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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(11): 647-652

www.thepharmajournal.com
Received: 18-09-2021
Accepted: 22-10-2021

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Progesterone profile during breeding and non-breeding season in ewes treated with Ovsynch plus progesterone impregnated vaginal sponge

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Abstract
The study evaluated 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. Application of intravaginal P_4 sponges resulted in significant increase in serum P_4 concentration on the day of sponge withdrawal compared to the day of sponge insertion in each group. On 28th day post-mating, serum P_4 concentration was highest in ewes treated with protocol-B during non-breeding (5.03±0.89 ng/mL) as well as during breeding season (6.42±1.66 ng/mL). In conclusion, the modified estrus synchronization protocol with intermediate duration of P_4 therapy (8 days, protocol-B) yielded better results [compared to short (5 days, protocol-A) and long-term (12 days, protocol-C) duration therapy]in the ewes in terms of outcome of improvement in serum progesterone profiles.

Keywords: Progesterone, Ewe, Season, AVIKESIL-S ® sponges, Duration

Introduction
Small ruminants play an important role in sustainable livelihood of arid, semiarid and mountain areas; especially of landless and small holder farmers and hence are important economic component of the livestock sector in India [1]. The reproductive seasonality is one of the constraints in achieving higher reproductive efficiency in ewes. Ovarian cyclical activity is a result of interaction and coordinated effect of multiple hormones in the biological system. The cyclical reproductive activities are affected by the hormones at various stages which enable the concept of using various exogenous hormones either alone or in combination for the estrus synchronization and improvement of reproductive efficiency in ewes [2].The success of an estrus synchronization protocol depends on establishing compact synchrony and achieving acceptable fertility rates [21], one of which is the poor reproductive condition of the ewe [12]. Therefore, the efficiency of most sheep production systems can be improved by nutritional andor hormonal treatments, resulting in higher estrus responses and subsequent conception rates. Progesterone can prevent ovulation during the period in which spontaneous luteolysis may occur in animals whose dominant follicles are not responsive to GnRH injection. Treatment with progestagen in conjunction with GnRH produced results comparable with those of eCG treatment [21].Improvement in pregnancy rates were reported in cattle when progesterone was applied during estrus synchronization with GnRH– PGF2α protocol (4, 8).The controlled progesterone releasing devices like sponges are very effective for estrus induction during the non-breeding season in small ruminants and their use along with eCG improved estrus response [16].

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). The animals had access to natural grazing area during day time and were kept indoor at night. Experimental animals were given the diet as per the nutritional recommendations of Central Sheep Breeding Farm, Hisar along with ad libitum water and mineral licks. All the ewes were dewormed for ecto- and endo-parasites before enrollment into the experiments. Animals were identified with ear tags and kept under iso-managerial conditions.

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).

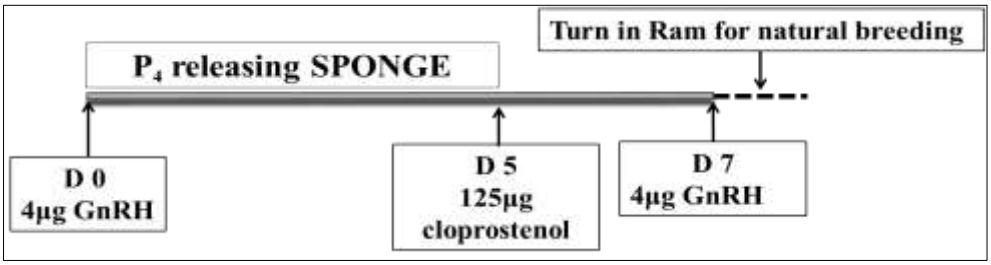


Fig 1: Schematic diagram representing experimental design in Protocol-A

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).

