$.

Results and Discussion

Effect of Stimulation on Follicular Population

On day 1, the mean follicular population recorded in non-stimulation and stimulated Sahiwal cows had no significant difference in different classes of follicles, *i.e.,* small, medium and large (Table 1). However, on day 2, a significantly higher numbers of medium (3.63±0.53 Vs. 1.75±0.29) and large follicles (1.25±0.15 Vs. 0.50±0.18) were recorded in stimulated as compared to non-stimulated group. Furthermore, on the day of OPU, a significantly lesser number of small (7.50±0.87 Vs. 16.63±0.81) and a greater number of medium (7.38±0.56 Vs. 2.25±0.23) and large (3.75±0.39 Vs. 0.86±0.23) follicles were recorded in stimulated than non-stimulated Sahiwal cows (Table. 1).

On the day of OPU, the size of the large (≥9 mm; LF), medium (≥5-9 mm; MF) and small (≥2.5-5mm; SF) follicle did not show any significant difference ($p>0.05$) between non-stimulated and stimulated Sahiwal cows (Table 1). Similar findings have been reported by earlier workers in cattle (Ongaratto *et al.,* 2015; Vennapureddy *et al.,* 2024). Similarly, Patil *et al.* (2022) also did not find any beneficial effect of FSH stimulation on the number of follicles in buffaloes. This could be due to different breed/ species used in the study along with the hormone used for stimulation. The most plausible reason behind this finding is the faster growth of small category follicle to become medium and large size follicle in the presence of gonadotropin (pFSH) preventing their atresia (Purwantara *et al*., 1993).

Effect of FSH Stimulation on Oocyte Recovery and Quality

The total numbers of oocytes recovered were 54 and 82 in non-stimulated and FSH stimulated group, with a recovery rate of 54.54% and 59.85%, respectively. The mean numbers of oocytes recovered per OPU session were significantly ($p< 0.05$) higher in FSH stimulated as compared to non-stimulated group (10.25±0.98 Vs. 6.75±0.60) (Table 2). Further, significantly higher viable oocytes were recorded in stimulated (91.46%) as compared to the non-simulated (83.33%) group. These findings were in agreement with the earlier observation of Vennapureddy *et al.* (2024). On the contrary, no effect of superstimulation on the number of

Table. 1: Comparative follicular dynamics at different days of OPU protocol in non-stimulated and stimulated Sahiwal cows

Follicles / CL	D1 FSH			D2 FSH			OPU		
	Non-stimulated	**Stimulated**	***P* value**	**Non-stimulated**	**Stimulated**	***P* value**	**Non-stimulated**	**Stimulated**	***P* value**
Larger follicles (≥9 mm; LF)									
i. Mean no. of LF	0.38±0.17	0.43±0.17	>0.05	0.50±0.18	1.25±0.15	<0.05	0.86±0.23	3.75±0.39	<0.05
ii. Mean diameter LF (mm)	10.60±0.61	9.48±0.18	>0.05	11.20±0.34	10.78±0.48	>0.05	9.51±0.19	10.23±0.24	>0.05
Medium follicles (≥5 –9 mm; MF)									
i. Mean no. of MF	1.25±0.15	1.25±0.15	>0.05	1.75±0.29	3.63±0.53	<0.05	2.25±0.23	7.38±0.56	<0.05
ii. Mean diameter MF (mm)	7.14±0.30	7.23±0.25	>0.05	7.24±0.27	7.28±0.25	>0.05	7.21±0.23	7.39±0.17	>0.05
Small follicles (≥2.5 –5 mm; SF)									
i. Mean no. of SF	19.88±0.99	17.88±1.82	>0.05	17.38±0.71	14.88±2.25	>0.05	16.63±0.81	7.50±0.87	<0.05
ii. Mean diameter SF (mm)	4.05±0.17	4.31±0.09	>0.05	4.39±0.09	4.38±0.08	>0.05	4.05±0.04	4.44±0.06	>0.05

follicles available for aspiration and oocyte recovery was reported in some of the earlier studies (De Carvalho *et al.,* 2019; Patil *et al.,* 2022).

In stimulated Sahiwal cows, a significantly ($p<0.05$) greater number of Grade-A oocytes (60.98±0.49%) was recovered as compared to the non-stimulated cows (24.07±0.40%)(Table 2). Conversely, the non-stimulated group had a higher percentage of Grade-C oocytes (31.48±0.28%) than the FSH stimulated cows (12.20±0.17%). However, we could not find any significant difference with respect to the numbers of Grade-B and D oocytes among the groups. Earlier reports also suggested recovery of more good quality oocytes in FSH stimulated group than non-stimulated cows (Aller *et al.,* 2010; Ciftci and Dinc, 2023). The good quality oocytes recovered in the present study concurred with the report of Gimenes *et al.* (2015). The possible reason for better oocyte recovery in FSH stimulated group may be that FSH treatment prevented the disruption of cell connection between oocytes, COCs, and individual cumulus cell (Sakaguchi *et al.,* 2019). Therefore, high numbers of Grade-A oocytes were obtained in the stimulated group.

Effect of Super-Stimulation on IVM and IVF

In the present study, significantly higher ($p<0.05$) matured occytes were observed (8.13±0.62 Vs. 5.38±0.53) per OPU session in stimulated than non-stimulated group, however maturation rate though high in stimulated group did not differ statistically between two (84.41% Vs. 81.10%)(Table 2). Similar higher maturation rate was recorded in FSH stimulated cows than non-stimulated by others (Blondin *et al.,* 2002; Vennapureddy *et al.,* 2024). Silvestri *et al.* (2021) suggested that oocytes having (>3) COCs layers exhibited significantly higher ($p<0.05$) *in-vitro* maturation than partial cumulus layered and denuded oocytes.

