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Effect of administration of human chorionic gonadotropin in ovsynch ovulation synchronisation protocol for the management of repeat breeding syndrome in cows

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Abstract

The objectives of the present study were to assess the effect of administration of human chorionic gonadotropin (hCG) instead of second GnRH in the Ovsynch ovulation protocol on the fertility of repeat breeding cows. Twenty-four repeat breeder (RB) cows free of any detectable anatomical abnormalities and subclinical endometritis were selected and randomly divided into three groups, each with eight animals, group I (Ovsynch), group II (modified Ovsynch) and group III (Control). Group I and group II RB cows received Ovsynch protocol and modified Ovsynch protocol respectively, initiated on day 5 of oestrous cycle, in which group II animals received hCG instead of second GnRH, followed by timed artificial insemination 18 h after second GnRH or hCG injection. Group III animals were inseminated on the day of standing oestrus. Blood samples were collected from RB cows for serum oestrogen and serum progesterone estimation. Ultrasonographic examination of uterus and ovaries were done to assess endometrial thickness, number and type of follicles and diameter of corpus luteum. All RB cows in treatment groups responded to protocols. There was no significant difference in the duration and time taken for onset of oestrus. The serum progesterone concentration in group II day 10 post AI was significantly higher ($p < 0.05$) than group I and III. The overall conception rate in group I, group II and group III were 50, 50 and 25 percent, respectively. It can be concluded modified Ovsynch protocol is effective in the management of repeat breeding in cattle with potential advantage by enhancing corpus luteum development and serum progesterone level.

Keywords: hCG, Ovsynch, repeat breeder, progesterone, oestrogen, ultrasonography

Introduction

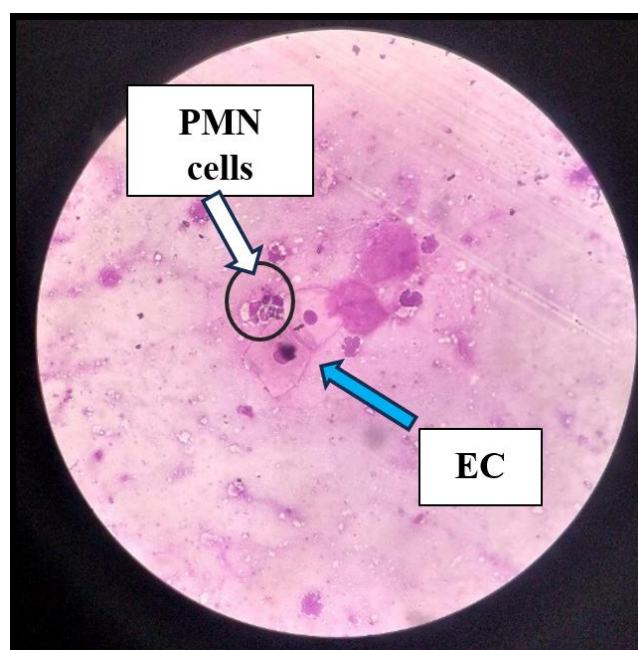
Fertility is the key to a cow's lifetime output, influencing both profitability and overall milk production. Achieving optimal fertility helps shorten calving intervals and reduces the days open. However, uterine infections during the post-partum period can significantly hinder reproductive efficiency. In Indian context, reproductive disorders such as endometritis, anoestrus, and repeat breeding are major factors resulting in reduced dairy productivity. Repeat breeding is characterised by cows failing to conceive despite multiple attempts. Repeat breeder cows have normal or nearly oestrous cycle, yet failed to conceive even after being bred three or more times to a fertile bull or inseminated with fertile semen (Roberts, 1986). The major risk factors of repeat breeding are nutritional imbalances, hormonal aberrations, reproductive tract infections, genetic predispositions and karyotypic aberrations. Subclinical endometritis, untimely AI, delayed ovulation, fertilisation failure and early embryonic mortality are the major identified causes of repeat breeding. Certain causes of repeat breeding such as, untimely AI and delayed ovulation can be managed using hormonal protocols. In field condition, most commonly used hormonal protocols are Ovsynch protocols and its modifications. Ovsynch protocol involves the administration of gonadotropin releasing hormone (GnRH) in the early luteal phase, followed by administration prostaglandin $F_{2\alpha}$ seven days' administration of GnRH and again a second dose of GnRH 48 h after administration of $PGF_{2\alpha}$. Many studies

have used Ovsynch protocols for improving pregnancy rate in repeat breeder cows with pregnancy rate varying from 40 to 60 percent. But there are only few studies using hCG in repeat breeder cows. Human chorionic gonadotropin can act directly on the ovary and is independent of pituitary gland for inducing ovulation. The effect of hCG is longer than endogenous LH and also elevates the progesterone concentration than GnRH treatment (Binversie *et al.*, 2012)^[8]. Hence the present study was done to study the effect of administration of hCG in Ovsynch ovulation synchronisation protocol for improving the fertility of the repeat breeder cows.

