Effect of Inoculation Place of Frozen Semen on Fertility Rate in Synchronized Afshari Ewes

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Summary

The aim of the present study was to perform laparoscopic inoculation of frozen semen and to determine the fertility rate after laparoscopic inoculation into one or both uterine horns regardless of the ovulation site in Afshari ewes. This study was performed in Tehran, Alborz and Qazvin provinces for about 4 years in- and out-of-breeding season. The studied animals included 11533 Afshari ewes (age range 2-6 years) with an average weight of 57.3 ± 4.1 kg and the body condition score (BCS) of 3.1 ± 0.1. The results of this study showed that fertility varied between years, but these changes were not significant. Significant differences were found between seasons. The highest fertility was observed in spring and summer compared to autumn and winter ($P < 0.05$). There was no significant difference between left, right, and both horns of the uterus using frozen sperm. Despite the significant difference of fertility between left, right, and both horns of the uterus using GLM procedure, the difference of fertility between left, right, and both horns was negligible. In this experiment, the percentage of pregnancies resulting from insemination in the left, right or both horns were 51.46, 51.36, and 51.74%, respectively. Thus, for more certainty, the mean comparison between inoculation to one or two horns of uterus was performed by T-test. The T-test did not indicate any significant difference between fertility rate after semen inoculation into one or both uterine horns. Therefore, it can be concluded that injection in any of the uterine horns, which is easier to access by the operator, increases the speed and reduces time of insemination and consequently increases the efficiency of artificial insemination.

Key words

Afshari ewes, frozen semen, laparoscopic, ovulation, uterine horns

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Introduction

Mutton is a common source of protein in Iran and its consumption is high compared to beef and goat. At present, every year about 300 thousand tons (41%) of the total meat produced in the country is by approximately 50 million sheep in the form of 27 breeds adapted to the climatic, cultural, economic and social conditions of different regions. Genetic selection and proper nutrition, artificial insemination (AI), sperm and management along with improvement of reproductive factors can increase the reproductive efficiency in sheep. Increasing reproductive productivity leads to more lamb production per ewe and ultimately increases livestock income (McCappin and Murray, 2011).

AI is used as a scientific method to advance reproductive goals and increase genetic efficiency in sheep and is also used to prevent the spread of infectious diseases (Noakes et al., 2018; Agrebi and Larbi, 2020; Bergstein-Galan et al., 2022). The advantage of AI is the greater use of superior rams and the control of infectious diseases among herds. Using frozen semen, the genetic potential of a superior ram can be used for many years, but due to the sensitivity of ram sperm to the freezing process, a large percentage of sperm in the semen and their ability to move and reach the fertilization site is damaged. Finally, the fertility rate from AI with frozen sperm decreases (Salamon and Maxwell, 1995). However, by injecting the frozen sperm near the fertilization site (uterus), the chance of fertilization can be increased.

Various methods for AI in sheep have been introduced, including vaginal, cervical, trans-cervical and laparoscopic methods without surgery, which have different reproductive efficiencies depending on the existing conditions, such as sperm type. Pregnancy caused by AI offers different solutions using various methods, one of these methods being laparoscopic inoculation (Faigl et al., 2012). Laparoscopic intrauterine insemination has been performed in sheep since the 1980s (Maxwell and Hewitt, 1986). Today, the use of laparoscopic method for AI and embryo transfer in sheep is common and has a good efficiency of pregnancy and embryo reception (Aybazov et al., 2019). For laparoscopic inoculation, the abdomen is pierced in two areas, with the first hole about 8 cm below the left udder and the second hole about 5 cm to the right of the first hole. The aim of the present study was to compare the fertility rates of laparoscopic AI using frozen sperm in the left, right or both horns in- and out-of-breeding season in Afshari sheep.

Materials and Methods

Time and Place of Testing

The study was carried out during 4 continuous years (in- and out-of-breeding seasons i.e. spring, summer, autumn, and winter) on mixed Brula Afshar sheep bred in 23 animal husbandry complexes located in Tehran, Alborz and Qazvin provinces.

