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Estradiol benzoate as substitute for GnRH in protocols for synchronizing ovulation in postpartum anestrum cows

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Abstract
The effect of substituting estrogen for the 2nd dose GnRH on the synchronization of ovulation was studied. Healthy pluriparous crossbred cows were selected and randomly divided into two groups (A and B) of six cows each. Cows from group A were treated with injection of 10 µg of GnRH on day 0, 500 µg of $PGF_{2\alpha}$ on day 7 and another 10 µg of GnRH 48 h after PGF2α (day 9) and timed artificial insemination (TAI) was done on day 9 and 10. In group B, the second dose of GnRH was replaced with 1mg of estradiol benzoate injection 24 h after PGF2α (day 8). Intensity of estrum was significantly ($P<0.01$) higher in group B Cows. Though ovulation rate was higher in group A, no significant difference in overall conception rate was observed. The concentration of progesterone was higher among cows which ovulated and in cows conceived at 1st service. The estradiol-17β concentration was highest at estrum (63.17±3.62 pg/ml) and it was high in cows which ovulated and in cows which become pregnant. Concentration of IGF-I was significantly lower in group A at all stages of experiment. The concentration was lower during estrus in cows which ovulated (149.21±16.72 vs. 175.99±25.39). Increase in estradiol and decrease in IGF-1 and progesterone at estrus has positive results on the establishment of pregnancy. Though the results of replacing the 2nd dose of GnRH with estrogen injection were similar to that of group A, less cost of estrogen, improved estrous expression and ease of AI favours this treatment.

Keywords: Estradiol benzoate, Cosynch, progesterone, estradiol-17β, IGF-I, ovulation and conception rate

1. Introduction
Estrous synchronization and fixed time AI has been considered a better alternative to overcome the postpartum anestrum in cows. Ovsynch is one of the estrous synchronization protocol consisting of two injection of GnRH combined with single $PGF_{2}\alpha$ [1]. The efficacy of the protocol varies and various modifications have been made to improve conception rate in cows [2, 3, 4, 5] Macmillan *et al*., (1997) reported that a small dose of estradiol benzoate (1 mg) injected 12, 13 or 14 d after GnRH injection increased fertility and pregnancy rates. Heatsynch protocol involves replacing second GnRH in the Ovsynch protocol with estradiol benzoate on day 8th followed by FTAI 48 hours of $PGF_{2}\alpha$ [6]. Supplementation of estradiol increase pregnancy rate by ovulating medium size follicle (10-15 mm in diameter) [7]. Pancarci *et al*., (2002) recorded that the cows subjected for Heatsynch protocol had greater uterine tone, ease of insemination and more occurrence of estrous. The effect of estrogen on the synchronization of ovulation was not studied in detail in cattle under Indian condition. Further comparison of serum progesterone, estradiol-17β and IGF-I levels and ovarian changes through rectal and ultrasonographic examinations before, during and after synchronization of ovulation treatment protocols in dairy cows injected with estradiol may help to elucidate the possible association of these parameters with fertility.

2. Materials and Methods
Healthy pluriparous crossbred cows in their second to fifth lactation at 60 days post-partum were selected from Large Animal Gynaecology unit of Teaching Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal and from Veterinary Dispensaries located in and around Namakkal district of Tamil Nadu and were randomly divided into two groups (Group A and B) 6 cows each. Cows from group A were treated with intramuscular injection of 10 µg of GnRH (2.5 ml of Buserelin acetate, Gynarich, INTAS Pharmaceuticals, Ahmedabad, India) on the day of starting of experiment (d 0), 500 µg of $PGF_{2}\alpha$ (2ml,

Cloprostenol, Pregma, INTAS Pharmaceuticals, Ahmedabad, India) seven days later (d 7) and another 10 µg of GnRH (2nd GnRH) 48 hours after the $PGF_2\alpha$ (d 9) and timed artificial insemination (TAI) was done on day 9 and 10 respectively. It was similar to the protocol of Co-Synch described by [8] Lamb *et al.* (2001) in cows with a modification of double AI at 24 hours interval. This group served as treated control.

In group B, the second dose of GnRH (10 µg) was replaced with intramuscular injection of 1mg of estradiol benzoate (2 ml of estradiol benzoate injection IP, Pregheat, Virbac Animal Health India Pvt Ltd., Mumbai) 24 hours after $PGF_2\alpha$ (day 8) and AI was performed on day 9 and 10. The protocol followed was similar to the heatsynch recommended by [9] Cevik *et al.* (2010) with a modification of double AI at 24 hours interval during induced estrus.

