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Follicular development and ovarian changes during superovulation and post-flushing luteolysis pattern in Sahiwal (*Bos indicus*) cows

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Abstract
The present study aimed to find the follicular and ovarian changes during superovulation and post-flushing luteolysis pattern. Sahiwal donor cows (n=10) were superstimulated using (STIMUFOL®/pFSH) during the mid-luteal phase and were monitored for follicular development during superovulation and post-flushing luteolysis changes were recorded using trans-rectal ultrasonography. At the initiation of FSH administration (10th day), the mean number of small follicles was significantly high, whereas on the day of prostaglandin (PG) injection (12th day), a significantly greater number of medium follicles were observed (p<0.001) and at superovulation estrus (SOE/14th day) significantly higher number of large follicles were recorded. Also, a significantly higher number of corpora luteum and the luteal areas was recorded on the day of flushing compared to any other day of superovulation protocol. Similarly, ovarian size was significantly larger on the day of flushing for both the right and left ovaries. Post-flushing administration of PGF_2 alpha to Sahiwal donors leads to a significant reduction in the area of corpus luteum within 72 hrs and ovaries returned to normal, non-stimulated size by day 6 post-flushing. The majority of Sahiwal donors exhibited estrus within 9.90±1.06 days post-flushing. However, 70% of Sahiwal exhibited silent estrus. The mean interval from flushing to subsequent successful breeding was 29.86±5.24 days.

Keywords: Follicular development, superovulation, post-flushing luteolysis, ultrasonography, ovarian changes

Introduction
According to latest livestock census (Anon, 2019; 20th Livestock Census, 2019), the cattle population in India is 192.49 million which is an increase of 0.8% over the last census. The animal market plays a diversified role in the socio-economic development of the society. Hence, it is necessary to research, discover, innovate, and transfer new knowledge, novel practices, and other alternatives from lab to land that can improve animal reproduction and production. The government of India is running many programs for the conservation of Indigenous breeds and some of the programs are; Rashtriya Gokul Mission (RGM); National Programme for Bovine Breeding, National Kamdhenu Breeding Centres, and National Mission on Bovine Productivity. Rashtriya Gokul Mission (RGM) was launched in 2014 by the Ministry of Agriculture for the development and conservation of indigenous bovine breeds to increase milk production and productivity. After artificial insemination, ETT emerged as the most widely accepted reproductive biotechnology in animals and commercial embryo transfer in cattle has become a well-established industry throughout the world. Superovulation (SO) is an effective technique for extracting progeny from genetically desirable females. Since the second follicular wave starts around 8-12 days in cattle so maximum of the superovulation treatment protocol is pragmatic between these days (Purwantara *et al*., 1993) [8] also in the mid-luteal phase CL is most responsive to prostaglandin treatment that's why the superovulation treatment using pFSH (STIMUFOL®) is started in mid cycle preferably between day 8-12 of cycle The primary goal of superovulation is to recover a greater number of transferable embryos from elite animal. The ovarian response is determined by the number of gonadotropin-responsive follicles present at the initiation of superovulatory treatment (Driancourt, 2001) [1]. The superovulation response is variable, each animal show variation in its superovulatory response which later ultimately affects embryo production.

It has been found that the number of small follicles (SF) and the overall follicular population at the initiation of the superovulation treatment is positively correlated to super-stimulatory response and embryo production. The information regarding follicular development during superovulation and post-flushing luteolysis, and successful breeding pattern has not been detailed in Sahiwal (*Bos indicus*) under subtropical condition further majority of the literature state that there should be a minimum of 60 days interval between two flushing that makes multiple ovulation and embryo transfer (MOET) costly to maintain the donors without breeding. Therefore, the present study was designed to document follicular development and ovarian changes during superovulation and subsequent successful breeding after post-flushing induced luteolysis in Sahiwal cows.