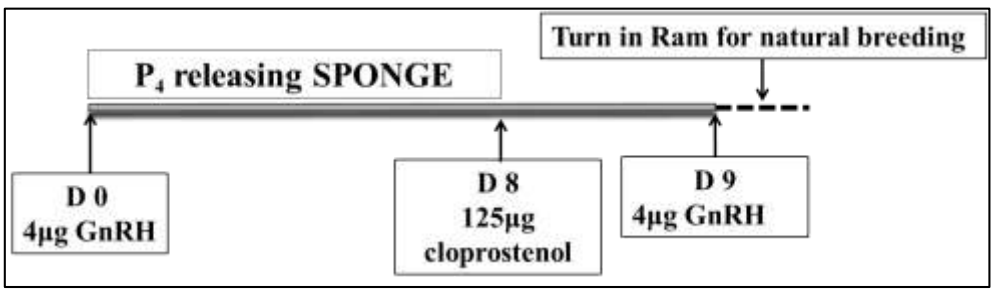


Fig 2: Schematic diagram representing experimental design in Protocol-B

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).

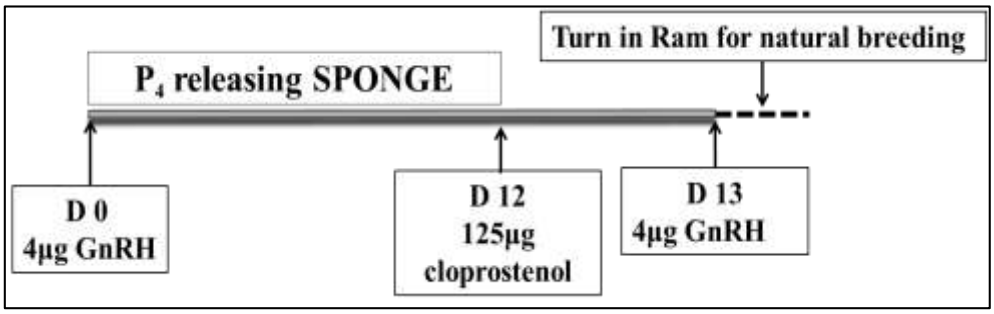
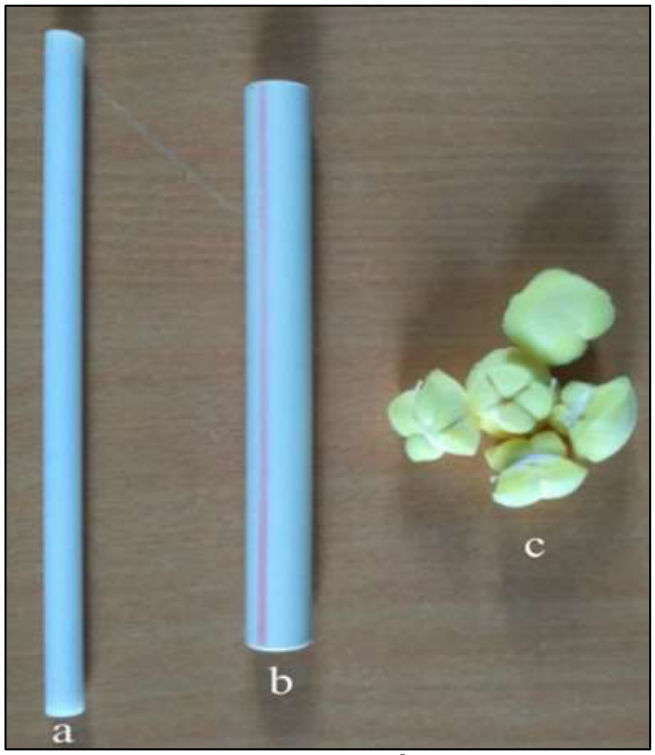


Fig 3: Schematic diagram representing experimental design in Protocol-C

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

Blood samples were collected by jugular veinupuncture from randomly selected 15 ewes from each treatment group on days of intervention as per protocol as well as post-mating on days 14 and 28 to study the luteal profiles and pregnancy diagnosis. 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 progesterone concentrations.

Serum progesterone concentrations were estimated by using a commercially available direct immune-enzymatic assay kit (Calbiotech, USA).