In stimulated group, significantly ($p<0.05$) higher number (6.38±0.81) of oocytes were fertilized as compared to the non-stimulated group (3.88±0.51) (Table 3). The IVF percentage in stimulated Sahiwal cows was higher than non-stimulated cows (79.46 Vs. 72.08; $p>0.05$). In the present study, the mean numbers of fertilized oocytes in pFSH stimulated cows were in consonance with the previous reports on HF and Sahiwal cows (Vennapureddy *et al.,* 2024). However, the IVF rate in the present study was higher than earlier report on Sahiwal cows (Nawaz *et al.,* 2022). The findings of the present study also suggested good fertilization rates in FSH stimulated group as reported earlier by Vieira *et al.* (2014).

Effect of Stimulation on *In-Vitro* Culture (IVC)

The embryo development rate was observed in terms of different cell stages and the data pertaining to various IVF parameters in both non-stimulated and stimulated Sahiwal cows are presented in Table 3. The cleavage rates observed in present experiment were 73.44% and 61.25% in FSH stimulated and non-stimulated group, respectively.

Table. 2: Follicular attributes, mean oocytes recovered, viable oocyte yield and the quality of oocytes in stimulated and non-stimulated Sahiwal cows

Parameters	Non-Stimulated (n=8)	Stimulated (n=8)	*p* value
Antral follicular population	19.88±0.99[a] (n=159)	17.88±1.8[a] (n=143)	>0.05
Total follicles aspirated/OPU	12.38±0.85[a] (n=99)	17.13±1.87[b] (n=137)	<0.05
Total oocytes recovered/OPU	6.75±0.63[a] (n=54)	10.25±0.98[b] (n=82)	<0.05
Recovery efficiency (%)	54.54[a] (54/99)	59.85[a] (82/137)	>0.05
Viable oocytes (%)	83.33[a] (45/54)	91.46[b] (75/82)	<0.05
Total oocytes recovered	54	82	-
a. Grade-A (%)	24.07±0.40 (13)	60.98±0.49 (50)	<0.05
b. Grade-B (%)	27.78±0.33 (15)	20.73±0.37 (17)	>0.05
c. Grade-C (%)	31.48±0.28 (17)	12.20±0.17 (10)	<0.05
d. Grade-D (%)	16.67±0.12 (09)	6.10±0.00 (05)	>0.05

Table. 3: Comparative *in-vitro* maturation, *in-vitro* fertilization, and cleavage percentage in non-stimulated and stimulated Sahiwal cows

Parameters	Sahiwal		
	Non-Stimulated (n=8)	Stimulated (n=8)	*p* value
Mean oocytes recovered	6.75±0.63[a] (n=54)	10.25±0.98[b] (n=82)	<0.05
Mean oocytes kept for maturation	6.63±0.64[a] (n=53)	9.63±0.89[b] (n=77)	<0.05
Mean *in vitro* matured oocytes	5.38±0.53[a] (n=43)	8.13±0.62[b] (n=65)	<0.05
Maturation rate (%)	81.10[a] (n=43/53)	84.41[a] (n=65/77)	>0.05
Mean *in vitro* fertilized oocyte	3.88±0.51[a] (n=31)	6.38±0.81[b] (n=51)	<0.05
IVF percentage	72.08[a] (31/43)	79.46[a] (51/65)	>0.05
Presumptive zygote, cleavage percentage and embryo developed stages			
Embryo produced	2.38±0.43[a] (n=19)	4.50±0.50[b] (n=36)	<0.05
Cleavage rate (%)	61.25[a] (19/31)	73.44[a] (36/51)	>0.05
a. 2-cell stage (%)	100[a] (19/19)	100[a] (36/36)	>0.05
b. 4-cell stage (%)	63.16[a] (12/19)	91.67[b] (33/36)	<0.05
c. 8-cell stage (%)	26.32[a] (5/19)	58.33[b] (21/36)	<0.05
d. 16-cell stage (%)	10.53[a] (2/19)	25.00[a] (9/36)	>0.05
e. Compact morula (%)	5.26[a] (1/19)	13.89[a] (5/36)	>0.05

Indeed, in the present study, gonadotropin stimulation had a positive impact on embryo production in Sahiwal cows. A total of 36 and 19 embryos were produced in stimulated and non-stimulated Sahiwal cows, respectively. In stimulated group, significantly ($p<0.05$) more embryo production (4.50±0.50/ OPU session) was recorded as compared to the non-stimulated cows (2.38±0.43/ OPU session) with cleavage rate of 73.44% Vs. 61.25%. When the presumptive zygotes were kept for culture, all the zygotes cleaved into 2-cell stage in both the groups, but subsequently significantly ($p<0.05$) more 4-cell stage, 8-cell stage and numerically higher 16-cell stage and compact morula (13.89% vs 5.26%) were recorded in stimulated than in non-stimulated Sahiwal cows (Table 3). The FSH stimulation before OPU resulted in two-fold increase in embryo production rate in stimulated cows as compared to non-stimulated cows. Previous reports also recorded a greater number of mean embryos produced in FSH stimulated group (De Roover *et al.,* 2008; Vieira *et al.,* 2014). However, Patil *et al.*

(2022) reported no influence of stimulation on mean embryo production in buffalo. The plausible reason for more embryo recovery in stimulated group may be a healthier follicular environment of oocytes as evidenced from large follicular size leading to better developmental competence, *in-vitro* maturation, and *in-vitro* fertilization rates.

Conclusions

In conclusion, the FSH pre-stimulation prior to OPU-IVF increased the number of medium and large follicles, oocyte recovery and embryo quality in Sahiwal cows.

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