Location and Experimental Animals

The experiment was conducted at Cattle and Buffalo Farm, ICAR-IVRI, Izatnagar, located at Bareilly U.P. Ten parous (1 to 4 parity) cows from the Sahiwal herd maintained under iso-managerial conditions, apparently free from any reproductive disorders with good body condition score (3.5–4) and crossed a minimum of 60 days post-partum were selected as donors.

Superovulation protocol

These donors were superstimulated on day 10th of the cycle following administration of STIMUFOL® (containing pLH 100 µg and pFSH 500 µg, manufactured by Reprobiol SPRL, Belgium) with a total dose of 240 µg in eight divided doses (90, 70, 50, 30 µg morning and evening in a tapering manner). Prostaglandin was given on the 12th day of the cycle along with the 5th dose of pFSH and all the donors exhibited super stimulatory estrus on the 14th day (Fig. 1). Oestrus characteristics were monitored through visual observation thrice a day and by teaser bull parading during morning and evening and estrus female were inseminated with good and fertile frozen bull semen.

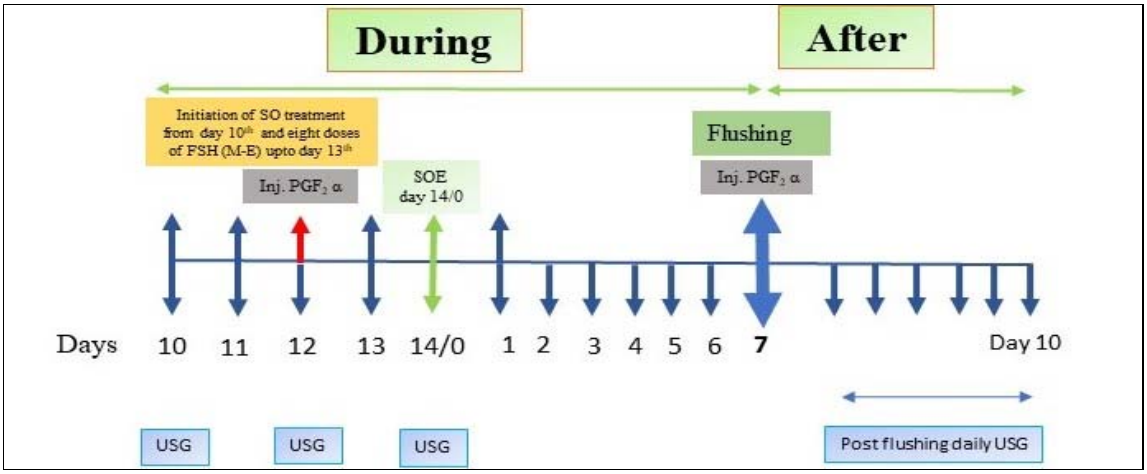


Fig 1: Schematic representation of superovulation protocol and USG monitoring during and after SO treatment

Ultrasonography and blood sampling schedule

The selected donor Sahiwal cows were monitored for follicular and luteal parameters by transrectal ultrasonography during the initiation of super stimulatory treatment. Ovaries of the Sahiwal cows were examined by real-time transrectal ultrasonography (Exago ECM, France) from ovulation (day 0) and on the 10th, 12^{th,} and 14th day of oestrus cycle and also on the day of flushing and continuously for 10 days post flushing or subsequent estrus which so ever early by the same operator at an interval of 24 hrs using 7.5-MHz per rectal probe (Fig. 2). The size and locations of the small (≥2- 4.9 mm), medium (≥5-8.9 mm), and large (≥9 mm) follicles were recorded on a sketch of each ovary, and the regions of interest were measured (Manik *et al.,* 1998) [5]. Further, the luteal area was calculated based on the formula (D1/2 X D2/2) π (Jaskowski *et al.,* 2021) [3].

Statistical analysis

All data pertaining to follicular development and post-flushing luteolytic pattern were presented as mean ± SEM. The metric variables were statistically analysed using the SPSS software package for Windows (version 20.0) using unpaired 't'-test, one-way ANOVA within the groups, and between the groups. The level of significance was evaluated at different levels i.e.; (p<0.05, p<0.01, and p<0.001).

