



## Effect of superstimulation on follicular growth in Hariana and Sahiwal cows under different OPU schedules

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### ABSTRACT

Superstimulation using exogenous equine chorionic gonadotropin (eCG) can enhance ovarian follicular development, which is crucial for improving reproductive outcomes in cattle. This investigation evaluated the impact of eCG (1000 IU, intramuscular) on follicular dynamics in Hariana and Sahiwal cows under weekly and bi-weekly ovum pick-up (OPU) protocols. Twelve repeat-breeding cows (n=6 Hariana, n=6 Sahiwal) were assigned to either weekly or bi-weekly OPU protocols (3 Hariana and 3 Sahiwal per schedule). Ovarian follicles were classified as small (<4 mm), medium (4–8 mm), and large (>8 mm) and counted by ultrasonography before and 72 hours after eCG injection. Statistical comparisons were performed using one-way ANOVA, with significance set at  $P<0.05$  and  $P<0.01$ . Results revealed a significant increase in medium and large follicles post-superstimulation in both breeds, with Hariana cows showing a more robust increase in large follicles. These findings support the need for tailored superstimulation protocols for indigenous breeds to maximize oocyte yield.

**Keywords:** eCG, OPU, Follicular dynamics, Hariana, Indigenous breeds, Sahiwal, Superstimulation

Reproductive performance is critical for livestock productivity and genetic improvement (Manik *et al.* 2003). Enhancing reproductive efficiency is particularly crucial for indigenous cattle breeds like Hariana and Sahiwal, known for their resilience and milk production, but often facing reproductive inefficiencies (Sarwar *et al.* 2020). Superstimulation protocols, especially those using (eCG), have demonstrated effectiveness in boosting follicular recruitment and oocyte recovery. These protocols involve hormonal administration to stimulate the ovaries, leading to the development of multiple follicles simultaneously, which increases the chances of successful fertilization and embryo development (Miyamoto *et al.* 2016).

Indigenous breeds such as Hariana and Sahiwal have distinct genetic characteristics that include disease resistance and suitability to local environments. Despite these advantages, they often lag behind exotic breeds in terms of reproductive efficiency (Singh *et al.* 2012). Research has shown that while superstimulation techniques are well-established for high-yielding breeds like Holstein and Jersey cows, their application in native breeds like Hariana and Sahiwal is not as well-documented

(Gumen *et al.* 2011).

Pre-OPU superstimulation aims to increase the number of follicles available for (OPU), rather than inducing multiple ovulations, necessitating adjustments in gonadotropin dosage and timing (Bols and Stout, 2018). Through the development of superstimulation methods specific to these breeds, it may be possible to overcome their reproductive inefficiencies and unlock their full genetic potential, leading to significant advancements in genetic improvement and livestock productivity (Looney *et al.* 2018). This study investigated the impact of superstimulation on ovarian follicular dynamics in Hariana and Sahiwal cattle, providing insights into breed-specific responses and the optimization of hormonal protocols (Lopez *et al.* 2020).

### MATERIALS AND METHODS

*Experimental Design:* The study was conducted on 12 repeat-breeding indigenous cows, comprising six Hariana and six Sahiwal animals, maintained at the institutional herd under uniform nutritional and managemental conditions. All animals were clinically examined and confirmed to have normal cyclic ovarian activity prior to inclusion.

The animals were randomly allocated into two groups (n = 6 per group), each containing an equal number of Hariana and Sahiwal cows. Superstimulation was performed using equine chorionic gonadotropin (eCG; 1000 IU, intramuscular), administered irrespective of the stage of the estrous cycle.

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- Group I (Bi-weekly protocol): 6 cows (3 Hariana, 3 Sahiwal) received superstimulation twice per week (minimum 72h between OPU sessions).
- Group II (Weekly protocol): 6 cows (3 Hariana, 3 Sahiwal) received superstimulation once a week, with OPU 72h post-eCG each time.

**Follicular Dynamics Monitoring:** Each ovary's follicle count was noted and by freezing the sonographic image. The diameter of the follicles was determined by averaging the lengths of the long and short axes of follicles using the built-in calliper (Nagai *et al.* 2015). The follicles were categorized as small (<4mm), medium (4- 8 mm) and large (≥8 mm) as per the method of Ginther & Associates (1989). The number and distribution of follicles in each category were recorded before and after superstimulation to evaluate the treatment's effect.

