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Circulating Estradiol-17β levels during breeding and non-breeding season in ewes treated with GnRH-PG-GnRH plus Progesterone impregnated Vaginal Sponge

Hariom, Harpreet Singh, Ravi Dutt, Sonu and LC Ranga

Abstract
The study was attempted to evaluate the efficacy of the (Ovsynch) modified estrus synchronization protocols in combination with different durations of progesterone (P_4) therapy (using P_4 impregnated intravaginal sponges) during breeding (November-January; autumn season) and non-breeding (May-July; summer season) seasons in Nali×Rambuillet crossbred ewes. Three GnRH-$PGF_{2\alpha}$-GnRH protocols were employed with modifications in three groups (during one breeding and one non-breeding season) sequentially on days 0, 5, 7 in protocol-A; on days 0, 8, 9 in protocol-B and on days 0, 12, 13 in protocol-C combined with different durations of P_4 therapy (5, 8 and 12 days of sponge insertion in protocol-A, -B and -C, respectively). Each experimental group comprised of randomly selected 50 ewes. A total of 5 proven breeding rams in each group were used for mating with the ewes exhibiting estrus. Serum estradiol-17β concentration on the day of estrus during non-breeding season was significantly ($p<0.05$) higher in ewes treated with protocol-B compared to those with protocol-A. Similarly, during breeding season also, the estradiol concentration was significantly ($p<0.05$) higher on the day of estrus in ewes subjected to protocol-B compared to those with protocol-C.

Keywords: Estradiol-17β, Ewe, Season, AVIKESIL-S ® sponges, GnRH

Introduction
Sheep constitute a very important species of livestock in India, mainly due to their short generation intervals, higher prolificacy rates and the ease with which their products *viz*, meat, wool and milk can be marketed. The increasing global demand for livestock products is an opportunity for India to increase its exports. Meat exported from India is risk-free, lean, nutritious and competitive priced meat. India is the largest exporter of sheep meat in the world. Sheep play an important role in the Indian economy by providing employment to a large population of marginal and landless farmers. The production from native breeds is relatively low due to their poor reproductive efficiency. Reproduction is a pre-requisite for any animal production system. However, the reproductive efficiency of different breeds of sheep in India is relatively low [1]. To overcome the major problem of seasonality in sheep reproduction, different hormones and estrus synchronization protocols are used. Induction of cyclicity in out of breeding season and controlling the estrus cycle during the breeding season in an effective and economical way is a very challengingestradiol-17β treatment could increase fertility of anestrus ewes primed with used CIDRs and stimulated with the ram-effect. Sheep is a seasonally polyestrous animal and normal ovulatory cycles occurs in winter. There are typically 3 or 4 waves of follicle development during the inter-ovulatory interval. A wave-like pattern of antral follicle development in sheep is manifested as a synchronized growth of 1-4 small (i.e., 2–3 mm) follicles to ostensibly ovulatory sizes, in both ovaries, following a rise in mean serum concentrations of FSH [4]. Observed that on the day before estrusestradiol-17β concentration in blood plasma ranges between 10 to 20 pg/ml during spring and autumn season respectively, because one or more than one follicles grow rapidly. Estradiol in the presence of progesterone may be more effective in suppressing gonadotropic secretion, and therefore terminating follicle growth in anestrous ewes, similar to cattle [5].

Materials and Methods
This study was conducted at Central Sheep Breeding Farm, Hisar, (Haryana) India. The institute is located at latitude 29^0 N and longitude 75^0 E with average elevation of 215 m from the sea level.

The institute is located at the place where mainly continental climatic conditions are present with a significant annual variation in the temperature during summers and winters. Crossbred (Nali×Rambuillet) ewes were identified using flock records and monitored for general health (animals were observed closely for change in behaviour or feeding pattern to detect any disease/disorder) and body condition score (BCS) before inclusion in the experiment. Finally, a total of the 300 Nali×Rambuillet crossbred ewes (150 during breeding and 150 during non-breeding season) aged between 3-5 years, weighing 34-45 kg were selected on the basis of their previous breeding history with absence of any reproductive illness. All the ewes had previously lambed and weaned their last lambs. The ewes had been maintained isolated from any ram at least two months prior to the experiment. Ultrasonography of all the selected ewes was performed before enrolling into the experiment to confirm their non-pregnant status. Twenty healthy crossbred (Nali×Rambuillet) breeding rams aged 3-4 years, weighing 50-60 kg were also selected on the basis of their previous breeding performance with absence of any reproductive illness for each season. The study was conducted in two phases viz. the non-breeding (May to July months; summer season) and natural breeding season (November to January months; autumn season).

