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Comparative evaluation of natural tupping with fixed-time laparoscopic and cervical insemination techniques using chilled semen in estrous synchronized sheep

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ABSTRACT

The present study was conducted in crossbred ewes to compare the efficiency of different artificial insemination techniques with natural tupping following fixed time artificial insemination using fresh semen. Crossbred ewes (n=29) were randomly divided into three groups with 10 animals in natural tupping (NT) and laparoscopic artificial insemination (LAI) groups and nine animals in cervical artificial insemination (CAI) group. Ewes in all the treatment groups were subjected to the same estrus induction protocol: insertion of intravaginal progesterone sponges for 10 days followed by an intramuscular injection of 500 IU eCG at the time of sponge withdrawal. Immediately after removal of sponges, animals of NT group were kept with proven breeding rams up to 72 h. Fixed time cervical or laparoscopic insemination was done at 48 h after sponge removal. The insemination was repeated 12 h later in CAI group. The pregnancy and lambing rates were significantly higher in NT group (90%, 90%), than CAI group (55.5%, 55.5%) and LAI group (20%, 10%). The prolificacy rate was significantly higher in CAI group (180%) than NT group (111.1%) and LAI group (100%). The serum progesterone concentration was higher in pregnant ewes on day 10, day 17 and day 35 than non-pregnant ewes. The fixed time cervical insemination following intravaginal progesterone sponges for 10 day+eCG protocol resulted in better pregnancy rate and prolificacy rate in crossbred ewes during breeding season.

Keywords: Estrous synchronization, Ewes, Fertility, Fixed time artificial insemination

Artificial insemination is the cheapest means to spread superior genetic traits of elite males across long distances and up to several decades from the demise of the sire (Foote *et al.* 2002). The development of artificial insemination and subsequent genetic improvement in farm animals has led to remarkable increase in the productivity of the livestock (Najafi *et al.* 2014, Gibbons *et al.* 2019). It makes more extensive use of the best rams possible and reduces the chances of genital infections commonly encountered following natural mating (Leethongdee *et al.* 2007). Moreover, it allows the use of dead, old or injured males and can prevent the transmission of sexual diseases (Hernandez Ballesteros *et al.* 2015). However, in sheep the limited success of the AI has been attributed to two main key factors (Salamon and Maxwell 2000, Anel *et al.* 2005). The structural complexity of ewe cervix constitutes a physical barrier to the deposition of semen into the uterus (Kaabi *et al.* 2002) and the ram semen also shows low resistance to cryopreservation by deep freezing compared to other livestock species (Salamon and Maxwell 2000, Dinatolo 2011).

AI in sheep is carried out mainly with chilled semen. The conventional procedure for inseminating ewes involves deposition of the fresh or chilled semen at the external os of the cervical canal, however low pregnancy rate remains a major challenge. Frequent fertilization failure documented in artificially inseminated ewes has been attributed mainly to the faulty transport of spermatozoa through their tortuous cervix (Boland *et al.* 1983). The problem can however be overcome by intra-uterine deposition of semen through surgical or laparoscopic procedures (Ishwar and Memon 1996). Laparoscopic intrauterine insemination using frozen-thawed semen is gaining popularity as an alternative minimally invasive method of AI (Anel *et al.* 2005). But its high equipment cost and the relatively long curve of expertise to perform the procedure smoothly have been major hindrances in its use, particularly under field conditions (Sathe 2018).

In sheep, timed artificial insemination (TAI) is an important tool as estrus detection is not feasible (Oliver-Muzante *et al.* 2013). The importance of estrous synchronization together with TAI is increasing, however results from different insemination techniques have

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not been consistent. Therefore, the present study was conducted to compare the efficiency of different artificial insemination techniques with natural mating following fixed time artificial insemination using fresh semen on reproductive performance in crossbred ewes.