**Data Analysis:** Data were statistically analyzed using SPSS software. All data expressed as mean ± standard error (SE). One-way ANOVA employed for paired comparisons (before vs. after eCG) within each protocol and breed, Inter-breed comparisons within the same OPU schedule the Significancelevel were P<0.05 (\*) & P<0.01 (\*\*).

RESULTS AND DISCUSSION

The current study evaluated the effects of superstimulation using equine chorionic gonadotropin (eCG) on follicular dynamics in Hariana and Sahiwal cows subjected to weekly and bi-weekly ovum pick-up (OPU) protocols. The findings reveal significant breed-specific differences in ovarian response, underscoring the need for tailored hormonal protocols in indigenous breeds.

**Effect of Superstimulation in Hariana Cows:** Hariana cows showed a clear and highly significant increase in medium (4–8 mm) and large (>8 mm) follicle populations after eCG treatment in both weekly and bi-weekly OPU schedules (Tables 1 and 2). This robust ovarian response is consistent with earlier findings in Holstein (*Bos taurus*) by Ginther *et al.* (1989), where stimulation with gonadotropins led to synchronized new follicular waves and increased aspiratable follicles. Silva-Santos *et al.* (2014) further showed similar patterns in Nelore (*Bos indicus*) cows,

indicating the universality of this effect in both *Bos taurus* and *Bos indicus*, but demonstrating that individual breeds may exhibit remarkable differences in absolute follicle numbers and response amplitude.

The expanded population of large follicles is particularly significant, as follicle size is directly linked with oocyte cytoplasmic maturation, competence, and subsequent embryo development (Presicce *et al.* 2020; Watanabe *et al.* 2018). Similar enhancements in ovarian output, oocyte developmental potential, and blastocyst

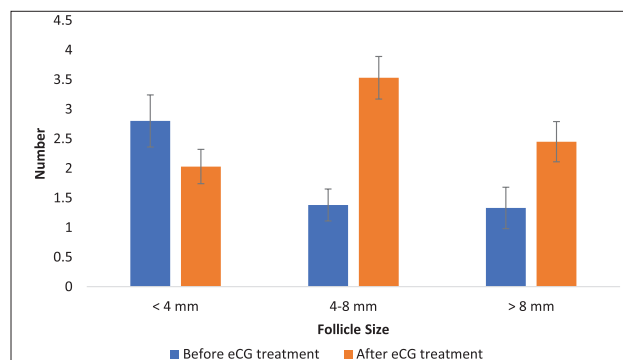


Fig. 1 Comparison of changes in follicle size following super-stimulation in Hariana cows subjected to OPU at weekly intervals

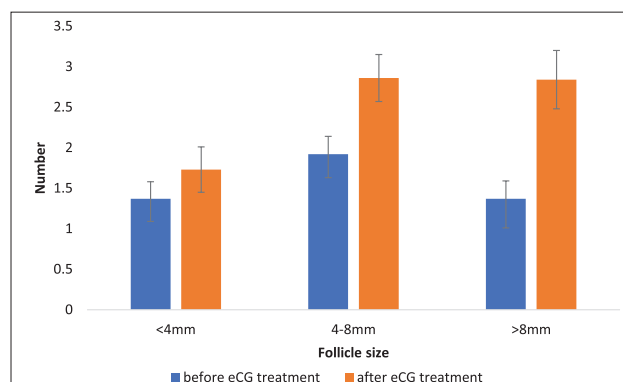


Fig. 1.1 Comparison of Changes in follicle size following super-stimulation in Hariana cows subjected to OPU at bi-weekly intervals

Table 1. Changes in the ovarian follicle status of Hariana cows (n=6) after eCG (1000 IU), OPU at weekly interval

Follicle category	Mean ± SEM (Before)	Mean ± SEM (After)	Range (Before)	Range (After)	N (Before)	N (After)
<4 mm	2.80 ± 0.44 <sup>a</sup>	2.03 ± 0.29 <sup>a</sup>	0–8	0–7	20	38
4–8 mm	1.38 ± 0.27 <sup>b</sup>	3.53 ± 0.36 <sup>c</sup>	0–4	0–8	21	38
>8 mm	1.33 ± 0.35 <sup>d</sup>	2.45 ± 0.34 <sup>e</sup>	0–5	0–8	21	38

