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Estrous synchronization and conception rate following insulin modified Co-synch protocol in postpartum anestrus cows

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Abstract
Negative energy balance in high producing cows reduces insulin and insulin like growth factor-I (IGF-I) and delays onset of estrous cycle. Under Indian conditions the addition of insulin in the co-sync protocol was not studied in detail. Twelve pluriparous crossbred cows were selected and equally divided into two groups I and II. All the cows were treated with 10 µg of GnRH injection on day 0, 500 µg of PGF2α 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. Cows from group II were injected with insulin (0.2 I.U/ kg bwt) subcutaneously on day 0, 1 and 2 of the experiment. Higher intensity and longer duration of estrum was observed in cows from group II. The ovulation rate was higher in group I cows (83.33 vs. 50.00 per cent) however, no significant difference in conception rate was observed. The concentration of progesterone was slightly lower on day 7 after AI in group II. The concentration of estrogen up to estrum was higher in cows which became pregnant and a significant decline on day 7 and 45 after AI. The concentration of IGF-I was lower during estrum in cows which ovulated. (149.21±16.72 vs. 175.99±25.39 ng/ml). Among the cows which became pregnant the concentration of IGF-I was higher during estrum, day 7 and 45 after AI. Ovulatory responses were effectively turned into pregnancy in insulin injected postpartum anestrum cows, the protocol can utilized under field condition.

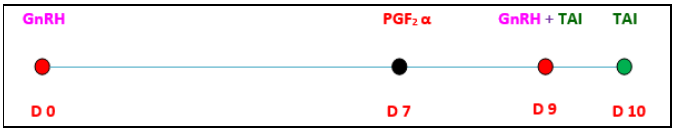
Keywords: Cosynch, insulin, progesterone, estradiol-17β, IGF-I, ovulation and conception rate

1. Introduction
One of the major contributing factors for the low productivity per cow in our country is infertility problem specifically in postpartum cows. The stress of pregnancy, parturition, onset of lactation and suckling in cows, negatively affect feed intake and there by expose the animals to negative energy balance [1] and result in prolonged interval from calving to first ovulation [2]. High producing cows experience a period of negative energy balance during the postpartum period (early lactation). Negative energy balance reduces insulin and insulin like growth factor-I (IGF-I) and delays estrous cycle, impaired oocyte quality and corpus luteum function in cows [3]. Insulin and IGF-I are important mediators of follicular development, steroidogenesis, oocyte maturation and subsequent embryo development. Administration of insulin increases peripheral as well as intra-follicular IGF-I levels [4], which potentiate the action of FSH on granulosa cell differentiation. High responsiveness of granulosa cell for FSH cause an increase in estrogen and it also causes an increase in progesterone concentration after ovulation [5].

Insulin directly plays an important role in follicular growth through its stimulatory effects on granulosa cells, estradiol production and indirectly by amplification of gonadotrophic actions[6]. Insulin injection in cows increased the steroidogenic capacity, the diameter of the largest follicle [4] and the ovulatory rate in response to superovulatory program [7]. Better estrous induction and conception was obtained by [8] Singh, (2013) injecting insulin in ovsynch protocol. However, [9] Maffi *et al*., (2019) observed that the application of a single dose of insulin does not promote an increase in follicular size, estrus presentation and conception rate in dairy cows.The effect of insulin on the Ovsynch protocol and possible association of the levels of serum progesterone, estrogen, IGF-I and glucose levels and ovarian changes on the fertility was the objective of the present study.

2. Materials and Methods
Twelve crossbred cows in their second to fifth lactation, with post-partum anestrum (not showing estrus symptoms for more than 90 days) 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 randomly divided into two groups of six cows each as Groups I and II.



Cows from group I 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 (2^{nd} 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 [10] Lamb *et al.* (2001) in cows with a modification of double AI at 24 hours interval. This group served as treated control.
Cows from group II were treated as per the treatment regimen followed in group I. Further all the cows were injected with insulin at the dose rate of 0.2 I.U/ kg body weight subcutaneously on day 0, 1 and 2 of the experiment (Isophane insulin injection i.p, Huminsulin, Eli Lilly and Company Pvt Ltd., Maharashtra, India).