Materials and Methods

The study was conducted on 34 Repeat Breeder (RB) cows in an organized farm, with a 20% occurrence of repeat breeding over one year. Clinico-gynaecological examination revealed two cows with abnormalities (follicular cyst and cervical abscess), which were excluded from the study. The remaining 32 cows were screened for Subclinical Endometritis (SCE) using the Whiteside test, which identified 7 positive animals. These cows were further tested for cytological endometritis using modified cytobrush technique, with a threshold of $\geq 5\%$ polymorphonuclear cells (PMN). Twenty-four SCE-free cows were selected and divided into three groups: Group I

(Ovsynch protocol), Group II (modified Ovsynch protocol), and Group III (control). Group I cows received 10 μg of buserelin acetate on day 5, 500 μg of cloprostenol sodium on day 12, and a second dose of 10 μg buserelin acetate on day 14. Timed artificial insemination (TAI) was performed 18 hours after the second GnRH dose. Group II cows followed a similar protocol, but on day 14, 1500 IU of hCG was administered instead of the second buserelin dose, with TAI 18 hours later. Group III cows were inseminated on the observed day of oestrus. The onset and duration of oestrus were recorded, and blood samples were collected to evaluate serum oestrogen and progesterone levels. Serum samples were taken on the day of oestrus (for oestrogen), day 10 and 21 post-AI, and on days 5, 12, and 14 of the oestrous cycle (for progesterone). The samples were analyzed for hormone levels using Radioimmunoassay (RIA). Ultrasonographic examinations were conducted using a 5 MHz transrectal probe to study ovarian and uterine structures. Measurements included endometrial thickness on days of oestrus, day 5, day 12, and day 14 of the oestrous cycle, as well as follicle size and corpus luteum (CL) diameter on different days. Pregnancy was diagnosed ultrasonographically on day 30 post-AI and confirmed on day 50 by per-rectal examination.



EC- Endometrial epithelial cells

Fig 1: Endometrial cytology (400x); PMN cells

Results and Discussion

Occurrence of subclinical endometritis among repeat breeder cows

The overall occurrence of SCE was 21.87 percent, while the occurrence of cytological endometritis was 9.37 percent. Zarekar (2014)^[40] and Parikh (2021)^[24] reported a similar occurrence of 20.62 and 18.42 percent of SCE in RB cows. Pothmann *et al.* (2015)^[25] reported a 12.70 percent prevalence of cytological endometritis in RB cows, with a threshold of PMN cells ≥ 5 percent. In contrary to this, Gupta *et al.* (2011)^[13], Raja *et al.* (2012)^[28] and Bhat *et al.* (2014)^[6] diagnosed SCE in RB cows using white side test and found an incidence rate of 45.83, 72 and 71.25 percent, respectively. In this study, the lower occurrence of SCE might be attributed to the reduced frequency of reproductive and metabolic issues in the cattle. Additionally, better hygiene practices, optimal

breeding management, or the use of repeated treatments and therapeutic interventions may have contributed to the prevention and control of uterine infections.

Time taken for onset of oestrus in group I and group II

All the RB cows in group I and group II responded to Ovsynch and modified Ovsynch ovulation synchronisation protocols. the time taken for onset of oestrus following $\text{PGF}_{2\alpha}$ administration for cows in group I was group II was 59.38 ± 1.21 h, while for group II, it was 57.13 ± 1.06 h. No significant difference was observed in the time taken for the time taken for onset of oestrus between RB cows in group I and II (Table 1). Similarly, Ahmed *et al.* (2016)^[2] and Aruna (2021)^[2] reported that Ovsynch treatment in cows resulted in 100 percent induction of oestrus after the $\text{PGF}_{2\alpha}$ injection. Selvaraju *et al.* (2008)^[31] noted that the time taken for the

onset of oestrus after treatment with PGF₂ α in RB cows was 59.38 ± 0.81 , which was in accordance with the present findings. Similarly, Krishnakumar and Chandrahasan (2012) [19] observed a similar time period of 60.20 ± 1.41 h for onset of oestrus after PGF₂ α administration in RB cows. But, Prajapati *et al.* (2015) [26] observed a slightly higher duration

of 62.10 ± 2.26 h in RB cows treated with Ovsynch protocol. Whereas, Ahmed *et al.* (2016) [2] observed that RB cows treated with Ovsynch protocol had a shorter period for induction of oestrus (48.750 ± 0.71 h) in RB cows after PGF₂ α administration.