Considering the importance of nutrition on reproduction and the effect of flushing on increasing the performance of sheep, all the sheep were fed 3 times a day, from three weeks before to two weeks after the time of inoculation, with about one kilogram of concentrate (Table 1), as well as one kilogram of an equal combination of alfalfa and wheat straw. It should be noted that the selected sheep were adapted to flushing concentrate one week before the start of synchronization.

Table 1. Used sheep flushing concentrate

Ingredients*	Amount	Unit
NED	2.5 ± 0.5	Mcal kg^{-1}
NEM	1.7 ± 0.5	Mcal kg^{-1}
NEG	1.1 ± 0.5	Mcal kg^{-1}
DM	90	%
CP	13.5 ± 0.5	%
EE	4 ± 0.2	%
Ash	7 ± 0.5	%
Ca	0.7	%
P	0.3	%

Note: NED – net energy for weight gain in addition to maintenance, NEM – net energy for maintenance, NEG – net energy for gain, DM – dry matter, CP – crude protein, EE – ether extract, Ca – calcium, P – phosphorus

Animals

The animals studied in this study included 11533 Afshari ewes (age range 2-6 years). The mean weight and body condition score (BCS) were 57.3 ± 4.1 kg and 3.1 ± 0.1, respectively. All ewes received the same diet and access to the fresh water. Descriptive statistics for the number of inoculated and conceived animals by season and inoculated uterine horn are presented in Table 2.

Synchronization of Estrus

All ewes received GnRH (25 µg of alarelin acetate, Vetaroline, Aboureihan, Iran) and ESPONJAVET (Polyurethane sponge soaked with 60 mg of Medroxyprogesterone acetate/sponge, Hipra, Spain) was inserted into the vagina for 12 d, then removed and PGF2α (75 µg d-cloprostenol, Vetaglandin, Aboureihan, Iran) plus eCG were administered. (500 IU; Gonaser, Hipra, Spain). All the ewes were inseminated based on fixed time laparoscopic AI at 48 hours after administration of the eCG and PGF2α (Youngquist and Threlfall, 2006). To this end, the ewes were deprived of food and water for 24 h prior to laparoscopy.

Collection of Semen

12 "Afshar Booroola" cross ram was selected each time pooled semen, from three rams collected with artificial vagina, was used. Semen was extended at 35 °C in an Triladyl-based extender (MINITUBE, Germany) to a concentration of 20×10^6 spermatozoa 0.25 mL^{-1}. Triladyl® contains TRIS, citric acid, sugar, buffers, glycerol, and the purest water and antibiotics according to the EU Directive 88/407 (Tylosin, Gentamicin, Spectinomycin, Lincomycin). Extended semen was frozen to 5 °C in 2 – 3 h within a water jacket. Frozen semen, rewarmed at the time of insemination to 35 °C, was used for laparoscopic AI and a single uterine horn or both uterine horn was inseminated without examining ovaries.

Table 2. Fertility rate of synchronized and laparoscopically inseminated Afshari ewes in deferent seasons

	Spring	Summer	Autumn	Winter	Total
Right horn	1063 (723)	1339 (776)	754 (324)	695(257)	3851 (2080)
Left horn	1060 (721)	1349 (781)	776 (332)	696 (257)	3881 (2091)
Both horn	1047 (713)	1331 (773)	739 (322)	684 (254)	3801 (2062)
Total	3170 (2157)	4019 (2330)	2269 (978)	2075 (768)	11533 (6233)

Laparoscopic AI

Animals were sedated with 0.3 ml acepromazine maleate (Neurotanq; 10 mg mL^{-1}, Alfasan, Holland) 10 to 30 minutes before insemination (Sathe, 2018). Ewes were placed in laparoscopic cradles and kept in dorsal recumbences. Local anesthesia was performed by administration of 2 mL Lidonalin (each mL contains 20 mg lidocaine HCl, 0.08 mg Noradrenaline, Nasr, Iran) in the site of abdominal puncture 5 min prior to insemination (Sathe, 2018). Two trocars were inserted about 3 cm on either side of the midline and about 3 cm cranial to udder to allow for introducing telescope and insemination pipette (Sathe, 2018). Following observation of uterine horns, half of the volume of frozen semen (20×10^6 spermatozoa) was inseminated in each uterine horn or (40×10^6 spermatozoa) inseminated just in one horn. After insemination, all trocar puncture wounds were sprayed with a combined antiseptic and insect.