Means with different superscripts within a row differ significantly. b vs. c: P<0.01, F=15.95 d vs. e: P<0.05, F=4.39

Table 2. Changes in the ovarian follicle status of Hariana cows (n=6) after eCG (1000 IU), OPU at bi-weekly interval

Follicle category	Mean ± SEM (Before)	Mean ± SEM (After)	Range (Before)	Range (After)	N (Before)	N (After)
<4 mm	1.37 ± 0.21 <sup>a</sup>	1.73 ± 0.28 <sup>a</sup>	0–5	0–7	51	56
4–8 mm	1.92 ± 0.22 <sup>b</sup>	2.86 ± 0.29 <sup>c</sup>	0–7	0–8	51	56
>8 mm	1.37 ± 0.22 <sup>d</sup>	2.84 ± 0.36 <sup>e</sup>	0–5	0–13	51	56

Means with different superscripts within a row differ significantly. b vs. c: P<0.05, F=6.35 d vs. e: P<0.01, F=11.65

Table 3. Changes in the ovarian follicle status of Sahiwal cows (n=6) after eCG (1000 IU), OPU at weekly interval

Follicle category	Mean ± SEM (Before)	Mean ± SEM (After)	Range (Before)	Range (After)	N (Before)	N (After)
<4 mm	2.95 ± 0.40 <sup>a</sup>	1.90 ± 0.35 <sup>a</sup>	0–7	0–6	21	20
4–8 mm	0.59 ± 0.17 <sup>b</sup>	2.45 ± 0.48 <sup>c</sup>	0–2	0–6	22	20
>8 mm	0.27 ± 0.13 <sup>d</sup>	1.05 ± 0.26 <sup>c</sup>	0–2	0–3	22	20

Means with different superscripts within a row differ significantly. b vs. c: P<0.01, F=14.16 d vs. e: P<0.01, F=7.60

Table 4. Changes in the ovarian follicle status of Sahiwal cows (n=6) after eCG (1000 IU), OPU at bi-weekly interval

Follicle category	Mean ± SEM (Before)	Mean ± SEM (After)	Range (Before)	Range (After)	N (Before)	N (After)
<4 mm	0.96 ± 0.24 <sup>a</sup>	1.46 ± 0.28 <sup>a</sup>	0–5	0–5	28	26
4–8 mm	1.25 ± 0.23 <sup>b</sup>	3.19 ± 0.38 <sup>c</sup>	0–4	0–8	28	26
>8 mm	1.30 ± 0.24 <sup>d</sup>	2.73 ± 0.33 <sup>c</sup>	0–4	0–6	27	26

Means with different superscripts within a row differ significantly. b vs. c: P<0.01, F=20.14 d vs. e: P<0.01, F=12.33

rates after optimized superstimulation protocols have been reported by Farias *et al.* (2023) and Ciftci and Dinc (2023), underscoring the reliability of these approaches across breeds and countries

**Effect of Superstimulation in Sahiwal Cows:** Sahiwal cows also responded favorably to eCG-induced superstimulation, with significant increases in both medium and large follicle categories across weekly and bi-weekly protocols (Tables 3 and 4). However, the magnitude of response was more moderate than in Harijana cows. This observation mirrors other reports in Sahiwal

(Khatun *et al.* 2022), Nelore, and Gir cattle (Silva-Santos *et al.* 2014; Presicce *et al.* 2020), and may be attributed to breed-intrinsic ovarian reserves, follicle sensitivity to gonadotropins, and differences in endocrine environment (Singh *et al.* 2012; Sarwar *et al.* 2020).

Ongaratto *et al.* (2020) used FSH or eCG superstimulation protocols with *Bos indicus* and *Bos indicus* × *Bos taurus* donors to achieve similar follicle population growth which validated cross-breed applicability yet required protocol modifications for maximum results.