The experimental cows were closely observed for oestrus signs during the period of treatment and estrus response in percentage, intensity of oestrus and duration of oestrus were calculated. Ovulatory response was assessed by rectal examination performed 7 days after estrus by the palpation of the corpus luteum in any one of the ovaries and it was further confirmed by ultrasonography.

Conception rate was calculated as percentage of animals that conceived to AI at induced estrus (First service conception rate) and subsequent oestrus (Second service conception rate) divided by number of animals treated in each group. Pregnancy was confirmed by ultrasonography on day 32 post AI and later on confirmed by rectal palpation at 60 days post AI.

Blood samples were collected on the day of initiation of experiment, on day 7, at estrum, on day 7 and 45 days post AI. The concentration of progesterone, oestradiol-17β and IGF – I were analyzed by radio immune assay method.

The data collected were analyzed using SPSS 21 software package. Post hoc analysis was done by Tukey's Honestly Significance Difference. Pair wise comparisons were done using Duncan's Multiple Range Test and one-way ANOVA was done for continuous traits such as Duration of estrum and hormone levels at different days of collection. The ordinal score traits were analyzed using non-parametric Kruskal Wallis test, for which significant pair wise differences were done using Mann-Whitney test. The mean ranks were used to compare different treatment groups.

3. Results and Discussion

All the cows subjected to treatment exhibited behavioural, physiological and gynaecological signs after injection of $PGF_2\alpha$. Indicating that the treatment were highly effective to induce oestrus in postpartum anestrus cows. The cows from group B showed intense estrum (66.67 per cent) than group A (50.00 per cent). Group B had significantly (P<0.01) higher ranking in behavioural, physiological and gynaecological changes and in intensity of estrum (Table 1).

Cows from group A had 83.33 per cent of palpable corpus luteum in one or both ovaries. Whereas, the cows from group B had 66.66 per cent ovulation. In group B cows the ovulation may have happened in small follicles which may not have resulted in mature corpus luteum noticeable on day 7 of the cycle. In addition, exogenous estradiol rarely stimulated an LH surge in the presence of elevated progesterone concentration [10, 11].

Overall conception rate was 50.00 per cent. No significant difference was observed in the overall conception rate among the cows in both the groups. However, the 1st service conception was lower in group B (33.33 per cent) than group A (50.00 per cent). The increase in duration of estrus, behavioural and gynaecological changes in estrus coupled with lower rate of estradiol in comparison to GnRH make the protocol followed in group B superior to group A. Similarly [7] Pancarci *et al*. (2002) have observed that the cows in Heatsynch program had greater uterine tone, ease of insemination and better estrous behaviour. Though the ovulation rate was 66.66 per cent in this group the corresponding conception rate was 50.00 per cent. The reduction in the conception rate over ovulation rate may be due to the ovulation of small follicles by estradiol [12] with smaller corpus luteum that reduce circulating concentration of progesterone result in impairment of embryonic development [13].

The concentration of progesterone were low towards the basal values on the day on selection in all cows (0.56±0.73 ng/ml) indicating that all the cows selected for the experiments were in true anestrum (Table 2). The concentration was decreased at the time of estrum. The progesterone concentration was significantly (P<0.01) higher among cows with ovulation in all stages of experiment except on the day of estrum (0.62±0.76 vs 1.35±1.03). The presence of well-developed corpus luteum after ovulation may be responsible for the increased progesterone secretion in these cows. The results observed in this study were similar to the results presented by [14] Ammu *et al*, (2012) [15], Bhoraniya *et al*. (2012) and [16] Buhecha *et al*. (2015).

The cows which conceived in 1st service had significantly higher concentration of progesterone at all stages of the experiment. However, the concentration of progesterone at estrum was significantly (P<0.01) lower in cows, conceived during 1st service (0.50 ± 0.99 vs 1.06 ± 0.76). The cows which were having higher concentration of progesterone at estrum had less ovulation and lower conception rate in the present study. The cows with insufficient corpus luteum lysis because of immature corpus luteum may be the cause of increased progesterone at estrum. The presence of high progesterone at estrum may lead to lower ovulation and conception rate [10, 12, 11]. The estrogen secreted from the follicles or estrogen administered exogenously in group B cows, rarely stimulate an LH surge in the presence of elevated progesterone concentration [10]. Hence there will be no ovulation and subsequent pregnancy.