Results and Discussion

The superovulation treatment resulted in a significantly higher (p<0.01) number of large-size follicles (LF) at super ovulatory estrus as compared to the day of initiation, the day of prostaglandin administration, and also on the day of flushing (Table 1). A significantly higher (p<0.001) number of medium size follicles (MF) were recorded on the day of PG administration than on other days of the superovulation treatment period (Table 2). Further, a significantly higher (p<0.001)) number of small follicles were reported on the day of initiation of SO treatment (Table 3). No difference was noticed between the diameter of the small follicle on the day of initiation of SO treatment and prostaglandin administration. After administration of FSH injection, the small follicle grows at a significantly faster growth rate to form a medium size follicle pool as compared to the medium size follicle's growth rate to become large follicle (Table 3). The number of corpora lutea was significantly higher (p<0.001) on the day of flushing than on other days of SO treatment, however, the mean diameter of CL (13.63±0.56 mm) was found to be significantly lower (p<0.05) than day of FSH administration (17.54±0.90 mm) and day of PG administration (17.63±0.77 mm). Similarly, the total luteal area was significantly (p<0.001) high on the day of flushing (Table 4).

Table 1: Large follicle development pattern during the superovulation program in Sahiwal donor

Larger follicles (≥9 mm) (LF)	D1 FSH	Day of PG	SOE	Flushing	P value
Mean no. of LF	0.7±0.21[a]	1.8±0.53 [a]	13.0±2.24[b]	5.3±1.27 [a]	<0.01
Mean diameter LF (mm)	11.15±0.86[a]	10.09±0.44[a]	10.01±0.16[a]	14.52±1.19 [b]	< 0.05
Mean growth rate of LF	-	-	0.73±0.17	-	

p<0.05-statistically significant; p<0.01-statistically highly significant

Table 2: Medium follicle development pattern during the superovulation program in Sahiwal donor

Medium follicles (≥5 m–9 mm) (MF)	D1 FSH	Day of PG	SOE	Flushing	P value
Mean no. of MF	0.80±0.20[a]	12.3±3.06[b]	1.60±0.67[a]	0.30±0.16 [a]	<0.001
Mean diameter MF	7.17±0.43	7.82±0.14	7.92±0.28	7.66±0.54	> 0.05
Mean growth rate of MF	-	1.09±0.08	-	-	-

p<0.05-statistically significant; p<0.001-statistically highly significant

Table 3: Small follicle development pattern during the superovulation program in Sahiwal donor

Small follicles (≥2.5 mm–5 mm) (SF)	D1 FSH	Day of PG	SOE	Flushing	P value
Mean no. of SF	17.6±2.51[b]	2.9±1.32[a]	0.00±0.00[a]	0.00±0.00 [a]	<0.001
Mean diameter SF	3.59±0.14	3.13±0.12	-	-	>0.05
The mean growth rate of SF	2.18±0.08	-	-	-	

p<0.05-statistically significant; p<0.001-statistically highly significant

Results from the current study revealed that when gonadotropin treatment was given on the 10th day of the estrous cycle (mid-luteal phase) there was an increase in the total number of medium size follicle pools on the day of PG and increase in large-size follicle pools on the day of SOE. The Sahiwal donor responded positively to the gonadotropin treatment which resulted in the majority of the small follicle on the day of SO treatment being converted into a large size follicle pool on the day of SOE. This supports the hypothesis that gonadotropin treatment increases the number of antral follicles, favours the growth of small and medium follicles, and protects the small follicles (>1.7 mm) undergoing atresia (Monniaux *et al*., 1983) [7]. During super stimulation on day of administration of PG (day 3), there is an increase in medium size follicles (>5mm), this finding is similar to that of Manik *et al.* (1998) [5], Purwantara *et al*. (1993) [8] and Kurhe, (2019) [4]. It occurs due to the rapid growth of the small follicle category to become medium in the presence of gonadotropin (FSH) which supports the growth of the small follicles and prevent their atresia. Further, on the day of SOE (day 5) the higher number of large follicles were recorded due to the shift of MF to LF category, whether or not they were destined to ovulate. Similar observations were recorded by a number of researchers (Manik *et al.,* 1998; Satheshkumar, 2015) [5, 10]. In fact, the increase in the large follicle population on the day of SOE was due to shifting of small to large follicles also supported by various studies (Grasso *et al*., 1989; Purwantara *et al*., 1993; Singh *et al*., 2004) [2,8,12].