Experimental design

The study was in two phases i.e. during one breeding season and one non-breeding season. The experimental ewes were enrolled into 3 groups during breeding season (3 treatment groups) and similarly 3 groups during non-breeding season (3 treatment). Each treatment as well as control group comprised of randomly (parity-wise) selected 50 ewes. The Groups-I, -II and III. Groups-Ia, -IIa, and IIIa were used in breeding and non-breeding season, respectively. Three protocols (each protocol in 1 group during breeding as well as in 1 group during non-breeding seasons) were applied in the treatment groups of breeding: Groups I, II and III, non-breeding: Groups-Ia, IIa and IIIa seasons, as detailed below: Protocol-A: (Group-I during breeding season and Group-Ia during non-breeding season) Protocol-B: (Group-II during breeding season and Group-IIa during non-breeding season) Protocol-C: (Group-III during breeding season and Group-IIIa during non-breeding season) the respective season(n=50 in each group).

Treatment Groups

Protocol-A (for Groups-I, Ia): All ewes were administered with GnRH analogue (Buserelin acetate, 4µg, IM. Receptal®Vet MSD animal health, India) on day 0, PGF2α (cloprostenol, 125µg, IM Estrumate™ MSD animal health India) on day 5, and second dose of GnRH analogue (Buserelin acetate, 4µg, IM) on day 7, plus progesterone supplementation with intravaginal AVIKESIL-S ® sponges (each containing 350 mg progesterone) from day 0 to day 5 (i.e for 5 days).

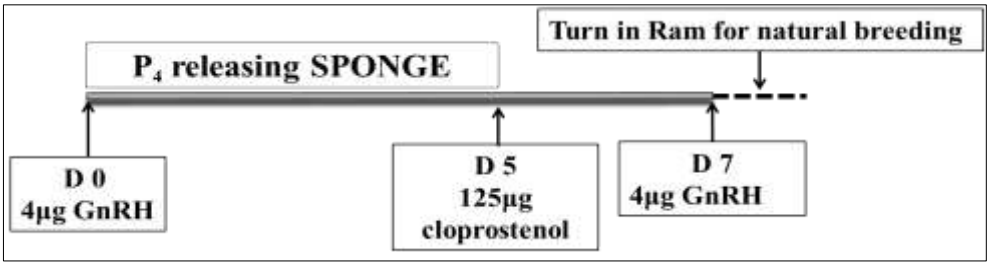


Fig 1: Schematic diagram representing experimental design in Protocol-A

Protocol-B (for Groups-II, IIa): Ewes were administered with GnRH analogue (Buserelin acetate, 4µg, IM) on day 0, a dose of PGF2α (cloprostenol, 125µg, i.m.) on day 8 and second dose of GnRH analogue (Buserelin acetate, 4µg, IM) on day 9 plus progesterone supplementation with intravaginal AVIKESIL-S® sponge (each containing 350 mg progesterone) from day 0 to day 8 (i.e for 8 days).

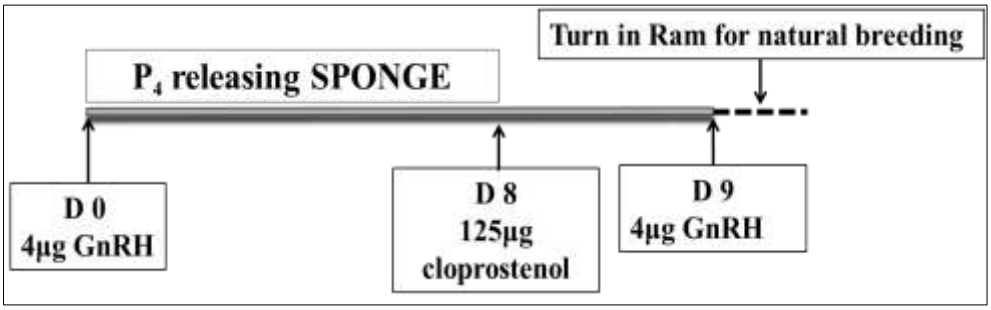


Fig 2: Schematic diagram representing experimental design in Protocol-B

Protocol-C (for Groups-III, IIIa): Ewes were administered with GnRH analogue (Buserelin acetate, 4µg, IM) on day 0 and a dose of PGF2α (cloprostenol, 125µg, i.m.) on day 12 and second dose of GnRH analogue (Buserelin acetate, 4µg, IM) on day 13 plus progesterone supplementation with intravaginal AVIKESIL-S sponge (each containing 350 mg progesterone) from day 0 to day 12 (i.e. for 12 days).

