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Research Article

COMPARATIVE STUDIES ON TUPPING, FIXED-TIME LAPAROSCOPIC AND CERVICAL INSEMINATION TECHNIQUES IN ESTRUS SYNCHRONIZED EWES DURING NON-BREEDING SEASON

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ABSTRACT

The present study was designed to compare the fertility in cross bred ewes following natural mating and different insemination methods during non-breeding season. Healthy cross bred ewes (n=24) were randomly allotted to three groups during non-breeding season with six, eight and ten animals in tupping (T), cervical (CAI) and laparoscopic artificial insemination (LAI) groups, respectively. Ewes in all the treatment groups were subjected to the same estrus induction protocol: insertion of intravaginal progesterone sponge for 10 days followed by eCG (500 IU, I.M.) at the time of sponge withdrawal. After removal of the sponges, animals of T group were kept with proven breeding rams upto 72 hours. In animals belonging to CAI and LAI groups, fixed time cervical and laparoscopic insemination was done at 48 hours after sponge removal. The cervical insemination was repeated 12 hour later in all ewes of CAI group. The pregnancy and lambing rates were non-significantly higher in animals subjected to natural tupping (50%, 50%) than those of cervical (25%, 25%) and laparoscopic insemination (10%, 10%) groups. The serum progesterone levels were significantly higher ($P<0.05$) in pregnant ewes on day 10, day 17 and day 35 than non-pregnant ewes. The intravaginal progesterone sponges for 10 days with eCG regimen successfully induced estrus in ewes during out-of-breeding season. The protocol improved reproductive performance of anestrus ewes.

Keywords: Artificial insemination, Estrous synchronization, Ewes, Fertility, Non-breeding season

Induction of estrus in ewes during non-breeding season to achieve lambing in fall has been attempted with limited success. Such programmes have been aimed at increasing the number of lambings per year (3 lambings/2 years), for getting high prices during specific seasons and for making the efficient use of labour and other resources (deNicolo, 2007). Several protocols have been used to induce and synchronize estrus and ovulation in small ruminants (Kusina *et al.*, 2000). Since efficacy of PGF2α is limited to the breeding season due to active corpus luteum (CL), different protocols using combination of progesterone and gonadotrophin Releasing Hormone (GnRH) or human Chorionic Gonadotropin (hCG) have been recommended for estrus synchronization outside the breeding season (Martinez *et al.*, 2015). A precise estrus synchronization protocol has been formulated using intravaginal sponges impregnated with progesterone for 10-16 days, followed by administration of equine Chorionic Gonadotropin (eCG) (Swelum *et al.*, 2015). The use of eCG improves estrus synchronization, follicular maturation, ovulation rate and fertility. 300 to 600 IU of eCG have been administered in different breeds of sheep of different body weight during various seasons of the year (Dogan and Nur, 2006).

Artificial insemination (AI) in sheep has been poorly implemented and is carried out mainly with chilled semen because of the low fertility results obtained when using frozen-thawed semen (Salamon and Maxwell, 2000). The AI with cooled or chilled semen is usually performed vaginally, by deposition at the external os of the cervical canal (Gibbons *et al.*, 2019). Frequent fertilization failure in artificially inseminated ewes have been attributed mainly to the faulty transport of spermatozoa through their tortuous cervix (Boland *et al.*, 1983). This problem can be overcome by direct transfer of semen in the lumen of uterus by laparoscopy (Cseh *et al.*, 2012). Consequently the laparoscopic intrauterine insemination using frozen-thawed semen is gaining popularity as an alternative method of AI (Anel *et al.*, 2005). But high initial equipment cost, need for trained labour and the relatively steep curve of surgical expertise have been major hindrances in its use under field conditions (Cseh *et al.*, 2012).

Fixed time artificial insemination (FTAI) is an important tool as estrus detection is not feasible in sheep (Olivera-Muzante *et al.*, 2013). The application of estrus synchronization together with FTAI is steadily increasing, however results of different insemination techniques have not been consistent during different seasons. To the authors knowledge, limited number of studies have been carried out in sheep during off-season to compare the fertility following various insemination techniques. Therefore, the current study was designed to compare the efficiency of fixed time cervical and laparoscopic artificial insemination with natural matings in cross bred ewes during non-breeding season.

