

# Presynchronization with simultaneous administration of GnRH and PGF<sub>2α</sub> 7 days prior to Ovsynch improves reproductive profile in Haryana zebu cow

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## Research Article

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## Abstract

This study aimed to assess the impact of simultaneous administration of GnRH and PGF<sub>2α</sub> 7 days prior to Ovsynch in Haryana cow. Two hundred cyclic cows (> 4 months postpartum) were assigned to Control ( $n = 54$ ) and Pre-OV ( $n = 146$ ). As per Ovsynch protocol, Buserelin acetate (10μg), Cloprostenol (500μg) and Buserelin acetate (10μg) were injected i.m. on day 0, 7 and 9, respectively in cows irrespective of treatment. But in Pre-OV cows, Buserelin acetate (10μg) and Cloprostenol (500μg) were also injected i.m. simultaneously 7 days prior to initiate Ovsynch protocol. Artificial insemination was performed between 18–24 hours after 2nd GnRH of Ovsynch in the both treatments. Ultrasonography and blood sampling for hormonal analysis was done on each day of treatment, on day of AI and 12 days post-AI. Pre-OV treatment resulted to increased (45.20% vs 29.62%;  $P < 0.05$ ) pregnancy outcomes, higher ( $P < 0.01$ ) ovulation rate to first GnRH of Ovsynch than control. Cows showing complete luteolysis in response to PGF<sub>2α</sub> of Ovsynch were also higher ( $P < 0.05$ ) in Pre-OV than control. Greater ( $P < 0.05$ ) synchronization rate was recorded in Pre-OV than control (86.76% and 68.75%). The circulating conc. of estradiol on day of AI and progesterone on day 12 post-AI were higher ( $P < 0.01$ ) in cows diagnosed pregnant than non-pregnant in both control and Pre-OV treatment. In conclusion, simultaneous administration of GnRH and PGF<sub>2α</sub> 7 days before Ovsynch improved the synchronization rate, luteal profile in terms of CL area and hence resulted in higher conception rate in Haryana zebu cow.

## Introduction

*Bos indicus* cows are well adapted to hot and humid climatic conditions prevailing in the tropics especially south-east Asia (Li et al. 2011; Sodhi et al. 2013). *Bos indicus* has various physiological differences from *Bos taurus* probably due to their adaptation to two different agro-climatic conditions (Sartori et al. 2016). But, the overall low reproductive efficiency of *Bos indicus* cattle remains a bottleneck in the flourishing dairy industry in India (Srivastava et al. 2019). To attain the optimum reproductive efficiency, it is impeded that one calf crop per year is produced which ultimately depends upon the timely postpartum ovarian rebound. *Bos indicus* cattle have longer postpartum anestrus period as compared to *Bos taurus* with reported incidence of 48.7% between 40- and 60-days post-partum (Abeygunawardena and Dematawewa 2004), which hampers the profitability of dairy business. Further, hormonal disturbances were found to be primary cause that contribute to more than 47% cases of postpartum anestrus in *Bos indicus* (Abeygunawardena and Dematawewa 2004; Kumar et al. 2014). Average calving to first standing estrus duration in *Bos indicus* was 72 days as compared to 45 days in *Bos taurus* (Henao et al. 2000; Ambrose 2021) but by judicious monitoring and management, *Bos indicus* can also have postpartum ovarian activity comparable to *Bos taurus* (Venkantanaidu et al. 2007; Brar and Nanda 2008).

Various researchers have reported the varying success rates to decrease the postpartum anestrus period and thus, improving the reproductive traits in *Bos indicus* cattle using the fixed time artificial insemination (FTAI) protocols with synchronization of estrus (Junior et al. 2016; Naikoo et al. 2016; Sahithi et al. 2019). FTAI protocols eliminate the need of estrus detection; therefore, prefixed timing of artificial insemination (AI) is a key to improve reproductive efficiency in *Bos indicus* animals with poor estrus expression (Baruseli et al. 2004). The advent of Ovsynch protocol by Pursley et al. (1995) made a breakthrough in the dairy industry. However, Ovsynch was unable to produce perfect synchrony of ovulation in all treated cows and pregnancy rate per AI (PR/AI) recorded lesser (Navanukraw et al. 2004; Singh et al. 2020). Pregnancy rate reported higher if ovulation increased in response to first gonadotrophin releasing hormone (GnRH) of the Ovsynch protocol (Bello et al. 2006). Initiation of Ovsynch on 6-7th day resulted in increased number of cows with a new accessory corpus luteum (CL) at the time of prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) of Ovsynch, greater control of progesterone (P<sub>4</sub>) milieu, subsequent luteolysis, antral age of the ovulatory follicle (Bello et al. 2006; Pursley and Martins 2011) and improved pregnancy per AI (P/AI) (Vasconcelos et al. 2013). Thus, concept of presynchronization came into existence and modified protocols like G7G (Dirandeh et al. 2015), G6G (Bello et al. 2006), Double Ovsynch (Souza et al. 2008) and Presynch-11 (Galvão et al. 2007) were evolved to presynchronize cows to 6-7th day of estrous cycle before the start of Ovsynch protocol (Wiltbank and Pursley 2014). But, the longer duration of these presynchronization protocols and involvement of more labour cost make them practically non-feasible (Martins et al. 2017). Among earlier mentioned presynchronization protocols, G6G is having shortest duration of treatment protocol and resulted in comparable P/AI when compared to longer protocol like G7G (Dirandeh et al. 2015). Nevertheless, the protocol needs higher number of treatment days for effective estrus synchronization seems cumbersome to implement in effective manner. Further, Yousaf et al. (2016) reported a simpler presynchronization strategy of injecting PGF<sub>2α</sub> and GnRH in combination on 7 days before the start of Ovsynch in lactating Holstein dairy cows, resulted in PR/AI comparable to G6G (50% vs 57%) in addition to decreasing number of treatment days. Moreover, Martins et al. (2017) observed similar P/AI (43% vs 43%) with Presynch-10 compared to presynchronization by injecting PGF<sub>2α</sub> and GnRH in combination on 7 days before the start of Ovsynch in lactating dairy cows.