Pregnancy Diagnosis

Diagnosis of pregnancy was performed by transabdominal ultra-sonographic examination using a B-mode ultrasound machine (HONDA ELECTRONICS HS-2000, Japan) equipped with a 3.5 MHz convex probe, 40 to 50 days after laparoscopic AI.

Data Analysis

Factors affecting the percentage of pregnancies resulting from inoculation between different uterine horns were determined using SAS software GLM analysis. The following statistical model was used to analyze the variance:

$$Y = \mu + year + season + horn + e$$

Here, Y indicates pregnancy percentage for each groups of animals (categorized by year, season and inoculated horn), μ total mean, *year* is year, *season* is season, *horn* is horn or branches of the uterus in which the insemination was performed, and e is residual effects. The means were compared using the Duncan multi-domain method and significant means were identified at the $P \leq 0.05$ significance level.

Results and Discussion

In all ewes, the intravaginal sponge was removed from two days before laparoscopy and any inflammatory process of the vagina was examined at the end of the treatment. When removing the sponge, all ewes showed minimal vaginal mucus secretion. During the next 2 days, moderate hyperemia, edema and mucous secretions indicated optimal estrus of ewes. Regardless of having active ovaries or the degree of consistency and hyperemia of uterine tissue, 148 ewes were excluded from the study due to health problems and lack of fertility by artificial insemination.

At the end of surgery, none of the ewes showed any signs of apparent pain or compromised physiological function. Half an hour later, all the ewes began to feed. During 2 weeks after surgery, no infection was observed in the laparotomy wound and no contraction occurred.

The results of analysis of variance to investigate the factors affecting pregnancy percentage are presented in Table 3. In this table, the factors of year, season and place of insemination or uterine horn are inspected, and also the results of comparison of means are presented in Table 3.

Table 3. Effects of years, seasons and incubated horn on percentage of pregnant

Variable	Category	Pregnancy (%) mean	*P* value
Year	2019	45.954^{c}	0.328
	2020	51.623^{b}	
	2021	51.492^{b}	
	2022	67.967^{a}	
Season	Spring	68.023^{a}	<0.001
	Summer	57.968^{b}	
	Autumn	43.108^{c}	
	Winter	36.998^{d}	
Incubated horn	Right	51.463^{b}	0.002
	Left	51.361^{b}	
	Both	51.748^{a}	
Year × Season			0.399
Year × Incubated horn			0.220
Season × Incubated horn			0.199

Note: Means with different superscript letters in a column are significantly different ($P \leq 0.05$)

Table 4. Results of T-test for comparison of pregnancy rates when ewes inseminated by one or two horn

T-Tests					
P value	T Value	DF	Variances	Method	Variable
0.9401	0.08	34	Equal	Pooled	Pregnancy (%)
0.9407	0.08	21.8	Unequal	Satterthwaite	Pregnancy (%)
Equality of Variances					
P value	F Value	Den DF	Num DF	Method	Variable
0.8973	1.04	23	11	Folded F	Pregnancy (%)

The results of analysis of variance showed that the effect of inoculation site on pregnancy percentage was significant, but comparison of means by the Duncan test showed very little difference between inoculated horns (Table 3). The mean percentage of pregnancy at inoculation in the right, left and both horns was 51.46, 51.36 and 51.75, respectively. The mean percentage of pregnancy at inoculation in both horns was 0.55 and 0.75% higher than the right and left horns, respectively. In essence, despite the statistically significant differences, it can be claimed that there is no significant difference between the percentages of pregnancy at insemination in different horns of the uterus. For more reassurance, the difference in pregnancy percentage during insemination in both uterine horns and one uterine horn (right or left) was compared using T-test (Table 4). The results of T-test at the level of error percentage ($\alpha = 0.01$) did not show any significant difference between the pregnancy results of inoculation in one or both uterine horns.