Table 1: Time taken for onset of oestrus in repeat breeder cows in group I and group (n = 8)

Groups	Number of animals responded to treatment	Percent of animals responded to treatment	Time taken for onset of oestrus (h)
Group I	8	100	59.38 ± 1.21
Group II	8	100	57.13 ± 1.06
t - value (P value)			1.399 ^{ns} (0.183)

ns non-significant; Group I - Ovsynch, Group II - modified Ovsynch; Independent t - test

Duration of oestrus

Duration of spontaneous oestrus was 30.00 ± 3.49 , 27 ± 2.20 and 25.50 ± 1.96 h in group I, II and III, respectively. The duration of induced oestrus was observed to be 28.13 ± 2.70 and 26.13 ± 2.04 h in group I and group II, respectively. No significant difference ($p > 0.05$) was observed in the duration of spontaneous and induced oestrus between groups. There was no significant difference ($p > 0.05$) in the duration of spontaneous and induced oestrus between group I and II (Table 2). Kumar *et al.* (2008) [20] observed that the duration of oestrus in repeat breeder cattle was 28.90 ± 0.14 , which is in accordance with the findings of present study. Similarly, Layek *et al.* (2013) [22] observed that there was no significant difference ($p > 0.05$) between the duration of spontaneous and

induced oestrus. But, Bhat and Bhattacharya (2012) [5] observed a duration of 34.43 ± 3.67 h oestrus in RB cows, which is higher than that observed in present study. In contrary to this, Reddy *et al.* (2022) [29] observed a shorter duration of 22.94 ± 0.87 h in RB cows. In RB cows the extended duration of oestrus might be due to delayed ovulation, likely linked to suprabasal progesterone levels. This delay in ovulation reduced LH pulse frequency, resulting in prolonged oestrogen production and a longer oestrus period (Bigelow and Fortune, 1998) [7]. The shorter duration of oestrus after ovulation synchronisation protocols might be due to administration of hCG or GnRH after PGF₂ α , which accelerated ovulation, reduced the oestradiol peak, and shortened behavioural oestrus.

Table 2: Duration of spontaneous and induced oestrus in repeat breeder cows (n = 8)

Groups	Duration of oestrus		t - value (P - value)
	Spontaneous (h)	Induced (h)	
Group I	30.00 ± 3.49	28.13 ± 2.70	1.930 ^{ns} (0.095)
Group II	27.00 ± 2.20	26.13 ± 2.70	1.698 ^{ns} (0.133)
Group III	25.50 ± 1.96	-	-
F value (P - value)	0.756 ^{ns} (0.482)	-	-
t - value (P - value)	-	0.591 ^{ns} (0.564)	-

ns non-significant; Group I - Ovsynch, Group II - modified Ovsynch, Group III - Control; One-way ANOVA followed by DMRT, Independent t-test and paired t - test

Endometrial thickness on different days of oestrous cycle

There was significant difference ($p < 0.01$) in endometrial thickness on day 0, day 5 and day 12 of oestrous cycle within both group I and group II (Table 3 and Fig. 2 to 5). There was significant difference ($p < 0.05$) in the endometrial thickness on day 5 between group I (5.15 ± 0.25) and II (4.50 ± 0.09). This might be due to the higher progesterone level in animals in group II (8.94 ± 1.33) compared to animals in group I (6.12 ± 1.69). DesCoteaux *et al.* (2010) noted that ultrasonographic image of uterus during peri-oestrus phase showed noticeable swelling, more heterogenous appearance and less uniform

gray tones of the uterine wall, which is in accordance with the present study. Ahmadi *et al.* (2019) noted that uterus was thicker during the oestrus than dioestrus phase of the oestrous cycle. Increased endometrial thickness on day of oestrus might due to high level of oestrogen in circulation and reduced endometrial thickness during luteal period might be due to high circulating level of progesterone during luteal phase of oestrous cycle. DesCoteaux *et al.* (2010) observed that the high level of progesterone in circulation decreases the endometrial thickness.