The Haryana breed of indigenous cow is an important zebu cattle breed of India, which is mainly reared for milk purposes. Moreover, it is considered as dual-purpose breed, because of the good muscular strength of male animals in Northern part of India (Joshi et al. 1996).

The perusal of the literature revealed reports pertaining to use of this presynchronization strategy (GnRH and PGF<sub>2α</sub> on 7 days before Ovsynch) is lacking in the indigenous Haryana breed of zebu cow. Therefore, the present study was conducted with the hypothesis that merging PGF<sub>2α</sub> and GnRH in a presynchronization strategy one week before the start of Ovsynch would regress the existing corpus luteum (CL) in addition to ovulation of the largest follicle which would lead to initiation of the new cycle that would result in better synchrony of follicular events and improved pregnancy rate as compared to Ovsynch started at random stage of estrous cycle. The present investigation aimed: (i) to evaluate and compare the effect of presynchronization with GnRH and prostaglandin 7 days before Ovsynch on luteal profile (CL area and P<sub>4</sub> conc.), synchronization rate and first service conception rate (FSCR in Haryana zebu cow).

## Materials And Methods

### Sample size

Sample size was estimated to provide 80% power of getting a cyclic cow not bred for > 4 months post-partum with 95% confidence. According to record maintained at farm, a prevalence of 15% was estimated for such cows. Thus, on the basis of 15% prevalence with 5% error margin, sample size was calculated using an online computer software (Calculator.net) and sample size of minimum 196 cows was estimated to have confidence level of 95% that real value was within ± 5% of measured value. Thus, it was decided to include minimum of 196 cows in study.

### Selection of animals and management

The present investigation involved indigenous breed of Haryana zebu cow and was carried out between December, 2020 and April, 2021 with an approval of Institutional Animal Ethics Committee, LUVAS, Hisar (1669/GO/ReBiBt-S/Re-L/12/CPCSEA). The intensity of estrus response is weaker, and of shorter duration in case of zebu cattle (Wiltbank et al. 2002; Layek et al. 2011), and likely to show the estrus during night hours (Pinheiro et al. 1998); thus, it makes detection of estrus difficult, especially on farms where manual detection of estrus is undergoing as routine practice. Thus, increasing the chance of missed estrus and the chances of a normal cyclic cow is likely to remain unbred for extended post-partum period. Thus, present study was intended to design a FTAI protocol for normal cyclic cows which couldn't be bred even after extended post-partum period, and could tightly synchronize ovulation events, eliminate the need of estrus detection and could improve FSCR in Haryana zebu cow. A total of Two hundred and twenty cyclic cows (parity: 2nd to 5th, body condition score: ranged between 3 and 4 (5-point scale) as described by Edmonson et al. 1989) with history of not bred for > 4 months postpartum, with no reproductive abnormalities (on the basis of clinical examination including trans-rectal palpation and ultrasonography) were selected for this study. The selected animals were kept in loose housing system under group management practice in a single barn for allowing their free movement with provision of fresh *ad libitum* drinking water at a farm located at 28.6° N and 76.6° E. All the animals were milked twice a day, morning (0500 hour) and evening (1700 hour). Animals were fed with total mixed ration consisting of green fodder, roughage, concentrate and mineral supplements (Indian Council of Agricultural Research- National Institute of Animal Nutrition and Physiology, 2013). Before start of treatment protocol, ultrasonography was performed on all the animals and repeated after 11 days interval for the confirmation of presence of CL on either ovary. In addition, plasma progesterone conc. was also measured for confirmation of cyclicity of selected animals in paired samples collected on 11 days interval ( $\geq 1$  ng/mL on either of samples).

### Experimental design

As reports pertaining to use of Ovsynch in zebu cattle were numerous (Bhoraniya et al. 2012; Naikoo et al. 2016), but until date no report has been published regarding presynchronization strategy used in present study involving zebu cattle. So, it was decided to randomly allocate one cow in control after randomly allocating 3 cows to Pre-OV. At start of experiment, 55 cows were included in control, while 165 in Pre-OV treatment. However, during course of study, 1 cow from control and 19 from Pre-OV treatment were excluded from study due to shifting of these cows to another farm. Thus, 200 cyclic Haryana cows were included in the study, Control ( $n = 54$ ) or Pre-OV ( $n = 146$ ) treatments in weekly cohorts (Fig. 1). As per Ovsynch protocol, 10 $\mu$ g Buserelin acetate (Ashored®, Carus Laboratories Pvt. Ltd., India), 500 $\mu$ g Cloprostenol sodium (Zolcol®, Carus Laboratories Pvt. Ltd., India) and 10 $\mu$ g Buserelin acetate were injected i.m. on day 0, 7 and 9, respectively in cows irrespective of treatment. But in Pre-OV cows, Buserelin acetate (10 $\mu$ g) and Cloprostenol (500 $\mu$ g) was also injected i.m. simultaneously, 7 days prior to initiate Ovsynch protocol. Timed artificial insemination (TAI) was performed by one trained technician blind to treatment protocol used with frozen thawed semen of one progeny tested bull at 18–24 hours after administration of 2nd dose of GnRH of Ovsynch in cows of both the treatment protocols.