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MATERIALS AND METHODS

Study Period and Area: The present study was conducted at Mountain Research Centre for Sheep and Goats (MRCSG), FVSc & AH, Shuhama, SKUAST - Kashmir located in Srinagar city, Kashmir province, India (34° 08′ N 74° 28′ E). The treatment was initiated during Spring (March-May) the off-season for ovine breeding in the temperate climatic conditions of Jammu and Kashmir. Twenty four multiparous healthy cross bred ewes (NARI-Swarna ram x non-descript ewes) weighing 36.41 ± 4.25 kg with body condition scores ranging from 2.5-3.5 were used. All the ewes had completed third or fourth parity. They were maintained under uniform management conditions.

Experimental design: Cross bred ewes (n=24) were randomly allotted to three groups: tupping (T), cervical artificial (CAI) and laparoscopic artificial insemination (LAI). The number of animals in these groups were 6, 8 and 10, respectively. Progesterone impregnated intravaginal sponges (AVIKESIL-S®, CSWRI, Avikanagar, India) with the help of speculum and introducer placed in the vagina of all the ewes included in the study on day 1.500 IU eCG (Folligon®, MSD Animal Health, Pune, India) was given intramuscularly to all the animals at the time of sponge withdrawal on day 10 (Fig. 1). The animals undergoing laparoscopic insemination were synchronized in such a way that only two animals were ready for insemination per day.

Immediately after removal of sponges, all the ewes of T group were kept with proven breeding ram upto 72 h for tupping. Ewes were observed twice daily for breeding marks every morning and evening.

Semen collection and processing: Four healthy cross bred rams were selected for semen collection. Semen was collected by artificial vagina method. The semen collected was extended with standard Tris-fructose-egg yolk extender in the ratio of 7:1. The semen samples were checked for quality by estimation of initial motility. The samples having motility more than 70% were pooled. The concentration of the pooled semen was determined and adjusted at 300 million per ml. It was then maintained at 4 °C for 3-4 h before its use for AI.

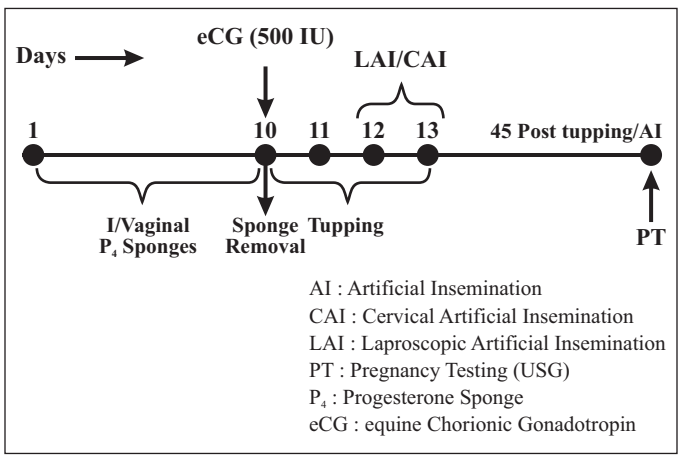

Fig. 1. Experimental design and timeline for treatment distribution

Cervical and Laparoscopic artificial insemination: In ewes belonging to CAI group, timed cervical insemination was performed using chilled semen at 48 h (day 12 evening) after sponge removal and repeated after 12 h interval. The insemination dose of semen was 0.5 ml (150 million sperms). In ewes, belonging to LAI, food and water were withheld to the ewes for 36 h and 24 h, respectively. Timed laparoscopic insemination was performed with chilled semen 48 h after sponge withdrawal.

Pregnancy diagnosis: The pregnancy was confirmed trans-abdominally on day 45 post-tupping or AI using real time B-mode ultrasonography (esoate MyLabTM40 VET) equipped with a 3.5-10.00 MHz sector array transducer.

Blood sampling: The blood samples were collected on day of start of the treatment, day of insemination/tupping (day 0), day 10, day 17 and day 35 post-tupping. Blood samples of 8.0 ml was collected by jugular venipuncture into 15 ml centrifuge tubes without anticoagulant. Serum was later harvested after centrifugation at 1500 × g at 4 °C for 15 min and stored in 2 ml vials in duplicate at -20 °C till further analysis.