MATERIALS AND METHODS

Animals, location and management: The present study was conducted at FVSc & AH, SKUAST-Kashmir, Shuhama Srinagar, Kashmir, India (34°08′N 74°28′E). The study was initiated after the onset of the breeding season which corresponds to autumn (September-November). Twenty nine multiparous crossbred healthy ewes (NARI-Swarna ram × non-descript ewes) weighing 36.41±4.25 kg (Mean±S.E.M) and body condition score between 2.5-3.5 (on a scale of 1~5) were included. The ewes used were in the third or fourth parity only were included. All the ewes were maintained under uniform management conditions at Mountain Research Centre for Sheep and Goats (MRCSG), FVSc & AH. Animals were allowed grazing during the day (9:00 AM to 3:00 PM) and housed in closed pens at night up to November 15. Subsequently they were additionally provided hay (oats+sorghum) @1.5 kg and concentrate feed (Agrofeed Industries J&K Ltd) @300 g till 31st December. From January onwards the concentrate feed was increased up to 600 g/day/pregnant ewe. Free access to drinking water was provided to the animals throughout the study period.

Experimental design: Crossbred ewes (n=29) were randomly allotted to three groups; 10 animals each in natural tupping (NT) and laparoscopic artificial insemination (LAI) groups and 9 in cervical artificial insemination (CAI) group. Ewes in all the treatment groups were subjected to the same estrus induction protocol: insertion of intravaginal progesterone sponges (AVIKESIL-S, CSWRI, Avikanagar, India) for 10 days. 500 IU eCG (Folligon®, MSD Animal Health) was given intramuscularly to all the animals at the time of sponge withdrawal (Fig. 1). The animals to be used for laparoscopic insemination were synchronized in such a

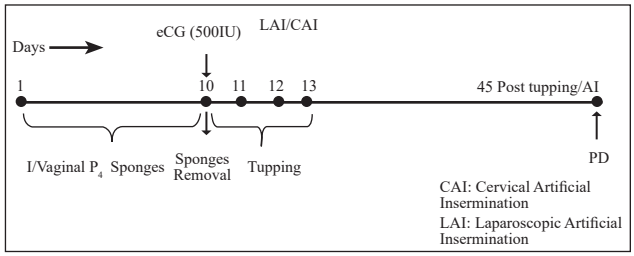

Fig. 1. Experimental design and timeline for treatment distribution.

way that only two animals were ready for insemination per day. After removal of sponges, animals of control group (NT) were kept with one proven breeding ram (10:1) for tupping up to 72 h. Ewes with colour mark on the rump were considered as tupped.

Semen collection, evaluation and dilution: Four healthy crossbred rams were selected and semen was collected by artificial vagina method. The semen obtained was extended (ratio 7:1) in standard Tris-fructose-egg yolk extender. The extended semen with motility more than 70% only was pooled. The concentration of the pooled semen was again determined and adjusted at 300 million spermatozoa per ml before storing it at 4°C for 3-4 h.

Cervical artificial insemination (CAI): In ewes belonging to this group, timed cervical insemination was done using chilled semen 48 h (day 12 evening) after sponge removal. The AI was repeated after 12 h interval, i.e day 13 morning in all these animals. 0.5 ml of the semen (150 million sperms) was used per insemination. Sterile disposable bovine AI sheath attached with a 5.0 ml disposable syringe was used for depositing semen. At the time of insemination, the hind-quarters of the ewe was raised above the ground level by an attendant and os cervix was located using a vaginal speculum with light source. The free end of the semen loaded sheath was then introduced into the vagina and the semen was deposited at external os of the cervix. The animal was released after lowering its hind-quarters.

Laparoscopic artificial insemination (LAI): Prior to laparoscopic insemination 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. The procedure was performed in laparoscopy theatre of the Division of Animal Biotechnology, FVSc & AH, Shuhama. The animals included in this group were sedated using injection of xylazine hydrochloride (0.05 mg/kg; IM). The abdominal region caudal to the umblicus was surgically prepared by shearing the wool, shaving and disinfecting the skin.