### Ovarian ultrasonography (USG) and pregnancy diagnosis (PD)

Transrectal ovarian USG was carried out for selection of cyclic animals using real time B-mode ultrasound scanner (SonoScapeS6®, SonoScape Medical corp., China) equipped with inbuilt interchangeable 5/7.5 MHz linear-array rectal transducer. USG was performed in a

subset of 100 cows (Control,  $n = 32$ ; Pre-OV,  $n = 64$ ) from the day of onset of the protocol, on each day of treatment and, at the time of AI (Fig. 1). Ovaries were systematically scanned; scanned images were frozen and recorded. The size of the follicles/CL was determined by measurement of the largest and widest diameter of the follicles/CL and thereafter, average diameter calculated (Pierson and Ginther 1985). Ovulation was characterized by appearance of a new CL in place of previous ovulatory follicle on either ovary (Pandey et al. 2018) while, luteolysis was determined by > 70% reduction in progesterone concentration (recorded on day of PGF<sub>2α</sub> injection) 2 days after PGF<sub>2α</sub> administration in addition to disappearance or reduction in size of CL observed at previous USG examination on day of PGF<sub>2α</sub> administration. In addition, CL diameter was measured through USG on day 12 post-AI also, and CL area was calculated using formula  $\pi \times (\text{diameter of CL}/2)^2$ . Synchronization rate was defined as number of cows showing > 1 ng/mL plasma P<sub>4</sub> on day of PGF<sub>2α</sub> injection of Ovsynch and had responded to PGF<sub>2α</sub> injection in terms of complete luteolysis (Carvalho et al. 2018). Ultrasonography was performed at day 35 and 45 post-AI for detection of fluid in uterus in addition to fetal heart beat for diagnosis of pregnancy. First service conception rate (FSCR; %) was defined as number of cows found pregnant on 45 days post-TAI out of total cows inseminated.

### Blood collection and hormonal analysis

Ten mL blood was collected from jugular venipuncture in heparinised 15 mL centrifuge vials on each day of treatment, day of AI and on day 12 post-AI (Fig. 1) in subset of 100 cows randomly selected from both treatments (Control,  $n = 32$ ; Pre-OV,  $n = 64$ ) and was centrifuged at 1500 x g for 15 minutes to separate plasma. The plasma aliquots were stored in duplicates at -20<sup>0</sup>C until estimation of estradiol (E<sub>2</sub>) and progesterone (P<sub>4</sub>) conc. Plasma P<sub>4</sub> was estimated using a commercially available ELISA Kit (Calbiotech Inc; California, USA). The sensitivity of the assay was 0.112ng/mL. The intra- and inter-assay coefficient of variation was 5.36 and 9.68%, respectively. The plasma Estradiol (E<sub>2</sub>) conc. were analyzed through commercially available ELISA Kit (Calbiotech Inc; California, USA) having sensitivity of 3.94pg/mL. The inter- and intra-assay coefficient of variation was 8.2 and 5.8%, respectively.

### Statistical analysis

All statistical analysis was performed in computer-based SPSS 20 version software. Numerical data were presented as mean ± SE. Effect of treatment and synchronization on conception rates was determined using Chi-square test. The means of various parameters (follicle size, plasma E<sub>2</sub> conc., CL area and plasma P<sub>4</sub> conc.) between two treatments (Control vs Pre-OV), pregnancy status (pregnant vs non-pregnant) and their interactions were compared using two-way ANOVA using following linear model-

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \epsilon_{ijk}$$

where,  $Y_{ijk}$  = repeated observation;

$\mu$  = grandmean;

$\alpha_i$  = effect of  $i^{\text{th}}$  treatment group;

$\beta_j$  = effect of  $j^{\text{th}}$  pregnancy status;

$\gamma_{ij}$  = interactions;

$\epsilon_{ijk}$  = random error with mean 0

Also, two-way ANOVA was employed for determining any significant difference between the pregnancy status and their interactions for various parameters. In addition to this, two-way ANOVA with repeated measures was used to compare control and Pre-OV at different time points as well as to compare various time points (day 0, 7, 9, and day 12 post-AI) for both treatments. Pearson correlation analysis was used to ascertain the correlation between LPF size on day of estrus, and E<sub>2</sub> conc. on day of estrus, LPF size on day of estrus and CL area on day 12 post-AI, CL area and P<sub>4</sub> conc. 12 days post-AI. Estimated variance for plasma P<sub>4</sub> conc. (during first GnRH of Ovsynch) and size of the largest follicle (LF) on day of first and second GnRH of Ovsynch was tested using two samples T-test. Binomial logistic regression for determining predicted probability of pregnancy on day 45 post-AI with respect to various factors like P<sub>4</sub> on different days of treatment, LPF size on day of AI was also performed using following model:

$$\text{logit}(P) = \log \left( \frac{P}{1 - P} \right) = \beta_0 + \beta_i X_i$$

where;  $P$  is probability of pregnancy;

$\beta_0 = \text{intercept}$ ;

$\beta_i = \text{logodds}$ ;

$X_i = \text{predictedvariable}$

$\text{oddsratio} = \text{Exp}(\beta)$

Statistical significances were set at  $P < 0.05$ . Additionally, statistical tendency as numerical trends at  $P \leq 0.1$  was also considered in the study.

## Results

### Ovarian response vis-à-vis endocrine profile subsequent to presynchronization strategy

In response to simultaneous injection of PGF<sub>2α</sub> with GnRH at random stage of estrous cycle in Pre-OV treatment, 42.64% (29/68) cows exhibited luteolysis while, ovulation of LF occurred in 51.47% (35/68) cows.

#### At 1<sup>st</sup> GnRH of Ovsynch

Presynchronization strategy with simultaneous administration of PGF<sub>2α</sub> and GnRH 7 days before start of Ovsynch resulted in follicle with larger diameter ( $P < 0.07$ ), smaller mean area of CL ( $P < 0.01$ ) and elevated ovulatory response ( $P < 0.01$ ; 60.29 vs 31.25%) on first GnRH of Ovsynch in Pre-OV in comparison to control (Table 1). Plasma P<sub>4</sub> conc. (ng/mL) were found to be similar ( $P > 0.05$ ) in both treatments (Fig. 2a). The number of cows with follicle size smaller than 8 mm were significantly higher ( $P < 0.05$ ) in control as compared to Pre-OV (8/32 vs 2/68).

Table 1

LF size (mm) on different days of treatment protocols, mean CL area (mm) at the time of first GnRH and PGF<sub>2α</sub> of Ovsynch, number of follicle ovulated (%) in response to first GnRH of Ovsynch, number of CL present at the time of PGF<sub>2α</sub> of Ovsynch, synchronization rate (%), and FSCR (%) of cows treated with Control (Ovsynch started at random stage of estrous cycle) or Pre-OV treatment (GnRH and PGF<sub>2α</sub> simultaneously 7 days before start of Ovsynch, followed by Ovsynch).