Fig 2: Ultrasonographic image of endometrial thickness on day of oestrus

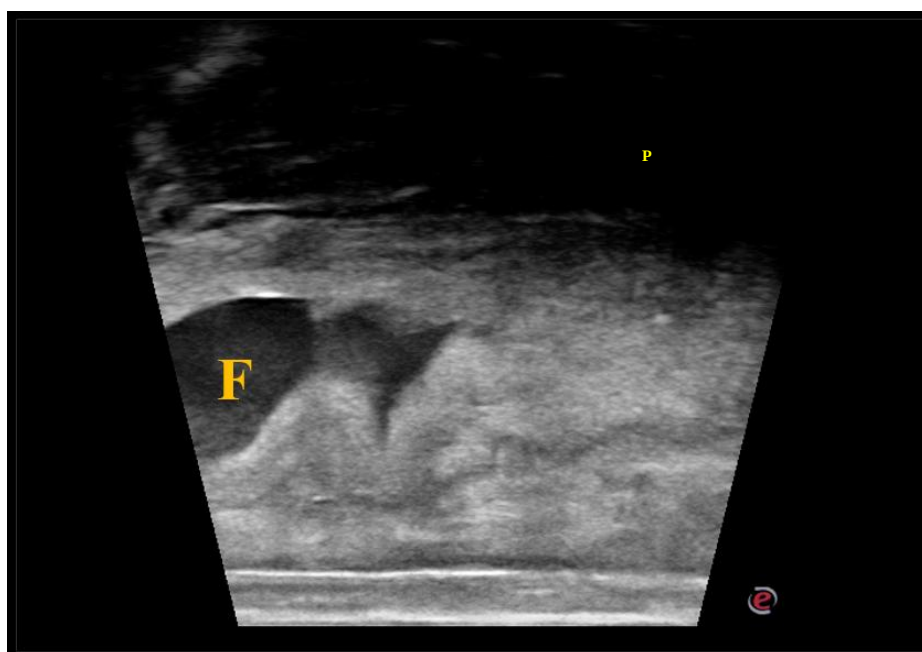
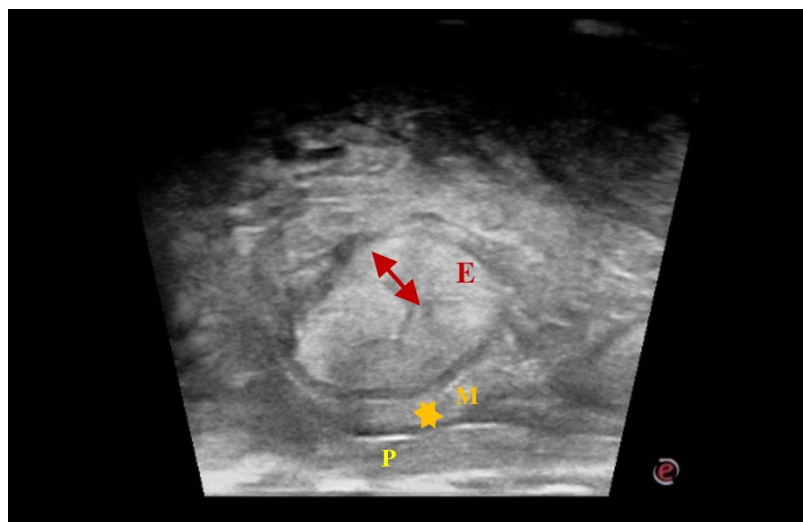


Fig 3: Ultrasonographic image of oestral mucus accumulation in uterine lumen during day of oestrus (D0)



Fig 4: Ultrasonographic image of reduction in the endometrial thickness on day 12 of oestrous cycle



E - Endometrium, M - Myometrium, P - Perimetrium

Fig 5: Ultrasonographic image of appearance of uterus during day of oestrus (D0)**Table 3:** Ultrasonographic examination of endometrial thickness (mm) on different days of oestrous cycle in repeat breeder cows