Table 4: Corpus luteum development pattern during the superovulation program in Sahiwal donor

Corpus luteum	D1 FSH	Day of PG	SOE	Flushing	P value
Mean no. of CL	1.00±0.00[a]	1.00±0.00[a]	0.00±0.00[a]	8.60±1.23[b]	<0.001
Mean diameter of CL	17.54±0.90[a]	17.63±0.77[a]	-	13.63±0.56[b]	<0.05
Luteal area (mm^2)	247.08±24.76[a]	248.02±21.26[a]	-	1233.7±184.4[b]	<0.001

p<0.05-statistically significant; p<0.001-statistically highly significant

The present investigation recorded when superovulation treatment was given during the mid-luteal phase and initially, the diameter of CL was found constant, after PGF2α injection on 3rd day it started to regress as observed by various authors (Purwantara *et al*., 1993; Kurhe, 2019) [8, 4]. Present study recorded 8.60 CL per animal at flushing by USG, almost similar to earlier reports in Sahiwal cows (Mishra *et al*., 1996) [6]. Similar to our study, Ullah *et al*. (1988) [13] recorded 7.6 CL in Sahiwal cows on the day of flushing. However, a low number of corpora lutea (5.67) was recorded by Siddiqui *et al*. (2011) [11] in Sahiwal cows. Moreover, Kurhe, (2019) [4] observed 7.20 CL in crossbred cows. The diameter of corpora lutea observed on the day of flushing was smaller than normal cyclic corpora lutea at mid luteal phase of non-stimulated cows (Kurhe, 2019) [4] may be due to the ovulation of small-size preovulatory follicles as a result of ovarian overcrowding (Robertson *et al.,* 1993) [9]. The number of CL palpated manually was more when compared with ultrasonography, which might be due to the presence of anovulatory follicles which often give a false picture of corpora lutea, termed as pseudo corpora lutea by Monniaux *et al*. (1983) [7]. And also, through USG we are getting only a 2D image of ovaries which may lead to non-visualization of CL located on the other side of the ovary.

Post-flushing luteal characteristics were closely monitored to complete luteal regression. The number of corpora lutea was reduced from day one to six post-flushing. The reduction in the number of CL from day three to six was statistically significant (p<0.05), whereas, the CL number on days 1 and 2 post flushing was statistically non-significant (p>0.05). Similarly, a significant (p<0.05) decrease in the mean diameter of CL was observed from day 1 to 6 post-flushing. Further, a significant reduction in the total luteal area was also seen from the 3rd day onwards (Fig. 3).

In the present study, luteal tissue was remarkably high on day of flushing as compared to after flushing, which may be due to a greater number of CL present on the day of flushing, which receives support from the previous findings of Kurhe, (2019) [4]. Interestingly, from 3rd day post-flushing, a significant reduction in mass and number of corpora lutea was

recorded and complete regression was observed on day 6 post-flushing.

The changes in ovarian size during super stimulatory treatment and day of flushing were monitored and the mean values are shown in Table 5. On the day of flushing, the size of both left (36.89±2.53 mm) and right ovaries (40.5±2.41 mm) were significantly larger ($p<0.05$) than the other days i.e; D1 FSH, day of PG administration and day of SOE during which no significant difference was observed. Studies in bovine model reported that the ovarian dimensions significantly correlated with the antral follicle count, an outcome of superovulatory responses (U-krit *et al*., 2022) [14]. Post flushing majority of the animals (80%) exhibited silent estrus (70%) within 0 days post flushing followed by successful breeding within a month (Table 6). Similar observations were recorded in crossbred cattle (Kurhe, 2019) [4].