Parameters	Groups		P value
	Control (n = 32)	Pre-OV (n = 68)	
LF size (mm) at the time of first GnRH of Ovsynch	10.54 ± 0.62	11.53 ± 0.23	< 0.07
Mean CL area (mm) at the time of first GnRH of Ovsynch	130.02 ± 6.65	103.02 ± 6.10	< 0.01
Follicles ovulated (%) in response to first GnRH of Ovsynch	31.25 (10/32)	60.29 (41/68)	< 0.01
LF size (mm) at the time of PGF <sub>2α</sub> of Ovsynch	8.67 ± 0.30	9.88 ± 0.25	< 0.01
Mean CL area (mm) at the time of PGF <sub>2α</sub> of Ovsynch	147.52 ± 8.58	132.10 ± 3.11	< 0.07
Number of CL present at the time of PGF <sub>2α</sub> of Ovsynch	1.31 ± 0.07	1.60 ± 0.06	< 0.01
Synchronization rate (%)	68.75(22/32)	86.76 (59/68)	< 0.05
LF size (mm) at the time of second GnRH of Ovsynch	12.65 ± 0.27	13.29 ± 0.17	< 0.05
FSCR (%)	29.62 (16/54)	45.20 (66/146)	< 0.05

Control: (day 0: (10µg) Buserelin acetate; day 7: (500µg) Cloprostenol; day 9: (10µg) Buserelin acetate); Pre-OV: (day (-7): (10µg) Buserelin acetate and (500µg) Cloprostenol simultaneously followed by similar protocol from day 0 as in control); CL: Corpus luteum; FSCR: First service conception rate; GnRH: Gonadotropin releasing hormone; LF: Largest follicle; PGF<sub>2α</sub>: Prostaglandin F<sub>2</sub> alpha; mm: millimeter; n: number of cows;

#### At PGF<sub>2α</sub> of Ovsynch

The size of LF on day of PGF<sub>2α</sub> administration differed ( $P < 0.01$ ) between treatments with larger size in Pre-OV as compared to control (Table 1). Mean number of CL was significantly higher ( $P < 0.01$ ) in Pre-OV than control (Table 1). Subsequent to administration of PGF<sub>2α</sub>,

86.76% cows from Pre-OV and 68.75% ( $\chi^2 = 4.59$ ;  $P < 0.05$ ) cows from control showed complete luteolysis. The synchronization rate was higher ( $\chi^2 = 4.59$ ;  $P < 0.05$ ) in Pre-OV than control (Table 1). The mean plasma P<sub>4</sub> conc. on day of PGF<sub>2 $\alpha$</sub>  administration of Ovsynch was higher ( $P < 0.01$ ) in Pre-OV than (2.39  $\pm$  0.07 vs 2.0  $\pm$  0.05 ng/mL) control (Fig. 2a). On the basis of logistic regression analysis, P<sub>4</sub> concentration on day of PGF<sub>2 $\alpha$</sub>  administration was identified as a probable predictor of pregnancy 45 days post-AI with odds ratio of 2.9, and chances of pregnancy increased ( $P < 0.05$ ) with increasing conc. of P<sub>4</sub> on this day.

### At 2<sup>nd</sup> GnRH of Ovsynch

Average LF size was greater ( $P < 0.05$ ) in Pre-OV (13.29  $\pm$  0.17 mm) as compared to control (12.65  $\pm$  0.27 mm) (Table 1). Nevertheless, the difference between estimated variance in follicular size at 2nd GnRH was lower in Pre-OV (2.0 vs 2.3;  $P < 0.05$ ) than control. The mean plasma P<sub>4</sub> concentration did not differ significantly ( $P > 0.05$ ) between Pre-OV and control (0.48  $\pm$  0.02 vs 0.46  $\pm$  0.03 ng/mL; Pre-OV vs control; Fig. 2a). Moreover, higher ( $P < 0.05$ ) plasma P<sub>4</sub> conc. were found in non-pregnant cows (0.52  $\pm$  0.03 vs 0.41  $\pm$  0.02 ng/mL) as compared to their pregnant counterparts in Pre-OV (Fig. 2b). Results of logistic regression analysis revealed probable chances of pregnancy increased ( $P < 0.05$ ) with decrease in plasma P<sub>4</sub> concentration on day of 2nd GnRH administration with odds ratio of 0.04.

### At the time of AI

Mean largest preovulatory follicle diameter (LPF) on the day of AI tended to be greater ( $P < 0.08$ ) in Pre-OV compared with control (13.47  $\pm$  0.17 and 12.92  $\pm$  0.27 mm, respectively). Moreover, LPF diameter was found to be greater ( $P < 0.01$ ) in cows diagnosed pregnant than for those diagnosed non-pregnant in both control and Pre-OV treatment (Table 2). The mean plasma E<sub>2</sub> conc. on day of AI were higher ( $P < 0.05$ ) in Pre-OV treatment (5.38  $\pm$  0.18 pg/mL) compared with controls (4.72  $\pm$  0.22 pg/mL; Table 2). Further retrospective analysis revealed higher ( $P < 0.01$ ) plasma estradiol concentration on the day of AI in cows that diagnosed pregnant than cows diagnosed non-pregnant in both control (5.29  $\pm$  0.33 vs 4.15  $\pm$  0.24 pg/mL, respectively) and Pre-OV treatment (5.92  $\pm$  0.26 vs 4.84  $\pm$  0.20 pg/mL, respectively) (Table 2). Additionally, the data were compared between control and Pre-OV treatment cows of same pregnancy status, the LPF diameter and plasma E<sub>2</sub> conc. were found to be similar ( $P > 0.05$ ) in pregnant cows; however, in non-pregnant cows, the LPF diameter and plasma E<sub>2</sub> conc. were significantly higher ( $P < 0.05$ ) in Pre-OV than controls (Table 2).

Table 2

Largest pre-ovulatory follicle (LPF) size and plasma estradiol (E<sub>2</sub>) concentrations on the day of AI, corpus luteum (CL) area and plasma progesterone (P<sub>4</sub>) concentrations on day 12 post-AI of cows diagnosed either pregnant (P) or non-pregnant (NP) 45 days after AI, in response to Control (Ovsynch started at random stage of estrous cycle) or Pre-OV treatment (GnRH and PGF<sub>2 $\alpha$</sub>  simultaneously 7 days before start of Ovsynch, followed by Ovsynch).