Principle of assay:

The anti-progesterone antibodies were coated on plate microwells. Progesterone in the sample competes with HRP-labelled progesterone for binding to the coated antibody. After incubation and washing, unbound conjugate was washed off and an enzyme substrate was added. Then, the enzyme HRP in the bound fraction reacts with the substrate and gives colour. The amount of progesterone in the sample is inversely proportional to the enzyme activity and the reaction is terminated by adding stopping solution. Absorbance was measured on a micro plate reader (Multiskan FC, Thermo Fisher Scientific). The colour intensity is inversely proportional to the progesterone concentration in the sample. The protocol used (as per the guideline of manufacturer) for the estimation of plasma concentrations of progesterone is as under:

1. All components of the kit and samples were brought to room temperature.
2. Microtitre plate wells for each calibrator, control serum and samples to be assayed were formatted.
3. 10µl of calibrator, control serum and samples were dispensed into appropriate wells.
4. 200µl of conjugate was added to each well.
5. Microtitre plate was gently swirled for 30 seconds.
6. The plate was covered with protective film and incubated for 60 minutes at 20-25ºC.
7. Afterwards, wells were washed five times with 300µl working washing solution per well.
8. The plate was tapped firmly against absorbent paper to ensure that plate was dry.
9. 100µl of TMB substrate was added to each well at timed intervals.
10. Plate was incubated in a dark place at room temperature for 15 minutes.
11. 50µl of stopping reagent was added to each well.
12. Microtitre plate was gently swirled for 5-10 seconds to mix content.
13. Absorbance was recorded on microplate reader at 450 nm within 10 minutes.
14. Following the reading of optical density, four parameters logistic curve was prepared and concentration (ng/ml) of each sample was calculated.

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 [15]. 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 [17]. 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 [18]. 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 [19, 6]. Reducing the period of sponge insertion may maintain higher circulatory P4 levels upon removal of sponges and may reduce the chance of vaginal contamination.

Progesterone concentrations

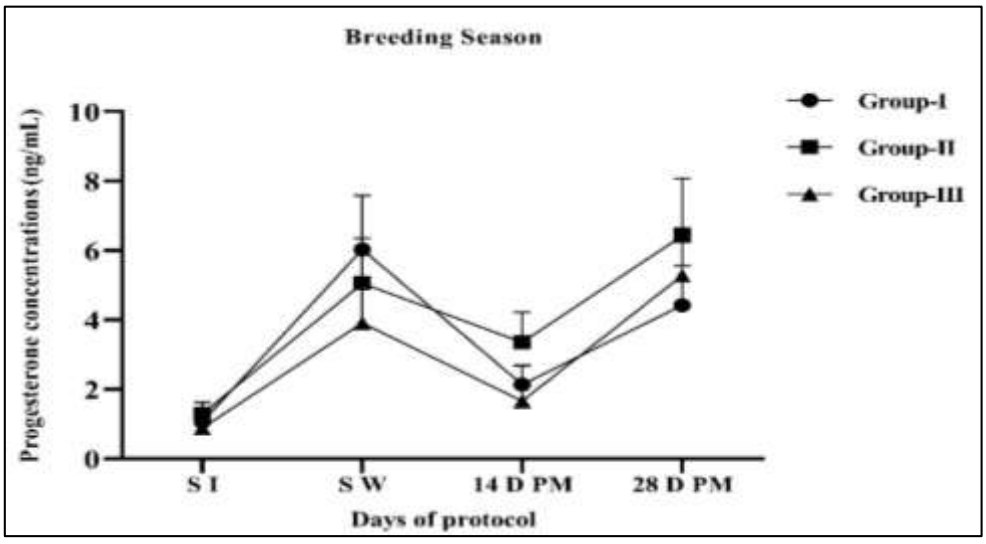
Progesterone concentrations were estimated on different days i.e. day of sponge insertion (SI), day of sponge withdrawal (SW), day 14 post-mating (14 D PM) and day 28 post-mating (28 D PM) during breeding as well as non-breeding seasons and results are discussed below:

Serum progesterone concentrations during the breeding season

The blood progesterone concentrations in group-I (G0-P5-G7 plus AVIKESIL-S® for 5 days), groups-II (G0-P8-G9 plus AVIKESIL-S® for 8 days) and groups-III (G0-P12-G13 plus AVIKESIL-S® for 12 days) were 1.06±0.27, 1.29±0.33 and 0.89±0.23 ng/mL on day 0; 6.03±01.56, 5.05±1.30 and 3.91±1.01 ng/mL on day of sponge removal; 2.14±0.55, 3.35±0.86 and 1.66±0.43 ng/mL on day 14 post-mating and 4.42±1.14, 6.42±1.66 and 5.28±1.36 ng/mL on day 28 post-mating, respectively (Fig. 5, Table. 1). The higher values on day 0 in treatment groups compared to non-breeding season indicate follicular activity and presence of luteal structures. The findings of group-III in the current study are in agreement with [14] as they recorded blood progesterone levels as 3.82±0.60 ng/ml on 12th day (i.e. the day of CIDR implant removal during breeding season.