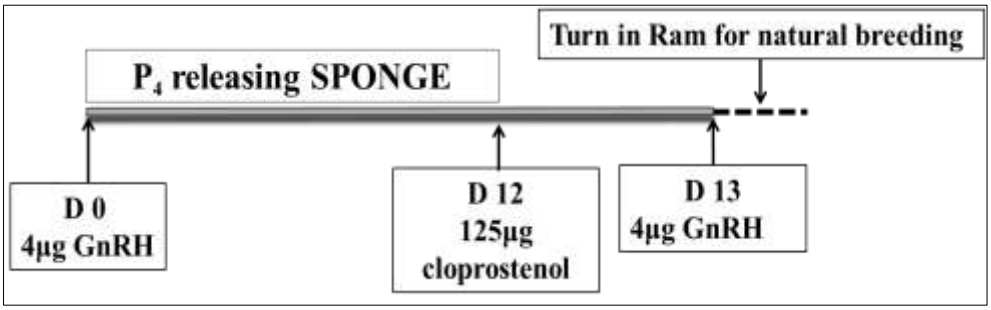
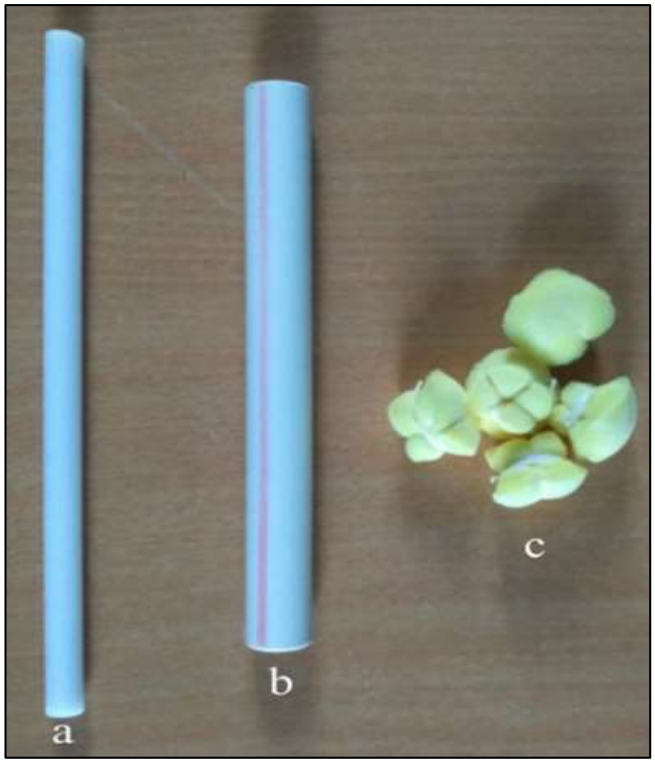


Fig. 3: Schematic diagram representing experimental design in Protocol-C

Application of intra-vaginal progesterone sponge



a) Plunger, b) Speculum, c) AVIKESIL-S® (Intravaginal P_4 sponge)

Fig 4: Sponge applicator device with sponge

Blood sampling

Fig 5: Blood collection

Fig 6: Serum harvesting

Blood samples were collected by jugular veinupuncture from randomly selected 15 ewes from each treatment group on different days i.e. day of sponge insertion, day of sponge withdrawal and on the day of the estrus during breeding as well as non-breeding seasons. Immediately after blood sample collection, the samples were centrifuged at 3000 rpm for 20 minutes and serum harvested was stored in aliquots at -20°C until hormone estimations.