Day	Parameters	Treatments									P-value (Control vs Pre-OV within P cows)	P-value (Control vs Pre-OV within NP cows)
		Control (n = 32)	Pre-OV (n = 68)	P-value (Control vs Pre-OV)	Control (n = 32)			Pre-OV (n = 68)				
					P	NP	P-value (P vs NP)	P	NP	P-value (P vs NP)		
		(n = 32)	(n = 68)		(n = 16)	(n = 16)		(n = 34)	(n = 34)			
Day of AI	LPF (mm)	12.92 $\pm$ 0.27	13.47 $\pm$ 0.17	< 0.08	13.71 $\pm$ 0.36	12.13 $\pm$ 0.32	< 0.01	13.95 $\pm$ 0.23	12.99 $\pm$ 0.22	< 0.01	> 0.1	= 0.03
	Plasma E <sub>2</sub> (pg/mL)	4.72 $\pm$ 0.22	5.38 $\pm$ 0.18	< 0.05	5.29 $\pm$ 0.33	4.15 $\pm$ 0.24	< 0.01	5.92 $\pm$ 0.26	4.84 $\pm$ 0.20	< 0.01	> 0.1	= 0.05
Day 12 post-AI	CL area (mm)	131.70 $\pm$ 10.72	159.21 $\pm$ 5.70	< 0.01	164.58 $\pm$ 14.0	98.84 $\pm$ 11.63	< 0.01	186.03 $\pm$ 7.11	132.39 $\pm$ 6.14	< 0.01	= 0.1	< 0.01
	Plasma P <sub>4</sub> (ng/mL)	1.79 $\pm$ 0.14	2.03 $\pm$ 0.09	> 0.1	2.21 $\pm$ 0.17	1.36 $\pm$ 0.16	< 0.01	2.47 $\pm$ 0.10	1.58 $\pm$ 0.10	< 0.01	> 0.1	> 0.1

Control: (day 0: (10 $\mu$ g) Buserelin acetate; day 7: (500 $\mu$ g) Cloprostenol; day 9: (10 $\mu$ g) Buserelin acetate); Pre-OV: (day (-7): (10 $\mu$ g) Buserelin acetate and (500 $\mu$ g) Cloprostenol simultaneously followed by similar protocol from day 0 as in control), AI: Artificial insemination; mm: millimeter; n: number of cows; NP: Non-pregnant; P: Pregnant.

### At 12-day post-AI

On day 12 post-AI, cows treated with Pre-OV had greater ( $P < 0.01$ ) CL area compared with control (Table 2). However, plasma  $P_4$  concentration did not differ significantly ( $P > 0.05$ ) between Pre-OV and control cows (Table 2). The pregnant cows of both the treatments (Pre-OV and control) had greater ( $P < 0.01$ ) CL area and exhibited higher ( $P < 0.01$ ) plasma  $P_4$  conc. on day 12 post-AI, as compared to their non-pregnant counterparts (Table 2). Additionally, within pregnant cows, the CL area and plasma progesterone conc. did not differ significantly ( $P > 0.05$ ) between control and Pre-OV treatment; however, in non-pregnant cows, the CL area was greater ( $P < 0.01$ ) in Pre-OV than controls (Table 2).

### First service conception rate (FSCR)

No embryonic mortality was observed, as equal numbers of cows were found to be pregnant on day 35 and 45 post-AI in control and Pre-OV treatments. Presynchronization with GnRH and  $PGF_{2\alpha}$  increased FSCR (1.53 times) compared with control (45.20% vs 29.62%;  $\chi^2 = 3.95$ ,  $P < 0.05$ ; Table 1). It was further observed that FSCR was tended to be higher ( $P < 0.07$ ) in synchronized as compared to non-synchronized cows in both Pre-OV: 52.54% (31/59) vs 33% (3/9), and control: 59.09% (13/22) vs 30% (3/10). Additionally, regardless of treatment instituted, FSCR in synchronized cows had tendency to be higher than cows that did not synchronized (54.32%, 44/81 vs 31.57%, 6/19;  $\chi^2 = 3.18$ ,  $P < 0.07$ , respectively).

### Relationship among LPF diameter, plasma $E_2$ conc. on the day of AI, luteal profiles on day 12 post-AI and FSCR

A positive linear relationship ( $r = 0.899$ ,  $P < 0.01$ ) was identified between LPF diameter and plasma  $E_2$  conc. on the day of AI. The CL area on day 12 post-AI, was positively correlated ( $r = 0.441$ ,  $P < 0.01$ ) with LPF diameter on the day of AI. In addition, plasma  $P_4$  was also positively correlated ( $r = 0.81$ ,  $P < 0.01$ ) with the CL area on day 12 post-AI.