Reproductive performance: Reproductive performance was calculated using the following formulae: ***(1) Pregnancy rate:*** number of ewes found pregnant on day 45/total number of ewes tupped/AI/LAI; ***(2) Lambing rate:*** number of ewes lambed/total number of ewes tupped/AI/LAI; (***3) Prolificacy rate:*** total number of lambs born/total number of ewes lambed.

Blood hormone analysis: The serum concentrations of progesterone were measured using solid phase competitive enzyme immunoassay kits obtained from Calbiotech Inc (Cordell Ct., El Cajon, CA). Sensitivity of the assay was 0.22 ng/ml; intra-assay and inter-assay variation coefficients were 5.36% and 9.68%, respectively.

Statistical analysis: The data obtained in the study was analyzed using standard statistical procedures (Snedecor and Cochran, 1994) using SPSS version 20. The data obtained in respect of pregnancy rates, lambing rates and prolificacy were analyzed by Chi-square test. The data

obtained in respect of mean serum progesterone concentration at different days between pregnant and non-pregnant groups were analyzed by Independent t-test. However, variation in means at different days within pregnant and non-pregnant groups was analyzed by One way-ANOVA. The Post-hoc analysis was performed using DMRT. All the data are presented in the tables as mean ± SEM. The level of significance was set as $P<0.05$.

RESULTS AND DISCUSSION

The pregnancy and lambing rates were non-significantly ($P>0.05$) higher in T group (50%, 50%) than CAI (25%, 25%) and LAI group (10%, 10%). The prolificacy did not differ significantly among the three groups (Table 1).

The initial pretreatment day serum progesterone values were basal and almost similar in both pregnant and non-pregnant animals (0.66±0.08 ng/ml and 0.49±0.06 ng/ml), indicating the absence of cyclicity and presence of seasonal anestrus in ewes (Table 2). Progesterone concentration difference was non-significant on day 0 between pregnant and non-pregnant animals. But there was a significant difference in serum progesterone concentration between pregnant and non-pregnant ewes on day 17 (20.01 ± 2.39 ng/ml vs 4.87±0.94 ng/ml) and day 35 (20.98±2.96 ng/ml vs 4.34±0.78 ng/ml). In non-pregnant ewes, the progesterone concentration increased significantly ($P<0.05$) on day 10 compared to day 0, but later on again decreased significantly ($P<0.05$). However, in pregnant ewes, the serum progesterone concentration increased significantly ($P<0.05$) from day 0 (0.57±0.13 ng/ml) to day 10 (17.67±2.24ng/ml) and then varied non-significantly thereafter upto day 35.

Table 1

Fertility parameters (Pregnancy, Lambing and Prolificacy rate) in cross bred ewes following different insemination techniques during non-breeding season

Treatment group	Fertility parameters (%)			
	Treated ewes	Pregnancy rate	Lambing rate	Prolificacy rate
Tupping (T)	6	50	50	100
Cervical Insemination (CAI)	8	25	25	100
Laparoscopic Insemination (LAI)	10	10	10	100

Table 2

Serum progesterone concentration (Mean±S.E.M) in all pregnant and non-pregnant cross bred ewes during non-breeding season

Stage/Day	Progesterone Concentration (ng/ml)	
	Pregnant (n=6)	Non-Pregnant (n=18)
Pretreatment	0.66 ± 0.08^{aA}	0.49 ± 0.06^{aA}
Day 0	0.57 ± 0.13^{aA}	0.51 ± 0.07^{aA}
Day 10	17.67 ± 2.24^{bA}	11.90 ± 1.83^{cA}
Day 17	20.01 ± 2.39^{bA}	4.87 ± 0.94^{bB}
Day 35	20.98 ± 2.96^{bA}	4.34 ± 0.78^{abB}

Means bearing different superscript (a,b,c) within columns and (A,B) within rows differ significantly ($P<0.05$)