Estimation of serum estradiol-17β concentrations

Serum estradiol-17β concentrations were estimated by using a commercially available enzyme immunoassay Kit (DetectX®) by ARBOR ASSAYS

Principle of assay

The DetectX® Serum Estradiol Immunoassay kit uses a specifically generated antibody to measure estradiol in serum and plasma samples. The assay procedure was as below:

1. 100 µL of each sample or standard was pipetted into wells in the plate.
2. 125 µL of Assay Buffer added into the non-specific binding (NSB) wells.
3. 100 µL of Assay Buffer was added into the maximum binding (B0 or Zero standard) wells.
4. 4. 25 µL of the DetectX® serum estradiol conjugate was added to each well using a repeater pipette.
5. 25 µL of the DetectX® serum Estradiol Antibody was added to each well, except the NSB wells, using a repeater pipette.
6. The sides of the plate were gently tapped to ensure adequate mixing of the reagents. Then the plate was

covered with the plate sealer and shaken at room temperature for 2 hours.
7. Each well of plate was aspirated and then washed 4 times with 300 µL wash buffer. Then the plate was tapped on clean absorbent towels to dry.
8. 100 µL of the TMB Substrate was added to each well, using a repeater pipet.
9. Then plate was incubated at room temperature for 30 minutes without shaking.
10. 50 µL of stop solution was added to each well, using a repeater pipette.
11. Then the optical density was generated from each well in a plate reader at 450 nm.
12. Standard curve for estradiol was developed using manufacturer instructions and then OD for each sample was used to measure the concentration of estradiol in each sample.

Statistical analysis

Statistical software IBM SPSS Statistics 24.0 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism (Version 8, San Diego, CA) were used for the making the statistical comparisons of the data. The data obtained from the all groups (both in breeding and non-breeding season) were analysed using one-way ANOVA and Chi-square tests. The values were expressed as mean ± standard error of mean (SEM) or percentage.

Result and Discussion

Estrus synchronization in sheep is achieved by control of the luteal phase of the estrous cycle, either by providing exogenous progesterone or by inducing premature luteolysis. The latter approach is not applicable during seasonal anestrous, whereas exogenous progesterone in combination with gonadotropins can be used to induce and synchronize estrus in acyclic ewes. Progesterone has a "priming" effect on the central nervous system, which enhance the response to gonadotropins administered after the end of progesterone treatment (12). The withdrawal of progesterone therapy leads to decrease in progesterone level and restores the LH pulse frequency and amplitude. This allows the subsequent development of dominant follicle and leads to estrus and ovulation (13). Hence, supplementation of progesterone results in an induced final LH surge and a highly synchronous time to estrus, which allows for the use of timed AI without estrus detection (14). Short periods of progesterone treatment with sponges for 5 to 7 days have been reported to be successful in inducing and synchronizing estrus in ewes during breeding as well as non-breeding seasons (16, 2). Reducing the period of sponge insertion may maintain higher circulatory P_4 levels upon removal of sponges and may reduce the chance of vaginal contamination.

Serum estradiol-17β concentrations

The serum estradiol-17βconcentrations (pg/mL) were estimated on different days i.e. day of sponge insertion, day of sponge withdrawal and on the day of the estrus during breeding as well as non-breeding seasons.

Estradiol concentrations during breeding season

The serum estradiol (E_2) concentrations in group-I, -II and -III were 6.05±0.89, 4.36±0.57 and 3.71±0.41 pg/mL; 10.12±1.03, 8.38±1.23 and 6.15±0.72 pg/mL and 15.50±1.57, 18.48±1.30 and 12.96±1.41 pg/mL on day 0, day of sponge removal and day of estrus, respectively. The higher values of estradiol on day 0 in treatment groups during breeding compared to non-breeding season indicate evidence of follicular activity. The concentration observed in group-III is in accordance with (11) as they recorded estradiol levels of 6.62±3.58pg/ml at the day of CIDR implant removal (on 12th day) during breeding season (Fig.7) (Table 1).

