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Effects of hCG administration at day 6 of the estrous cycle on follicular and luteal studies of repeat breeder cross-bred cow

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Abstract
The present study was aimed to determine the effects of hCG (Chorulon®, 1500 I.U.) administration at day 6 of the estrus cycle on follicular and luteal dynamics, progesterone (P_4) concentration and conception rate in repeat breeder cross-bred cows. Twelve healthy and normal cyclic repeat breeder cross bred cows (n= 12) were used in this study. The plasma progesterone was estimated using RIA kit. Ultrasonography and blood collection was done on day 0, 6, 14 of estrous cycle. There was no significant difference in diameter of follicle between and within control and treatment groups. There was no significant difference in diameter and area of the corpus luteum between control and treatment group. No accessory corpus luteum was formed in cows of control group. The formation and retention of one accessory corpora lutea was obtained in treatment group. Progesterone concentration was significantly high on day 14 in treatment group in comparison to control group. The pregnancy rate was Nil in control group, whereas, 16.66% in treatment group. It was concluded that there was no significant effect of hCG administration on diameter of pre-ovulatory or dominant follicle, diameter and area of corpus luteum, however, the diameter and area of corpus luteum increased post hCG administration. The accessory corpus luteum could be produced by hCG administration on day 6 of the estrous cycle. After hCG administration there was significant rise in progesterone concentration, may be due to accessory corpus luteum formation or stimulatory effect on existing corpus luteum.

Keywords: Repeat breeding, hCG, Day 6, Accessory corpus luteum, progesterone

Introduction
Repeat breeder has been considered as one of the most important reproductive disorder which distresses fertility and results in huge economic losses to dairy industry. Repeat breeder animal is usually defined as a subfertile animal which has been served three or more times with a fertile bull or inseminated with fertile semen yet fails to conceive and continually returns to estrus in the absence of any obvious pathological disorder in the genital tract [1]. This condition has been extensively studied in cattle [2, 3]. Since several factors affect the incidence of the repeat breeding in dairy cows, it is difficult to make generalized statement regarding predominant causes but still in this syndrome impaired embryonic development, periovulatory abnormalities, damage to endometrium stress, and inadequate postovulatory progesterone concentrations are among few most important causes. Accordingly, many treatment regimens involving antibiotics [4], chemotherapeutic agents and hormones [5] have been reported with varying results [6]. In repeat-breeder cows, the time when the embryo enters the uterus and undergoes blastocoels formation (Day 6–8) has been suggested to be a critical period during which embryonic death occurs [7]. Also a slower rise and lower total progesterone concentration have been reported in low fertility cows and repeat breeder heifers in the first 6 days after estrus [7, 8]. It is well documented that elevation in progesterone (P_4) concentration during the first week of pregnancy plays a major role in reducing embryonic mortalities [9, 10] through increasing the secretion of adequate interferon tau (IFN-τ) [11]. Interferon-tau in turn is very important in extending the lifespan of the corpus luteum (CL) by suppressing estradiol and oxytocin receptor genes [12] and attenuating the endometrial secretion of $PGF_2\alpha$ [13]. Several approaches were used to increase P_4 concentration in early pregnancy in order to reduce the occurrence of embryonic mortalities either by inducing increased endogenous secretion or by administering exogenous P_4 [14]. Administration of P_4 exogenously includes application of progesterone, progesterone releasing intravaginal device (PRID), Controlled Internal Drug Release (CIDR) and Synchromate ear implants which are commercially

available [15]. Whereas, for endogenous production administration of GnRH or human chorionic gonadotropin (hCG) after AI can stimulate CL function and/or induce accessory CL formation which in turn increase P_4 and reduce estradiol production, with a consequent positive effect on embryo survival [14, 16]. Human chorionic gonadotropin has activity similar to LH, and after binding to LH receptors, causes small luteal cells to increase progesterone synthesis [17]. The GnRH and hCG treatments are equally effective in inducing an accessory CL, but the subsequent increase in progesterone concentrations was greater in hCG-treated heifers [18]. In continuation to our previous reported findings on ultrasonography studies on effects of hCG administration at day 5 of the estrous cycle on pregnancy rate of repeat breeder cross-bred cow [19]. The aim of the present study to determine the effect of hCG administration at 6th day of AI, on pregnancy in repeat breeder crossbred cows that are occurring due to luteal deficiency, resulting in accessory corpus luteum by luteinizing dominant follicle of 1st follicular wave thus producing more P_4, necessary for better embryo survival in luteal deficient repeat breeder cross-bred cows.