The ewe was restrained on a laparoscopy cradle in dorsal recumbency with head-down (Trendelenberg) position at 45° angle with the floor. Laparoscope (5.0 mm) and allied instruments of Karl Storz, DmbH, Germany were used. The local anaesthetic was infiltrated at portal sites (1.0 ml/port). Two ports 7-10 cm cranial to the udder and 5 cm lateral to the midline (*linea alba*) on either side were initially created. A scalpel blade was used to make a skin stab before passing the veress needle at one port site. Filtered room air was used to insufflate abdominal cavity up to 10-12 mm of Hg. After insertion of the first trocar and cannula, the trocar was withdrawn and laparoscope introduced. The pelvic cavity was explored to locate the uterus. Second port was created on the contra-lateral side of the *linea alba*. Through this port, the tissue forceps was passed to grasp the uterus. Third port was created 5-6 cm caudal to the second port. The loaded insemination gun was passed through this port and 0.5 ml of extended semen deposited in the body of the uterus. Instruments were then withdrawn from the abdominal cavity. The cannulas were removed only after evacuation of abdominal air. An antibiotic fly repellant spray was applied to the port sites. The animal was shifted from the cradle and released.

Ultrasonography and blood sampling: In all the ewes included in the study, the pregnancy was confirmed on

day 45 post-tupping/insemination using real time B-mode ultrasonography (esoate MyLab^{TM}40 VET) equipped with a 3.5 MHz to 10.0 MHz sector array transducer. The animals were placed on the table after cleaning the inguinal region. The ewes were examined trans-abdominally in right lateral recumbency. Standard ultrasonographic technique was followed. Visualization of embryonic vesicle, placentomes and pulsation of heart within the embryonic vesicle were assessed to confirm pregnancy. Blood samples (8.0 ml) were collected by jugular venipuncture into 15 ml centrifuge tubes without anticoagulant. The tubes were immediately stored in ice and placed in slanting position for 1-2 h. Serum was harvested by centrifugation at 1500 × g at 4°C for 15 min. Serum was removed from tubes with micropipette and stored in 2 ml storage vials in duplicate at -20°C until further analysis. The blood samples were collected on day of start of the treatment, on the day of insemination/tupping (day 0), day 10, day 17 and day 35 post tupping/AI.

Reproductive performance: Reproductive performance was calculated using the following formulas: Pregnancy rate: number of ewes found pregnant on day 45/total number of ewes tupped/CAI/LAI; lambing rate: number of ewes lambed/total number of ewes tupped/AI/LAI and 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 statistical software SPSS version 20. The data obtained in respect of pregnancy rates, lambing rates and prolificacy was analyzed by Chi-square test. The data obtained in respect of mean serum progesterone concentration at different days between pregnant and non-pregnant groups was analyzed by Independent samples 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 Duncan's multiple range tests. 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, lambing and prolificacy rate of ewes inseminated following different methods is presented in Table 1. The pregnancy and lambing rates were significantly ($P<0.05$) higher in NT group (90%, 90%) than LAI group (20%, 10%) and non-significantly higher than CAI group (55.5%, 55.5%). The prolificacy rate was significantly ($P<0.05$) higher in CAI group (180%) than NT group (111.1%) and LAI group (100%).

The ewes included in the experiment were cyclic as indicated by the higher progesterone levels in both pregnant and non-pregnant ewes on pre-treatment day. The mean serum progesterone concentration on pre-treatment day and day 0 varied non-significantly between pregnant (17.07±1.92 ng/ml; 0.58±0.06 ng/ml) and non-pregnant (21.07±3.38 ng/ml; 0.52±0.08 ng/ml) ewes. However, there was difference in mean progesterone concentration between pregnant and non-pregnant ewes on day 10, day 17 and day 35 (Table 2). In non-pregnant animals, there was significant ($P<0.05$) decrease in serum progesterone concentration from pre-treatment day (21.07±3.38 ng/ml) to the day 0 (0.52±0.08 ng/ml). However, on day 10 the concentration increased (14.29±2.45 ng/ml) and again decreased on day 35 (4.52±0.4 ng/ml). In pregnant animals, there was a significant ($P<0.05$) decrease in serum progesterone concentration on day 0 (0.58±0.06 ng/ml) compared to day of pre-treatment (17.07±1.92 ng/ml). Thereafter there was notable increase on day 10 (22.67±0.87 ng/ml) which further increased non-significantly ($P>0.05$) up to day 35 (25.22±2.32 ng/ml).