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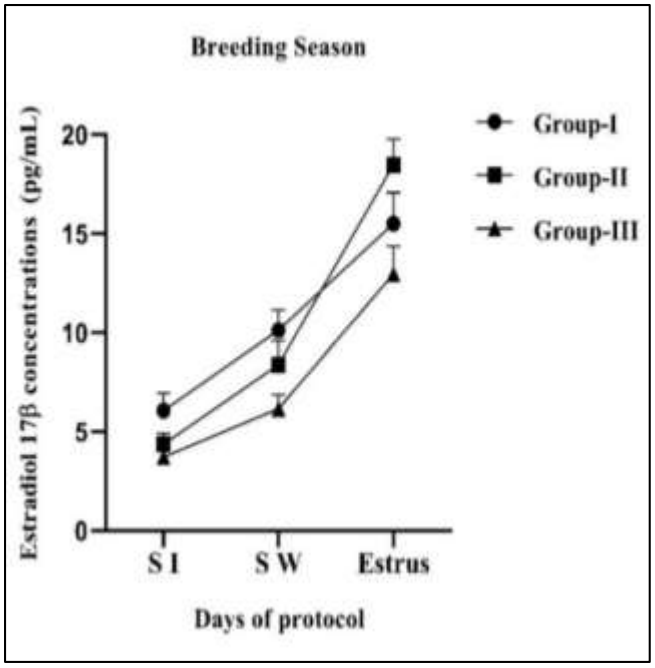


SI- Day of sponge insertion **SW**- Day of sponge withdrawal

Fig 7: Estradiol (pg/mL) profiles in ewes of different groups during the breeding season

Table 1: Estradiol-17β concentrations (pg/mL) on different days of protocols during breeding season

Groups	Estradiol-17β (pg/mL)		
	Day of sponge insertion	Day of sponge withdrawal	Day of estrus
Group-I	$6.05\pm0.89^{a, A}$	$10.12\pm1.03^{b, B}$	$15.50\pm1.57^{b, B}$
Group-II	$4.36\pm0.57^{b, A}$	$8.38\pm1.23^{a, b, B}$	$18.48\pm1.30^{b, C}$
Group-III	$3.71\pm0.41^{b, A}$	$6.15\pm0.72^{a, B}$	$12.96\pm1.41^{a, C}$

Values with different uppercase alphabet superscripts (A, B, C) within a row differ significantly ($p<0.01$)
Values with different lowercase alphabet superscripts (a, b, c) within a column differ significantly ($p<0.05$)

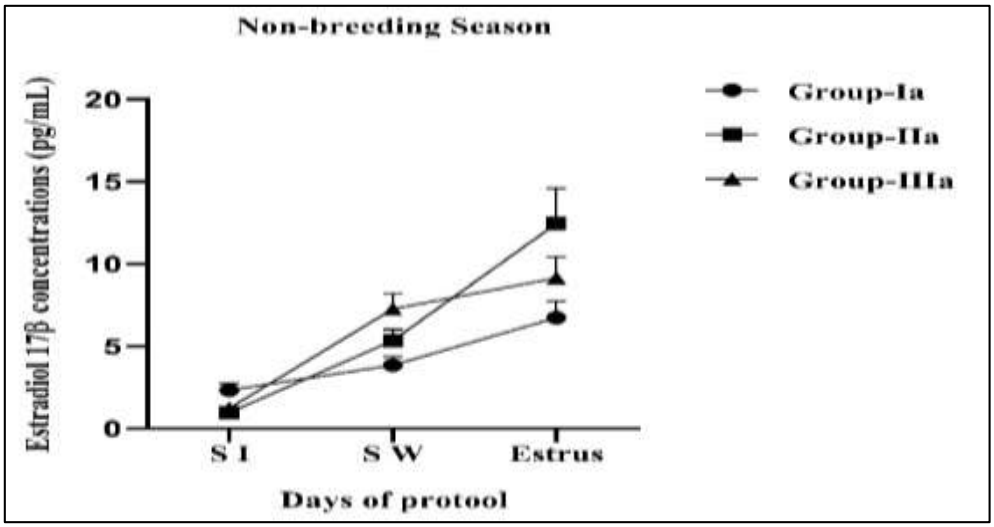
The mean concentration of E_2 at estrus was higher than the values on day 0 and day of sponge removal in treatment groups. This could be as a result of the development of ovulatory follicles and E_2 secretion by them (Carson *et al.*, 1981). The E_2 profiles on day of estrus were greater in group-II (Ovsynch, 0, 8, 9 plus AVIKESIL-S®for 8 days) compared with the other treatments was due to more number of ewes induced to estrus in this group. Similar estradiol-17β levels of 10.9±1.34 and 17.6±2.47 pg/ml at estrus and 1.2±0.42 and 2.1±0.58 pg/ml on day 0 in the control and treatment groups (8) have been observed during the breeding season in ewes. The values are lower than in the current study. This could be attributed to many factors including like breed variation, differences in climate, age, nutritional and hormonal status.
Short term treatments results in a series of benefits *viz* a better control of follicular response and ovulation, acceptable fertility rates (not lower than conventional progesterone treatments), shorter period for large scale implementation of