Materials and Methods

Animal and management

The experimental animals comprised of 12 healthy and cyclic crossbred repeat breeder cattle, aging 4-7 years, kept at Instructional livestock farm (ILFC), SKUAST-J, Jammu, organized dairy farms, commercial dairy farms and local nearby area, with a body condition score (BCS) between 3 and 4 (Scale 1 to 5; BCS 1 = emaciated; BCS 5 = obese/ extremely fat [20] at initiation of the study were selected for experimentation.

Experimental design

Animal at standing estrous, confirmed on the basis of records for regular estrous cycle, signs of estrus followed by trans-rectal ultrasonography when ovary contains pre ovulatory follicle of ≥ 10 mm diameter. All the animals at standing estrus negative to 'White Side Test' (a test of endometritis; [21], were selected for experimentation. All selected animals were bred twice by artificial insemination at 12 and 24 hrs after onset of estrus with fertile frozen semen and were randomly divided into 2 groups (each group containing 6 animals) as control group: inj. 5ml Normal Saline on day 6/ did not received any treatment and treatment group: inj. hCG (Chorulon®) 1500 IU i.m. at day 6 of AI.

Collection of blood

Blood samples were collected at each ultrasound session by jugular venipuncture in heparinized test tube on day 0, 6 and 14 and plasma was harvested by centrifuging at 3000 rpm for 15 minutes within 30 minutes of collection and stored at -20 °C pending progesterone assay.

Progesterone estimation

The progesterone estimation from blood plasma was done using progesterone RIA Kits. Analytical sensitivity: 0.03 ng/ml (0.10 nmol/L). Specificity: the antibody used in the immunoassay is highly specific for progesterone.

Ultrasonographic studies

Portable Ultrasound Diagnostic Imaging System, CHISON Model: ECO 1 VET equipped with a 5.0 MHz linear-array transducer was used for trans-rectal scanning (Figure 1) of ovaries, follicular study (Figure 2), luteal study and early pregnancy diagnosis (Figure 3). Ovarian maps were drawn to record size and position of the follicles and corpus luteum as per [22] Savio *et al*. (1988) and pregnancy diagnosis on day 45 post AI with inbuilt calipers.

Statistical analysis

All data were expressed as mean ± standard error of mean of six animals. The data were analyzed statistically using Analysis of Variance (ANOVA) [23].

Results and Discussion

Follicular development

The effect of hCG administration on day 6 on follicular development at day 0, 6 and 14 are given in table 1. The follicle diameter (mm) in control and treatment group on day 0, 6 and 14 were 12.27 ± 1.30, 12.65 ± 1.00, 10.70 ± 0.49 and 12.12 ± 0.61, 12.30 ± 1.09, 10.59 ± 0.43, respectively. There was no significant difference in follicular diameter (mm) between and within the control and treatment group on various days of observation i.e. day 0, day 6 and day 14 of the estrous cycle. The diameter (mm) of follicle present on day 0, 14 in both the groups i.e. control group and treatment group was ≥ 10 mm. This finding was in agreement with finding of [24] Sianangama and Rajamahendran (1996) who reported that the preovulatory follicle (day 0) or dominant follicle (day 7 and day 14) are of greater than 11 mm, and findings of [25] Ginther *et al.* (2003) who defined dominant follicle that has reached diameter > 10 mm. The larger diameter in both control and treatment group on day 6 might be due to presence of a dominant follicle of first follicular wave.