Day of oestrus	Group I	Group II	Group III	t-value (P-value)	F- value
Day 0	7.05 ± 0.30 ^a	7.48 ± 0.26 ^a	7.29 ± 0.14 ^a	1.059 ^{ns} (0.308)	0.759 ^{ns} (0.481)
Day 5	5.15 ± 0.25 ^b	4.50 ± 0.09 ^d	-	2.459* (0.037)	-
Day 12	4.13 ± 0.15 ^c	3.88 ± 0.12 ^c	-	1.351 ^{ns} (0.198)	-
Day 14	6.80 ± 0.12 ^a	6.74 ± 0.2 ^a	-	0.267 ^{ns} (0.794)	-
F-value (P-value)	37.99** (<0.001)	114.35** (<0.001)	-	-	-

** Significant at 0.01 level; * Significant at 0.05 level; ns - non-significant

Group I - Ovsynch, Group II - modified Ovsynch, Group III - Control

One-way ANOVA followed by DMRT, Independent t-test, Repeated Measures ANOVA followed by LSD

Number and diameter of follicles on different days of oestrous cycle in RB cows

The number of small follicles in group III on day 0 was significantly higher ($p < 0.01$) than group I and II. There was significant difference ($p < 0.05$) in the diameter of medium sized follicle in group II with group I. There was no significant difference in the number and diameter of medium and large sized follicles on day 0 between groups. On day 5 of and day 12 oestrous cycle, there was no significant difference ($p > 0.05$) in the number of different sized follicles and diameter of small and large sized follicles, between group I and group II. The size of the dominant follicle during induced oestrus was 12.06 ± 0.38 mm for group I and 12.16 ± 0.39 mm for group II. Diameter of dominant follicle on day of spontaneous oestrus was 10.96 ± 0.31 mm for group III and there was no

significant difference ($p > 0.05$) in the diameter of dominant follicle during induced oestrus in group I and II (Fig. 6 to 7). Bage *et al.* (2002)^[4] found that RB cows had more small follicles in their ovulatory wave compared to virgin heifers. Lamb *et al.* (2003)^[21] reported significantly higher pregnancy rates ($p < 0.01$) in cows with follicles larger than 12 mm. Similarly, Keskin *et al.* (2010)^[18] noted that cows with ovulatory follicles between 13.50 and 16.50 mm had higher conception rates and lower embryonic loss. Singh *et al.* (2019)^[34] observed larger dominant follicles was observed in Ovsynch treated RB cows compared to controls, aligning with findings of current study. Kashyap *et al.* (2020)^[16] observed that in repeat breeder cows treated with Ovsynch protocol the average diameter of preovulatory follicle measured 11.48 ± 0.44 mm, supporting the findings of the present study.

**Fig 6:** Dominant follicle and small follicle observed on day of oestrus

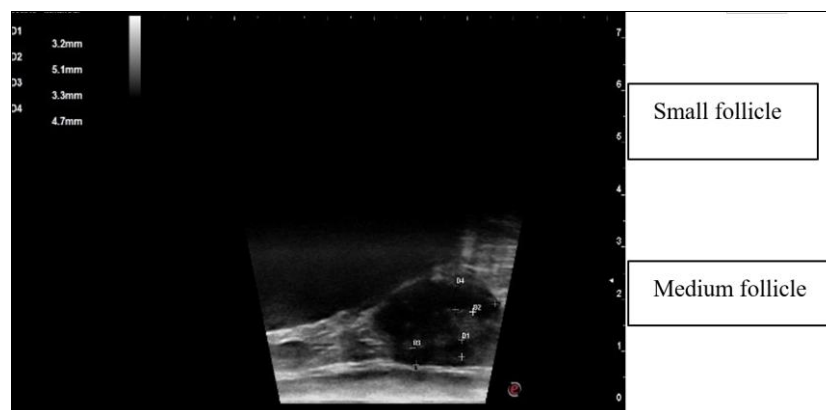


Fig 7: Medium follicle and small follicle observed on day five of oestrous cycle

Diameter of CL oestrous cycle on different days of oestrous cycle in RB cows

The diameter of CL on day 5 for group I and II was 9.96 ± 2.19 and 14.10 ± 0.43 mm, respectively and on day 12 it was 18.51 ± 0.73 and 19.31 ± 0.46 mm, respectively. There was no significant difference ($p > 0.05$) in the diameter of CL between group I and II on day 5 and day 12. Diameter of CL day 10 post AI in group I (19.28 ± 0.49) and II (19.76 ± 0.46) was significantly higher ($p < 0.01$) than group III (17.79 ± 0.47). There was no significant difference ($p > 0.05$) in the diameter of CL on day 21 post AI between post AI (Table 6