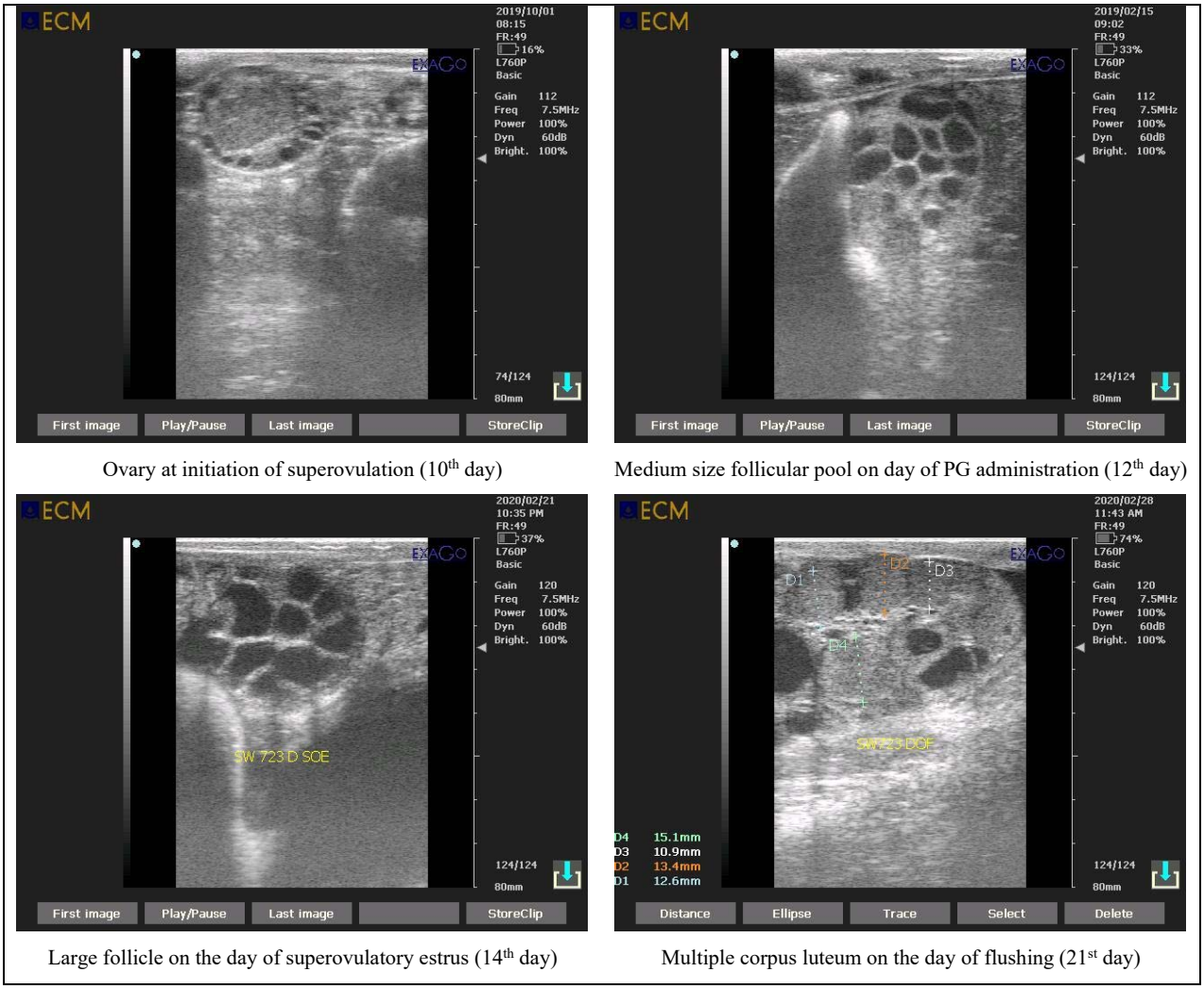
Table 5: Ovarian changes during superovulation program in Sahiwal donor