Table 2. Serum progesterone concentration (Mean±S.E.M) in pregnant and non-pregnant crossbred ewes from all the groups during breeding season

Stage/Day	Progesterone Concentration (ng/ml)	
	Pregnant (n=16)	Non-Pregnant (n=13)
Pretreatment	17.07 ± 1.92^{a}	21.07 ± 3.38^{a}
Day 0	0.58 ± 0.06^{b}	0.52 ± 0.08^{b}
Day 10	22.67 ± 0.87^{cA}	14.29 ± 2.45^{cB}
Day 17	24.03 ± 3.04^{cA}	12.60 ± 2.21^{cB}
Day 35	25.22 ± 2.32^{cA}	4.52 ± 0.4^{bB}

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

Artificial insemination (AI), an alternative to the natural tupping in ewes is adopted for the maximum utilization of sires of high genetic value (Rojero *et al.* 2009, Gibbons *et al.* 2019). However pregnancy following AI has been low, mainly due to the complex anatomy of sheep cervix and poor post-thaw quality of ram semen (Salamon and Maxwell 2000). The ovine cervix is a highly complex fibrous structure with multiple folds protruding into and

Table 1. Fertility parameters of cross-bred ewes following different insemination techniques during breeding season

Treatment group	Fertility parameter			
	Treated ewes *N*	Pregnancy rate (%)	Lambing rate (%)	Prolificacy rate (%)
Natural Tupping (NT)	10	90.0^{a}	90.0^{a}	111.11^{a}
Cervical Insemination (CAI)	9	55.5^{ab}	55.5^{ab}	180.00^{b}
Laparoscopic Insemination (LAI)	10	20.0^{b}	10.0^{b}	100.00^{a}

Means bearing different superscript within columns differ significantly ($P<0.05$).

obliterating the lumen. Generally the second fold is more misaligned than the first and third, thus narrowing the cervical lumen (Naqvi *et al.* 2005, Kaabi *et al.* 2006). Due to this morphological complexity of the cervix in sheep the insemination pipette can't be passed into the uterus. Therefore, the semen is deposited either at the entrance of the cervix or deep into the vagina. Such inseminations result in lower conception rate (Kershaw *et al.* 2005). For better conception rates intra-uterine deposition of semen by laparoscopy is advocated (Ishwar and Memon 1996). It has become the most popular procedure of AI in ewes (Salamon and Maxwell 1995). This technique makes possible the deposition of the semen directly into the uterus even near the oviduct (Evans and Maxwell 1990).

The use of eCG improves estrus synchronization, follicular maturation, ovulation rate and fertility. 300 to 600 IU of eCG was administered in different breeds of sheep with different body weight (Olivera-Muzante *et al.* 2011). Superovulatory effect and increased prolificacy has been noticed at the induced estrus (Quintero-Elisea *et al.* 2011). The efficacy of any treatment depends on several extrinsic and intrinsic factors. Extrinsic factors include the active principle of the drug, time of the year in which treatment is undertaken, the dose used, duration and the protocol followed (Knights *et al.* 2011). Intrinsic factors include breed and age of the animals. All of these factors independently as well as in combination, define the ovarian status at the time of application of the synchronization protocol and subsequent responses (Kareta *et al.* 2006). The FTAI using frozen semen in ewes exposed to MAP or FGA sponge along with eCG treatment (at the time of sponge removal) is generally performed 48-60 h after sponge removal (Aitkin *et al.* 1990). This FTAI abolishes the need of estrus detection as the estrus signs are less pronounced in sheep.