Corpus luteum development

The effect of hCG administration on day 6 on corpus luteum development on day 14 is given in table 2. The corpus luteum diameter (mm) in control and treatment group on day 14 was 17.19 ± 0.70 and 20.53 ± 2.34, respectively and the corpus luteum area (mm^2) in control and treatment group on day 14 was 317.02 ± 8.27 and 335.82 ± 2.91, respectively. There was no significant difference in corpus diameter (mm) and area (mm^2) between the control and treatment group. This finding of increase in corpus luteum diameter and area in treatment group might be due to luteotropic effect of hCG. This luteotropic effect of hCG on corpus luteum is also reported by various researchers [17, 18, 26-30].

Progesterone concentration

The progesterone concentration (ng/ml) in control and treatment group on day 0, 6, 14 were 0.13 ± 0.06, 0.73 ± 0.34, 5.11 ± 0.58 and 0.21 ± 0.16, 0.67 ± 0.09, 17.82 ± 5.6, respectively (Table 1). It was observed that there was no significant difference in the progesterone concentration (ng/ml) on day 0 and 6 between control and treatment group, whereas, the progesterone concentration was significantly higher (P<0.05) on day 14 of treated animal in comparison to control group. It was also observed that within control and treatment group the progesterone concentration (ng/ml) at day 14 was significantly high (P<0.05) in comparison with the levels at day 0 and day 6 within the same group. It was revealed that progesterone concentration (ng/ml) on day 0 and 6 in both the groups were below 1 ng/ml (Table 1). Whereas, on day 14 the concentrations increased more than 1 ng/ml in both groups. This finding was in agreement with [31] Hansel *et al.* (1973) who reported that corpus luteum takes approximately 4-5 days from ovulation to gain progesterone producing capacity in excess of 1 ng/ml. It was observed that within control and treatment group the progesterone concentration (ng/ml) at day 14 was significantly high (P<0.05) in comparison with the levels at day 0 and 6 within the same group. This finding was in agreement with [32] Patel *et al.* (2006) who reported that the level of plasma progesterone steadily increase from day 0 of estrous cycle and

attains peak on day 14. It was also observed that progesterone concentration was significantly higher ($P<0.05$) on day 14 in treatment group in comparison to control group this finding of increase in progesterone concentration in treatment group is in agreement with [33] Donaldson *et al.*, 1964; [34] Hansel and Seifart, 1967; [35] Moody and Hansel, 1971 and [36] Machado *et al.*, 2008, who reported that hCG administration during luteal phase of cattle increased the size and weight of already existing corpus luteum, as well as serum progesterone concentration.

Accessory corpus luteum (ACL):

Total number of ACL produced in control and treatment group were 0 and 1, respectively (Table 3). The formation and retention of ACL as a result of hCG inj. on day 6 in treatment group was 16.66 per cent. The diameter (mm) and area (mm^2) of ACL on day 14 was 16.16 and 263.20, respectively. The size of ACL both diameter and area were lower when compared with the size of primary CL on day 14 of the estrous cycle. It was observed that no ACL is formed in control group, while 1 ACL (16.66%) was produced in treatment group. The induction of ACL was less in our study in comparison to previous findings of [26] Helmer and Britt (1986) and [37] Price and Webb (1989) who reported that formation of ACL was found to be greater when hCG was given during early luteal stage (day 4 to 7) than during follicular (day 0 to 3) or mid luteal (day 8 – 12) stage of estrous cycle. Our findings were less than reported by others which might be due to change in hormonal profile of our repeat breeder animals in comparison to normal cattle. It was observed that average diameter and area of ACL was less than the primary CL in same group on the same day of estrous cycle. This finding was in agreement with the findings of [24] Sianangama and Rajamahendran, 1996 and [38] Stevenson *et al.*, 2008, who reported that induced accessory corpus luteum are smaller in size compared with primary corpus luteum.

Pregnancy rate

As a result of hCG inj. on day 6 the pregnancy rate was 16.66 percent in the treatment group (Table 3) in comparison to 0 in the control group. The higher 1st service conception rate (16.66%) might also be due to ACL production.