The elevated serum progesterone profile in the Ovsynch [0, 8, 9] plus AVIKESIL-S® for 8 days group compared with the remaining treatments was due to higher luteal activity on day 14 and pregnancy rates on day 28 subsequent to induced estrus and natural mating. The average plasma P4 concentrations (ng/mL) on day 0 varied non-significantly (p>0.05) among the groups. The luteal function of the ewes underwent treatment was maintained better in group-IIa as evidenced by serum P4 concentration on day 14 and 28 post-mating which was significantly (p<0.05) higher coinciding with better estrus response and pregnancy rate in group-IIa compared to group-Ia and -IIIa.

This decline in P4 concentrations during the period of insertion (in group-IIIa and group-III) may be due to drain out of progesterone from the sponges or the wash-out of the progesterone by vaginal fluid [20]. But the corresponding P4 values in non-breeding values were lower which may be due to presence of corpora lutea in the ewes during the breeding season. Therefore, the amount of P4 absorbed and circulatory P4 levels at the time of sponge removal in a 12-day treatment may not be sufficient to maintain normal patterns of follicular growth [10]. Consequently, persistence of low blood P4 concentrations have been associated with the formation of persistent follicles, prolonged luteal function and reduced fertility in sheep [13]. The longer period of progestogen treatment generally has been associated with low conception rates [19], attributed to impairment of sperm transport in the genital tract of progestogen treated ewes [11].



SI- Day of sponge insertion, **SW**- Day of sponge withdrawal, **14 D PM**- Day 14 post-mating**28 D PM**- Day 28 post-mating

Fig 5: Progesterone (ng/mL) profiles in different groups during breeding season

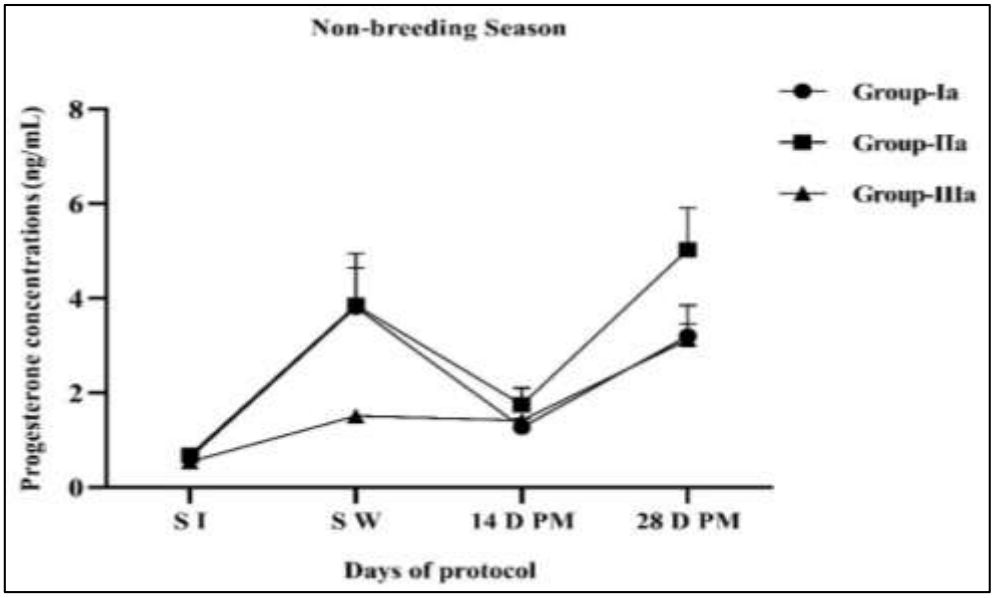
Table 1: Serum progesterone concentrations (ng/mL) on different days of protocol during breeding season