## Discussion

The main objective of this investigation was to compare conception rate between Ovsynch and a modest presynchronization strategy consisting of simultaneous administration of  $PGF_{2\alpha}$  and GnRH 7 days before Ovsynch (Pre-OV) in indigenous Haryana breed of zebu cow. High producing dairy cow subjected to presynchronization of estrous cycle before timed-AI resulted with increased fertility (Moreira et al. 2001). Additionally, the researchers have reported increased fertility response subsequent to presynchronization of estrous cycle compared to Ovsynch alone (Navanukraw et al. 2004; Galvão et al. 2007). In an experiment on dairy cows, proportion of cows found pregnant were tended to be higher ( $P < 0.08$ ) following presynchronization with G6G protocol ( $PGF_{2\alpha}$  followed 2 day later by GnRH, administered 6 days prior to start of Ovsynch protocol), in comparison with Ovsynch alone (50% vs 27%, Bello et al. 2006). Herlihy et al. (2012) observed that presynchronization either with double Ovsynch or double PG (14 days apart) followed by Ovsynch resulted in 46.3% and 38.2% conception rate in lactating dairy cows, respectively. Similarly, in present study, presynchronization with simultaneous administration of  $PGF_{2\alpha}$  and GnRH 7 days before Ovsynch resulted in better pregnancy outcomes than Ovsynch alone (Control). Additionally, previous studies based on similar presynchronization strategy, have reported comparable pregnancy outcomes as obtained with other laborious and logistically challenging presynchronization strategies like G6G and Presynch-10 (Yousaf et al. 2016; Martins et al. 2017). Moreover, FSCR obtained in presynchronized cows during present study was similar to previous reports. Contrary, Hubner et al. (2020) reported lower proportion of Holstein dairy cows pregnant (33.0%) subsequent to presynchronization with  $PGF_{2\alpha}$  and GnRH 7 days before Ovsynch. The discrepancies in the current and previous result could be attributed to involvement of post-partum cows in early stage (48 days post-partum) than current study. Thus, results of this study clearly show that presynchronization with simultaneous administration of GnRH and  $PGF_{2\alpha}$  7 days before Ovsynch improved the FSCR than Ovsynch alone (control), and underlying reasons could be: i) greater ovulation rate in response to 1st GnRH of Ovsynch, ii) greater number of CL and higher plasma progesterone present on day 7 of Ovsynch, iii) improved synchronization rate, iv) greater LPF size on day of AI. Thus, above findings support our hypothesis that Pre-OV treatment resulted in improved pregnancy rate as compared to control.

In present study, Pre-OV treated cows had smaller size of CL at start of Ovsynch as compared to Ovsynch initiated at random stage of estrous cycle in control. Also, on the day of first GnRH of Ovsynch, a tendency towards higher variation in plasma  $P_4$  conc. ( $\sigma^2 = 0.29$  vs  $0.08$ ;  $P < 0.1$ ; control vs Pre-OV) and size of LF ( $\sigma^2 = 12.7$  vs  $3.2$ ;  $P < 0.08$  control vs Pre-OV) was observed in control, which demarks randomness among control cows. Above findings implies that Pre-OV treatment had led to initiation of a new estrous cycle in cows and support our hypothesis that Pre-OV treatment would synchronize cows to 6-7th day of estrous cycle at the start of Ovsynch, while majority of cows might be in mid-cycle stage at initiation of treatment in controls. Indeed, greater CL size present on day 0 of Ovsynch in control than Pre-OV also indicates that the cows in control should have been at mid-cycle stage. In response to GnRH, ovulation of LF is size dependent (Sartori et al. 2001). The acquisition of ovulatory capacity of LF develops as it reaches  $> 8$ mm size in Nellore breed of zebu

cattle (Barros et al. 2008). In present study number of cows with LF of size < 8mm were higher (8 cows vs 2 cows;  $P < 0.05$ ) in control as compared with Pre-OV on day of initiation of Ovsynch. Indeed, presence of larger sized LF present at the time of 1st GnRH in Pre-OV compared with control resulted in higher ovulation rate in response to first GnRH of Ovsynch in Pre-OV than control. This observation forced us to speculate that Pre-OV treatment strategy might have increased the number of cows at 6-7th day of estrous cycle at start of Ovsynch which resulted in greater FSCR. Further, this high ovulatory response to first GnRH in Pre-OV and presence of younger functional CL at first GnRH of Ovsynch in a greater number of cows from presynchronized than control is the possible explanation of the higher FSCR in Pre-OV. Previous study has also shown that P/AI was higher when a young functional CL was present at first GnRH of Ovsynch (Carvalho et al. 2018). In response to first GnRH, ovulation rate observed in present study was similar to (68%) reported by Yousaf et al. (2016) using similar presynchronization strategy in Holstein Friesian cows. On contrary, Hubner et al. (2020) reported higher (90%) ovulation rate subsequent to presynchronization with PGF<sub>2α</sub> and GnRH 7 days before Ovsynch in lactating Holstein dairy cows. Moreover, such disparities in ovulation rate were previously reported for various presynchronization strategy like G6G (67–85%, Yousaf et al. 2016; Bello et al. 2006) and Double-Ovsynch (37.2–90.3%, Giordano et al. 2012b; Ozturk et al. 2010). Various factors like environment, nutrition and genetics of treated cows could account for such discrepancies (Hubner et al. 2020). While, ovulatory response to first GnRH in control cows of present study was similar to previous reports involving lactating dairy cows (Giordano et al. 2012b; Lopes et al. 2013). Giordano et al. (2012a) reported that progesterone levels at the time of GnRH administration suppress the LH surge which hampers the ovulation. Further suggested that, despite the capacity of follicles to ovulate, the reduced ovulation in response to GnRH, is associated with alterations in mechanism that triggers the ovulation process.

Although, mean CL area at the time of PGF<sub>2α</sub> of Ovsynch was smaller in Pre-OV compared to control but due to greater number of CLs observed in Pre-OV resulted into higher plasma P<sub>4</sub> in Pre-OV cows. As various studies have indicated that ovulation to first GnRH leads to formation of accessory CLs and resultant high P<sub>4</sub>, thus high ovulatory response to first GnRH can implied as reason for more CLs (Vasconcelos et al. 1999; Bello et al. 2006). Carvalho et al. (2018) speculated that cows experienced with elevated P<sub>4</sub> at the time of PGF<sub>2α</sub>, could have greater probability to become pregnant as also mentioned in previous reports (Bello et al. 2006; Wiltbank and Pursely 2014; Herlihy et al. 2012). Higher plasma P<sub>4</sub> in Pre-OV treatment could also be attributed to reduced chances of spontaneous luteolysis before PGF<sub>2α</sub> of Ovsynch in Pre-OV treated cows; as in the present study, PGF<sub>2α</sub> of presynchronization was intended to cause luteolysis of all mid and late-cycle CL, and a functional CL formed after ovulation to either GnRH of presynchronization or first GnRH of Ovsynch would persist until administration of PGF<sub>2α</sub> of Ovsynch.