Fig 1: Trans-rectal ultrasonography of bovine reproductive tract in progress

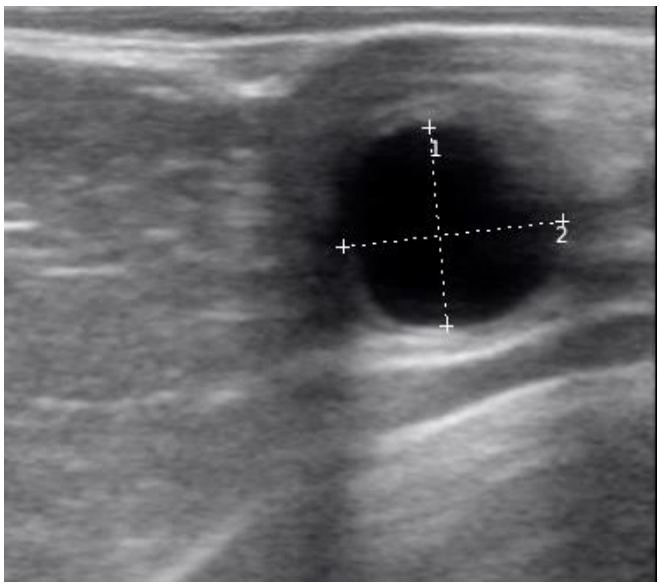

Fig 2: Ultrasonographic image of ovarian follicle

Fig 3: Ultrasonographic image of pregnant uterus

Table 1: Diameter (mm) of the largest follicle and plasma progesterone concentration (ng/ml) of control group versus treatment group (inj. hCG day 6) on day 0, 6 and 14 of the estrous cycle.

Days of estrous cycle	Diameter of the largest follicle (mm)		Progesterone concentration (ng/ml)	
	Control group	Treatment group	Control group	Treatment group
0	12.27 ± 1.30^{Aa}	12.12 ± 0.61^{Aa}	0.13 ± 0.06^{Aa}	0.21 ± 0.16^{Aa}
6	12.65 ± 1.00^{Aa}	12.30 ± 1.09^{Aa}	0.73 ± 0.34^{Aa}	0.67 ± 0.09^{Aa}
14	10.70 ± 0.49^{Aa}	10.59 ± 0.43^{Aa}	5.11 ± 0.58^{Ba}	17.82 ± 5.6^{Bb}

- Values are given as Mean ± S.E.M. of 6 animals.
- Values with different superscript (smaller alphabets) within row differ significantly ($P<0.05$)
- Values with different superscript (capital alphabets) within column differ significantly ($P<0.05$)

Table 2: Diameter (mm) and area (mm^2) of the corpus luteum of control group versus treatment group (inj. hCG day 6) on day 14 of the estrous cycle.

Days of estrous cycle	Diameter of the corpus luteum (mm)		Area (mm^2) of the corpus luteum	
	Control group	Treatment group	Control group	Treatment group
14	17.19 ± 0.70^{a}	20.53 ± 2.34^{a}	317.02 ± 8.27^{a}	335.82 ± 2.91^{a}

- Values are given as Mean ± S.E.M. of 6 animals.
- Values with different superscript (smaller alphabets) within row differ significantly ($P<0.05$)

Table 3: Induction of accessory corpus luteum (ACL) and pregnancy rate in control group versus treatment group (inj. hCG day 6) on day 14 of the estrous cycle.

Groups	Total no. of Accessory Corpus Luteum	Diameter (mm) of ACL	Area (mm^2) of ACL	Pregnancy	Pregnancy rate
Control	0	-	-	-	-
Treatment	1	16.16 (n=1)	263.20 (n=1)	1	16.66

- Values of ACL are given as Mean ± S.E.M. of number of ACL (n)

Conclusion

It was concluded that there was no significant effect of hCG administration on diameter of pre-ovulatory or dominant follicle, diameter and area of corpus luteum. Though, the diameter and area of corpus luteum increased post hCG administration. The accessory corpus luteum could be produced by hCG administration on day 6 of the estrous cycle, which is lesser in size. After hCG administration there was significant rise in progesterone concentration, may be due to accessory corpus luteum formation or stimulatory effect on existing corpus luteum. Further, more studies with larger populations and complete endocrine profile of the animals should be done in future studies so that we can firmly recommend our findings.