**Comparison Between Harijana and Sahiwal Cows:** The

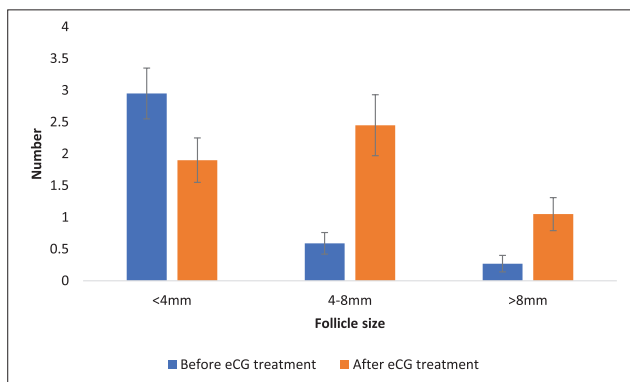


Fig. 2 Comparison of changes in follicle size following super-stimulation in Sahiwal cows subjected to OPU at weekly intervals

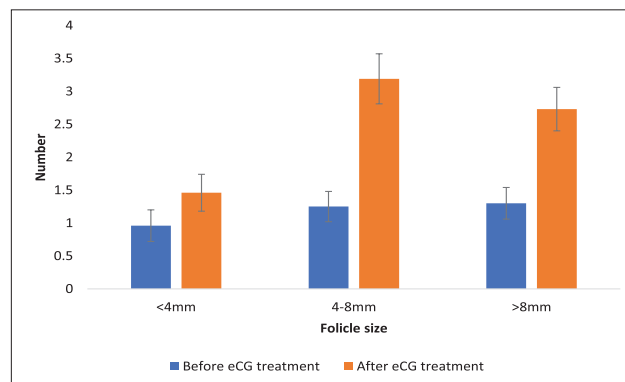


Fig. 2.1 Comparison of changes in follicle size following super-stimulation in Sahiwal cows subjected to OPU at bi-weekly intervals

Table 5. Comparison of super-stimulatory response of eCG (@1000 IU) between Harijana and Sahiwal cows for OPU at weekly interval. Sample size per group: n = 6

Follicle category	Harijana (Mean±SEM, Range, N)	Sahiwal (Mean±SEM, Range, N)	F Value	Statistical significance
<4 mm Before	2.80±0.44 (0–8, 20)	2.95±0.40 (0–7, 21)	0.06	ns
4–8 mm Before	1.38±0.27 <sup>b</sup> (0–4, 21)	0.59±0.17 <sup>a</sup> (0–2, 22)	5.94	*
>8 mm Before	1.33±0.35 <sup>b</sup> (0–5, 21)	0.27±0.13 <sup>a</sup> (0–2, 22)	8.12	**
<4 mm After	2.03±0.29 (0–7, 38)	1.90±0.35 (0–6, 20)	0.07	ns
4–8 mm After	3.53±0.36 (0–8, 38)	2.45±0.48 (0–6, 20)	3.05	ns
>8 mm After	2.45±0.34 <sup>b</sup> (0–8, 38)	1.05±0.26 <sup>a</sup> (0–3, 20)	7.53	**

Means with different superscripts within a row differ significantly (P < 0.05 or P < 0.01). \* and \*\* next to F Value indicate levels of statistical significance: \*P < 0.05; \*\*P < 0.01; ns = not significant. Superscripts a, b compare Harijana and Sahiwal means within each follicle category and time point. N: number of follicle counts per group

Table 5. Comparison of super-stimulatory response of eCG (@1000 IU) between Hariana and Sahiwal cows for OPU at bi-weekly interval. Sample size per group: n = 6

Follicle category	Hariana (Mean±SEM, Range, N)	Sahiwal (Mean±SEM, Range, N)	F Value	Statistical significance
<4 mm Before	1.37±0.21 (0–5, 51)	0.96±0.24 (0–5, 28)	1.54	ns
4–8 mm Before	1.92±0.22 <sup>b</sup> (0–7, 51)	1.25±0.23 <sup>a</sup> (0–4, 28)	3.90	*
>8 mm Before	1.37±0.22 (0–5, 51)	1.30±0.24 (0–4, 27)	0.05	ns
<4 mm After	1.73±0.28 (0–7, 56)	1.46±0.28 (0–5, 26)	0.35	ns
4–8 mm After	2.86±0.29 (0–8, 56)	3.19±0.38 (0–8, 26)	0.45	ns
>8 mm After	2.84±0.36 (0–13, 56)	2.73±0.33 (0–6, 26)	0.04	ns