The estradiol-17β concentration was highest at estrum (63.17 ± 3.62 pg/ml) (Table 3). The estradiol level at estrum was high in cows which ovulated (67.64 ± 3.71 vs. 58.70 ± 6.82) and in cows which become pregnant (66.96 ± 4.63 vs. 59.37 ± 3.92). The dominant follicle secretes estradiol that stimulates LH surge and ovulation and subsequently the cows become pregnant [17]. The cows with insufficient estrogen from small follicles may not be able to stimulate the LH and there by leads to anovulation.

The estradiol concentration on the day of estrum was highest in group B. However, the ovulation rate and conception rate were low in this group. Exogenously administered estrogen induce LH surge within 15-20 h after injection [18]. However, the supplemented estradiol increase ovulation rate by ovulating only medium sized follicle but not small or large follicles. The unovulated small or large follicle may continuously produce estrogen as observed in this study.

The concentration of IGF-I was significantly lower in group A at all stages of experiment when compared to group B (Table 4). This indicated that the cows treated with estradiol significantly increased the concentration of IGF-I. [19] Xia *et al*., (2001) observed the interactions between IGF-I and estradiol at pituitary level. [20] Michels, *et al*., (1993) reported that estradiol had prominent effect on components of IGF-I systems in the anterior pituitary. The greatest levels of pituitary IGF-I binding was demonstrated at pro estrous when estradiol level is at its maximum. The pituitary IGF-I receptors are regulated by estradiol [21]. Thus the administration of estrogen, up regulates the IGF-I receptors and there is increase in IGF-I concentration in group II. IGF-I and estradiol together facilitates the modulation of LH secretion [19].

The concentration of IGF-I was lower during estrus in cows which ovulated (149.21 ± 16.72 vs. 175.99 ± 25.39). Among the cows which become pregnant the concentration of IGF-I was higher during estrum, day 7 and 45 after AI (166.48 ± 17.69, 131.90 ± 17.69 and 241.25 ± 17.69 vs. 143.41 ± 24.18, 111.14 ± 24.18 and 234.90 ± 24.18). The concentration of IGF-I was lower during estrus in cows which ovulated (149.21 ± 16.72 vs. 175.99 ± 25.39). IGF-I sensitize the pituitary cells to estrogen action by up regulating estradiol receptor expression and together they facilitate the modulation of LH secretion [22]. They also found that there is a feedback mechanism between IGF-I and estrogen. Estrogen down regulates the concentration of IGF-I receptors and its ligands [22]. Hence the synergistic action of IGF-I and estrogen on GnRH stimulated LH secretion might occur predominantly at low concentration of IGF-I as observed in the present study.

Table 1: Mean Ranking of Behavioural, Physiological, Gynaecological Changes, Intensity of Estrum, Ovulatory Response and Conception Rate

Group	Behavioural changes	Physiological changes	Gynaecological changes	Intensity of estrum
I	11.50 [b]	7.58 [b]	11.50 [a]	10.50 [b]
II	12.42 [a]	13.83 [a]	11.58 [a]	12.50 [a]
Group	**Ovulatory response**	**1st service conception rate**	**2nd service conception rate**	**Over all conception rate**
I	83.33 [a] (5/6)	50.00 (3/6)	0.00 (0/6)	50.00 (3/6)
II	66.66 [b] (4/6)	33.33 (2/6)	18.33 (1/6)	50.00 (3/6)
Over all	75.00 (9/12)	41.67 (5/12)	8.33 (1/12)	50.00 (6/12)

Figures in the parentheses are number of animals, Mean rank values bearing different superscripts within the same column differ significantly (*P*<0.05).

Table 2: Mean (±Se) Serum Progesterone (Ng/Ml) Concentration in Correlation with Ovulatory Response and Conception Rate