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References

1. Purohit GN. Recent developments in the diagnosis and therapy of repeat breeding cows and buffaloes. CAB Reviews: Perspectives in Agriculture Veterinary Science Nutrition and Natural Resource. 2008; 3(62):1-34.
2. Reddy KRC, Reddy KCS, Reddy VCS. Efficacy of progesterone treatment in repeat breeding Ongole cattle. Indian Veterinary Medical Journal. 2001; 25(2):193.
3. Sharma A, Singh M, Vasishta NK. Studies on the effect of gonadotrophins releasing hormone administration on conception rate following artificial insemination in cattle maintained under sub-temperate climate. The Indian Journal of Animal Reproduction. 2006; 27(1):66-68.
4. Shukla MK, Wani NA. Treatment of repeat breeding condition using oxytetracycline and gentamicin therapy in cattle. Animal Reproduction Science. 2002; 26:269-270.
5. Stevenson JS, Call EP, Scoby RK, Pathak AP. Double insemination and gonadotropin releasing hormone treatment of repeat breeding dairy cattle. Journal of Dairy Science. 1990; 73:1766-1772.
6. Akhtar MS, Lodhi LA, Farooq AA, Ayaz MM, Murtaza S, Arshad M *et al*. Effect of different doses of hCG at AI on pregnancy rates of repeat breeder Nili-Ravi buffalo. Buffalo Bulletin. 2013; 32(2):362-365.
7. Shelton K, Gayeric De Abreu MF, Hunter MG, Parkinson TJ, Lamming GE. Luteal inadequacy during the early luteal phase of sub fertile cows. Journal of Reproduction and Fertility. 1990; 90:1-10.
8. Bage R, Gustafsson H, Larsson B, Forsberg M, Rodriguez-Mortinez H. Repeat breeding in dairy heifers: Follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. Theriogenology. 2002; 57:2257-2269.
9. Mann GE, Lamming GE. The influence of progesterone during early pregnancy in cattle. Reproduction in Domestic Animals. 1999; 34:269-274.
10. Barnes FL. The effects of the early uterine environment on the subsequent development of embryo and fetus. Theriogenology. 2000; 53:649-658.
11. Mann GE. Corpus luteum function and early embryonic death in the bovine. In: XXII World Buiatrics Congress. 2002; 18(23):300-306.
12. Spencer TE, Bazer FW. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology. 1996; 137:1144-1147.
13. Helmer SD, Hansen PJ, Thatcher WW, Johnson JW, Bazer FW. Intrauterine infusion of highly enriched bovine trophoblast protein 1 complex exerts an antiluteolytic effect to extend corpus luteum lifespan in cyclic cattles. Journal of Reproduction and Fertility. 1989; 87:89-101.
14. Alnimer MA, Shamoun AI. Treatment with hCG 4 or 6 days after TAI to improve pregnancy outcomes in repeat-breeding dairy cows. Animal Reproduction Science. 2015; 157:63-70.
15. Shams-Esfanabadi N, Shirazi A. Effects of supplementation of repeat breeder dairy cows with CIDR from 5-9 postinsemination on pregnancy rate. Pakistan Journal of Biological Science. 2006; 9:2173-2176.