Means with different superscripts within a row differ significantly ( $P < 0.05$  or  $P < 0.01$ ). \* and \*\* next to F Value indicate levels of statistical significance: \* $P < 0.05$ ; \*\* $P < 0.01$ ; ns = not significant. Superscripts a, b compare Hariana and Sahiwal means within each follicle category and time point. N: number of follicle counts per group

weekly OPU regimen produced the highest number of large follicles in Hariana cows when compared to Sahiwal cows according to direct breed comparisons (Table 5). The pre- and post-treatment assessment showed that the baseline and treatment effects resulted in differences between *Bos indicus* breeds regarding their ovarian responses. Research findings demonstrate that Sahiwal cows together with other native South Asian breeds have naturally smaller antral follicle numbers and reduced potential to develop follicles compared to more responsive breeds (Singh *et al.* 2012; Silva-Santos *et al.* 2014).

The observed breed-specific variations influence the development of superstimulation and OPU protocols. The Hariana donor population shows exceptional suitability for *in vitro* embryo production (IVEP). The significant post-superstimulation increase in Sahiwal cow oocyte yield indicates that customized treatment protocols and AFC-based donor selection methods can boost embryo collection success from every native breed (Khatun *et al.* 2022; Matoba *et al.* 2022).

The significant increases in medium and large follicles post-superstimulation are indicative of improved follicular recruitment, as also reported by Chaubal *et al.* (2007) and Michalovic *et al.* (2018). Larger follicles not only yield higher-quality oocytes but also enhance the likelihood of successful fertilization and embryo development (Watanabe *et al.* 2018).

Moreover, the breed-specific differences observed in this study emphasize the need for tailored superstimulation protocols. While Hariana cows responded robustly to eCG, Sahiwal cows demonstrated a more moderate response, suggesting that further optimization of hormonal regimens may be necessary for the latter.

It may be concluded that superstimulation using eCG is a promising strategy for enhancing ovarian follicular dynamics in indigenous cattle breeds. By improving oocyte yield and quality, such protocols can contribute significantly to genetic improvement programs and livestock productivity. These findings provide a foundation for developing breed-specific protocols, which could bridge the gap in reproductive efficiency between indigenous and exotic breeds.

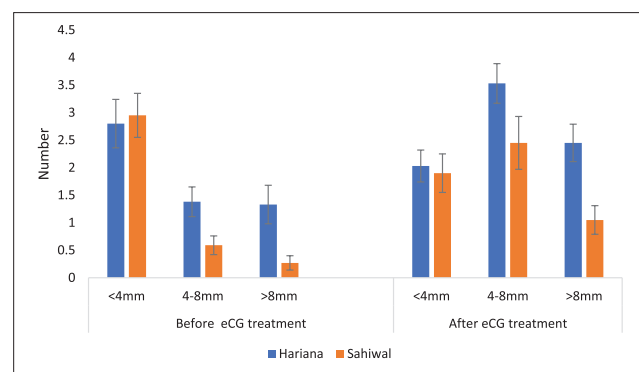


Fig. 3 Comparison of different category follicle (small, medium and large) between Hariana and Sahiwal cows following super-stimulation and subjected to OPU at weekly interval

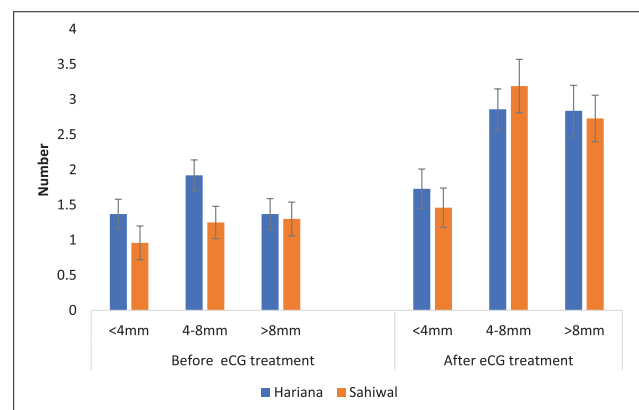


Fig. 3.1 Comparison of different category follicle (small, medium and large) between Hariana and Sahiwal cows following super-stimulation and subjected to OPU at bi-weekly interval

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