The experimental cows were closely observed for oestrus signs during the period of treatment and after $PGF_2\alpha$ injection. The estrus response in percentage, intensity of oestrus and duration of oestrus were calculated. Onset of oestrus was calculated in hours, from the time of $PGF_2\alpha$ administration. The intensity of oestrus was measured based on the manifestation of the estrual changes charted in the score card as described by [11] Rao and Rao (1981) with slight modifications 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 (Real time ultrasound scanner (SONORAY) equipped with liner array, 5-7.5 MHz frequency trans-rectal transducer).
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 cost of delayed pregnancy and economics of treatment were made.
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

Insulin is having a direct stimulatory effect on *in vitro* granulosa cell estradiol production and indirect stimulatory effect via amplification of gonadotropin action [12, 13]. However, reports demonstrating similar effects *in vivo* are less. This is probably due to insulin induced hypoglycaemia. The counter regulatory neuro-endocrine responses to hypoglycaemia may override the beneficial effects of insulin.
Most of the earlier studies utilizing insulin have used insulin at a higher dose rate of >0.5 IU/kg body weight daily for a longer period [7, 4, 5]. To prevent hypoglycaemia they administered intravenous glucose as a co treatment. However several experiments were carried out utilizing the beneficial effects of insulin at a lower dose of 0.2 IU/kg bodyweight on follicular development, steroidogenesis and ovulation rate without causing any hypoglycaemia and other effects [14, 15, 16].
In the current experiment blood samples were analysed to assess the glycaemic status of all cows (Table 1). The glucose level on day 7 of the experiment was increased over the concentration of glucose on the day of injection of insulin (6.50 and 26.39 per cent increase in group I and II respectively).
The level of glucose was elevated on day 7 of experiment in cows treated with insulin. There was a decrease in the glucose level at estrum and again it increased on day 7 post AI when compared to group I. This indicated that insulin administered was increasing the glucose level rather than causing hypoglycaemia and no side effects or any reactions were observed in any of the cows used in this experiment.
All the cows subjected to treatment from both groups expressed estrum (100 per cent induced estrum). The injection of GnRH recruits new follicles [17], insulin improves the growth of small follicles [5] and brings about estrus behaviour. However, [9] Maffi *et al*., (2019) observed no increase in the follicular diameter in dairy cows injected with single dose of insulin in Heatsynch protocol. The intensity of estrum and the mean ranking for physiological changes were significantly (P<0.01) higher in group II (12.50, 12.25 and 10.50, 7.58 respectively), which indicated that the addition of insulin had significant effect on the behavioural, physiological and gynaecological changes of estrum (Table 2). The addition of insulin improved the recruitment and growth of follicle to medium or large size and increase steroidogenesis thereby increase the estradiol concentration which responsible for the changes observed during estrum [5].
The ovulatory response was 83.33 per cent in group I (Table 3). The response was poor in cows injected with insulin (50.00). The less ovulatory response observed in this group might be due to negative energy balance of postpartum cows, that reduce the GHR 1A receptor and IGF-I mRNA expression in liver [18] and insulin administration during luteal phase, diminish the ovulatory process by high concentration of progesterone as opined by [19] Cox *et al*. (1987). Injection of insulin might have helped the follicles to grow from small to medium or large follicles. But, the required concentration of estrogen to induce preovulatory surge of LH might have

been low [20].

However, in group II the ovulation rate of 50 per cent was reflected in 50.00 per cent conception rate, 100 per cent conversion of ovulation into pregnancy. The above findings in the present study indicated a scope for improvement of conception rate in this group by increasing the ovulation rate. The body conditions of the cows at the time of selection may also act as a source for variation in ovulation rate in cows [21]. By selecting cows with BCS of 2-3 the ovulation rate may be increased.

The serum concentrations of progesterone (Table 3) 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.

The progesterone concentration on day 7 of the experiment and on day 7 and day 45 after AI were significantly higher among cows with ovulation and in cows which conceived in the first service. This indicated that the presence of functional large corpus luteum secreting progesterone in these cows. These results are in concurrence with the findings of [22] Ammu *et al.* (2012), [23] Bhoraniya *et al.* (2012) and [24] Buhecha *et al.* (2015).

