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FACTORS INFLUENCING FERTILITY IN LAPAROSCOPIC ARTIFICIAL INSEMINATION IN SHEEP

(Fatores que influenciam a fertilidade na inseminação artificial laparoscópica de ovelhas)

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ABSTRACT - Fertility following artificial insemination (AI) programs in sheep is affected by many variables. The aim of this study was to identify extrinsic (year, season), intrinsic (age and breed) and AI procedure related (number of AIs, synchronization protocols, semen preservation method) factors that influence fertility after laparoscopic AI in sheep. Data from 159 laparoscopic AI procedures were categorized according to year (from 2013 to 2017), season (Winter, Spring, Summer and Autumn), breed (Dorper, White Dorper, Texel, Cross-breed), age in months (≤12, 13-24, 25-36, 37-48, ≥49), estrus synchronization protocol (short-term or long-term), cumulative number of AIs (1 to 4) and semen preservation method (frozen or fresh). Frequency analysis using chi-square test was used. Year, age, number of AIs and synchronization protocols did not influence fertility after laparoscopic AI in sheep. Fertility was higher ($P<0.05$) in sheep inseminated in spring (64.28%) and summer (54.16%) when compared to winter (36.92%) and autumn (32.14%). Dorper breed fertility (58.02%) was higher ($P<0.05$) than White dorper (25%) and Cross-breed (30.23%). There was a lower ($P<0.05$) pregnancy rate in sheep inseminated with frozen semen (38.27%) than sheep inseminated with fresh semen (53.84%). In conclusion, season, breed and semen preservation method can influence fertility in sheep after laparoscopic AI.

Key words: season; pregnancy; estrus synchronization; ewe.

INTRODUCTION

Artificial insemination (AI) with the use of genetically superior sires allows accelerated genetic advancement in commercial flocks. In addition, when used in conjunction with estrus synchronization, it allows births to be timed for a commercially favorable season, the organization of the labor and a reduction in the seasonality effect in sheep(AKÉ-VILLANUEVA *et al.*, 2017; BERGSTEIN-GALAN *et al.*, 2018).

Laparoscopic AI procedures produce better results when compared to vaginal or transcervical insemination (CASALI et al., 2017; EL-BADRY et al., 2014). A mean conception rate of 44.89% was reported for laparoscopic AI in a study involving a large number of ewes under field conditions (ANEL et al., 2005). However, a number of factors may influence fertility after AI including: environmental, intrinsic ewe related, semen storage,

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and AI procedures (AKÉ-VILLANUEVA et al., 2017; ANEL et al., 2005; ARRÉBOLA et al., 2016; PALACÍN et al., 2012). In order to maximize the advantages of the procedure risk factors for the success of AI must be identified.

The aim of this work was to evaluate the effect of extrinsic factors (year, season), intrinsic factors (age and breed) and factors related to AI procedure (cumulative number of AIs, synchronization protocols, semen preservation method) on gestation rate after laparoscopic artificial insemination in sheep.

MATERIAL AND METHODS

Database

Data from AI procedures performed between the 2013 and 2017 at latitude 25°27'40.7"S 49°43'40.6"W, was used. In this period 159 AI procedures were performed on 127 sheep of the Dorper, White dorper, Texel and Cross-breed breeds, ranging from 7 to 121 months of age. The animals were grazed on native pasture and feed was supplemented with corn, soya and mineral premix. Water was available *ad libitum*.

AI procedures were categorized according: year (from 2013 to 2017), season (Winter, Spring, Summer and Autumn), breed (Dorper, White Dorper, Texel, Cross-breed), age in months (≤12, 13-24, 25-36, 37-48, ≥49), estrus synchronization protocol (short-term or long-term), cumulative number of AIs (1 to 4) and semen preservation method (frozen or fresh). The effect of each factor on fertilization rate after laparoscopic AI procedure was assessed (Figure 1).