In present study, high P<sub>4</sub> conc. at time of PGF<sub>2α</sub> of Ovsynch was identified as possible predictor of pregnancy at 45 days post-AI. During the development period of LF, elevated circulating P<sub>4</sub> conc. might have decreased LH pulsatility, likely leads to enhancing competency of LF which consequently, improved quality of the LPF (Mihm et al. 1994; Revah and Butler 1996), similar observations have been noticed in present study; however, LH conc. was not measured. Ovulatory response to first GnRH influences luteolysis at PGF<sub>2α</sub> of Ovsynch and synchronization rate with increased frequency of complete luteolysis in cows ovulated to first GnRH (Bello et al. 2006), and similar findings were observed in the present study as higher proportion of cows had complete luteolysis in Pre-OV. The luteolysis rate in present study (86.8%) was lower than reported by Yousaf et al. (2016) (97%) after using similar Pre-OV strategy in dairy cows. The higher luteolysis rate reported by Yousaf et al. (2016) might be owing to administration of an extra PGF<sub>2α</sub> injection on day 8 of Ovsynch. In present study, luteolytic response in control animals was less in comparison to previous reports 83.9% (Giordano et al. 2012b) and 83% (Carvalho et al. 2015). The exact reason for this discrepancy is not known, however, this difference could be attributed to the stage of estrous cycle at starting of Ovsynch and physiological differences between cows. Average ovulatory follicle diameter at 2nd GnRH of Ovsynch and at the time of AI was higher and found lesser variable in Pre-OV as compared to control. Less variability in ovulatory follicle size at 2nd GnRH of Ovsynch in Pre-OV was attributed to higher ovulatory response to first GnRH in this group, which increased the circulating P<sub>4</sub> conc. thus, allowing the development of the LF less variable and closer to the ideal size at the time of the second GnRH (Bello et al. 2006). Circulating conc. of P<sub>4</sub> at 2nd GnRH is identified as probable marker of fertility with decreasing P<sub>4</sub> resulting in increased likelihood of pregnancy at 45 days post-AI. This can be attributed to the fact that lower P<sub>4</sub> at the time of 2nd GnRH resulted in elevated expression of estrus and improves uterine environment for successful conception (Vasconcelos et al. 2013). The present study also confirmed the improved FSCR in cows that exhibited estrus than those did not.

In this study, plasma E<sub>2</sub> conc. on day of AI was found to be higher in Pre-OV cows as compared to control. Additionally, higher plasma E<sub>2</sub> conc. on day of AI was recorded in pregnant cows as compared to their non-pregnant counterparts in both treatments (Pre-OV and Control). Thus, high E<sub>2</sub> conc. in the present study was related to improve FSCR and the results were in accordance with the observations reported in earlier studies on dairy cows (Lopes et al. 2010; Vasconcelos et al. 2013) and buffalo (Pandey et al. 2018). It is well established fact that optimal E<sub>2</sub> conc. on day of estrus is a key for proper growth and coordination in important events like triggering LH surge, ovulation and

resumption of meiosis (Greenwald and Roy 1994). Further, increased  $E_2$  conc. on day of final GnRH of Ovsynch was also related to increasing probability of pregnancy at day 35 post-AI in cows receiving timed-AI (Bello et al. 2006). It seems logical that the higher plasma  $E_2$  on the day of AI would have led to the LH surge, consequently timely ovulation that might have resulted in increased conception rate.

In this study, LPF size on day of AI also affected luteal dynamics and function subsequent to ovulation on day 12 post-AI and a positive correlation was observed. The previous studies including dairy cows (Pursely and Martins 2011) and buffalo (Pandey et al. 2018) also showed constructive relationship between LPF diameter and luteal profiles. Many studies observed that  $P_4$  conc. affected the pregnancy status as increased plasma  $P_4$  concentration was conducive for establishment of pregnancy in cows (Grimard et al. 2006; Pursely and Martins 2011). It is noteworthy that, the pregnant cows had larger CL and higher plasma progesterone on day 12 post-AI compared to non-pregnant cows of respective treatment. Retrospective analysis of data obtained from both treatments (Pre-OV and control) indicated that ovulation in response to first GnRH of Ovsynch affects the outcome for each injection of Ovsynch; resultant luteal profiles and conception rate, which is a key determinant in success of FTAI programme based on Ovsynch strategy (Bello et al. 2006).

Based on this study, it can be concluded that simpler presynchronization strategy involving simultaneous administration of GnRH and  $PGF_{2\alpha}$  7 days before Ovsynch (Pre-OV) increased the ovulatory response to first GnRH of Ovsynch, increased CL area day 12 post-AI and resulted with higher conception rate. Plasma  $P_4$  conc. at  $PGF_{2\alpha}$  and at 2nd GnRH of Ovsynch was identified as significant predictor of pregnancy outcome at 45 days PAI. Further, it was determined that LPF size on day of AI influences luteal profiles and pregnancy outcomes post-AI

## Declarations

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### Competing Interests

“The authors have no relevant financial or non-financial interests to disclose.”

### Author Contributions

**Gitesh Saini:** Conceptualization, Investigation, Writing, Analysis, Drafting of manuscript. **Sandeep Kumar:** Conceptualization, Resources, Analysis, Review and editing of manuscript. **Anand Kumar Pandey:** Conceptualization, Analysis, Resources, Review and editing of manuscript. **Harender Singh:** Investigation. **Meenakshi Virmani:** Conceptualization, Methodology.

### Data Availability

“The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

### Ethics approval

The present investigation involved indigenous breed of Haryana zebu cow and was carried out between December, 2020 and April, 2021 with an approval of Institutional Animal Ethics Committee, LUVAS, Hisar (1669/GO/ReBiBt-S/Re-L/12/CPCSEA).

### Consent to participate

Authors have permission to participate.

### Consent for publication

Authors have permission for publication.