and Fig. 8 to 9). The larger diameter of CL in the cows treated with GnRH, along with the higher circulating progesterone levels compared to untreated animals could be due to an enhanced CL function, which might positively affect fertility (Hasan *et al.*, 2021) [14]. Treatment with hCG in cows has been linked to an increased number of large luteal cells and a reduction in small luteal cells, contributing to a larger CL (De Rensis *et al.*, 2008) [12]. The greater CL diameter day 10 post AI in group I and II compared to the group III, might be due to the luteotropic effect of GnRH or hCG administered in the treatment groups.



Fig 8: Ultrasonographic image of hyper-echoic corpus luteum on day 12 of oestrous cycle



Fig 9: Ultrasonographic image of corpus luteum on day 10 Post AI

Table 6: Diameter (mm) of corpus luteum on different days in treatment and control groups (Mean $\pm$ SE, n = 8)

Day of oestrous cycle	Group I	Group II	Group III	F-value (P - value)	t - value (P - value)
Day 5	9.96 $\pm$ 2.19	14.10 $\pm$ 0.43	-	-	3.443 ^{ns} (0.085)
Day 12	18.51 $\pm$ 0.73	19.31 $\pm$ 0.73	-	-	0.780 ^{ns} (0.448)
Day 10 post AI	19.28 $\pm$ 0.49 ^a	19.76 $\pm$ 0.46 ^a	17.69 $\pm$ 0.49 ^b	5.067** (< 0.001)	
t - value (P - value) Day 12 and day 10 post AI	0.816 ^{ns} (0.441)	0.461 ^{ns} (0.659)	-	-	

** Significant at 0.01 level; ns - non significant

Group I - Ovsynch, Group II - modified Ovsynch, Group III - Control

One-way ANOVA followed by DMRT, Independent t- test

Serum progesterone concentration on different days of oestrous cycle

The serum progesterone concentrations on the day of oestrus were 1.71 $\pm$ 0.44, 2.49 $\pm$ 0.22, and 2.21 $\pm$ 0.24 for groups I, II, and III, respectively. Suprabasal progesterone concentrations (>1 ng/mL) were observed in all three groups, with no significant difference across the groups ($p > 0.05$). Bage *et al.* (2002) [4] observed similar suprabasal progesterone levels in repeat breeders, and Singh *et al.* (2005) [32] also noted elevated levels in repeat breeder heifers. Suprabasal progesterone might result from incomplete regression of the Corpus Luteum (CL) and low prostaglandin levels, negatively affecting ovulation and LH surge, thus impacting fertility (Bigelow and Fortune, 1998) [7]. Additionally, higher progesterone levels might be produced by the adrenal glands during stress (Honparkhe *et al.*, 2010) [15].

On day 5 of the oestrous cycle, progesterone concentrations were 6.12 $\pm$ 1.69 and 8.94 $\pm$ 1.33 for groups I and II, respectively. The progesterone concentration on day 10 post AI was significantly higher in group II (12.83 $\pm$ 1.32) compared to groups I (8.22 $\pm$ 1.07) and III (7.97 $\pm$ 1.13), with $p < 0.05$. Progesterone levels further increased on day 12 (10.55 $\pm$ 2.99 and 15.07 $\pm$ 2.55 for groups I and II) and decreased by day 14 (1.40 $\pm$ 0.40 and 1.29 $\pm$ 0.45 for groups I and II). The findings were consistent with Murugavel *et al.* (2018), who reported lower progesterone levels on day 5 and higher concentrations on day 12 due to the functional CL or luteotropic effects of GnRH. On days 10 and 21 post AI, group II had significantly higher progesterone concentrations ($p < 0.05$) compared to the other groups. These findings align with studies by De Rensis and Peters (1999) [10] and Binversie *et al.* (2012) [8], who observed higher progesterone levels in cows treated with hCG compared to GnRH.