As mentioned before, the percentage of pregnancies resulting from insemination in the left, right or both horns was 51.46, 51.36 and 51.74%, respectively. The overall pregnancy rate is similar to that reported by other authors for the laparoscopic procedure. The site of inoculation had no effect on overall fertilization rate and no difference was found between the left, right, and both ovarian ducts, which was similar to the results of other researchers comparing the place of inoculation on the percentage of pregnancy (Perkins et al., 1996).

Discussion

Pregnancy rate varied between the studied years. The lowest pregnancy rate was observed in 2019 and the highest was in 2022. Although fertility varied between years, these changes were not significant ($P \geq 0.05$) (Table 2). Similar results have been reported in goats. The low winter temperatures are risk factors for reduced fertility after AI in Florida goats (Arrébola et al., 2016).

Significant differences were found between seasons. The highest fertility was observed in spring and summer as compared to autumn and winter (Table 2). Arrébola et al. (2012) also identified a decrease in the probability of pregnancy when goats were inseminated in autumn and winter, also they linked these results to temperature (Arrebola et al., 2012), which is consistent with our study.

Our result differs from the surveys on cervical or trans-cervical AI in sheep (Buckrell et al., 1994; Anel et al., 2005). In the study of Anel et al. (2005) when AI is performed in comparison with insemination by laparoscopy, the season factor shows a great impact, which may be due to changes in the quality of the cervical mucosa and thus a decrease in the quality of semen transport. Another hypothesis is that AI at the time of reduced food supply (winter and autumn) affects the growth of pregnancy due to lack of food and this factor may affect fertility.

In an investigation designed to identify external (year, season), internal (age and breed) and AI method (number of inseminations, synchronization protocols, semen conservation method) and factors affecting fertility after AI by laparoscopic method in sheep, experimental results showed that year, age, number of inseminations and synchronization protocols affected fertility after laparoscopy (Bergstein-Galan et al., 2022) Fertility in inoculated sheep in spring (64.28 %) and summer (54.16%) was higher than in winter (36.92%) and autumn (32.14 %) ($P \leq 0.05$).

Therefore, rejecting the hypothesis that injecting sperm into the both ewes' uterine horns can lead to spermatozoon distribution and the higher chance of fertilization or acceleration it can be said that the injection in any of the uterine horns that is easier to access by the operator will increase the speed of work (especially in synchronization of more than 100 heads) and consequently avoid the large time difference between inoculation of the ewes. Ultimately, this can increase the efficiency of AI by reducing the fatigue of the operator and assistant.

Additionally, by subtracting the manipulation and consequently the possibility of injury or bleeding during labor and related complications, the entire volume of spermatozoa can be injected into each of the uterine horns that are easily accessible.

Using laparoscopic intrauterine insemination, the pregnancy rate was 44.8 (Anel et al., 2005), 50.2 (Casali et al., 2017) and 63.7% (Pau et al., 2019) and was higher than other methods of AI in various investigations. In another study on 15 ewes that underwent laparotomy intrauterine inoculation with frozen semen, 60% of gestations were obtained (Sylla et al., 2021). Recently, a new surgical approach has been reported for cervical folds for intrauterine insemination through the cervix, which achieves 63.7% pregnancy (Pau et al., 2019).

In a survey with the aim to evaluate the effectiveness of laparoscopic intrauterine insemination compared to cervical insemination in ewes, and also to determine the optimal sperm count in each laparoscopic insemination the results showed that the rate of lambing after laparoscopic intrauterine insemination using 20×10^7 spermatozoa per mL displayed higher pregnancy rate than other methods, which is consistent with the results of the present study (DA et al., 2014).

Conclusion

Nowadays, the use of laparoscopic method for AI and embryo transfer in sheep is common and has a good efficiency of pregnancy and embryo reception. However, laparoscopy has disadvantages such as high cost, time consumption and the need for high expertise. This study was performed with a large number of samples and the results are highly reliable. The results of this study show that insemination of sperm in both uterine horns does not cause more pregnancy than insemination in one horn. In this way, by injecting in any of the uterine horns that are easier to access by the operator, the problems and disadvantages of the laparoscopic method can be overcome and in addition can lead to reducing costs, increasing the speed and time of insemination and consequently increasing the efficiency of AI.

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