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## Figures

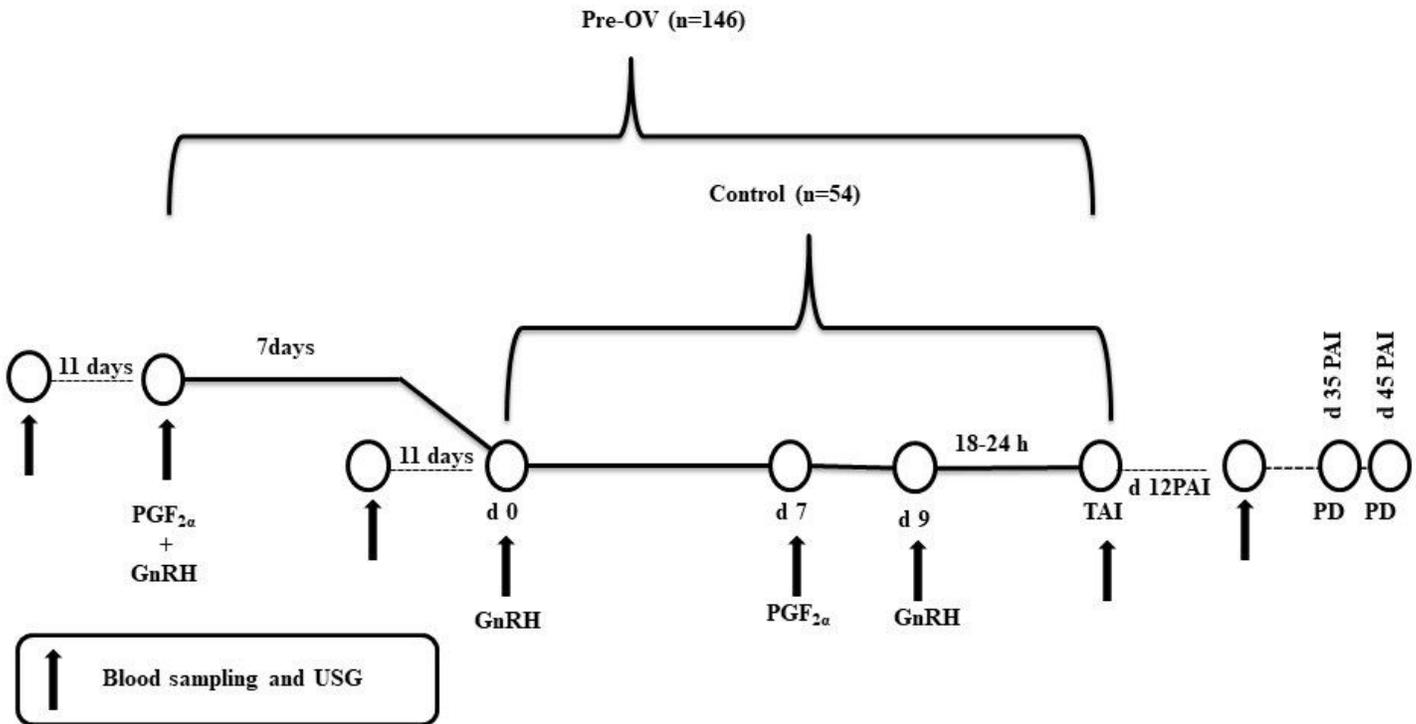


Figure 1

Schematic diagram for experiment schedule in cows. (d: day; GnRH: buserelin acetate; h: hours; n: number of cows; PAI: post-artificial insemination; PD: pregnancy diagnosis; PGF<sub>2α</sub>: cloprostenol sodium; TAI: timed artificial insemination).

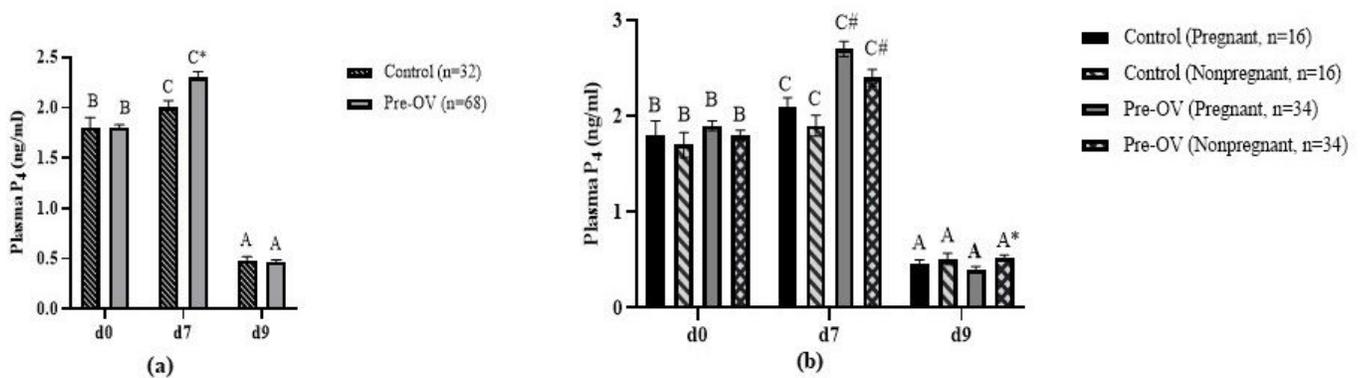


Figure 2

(a) Graph showing mean plasma progesterone concentration (ng/mL) of cows treated with Control (Ovsynch started at random stage of estrous cycle) or Pre-OV treatment (GnRH and PGF<sub>2α</sub> simultaneously 7 days before start of Ovsynch, followed by Ovsynch); [A, B, C ( $P < 0.05$ ) different superscripts differed significantly from each other within treatment; \* ( $P < 0.05$ ) differed significantly between control and Pre-OV treatment within a day]; (b) Mean plasma progesterone concentration (ng/mL) of cows diagnosed either pregnant (P) or non-pregnant (NP) 45 days after AI, in response to Control (Ovsynch started at random stage of estrous cycle) or Pre-OV treatment (GnRH and PGF<sub>2α</sub> simultaneously 7 days before start of Ovsynch, followed by Ovsynch); (A, B, C, ( $P < 0.05$ ) different superscripts differed significantly from

each other within treatment in a same pregnancy status; \*( $P < 0.05$ ) differed significantly between Pregnant (P) and Non-Pregnant (NP) of a treatment within a day; #( $P < 0.05$ ) differed significantly between control and Pre-OV treatment in a same pregnancy status within a day; d: Day; n= number of cows).