FTAI programs, and in some cases also allowing the re-utilization of intravaginal devices (10). But in the current study, Ovsynch (0,8,9) plus progesterone supplementation for 8 days exhibited significantly (p<0.05) longer duration of estrus compared to untreated control and non-statistically different (p>0.05) from short and long duration Ovsynch plus progesterone treatment regimens during the non-breeding season. Interestingly, the duration of estrus was significantly (p<0.05) lower duration of estrus compared to long duration Ovsynch plus progesterone during the breeding season. Incorporating a single intravaginal insert to the timed AI program increases progesterone concentrations, but did not benefit fertility in cows that have CL at the initiation of the Ovsynch (0-7-9) protocol (3). In the present study it seems that if ewes with luteal structures at the initiation of the Ovsynch protocol during breeding season are subjected to progesterone supplementation (of long duration), the large/dominant follicles might get suppressed leading to lesser estrus induction and pregnancy rates that could be significantly lower than of spontaneous estrus in breeding season. This variation from cattle can be due to species difference. Hence, the medium duration of protocol yielded better fertility response in terms of estrus synchronization rate, pregnancy rate, lambing rate, prolificacy and fecundity than the short duration protocol during breeding as well as non-breeding season. Further studies with follicular dynamics along with endocrinological interrelationships during such synchronization protocols are warranted to establish the effects.

Estradiol concentrations during non-breeding season

Estradiol concentrations on day of insertion of sponge were similar (p>0.05) in all groups during non-breeding season. However, estradiol concentrations on the day of sponge withdrawal were significantly (p<0.05) higher in Group-IIIa as compared to Group-Ia and Group-IIa. Further, estradiol concentrations on day of estrus were significantly (p<0.05) higher in Group-IIa as compared to Group-Ia.

The serum estradiol concentrations in group-Ia, -IIa and -IIIa were 1.78±0.24, 0.96±0.21 and 1.23±0.30 pg/mL; 3.84±0.52, 5.34±0.68 and 7.25±0.93 pg/mL and 6.72±1.01, 12.44±2.14 and 9.14±1.29 pg/mL on day 0, day of sponge removal and day of estrus, respectively. The estradiol concentrations observed after progesterone device removal in the present study were expected to reflect estradiol secretion by the ovarian follicles. The significantly higher serum estradiol concentration in the group-IIa (0,8,9, Ovsynch plus AVIKESIL-S®for 8 days) compared with the remaining treatment groups on the day of estrus was due to having more number of ewes with induced estrus in this group (Fig. 8) (Table 2).



SI- Day of sponge insertion, **SW**- Day of sponge withdrawal

Fig 8: Serum estradiol-17β profiles in ewes during non-breeding season

Table 2: Estradiol concentrations (pg/mL) on different days of protocols during the non-breeding season

Groups	Estradiol-17β (pg/mL)		
	Day of sponge insertion	Day of sponge withdrawal	Day of estrus
Group-Ia	1.78±0.24 ^{A}	3.84±0.52^{a}	6.72±1.01^{a}
Group-IIa	0.96±0.21^{A}	5.34±0.68$^{a, b, B}$	12.44±2.14$^{b, C}$
Group-IIIa	1.23±0.30^{A}	7.25±0.93$^{b, B}$	9.14±1.29$^{a, b, B}$

Values with different uppercase alphabet superscripts (A, B, C) within a row differ significantly (p<0.01)

Values with different lowercase alphabet superscripts (a, b, c) within a column differ significantly (p<0.05)

Conclusion

The synchronization protocol with 8-day long progesterone therapy resulted in significantly higher serum estradiol concentrations at the time of sponge withdrawal as well as at the time of estrus during non-breeding and the breeding season. Serum estradiol-17β concentration (pg/mL) was significantly (p<0.05) highest in Group-IIa (12.44±2.14) as compared to Group-Ia (6.72±1.01) on the day of estrus during non-breeding season.Serum estradiol-17β concentration (pg/mL) was significantly (p<0.05) highest in Group-IIa (18.48±1.30) as compared to Group-IIIa (12.96±1.41) on the day of estrus during breeding season.The modified G-P-G synchronization protocol with 8 days of progesterone therapy significantly improved the serum estradiol-17β hormone profiles in ewes.

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