The most common protocols to induce cyclicity in ewes involves the use of progesterone and eCG. Administration of gonadotrophins (eCG) stimulate follicular growth and maturation, increase ovulation rate and fertility (Yadav *et al.*, 2020). They also induce a tighter synchrony of ovulation in both anestrous and cycling sheep (Maurel *et al.*, 2003). During the off-season, eCG is required to develop the follicles to the ovulation stage. Following the use of eCG, superovulatory effect has been noticed in the induced estrus with the increase in prolificacy rate (Quintero-Elisea *et al.*, 2011). The FTAI using frozen semen in ewes treated with MAP or FGA sponge along with eCG treatment (at the time of sponge removal) is generally performed 48-60 h after sponge removal. This FTAI abolishes the need of estrus detection as the estrus signs are less pronounced in sheep.

The pregnancy rate was non-significantly ($P>0.05$) higher in ewes belonging to the T group (50%) than those of CAI (25%) and LAI (10%) insemination groups. No abortion or fetal death was recorded in animals included in this study. Consequently both lambing and pregnancy rates obtained were similar. The prolificacy rate also did not differ between the groups. The pregnancy rate obtained in the T group was in agreement with the findings of Husein and Kridi (2003) who obtained the pregnancy rate of 52.2% in Awassi ewes following tupping during non-breeding season. However, contrary to our findings, Moradi Kor *et al.* (2012) and Amer and Hazzaa (2009) obtained higher pregnancy rates of 62.0% and 70.8%, respectively during off-season. However, Almadaly *et al.* (2016) obtained lower pregnancy rate 44.4% following progesterone and eCG treatment during out-of-season breeding. The difference recorded may be attributed to the variation in the breed of the sheep and the type of the drugs used for synchronization of estrus.

The pregnancy rate recorded in non-breeding season in CAI group (25%) is in close agreement to the findings of Anel *et al.* (2005) who obtained a lambing rate of 29.79% following CAI in Churra ewes. However, Dogan and Nur

(2006) obtained high (76.5%) pregnancy rate following CAI in Kivircik ewes during off-season. The detrimental effects of synchronization on sperm transport and survival in female reproductive tract (Pearce and Robinson, 1985), differences in time of estrus (Baril *et al.*, 1993) and prolongation of the lifespan of the ovulatory follicles (Vinoles *et al.*,1999) may be responsible for the difference obtained in the pregnancy rates. The pregnancy rate (10%) following LAI in our animals is much less than the results reported in earlier studies. Anel *et al.* (2005) reported the lambing rate of 43.96% following laparoscopic insemination during non-breeding season. Changes in the photoperiod affect hormonal balance, causing seasonal reproductive variations (Anel *et al.*, 2005). Other factors also affect fertility, such as cervical mucus quality interfering with the sperm transport (Pearce and Robinson, 1985).

The initial pretreatment day serum progesterone concentrations were at basal level in both pregnant and non-pregnant animals (0.66±0.08 ng/ml and 0.49±0.06 ng/ml), indicating absence of cyclicity and seasonal anestrous in ewes. Amer and Hazzaa (2009) also found plasma progesterone concentration at basal level (<1ng/ml) in ewes during anestrous. The present study being conducted from March to May, during which cyclic activities generally cease in ewes. The seasonal absence of estrual behavior under similar climatic conditions has also been reported in Awassi ewes (Husein and Kridi, 2003). After day 0, progesterone concentration increased in both pregnant and non-pregnant ewes as reflected from the values obtained on day 10. This also indicated that all ewes ovulated during this period. There was a significant ($P<0.05$) difference in serum progesterone concentration between pregnant and non-pregnant ewes on day 17 which are in agreement with the findings of Amer and Hazzaa (2009). However, the values obtained in their study were lower than those obtained in ours trial. These differences in progesterone concentration may be due to various factors like breed and age of animals, season, analytical methods used (Mitchell *et al.*,1999) and also the level of stress in the ewes (Dobson *et al.*,1999).

CONCLUSION

In conclusion, intravaginal progesterone sponges for 10 days and eCG regimen successfully induced estrus in ewes during out-of-breeding season. The protocol followed by fixed time artificial insemination resulted in pregnancy of the animals. Experience with refinement in the technique may improve the outcome of the laparoscopic artificial insemination in sheep.

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