Progesterone concentrations (ng/mL)				
Group	**Day of sponge insertion**	**Day of sponge withdrawal**	**Day 14 post-mating**	**Day 28 post-mating**
Group-I	1.06 ± 0.27^{A}	6.03 ± 1.56^{D}	$2.14\pm0.55^{a, b, A, B}$	4.42 ± 1.14^{C}
Group-II	1.29 ± 0.33^{A}	$5.05\pm1.30^{C, D}$	$3.35\pm0.86^{b, B, C}$	6.42 ± 1.66^{D}
Group-III	0.89 ± 0.23^{A}	3.91 ± 1.01^{C}	$1.66\pm0.43^{a, A, B}$	5.28 ± 1.36^{D}

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

Serum progesterone concentrations during non-breeding season

The serum progesterone concentrations in group-Ia (G0-P5-G7 plus AVIKESIL-S® for 5 days), group-IIa (G0-P8-G9 plus AVIKESIL-S® for 8 days) and group-IIIa (G0-P12-G13 plus AVIKESIL-S® for 12 days) were 0.63±0.11, 0.68±0.11 and 0.54±0.09 ng/mL on day 0; 3.81±0.83, 3.85±1.10 and 1.51±0.10 ng/mL on day of sponge removal; 1.27±0.18, 1.75±0.35 and 1.42±0.10 ng/mL on day 14 post-mating and 3.20±0.65, 5.03±0.89 and 3.12±0.34 ng/mL on day 28 post-mating, respectively(Fig. 6, Table. 2). Initial serum P4 concentrations were basal and varied non-significantly ($p>0.05$) in all the groups and ranged between 0.54±0.09 ng/mL and 0.68±0.11 ng/mL indicating the absence of cyclicity and seasonal anestrous in the experimental ewes. This observation corroborates with that of [12]. Also, the values of serum P4 were comparable with [3] in non-breeding season. Similarly, the seasonal absence of estrual behavior has been previously reported under similar circumstances [5]. In non-breeding season ovulation do not occurs because the secretion of LH is low that there is absence of development of ovarian follicles and the corpora lutea and hence plasma progesterone concentrations remain very low [7]. The serum P4 values in the current experiment increased continuously and significantly ($p<0.01$) after sponge insertion to day 5, 8 in group-Ia and -IIa, respectively. In cases of long duration of sponge insertion for 12 days (group-IIIa), the values reduced significantly ($p<0.01$) on the day of sponge removal compared to the values on day 5 in group-Ia and day 8 in group-IIa. The elevated serum progesterone profiles on day 14 and 28 post-mating in the group-IIa (G0-P8-G9 plus AVIKESIL-S® for 8 days) were significantly ($p<0.05$) higher compared with the remaining treatments due to better luteal function and having more pregnant females from mating at the induced estrus. Notably, treatment regimens of a shorter interval resulted in better fertility responses in naturally mated ewes [19] and goats [9].



SI- Day of sponge insertion, SW- Day of sponge withdrawal, 14 D PM- Day 14 post-mating 28 D PM- Day 28 post-mating

Fig 6: Serum progesterone (ng/mL) profiles in ewes of different groups during non-breeding season

Table 2: Serum progesterone concentration in ewes on different days of protocol during non-breeding season

Progesterone concentrations (ng/mL)				
Groups	**Day of sponge insertion**	**Day of sponge withdrawal**	**Day 14 post-mating**	**Day 28 post-mating**
Group-Ia	0.63 ± 0.11^{A}	$3.81\pm0.83^{a, b, C}$	1.27 ± 0.18^{B}	$3.20\pm0.65^{B, C}$
Group-IIa	0.68 ± 0.11^{A}	$3.85\pm1.10^{b, B, C}$	1.75 ± 0.35^{A}	5.03 ± 0.89^{C}
Group-IIIa	0.54 ± 0.09^{A}	1.51 ± 0.10^{B}	1.42 ± 0.10^{B}	3.12 ± 0.34^{C}

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

Conclusion

Application of intravaginal progesterone sponges resulted in significant increase in serum progesterone concentration from the day of sponge insertion to the day of sponge withdrawal in each group. On 28th day post-mating, serum progesterone concentration (ng/mL) was highest in Group-IIa (5.03±0.89) and Group-II (6.42±1.66) during non-breeding and breeding season, respectively. The modified G-P-G synchronization protocol with 8 days of progesterone therapy significantly improved the serum progesterone hormone profiles in ewes.

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