Time period	Left ovary (size in mm)	Right ovary (size in mm)
Day of initiation of superovulatory treatment	22.74±0.96[a]	24.22±1.29[a]
Day of administration of PG	23.39±0.88[a]	24.61±1.35[a]
Superovulatory estrus	27.61±1.1[b]	29.05±1.24[b]
Day of flushing	36.89±2.53[c]	40.5±2.41[c]
P value	<0.05	<0.05

Mean bearing different superscripts (a, b, c) column-wise differ significantly.

Table 6: Post-flushing breeding pattern in Sahiwal donor

SI. No.	Particulars	Mean±SE
1.	Mean interval flushing to 1st estrus (days)	9.90±1.06
2.	Mean days of 1st CL visualization after flushing	12.40±1.12
3.	Silent estrus post flushing	70% (7/10)
4.	The mean interval from flushing to subsequent successful breeding (days)	29.86±5.24



Ovary at initiation of superovulation (10th day)

Medium size follicular pool on day of PG administration (12th day)

Large follicle on the day of superovulatory estrus (14th day)

Multiple corpus luteum on the day of flushing (21st day)

Fig 2: Ovarian changes during different days of the superovulation program

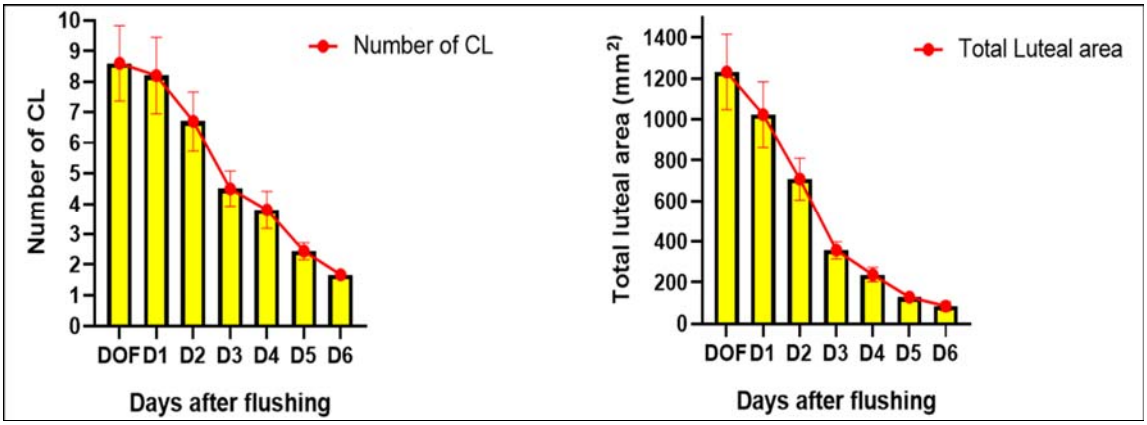


Fig 3: Post flushing PG induced luteolysis pattern in Sahiwal donors

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Conclusion
From this study, it is concluded that gonadotropin treatment increases the number of medium size follicles pool and subsequently converted them into large size follicle pools at superovulatory estrus (SOE) by preventing the atresia of small follicles and also propels the cohort of follicles in a continuous growth phase till superovulatory estrus. Post-flushing administration of PGF_2 alpha to Sahiwal donor leads to a significant reduction in the area of corpus luteum within 72 hrs and ovaries returned to normal non-stimulated size by day 6 post-flushing. And successful breeding was recorded within a month.

Conflict of interest
The authors declared no conflict of interest in relation to the research, authorship, and/or publication of this manuscript.

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