Groups / Days of collection		Day 0 Inj GnRH	Day 7 Inj PGF2α	Estrum	7th day post A.I	45th day post A.I.
Treatment groups	I	0.61 ± 1.43	2.97 ± 1.43	1.06 ± 1.43	2.50 ± 1.43	8.56 ± 1.43
	II	0.46 ± 1.37	1.28 ± 1.37	0.70 ± 1.37	4.29 ± 1.37	6.64 ± 1.37
Ovulatory response	Present	0.59 ± 0.76	2.76 ± 0.76	0.62 ± 0.76	3.79 ± 0.76	11.50 ± 0.76
	Absent	0.52 ± 1.03	1.81 ± 1.03	1.35 ± 1.03	2.44 ± 1.03	2.99 ± 1.03
Conception rate	Pregnant	0.63 ± 0.99	2.94 ± 0.99	0.50 ± 0.99	3.90 ± 0.99	14.36 ± 0.99
	Non pregnant	0.48 ± 0.76	2.18 ± 0.76	1.06 ± 0.76	3.07 ± 0.76	5.15 ± 0.76
Overall		0.56[D] ± 0.73	2.52[C] ± 0.73	0.80[D] ± 0.73	3.45[B] ± 0.73	9.37[A] ± 0.73

Mean values bearing different superscripts within the same row differ significantly (*P*<0.01)

Table 3: Mean (±SE) Serum Estradiol-17β (Pg/Ml) Concentration in Correlation with Ovulatory Response and Conception Rate

Groups / Days of collection		Day 0 Inj GnRH	Day 7 Inj PGF2α	Estrum	7th day post A.I	45th day post A.I.
Treatment groups	I	7.89 ± 6.62	24.59 ± 6.62	64.73 ± 6.62	31.51 ± 6.62	22.03 ± 6.62
	II	9.04 ± 6.10	8.05 ± 6.10	67.26 ± 6.10	44.70 ± 6.10	18.78 ± 6.10
Ovulatory response	Present	11.71 ± 3.71	22.08 ± 3.71	67.64 ± 3.71	30.64 ± 3.71	15.76 ± 3.71
	Absent	8.61 ± 6.82	15.27 ± 6.82	58.70 ± 6.82	44.92 ± 6.82	25.16 ± 6.82
Conception rate	Pregnant	13.96 ± 4.63	22.47 ± 4.63	66.96 ± 4.63	33.99 ± 4.63	16.66 ± 4.63
	Non pregnant	6.37 ± 3.92	14.88 ± 3.92	59.37 ± 3.92	41.58 ± 3.92	24.25 ± 3.92
Overall		10.16[D] ± 3.62	18.68[C] ± 3.62	63.17[A] ± 3.62	37.78[B] ± 3.62	20.46[C] ± 3.62

Mean values bearing different superscripts within the same row differ significantly (*P*<0.01)

Table 4: Mean (±SE) Serum igf1 (ng/ml) Concentration in Correlation with Ovulatory response and Conception Rate

Groups / Days of collection		Day 0 Inj GnRH	Day 7 Inj PGF2α	Estrum	7th day post A.I	45th day post A.I.
Treatment groups	I	42.23 ± 21.81	72.52± 21.81	142.01 ± 21.81	73.99 ± 21.81	177.08 ± 21.81
	II	46.18 ± 19.21	101.37 ± 19.21	162.46 ± 19.21	130.38 ± 19.21	217.51 ± 19.21
Ovulatory response	Present	47.09 ± 16.72	103.57 ± 16.72	149.21 ± 16.72	127.64 ± 16.72	239.43 ± 16.72
	Absent	44.91 ± 25.39	75.38 ± 25.39	175.99 ± 25.39	120.63 ± 25.39	235.06 ± 25.39
Conception rate	Pregnant	48.20 ± 17.69	90.43 ± 17.69	166.48 ± 17.69	131.90 ± 17.69	241.25 ± 17.69
	Non pregnant	44.59 ± 24.18	103.72 ± 24.18	143.41 ± 24.18	111.14 ± 24.18	234.90 ± 24.18
Overall		46.55[D] ± 16.50	96.52[C] ± 16.50	155.91[B] ± 16.50	122.38[BC] ± 16.50	238.34[A] ± 16.50

Mean values bearing different superscripts within the same column differ significantly (*P*<0.05)

4. Conclusion

The protocols followed in this experiment have improved the ovulation rate and conception rate to a considerable level in postpartum anestrus cows. Replacing the 2nd dose of GnRH with estrogen injection has similar results to that of group A. The cost of estrogen was less than GnRH, in addition there was improvement in estrous expression, gynaecological and physiological changes, which favours this treatment over group A. Increase in estradiol and decrease in IGF-1 and progesterone at estrus has positive results on the establishment of pregnancy. Special emphasis needs to be given for the follicular development with pre-treatment of cows in group B to make them having follicles of medium diameter for improvement of their ovulation and conception rate in future.

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