16. Thatcher WW, Guzeloglu A, Meikle A, Kamimura S, Bilby T, Kowalski AA. Regulation of embryo survival in cattle. Reproduction. 2003; 61:253-266.
17. Stevenson JS, Tenhouse DE, Portaluppi MA, Eborn DR, Kacuba S, De Jarnette JM. Interventions after artificial insemination: conception rates, pregnancy survival, and ovarian responses to gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone. Journal of Dairy Science. 2007; 90:331-340.
18. Schmitt EJP, Barros CM, Fields PA, Fields MJ, Diaz T, Kluge JM *et al*. A cellular and endocrine characterization of the original and induced corpus luteum after administration of a gonadotropin-releasing hormone agonist or human chorionic gonadotropin on day 5 of the estrous cycle. Journal of Animal Science. 1996; 74:1915–1929.
19. Singh H, Agarwal S, Singh P, Kumar S, Verma PK, Sharma U. Ultrasonography studies on effects of hCG administration at day 5 of the estrous cycle on pregnancy rate of repeat breeder cross-bred cow. Journal of Entomology and Zoology Studies. 2020; 8(4):864-868.
20. Edmonson AJ, Lean IJ, Weaver LD, Farver T, Webster G. A body scoring chart for Holstein cows. Journal of Dairy Science. 1989; 72(1):68-78.
21. Popov YN. Diagnosis of occult endometritis in cows using white side test on cervical mucus. Veterinarya Moscow. 1969; 4:85-87.
22. Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth dominant follicles during the oestrus cycle of heifers. Journal of Reproduction and Fertility. 1988; 83:663-671.
23. Snedecor GW, Cochran WG. Statistical Methods. 8th edition, Iowa State University Press, USA, 1989.
24. Sianangama PC, Rajamahendran R. Characteristics of corpus luteum formed from the first wave dominant follicle following hCG in cattle. Theriogenology. 1996; 45:977-990.
25. Ginther OJ, Beg MA, Donadeu FX, Bergfelt DR. Mechanism of follicle deviation in monovular farm species. A review. Animal Reproduction Science. 2003; 78:239-257.
26. Helmer SD, Britt JH. Fertility of dairy cattle treated with human chorionic gonadotrophin (hCG) to stimulate progesterone secretion. Theriogenology. 1986; 26:683-695.
27. Breuel KF, Spitzer JC, Henricks DM. Systemic progesterone concentration following human chorionic gonadotropin administration at various times during the estrous cycle in beef heifers. Journal of Animal Science.1989; 76:1564-1572.
28. Diaz T, Schmitt EJP, de la Sota RL, Thatcher MJ, Thatcher WW. Human chorionic gonadotropin-induced alterations in ovarian follicular dynamics during the estrous cycle of heifers. Journal of Animal Science. 1998; 76:1929-1936.
29. Santos JE, Thatcher WW, Pool L, Overton MW. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high-producing lactating Holstein dairy cows. Journal of Animal Science. 2001; 79:2881-2894.
30. De Rensis F, Valentini R, Gorrieri F, Bottareli E, Lopez-Gatius F. Inducing ovulation with hCG improves the fertility of dairy cows during the warm season. Theriogenology. 2008; 69:1077-1082.
31. Hansel W, Concannon PW, Lukaszewska SH. Corpora Lutea of the Large Domestic Animals. Biology of Reproduction. 1973; 8:222-232.
32. Patel PM, Dhami AJ, Hinsu TV, Ramani VP, Sarvaiya NP, Kavani FS. Comparative evaluation of blood biochemical and progesterone profile of fertile and infertile oestrous cycle in postpartum Holstein Friesian cows. Indian Journal of Animal Science. 2006; 76:191-195.
33. Donaldson LE, Hansel W, Vanvleck LD. Luteotropic properties luteinizing hormone and nature of oxytocin induced luteal inhibition in cattle. Journal of Dairy Science. 1964; 48:331-337.
34. Hansel W, Seifart KH. Maintenance of luteal function in the cow. Journal of Dairy Science. 1967; 50:1948-1958.
35. Moody EL, Hansel W. Effect of pretreating heifers with human chorionic gonadotropin and estradiol on subsequent in-vitro luteal tissue progesterone biosynthesis. Journal of Animal Science. 1971; 33:1032-1037.
36. Machado R, Bergamaschi MACM, Barbosa RT, de Oliveira CA, Binelli *M. ovarian* function in Nelore (Bos indicus) cows after post-ovulation hormonal treatment. Theriogenology. 2008; 69:798-804.
37. Price CA, Webb R. Ovarian response to hCG treatment during the oestrous cycle in heifers. Journal of Reproduction and Fertility. 1989; 86:303-308.
38. Stevenson JS, Tiffany SM, Inskeep EK. Maintenance of pregnancy in dairy cattle after treatment with human chorionic gonadotropin or gonadotropin-releasing hormone. Journal of Dairy Science. 2008; 91:3092-3101.