The estradiol-17β concentration was highest at estrum (63.17± 3.62 pg/ml) which were significantly higher than at any stage of experiment (Table 4). The increased serum estradiol levels during estrum in the present study coincided with the findings of [15] Gupta *et al.* (2011). The mature follicle under the influence of insulin, estradiol or insulin induced IGF-1 may secrete estradiol that is responsible for initiation of standing estrum and peak level of plasma estradiol [25].

In this current investigation estradiol level at estrum was high in cows which ovulated (67.64 ± 3.71 vs. 58.70 ± 6.82) and in cows which became pregnant (66.96 ± 4.63 vs. 59.37 ± 3.92). The dominant follicle secreted estradiol that stimulates LH surge and ovulation and subsequently the cows become pregnant [3]. The cows with insufficient estrogen from small follicles may not be able to stimulate the LH and there by leads to anovulation.

The concentration of IGF-I (Table 5) was significantly lower in group I during all stages of experiment when compared to group II. This indicated that the cows treated with insulin, significantly increase the concentration of IGF-I. The injection of insulin increases the concentration of IGF-I [26]. In addition insulin induced up regulation of IGF-I receptors in granulosa cell and facilitates increased ovarian steroidogenesis.

In this investigation concentration of IGF-I was low 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 facilitate the modulation of LH secretion [27]. They also found a feedback mechanism between IGF-I and estrogen. Estrogen down regulates the concentration of IGF-I receptors and its ligands [27]. Hence the synergistic action of IGF-I and estrogen on GnRH stimulated LH secretion might have occurred predominantly at low concentration of IGF-I. Increase in estradiol and decrease in IGF-1 and progesterone at estrus has positive results on the establishment of pregnancy.

Table 1: Mean (±Se) Blood Glucose (Mg/Dl) Levels Before, during and after Treatment of Postpartum Anestrus Cows

Days of collection		Day 0 Inj GnRH	Day 7 Inj PGF2α	Estrum	7th day post A.I	45th day post A.I.	Overall
Groups	I	48.49 ± 3.09	51.64^{b} ± 4.73	62.55^{a} ± 5.01	43.83^{b}± 8.49	61.27 ± 6.83	53.56 ± 2.81
	II	52.74^{qr} ± 6.63	66.66^{abpq} ± 6.18	37.48^{br} ± 5.26	83.37^{ap}± 4.44	51.73^{qr} ± 7.95	58.34 ± 3.87
Overall		49.32 ± 2.26	64.31 ± 3.05	55.42 ± 3.77	69.44 ± 4.36	58.71 ± 4.24	59.42 ± 3.36

Mean values bearing superscripts between rows with in the same column (a, b) and among column with in a row (p, q, r) differ significantly ($P \leq 0.05$).

Table 2: 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^{a}	7.58^{b}	11.50^{a}	10.50^{b}
II	11.50^{a}	12.25^{a}	10.25^{b}	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	50.00^{b} (3/6)	50.00 (3/6)	0.00 (0/6)	50.00 (3/6)
Over all	66.67 (8/12)	50.00 (6/12)	0.00 (0/12)	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 3: 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	0.55 ± 1.36	1.80 ± 1.36	0.76 ± 1.36	3.95 ± 1.36	9.71 ± 1.36
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

Table 4: 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	6.34 ± 5.94	23.35 ± 5.94	60.12 ± 5.94	38.14 ± 5.94	17.30 ± 5.94
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 5: 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	49.34 ± 18.65	73.93 ± 18.65	166.32 ± 18.65	123.85 ± 18.65	291.01 ± 18.65
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

4. Conclusion

Injection of insulin improved the ovulation rate and conception rate to a considerable level in postpartum anestrus cows. Hence, the protocol can be efficiently followed in field level for better ovulation and pregnancy. Special emphasis needs to be given for the follicular development with pre-treatment of cows to make them having follicles of medium diameter for improvement of the results.

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