Table 7: Serum progesterone concentration (ng/mL) on different days in repeat breeder cows (Mean $\pm$ SE, n = 8)

Groups	Serum progesterone concentration (ng/mL) at different days of modified Ovsynch and Ovsynch group					
	D0	D5 - First GnRH	D12 -PGF ₂ α	D14 -hCG/ second GnRH	Day 10 post AI	Day 21 post AI
Group I	1.71 $\pm$ 0.44	6.12 $\pm$ 1.69	10.55 $\pm$ 2.99	1.40 $\pm$ 0.40	8.22 $\pm$ 1.07 ^b	6.66 $\pm$ 1.38
Group II	2.49 $\pm$ 0.22	8.94 $\pm$ 1.33	15.07 $\pm$ 2.55	1.29 $\pm$ 0.45	12.83 $\pm$ 1.32 ^a	8.45 $\pm$ 2.43
Group III	2.21 $\pm$ 0.24	-	-	-	7.97 $\pm$ 1.13 ^c	4.89 $\pm$ 1.15
F - value	1.554 ^{ns}	-	-	-	5.413*	1.040 ^{ns}
t - value	-	1.312 ^{ns}	1.152 ^{ns}	0.180 ^{ns}	-	-
p - value	0.237	0.211	0.269	0.860	0.013	0.371

* Significant at 0.05 level; ns - non-significant

Group I - Ovsynch, Group II - modified Ovsynch, Group III - Control, One-way ANOVA followed by DMRT, Independent t-test

Serum oestrogen concentration

The mean serum oestrogen concentration was 11.76 $\pm$ 1.65, 12.46 $\pm$ 1.84 and 13.31 $\pm$ 1.45 pg/mL for group I, II and III, respectively. On day of oestrus, there was no significant difference ($p > 0.05$) in the level of oestrogen on the day of oestrus between groups. Sub basal oestrogen concentration (< 10 pg/mL) was observed in 37.50 percent of all RB cows. This finding is in accordance with observations of Sultana *et al.* (2022) [37], they found that the mean serum oestrogen level in RB cows as 15.49 $\pm$ 2.45 pg/mL. They also noted that the serum oestrogen in normal cycling cows (25.40 $\pm$ 4.07 pg/mL) was higher RB cows. But, Sood *et al.* (2015) [35] observed that RB cows had higher average plasma oestradiol levels (105.20 pg/mL) than normal cycling group, which had a lower average concentration of 85.60 pg/mL. The lower average concentration of oestrogen in RB cows when compared to the normal cycling cows might be due to poor nutrition in farm or metabolic disorders that could affect the endocrine system, resulting in poor follicular development and reduced oestrogen production. Increased steroid metabolism in liver due to lactational stress might be also a

reason for the lower oestrogen concentration in RB cows (Wiltbank *et al.*, 2006) [39].

Conception rate

The conception rate following the first AI were 37.50, 37.50 and 12.50 percent for group I, group II and group II, respectively. There was no significant difference ($p > 0.05$) in first AI conception rate and overall conception rate between the three groups (Table 9). Pursley *et al.* (1997) [27] reported a first AI conception rate of 37.80 percent using the Ovsynch protocol in dairy cows. Binversie *et al.* (2012) [8] later modified the double Ovsynch by incorporating hCG, resulting in first AI conception rates of 32.20 and 25 percent. The overall conception rates were 61.50 percent for double Ovsynch and 52.20 percent for the modified version. In contrast to these findings, Burns *et al.* (2008) [9] found that cows treated with GnRH before AI had a higher pregnancy rate (53.40 percent) compared to those treated with hCG (38.90 percent). The higher conception rate in treatment groups might be due to larger CL diameter, increased progesterone level and larger dominant follicles.

Table 9: First service and overall conception rate (percent) in repeat breeder cows treated with Ovsynch and modified Ovsynch protocol (n = 8)

Groups	No. of repeat breeders cows inseminated	No. of repeat breeders conceived in first AI	Conception rate (%)	No. of repeat breeders conceived after three consecutive AI	Overall conception rate (%)
Group I	8	3	37.50	4	50.00
Group II	8	3	37.50	4	50.00
Group III	8	1	12.50	2	25.00
P- value		0.446 ^{ns}		0.504 ^{ns}	

ns - non-significant

Group I - Ovsynch, Group II - modified Ovsynch, Group III - Control

Chi-square test

Conclusion

The study concludes that both Ovsynch and modified Ovsynch protocols are effective in managing repeat breeder syndrome in cows by successfully inducing oestrus and improving conception rate. The inclusion of the hCG in the Ovsynch protocol demonstrated a potential advantage by enhancing the serum progesterone level and CL development. Considering the CL diameter and progesterone concentration it can be inferred that modified Ovsynch protocol involving hCG is more effective than Ovsynch protocol.

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