The results of pregnancy rate (90%) of natural tupping group are in agreement with the findings of Waheeb *et al.* (2017) and Koyuncu and Alticekic (2010) who reported pregnancy and lambing rate of 90% and 86.2%, respectively in ewes during the breeding season. The outcome of the laparoscopic insemination is nearer to values (30%) reported by Gimenez-Diaz *et al.* (2011). However, our results are lower than the values of Alfaris *et al.* (2012), Rojero *et al.* (2009) and Anel *et al.* (2005). They reported conception rates of 71.4%, 75.0% and 44.89% respectively. The variation in the results may be due to the limited number of animals included in this study, variation in breed and season of the year. Additionally the use of three port laparoscopic techniques requiring comparatively longer procedural time might have put the animals to protracted distress. Besides low sperm count per insemination dose (150 million) might have decreased the pregnancy rate in this trial. Milczewski *et al.* (2000) recommended the inclusion of 250 million spermatozoa per dose in intrauterine insemination for getting higher pregnancy rates (69.56%). Although few of the earlier studies practiced LAI twice with a gap of 12 h, in this study, LAI was done only once per animal. In animals of the CAI group it was repeated again at 12 h interval and in natural tupping group the ewes were kept continuously with rams up to 72 h.

The lambing rate (55.5%) achieved after cervical insemination in this study was similar to the rates (55.56% and 57.0%) reported by El-Badry *et al.* (2014) and Ghalsasi and Nimbkar (1996), respectively but were lower than those (65.75% and 60.0%) reported by Donovan *et al.* (2000) and Nour *et al.* (2010), respectively. However our results are better than the rates (43.70%, 50.0% and 42.7%) reported by Rojero *et al.* (2009), Al-Wataar *et al.* (2009) and Menchaca and Queirolo (2005), respectively. The fertility rates obtained following cervical and laparoscopic insemination vary with the insemination technique, the farm location/size, age of the animals, the attributes pertaining to the male used for semen collection, number of inseminations per ewe, lambing-insemination interval, technician performing insemination, flock and management conditions (Anel *et al.* 2005, Paulenz et *al.* 2005).

Interestingly the prolificacy rate in the animals included in cervical insemination group was significantly ($P<0.05$) higher (180.0%) than those subjected to natural tupping (111.1%) and laparoscopic insemination (100.0%). The reason for this high prolificacy rate is the triplets born to two ewes in cervical insemination group.

The results of mean serum progesterone concentration are in accordance to the findings of earlier workers (Anghel *et al.* 2011, Rasool *et al.* 2019). The mean serum progesterone concentration was significantly ($P<0.05$) higher in pregnant than non-pregnant ewes on day 15 and day 35 (Rasool *et al.* 2019). In pregnant sheep, Anghel *et al.* (2011) obtained progesterone concentration of 14.4±1.52 ng/ml on day 25 post-mating. The values is in conformity to that obtained in pregnant animals of our study. However contrary to all the above described trials, progesterone level as low as 4.50±0.41 ng/ml has also been documented on day 18 in pregnant ewes (Weigl *et al.* 1975). This variation in the concentration may be due to breed, age and stress of the animal, season and the analytical method used (Dobson *et al.* 1999). In all the animals included in this study, the serum progesterone concentration decreased significantly ($P<0.05$) from day pretreatment to day 0. Progesterone values remain low (1.56±0.15 ng/ml) at the time of estrus (Rasool *et al.* 2019). The serum progesterone values decreased from day 10 to day 35 in non-pregnant animals due to the regression of corpus luteum. However, in pregnant ewes the values increased significantly ($P<0.05$) on day 10 which further increased non-significantly ($P>0.05$) up to day 35. The increase occurs due to the persistence of corpus luteum, the major source of progesterone during early pregnancy in ewes (Wiltbank *et al.* 2014). The progesterone plays a major role in pregnancy establishment and maintenance (Clemente *et al.* 2009).

In conclusion, fixed time cervical artificial insemination with liquid semen following intravaginal progesterone

sponges for 10 day+eCG protocol resulted in better pregnancy rate (55.5%) and prolificacy rate (180%) in crossbred ewes during breeding season. The low conception rate in ewes may be attributed to the distress on the animal by three port protracted laparoscopic procedure. Experience and refinement in the technique may improve the outcome.

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