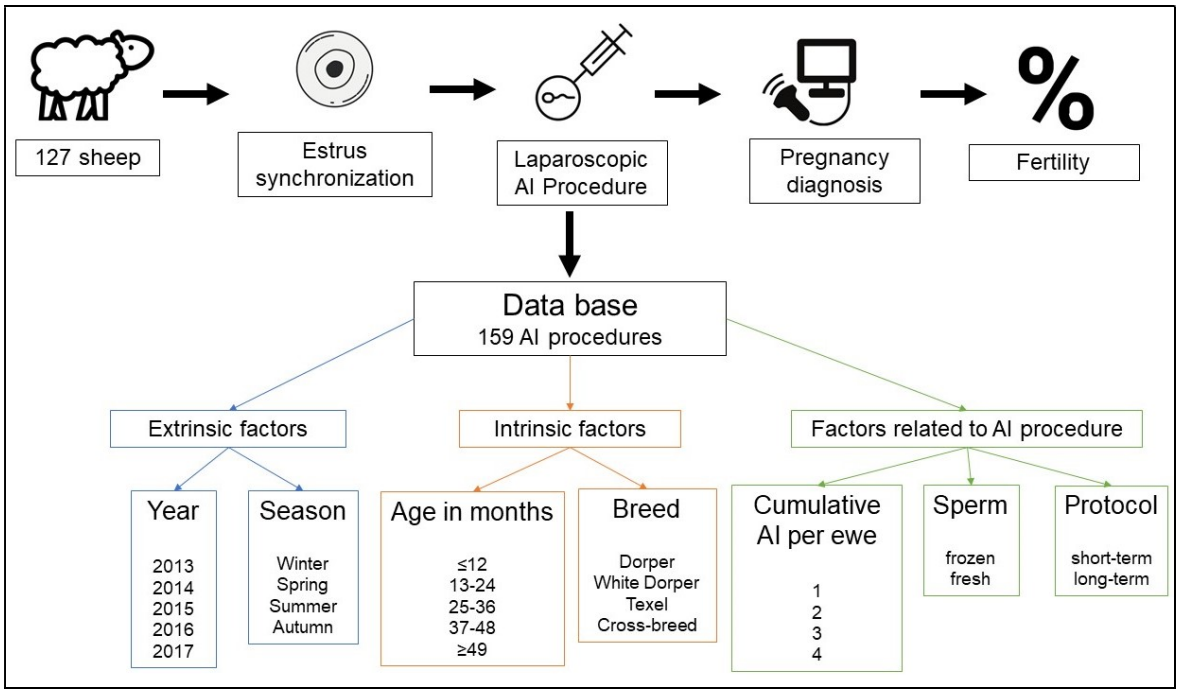

Figure 1- Diagram of the experimental design.

Synchronization protocol

A long-term synchronization protocol involved insertion of intravaginal implants impregnated with 60mg of medroxyprogesterone acetate (Progespon®, Zoetis, United States) for a period of 14 days. On the day of implant removal 500 IU of eCG (Novormon®, Zoetis, United States) was administered.

In the short-term protocol sheep received intravaginal implants impregnated with 60mg of medroxyprogesterone acetate (Progespon®, Zoetis, United States) for 6 days. One dose of 300 IU eCG (Novormon®, Zoetis, United States) and prostaglandin F2alpha analog (132 µg sodium cloprostenol, Ciosin®, Schering-Plough, New Jersey, USA) was given on Day 6 in the morning of device removal (CASALI et al., 2017).

In both protocols sheep remained with vasectomized rams for a period of 72 hours after progestagen withdrawal. The artificial insemination procedure was performed at a fixed time of 50 hours after withdrawal of the progesterone implant.

Semen analysis and inclusion criteria

Fresh semen was obtained from rams housed at Álamos Genetics Flushing Station using an artificial vagina. Frozen semen came from a variety of sources and consequently extender, concentration and freezing protocols were not standardized. In all cases straws were thawed in a water bath at 40°C for 20 seconds.

Semen analysis was performed under optical microscopy (Coleman, N 107, Brazil). Turbulence score (TS) varied from 0 to 5 and was evaluated by placing one drop of semen on a slide heated to 37°C and observed at 10x magnification. A drop of semen diluted to a concentration of 400 x 10^6 sperm per mL in Dulbecco's modified DMPBS Flush (PBS, Nutricell Nutrients Phones, Brazil) was deposited between the slide and cover slip warmed to 37°C and examined to assess total motility (TM), at 400x magnification. Sperm concentration was determined by cell counting with a Neubauer Chamber.

Fresh and frozen semen inclusion criteria were: TM ≥ 30% and TS ≥ 3. Inseminating dose per ewe was standardized to 200 x 10^6 total spermatozoa.

Artificial insemination and pregnancy diagnosis

Sheep were fasted (food and water) for at least 12 hours. The animals were sedated with acepromazine (0.05mg/kg) and placed in dorsal recumbency on barrows angled at 45° (Trendelenburg position). The uterine horns were visualized with a 5 mm and 30° angulated laparoscope (Karl Stroz, Tuttlingen, German). Semen was deposited

into the uterine lumen in the middle of the uterine horn using an Robertson pipet (Minitube Brasil, Porto Alegre, Brazil) (BERGSTEIN-GALAN et al., 2017).

Ultrasonographic pregnancy diagnosis was performed at 60 days after the AI procedure using a transabdominal 5mHz linear probe (DP2200 Vet, Mindray Medical International Limited, Shenzhen, China).

Statistics

Independent variables (i.e. year (from 2013 to 2017), season (Winter, Spring, Summer and Autumn), breed (Dorper, White Dorper, Texel, Cross-breed), age in months (≤12, 13-24, 25-36, 37-48, ≥49), estrus synchronization protocol (short-term or long-term), cumulative number of AIs (1 to 4) and semen preservation method (frozen or fresh)) were screened for univariate association with pregnancy rate using the Chi-square (Frequency procedure), significant level. The statistical program used was SPSS 25.0, with a significance level of 5% for all analysis.

RESULTS

Extrinsic factors

Fertility varied between the years studied, with 2016 having the lowest fertility rate. In 2013 fertility was 100%, however the number of inseminated sheep was small. Although fertility varied between years, this variation was not significant in the Chi-square test (Table 1).

Table 1- Fertility percentage, number of pregnant ewes, number of ewes inseminated, total number of ewes in the group and P value according to the year and season of the year when laparoscopic artificial insemination was performed.

Group	Category	*n*	% Fertility (Pregnant/Inseminated)	P value
Year	2017	159	53.8 (14/26)	0.3196
	2016		40.6 (37/91)	
	2015		55.5 (5/9)	
	2014		54.1 (13/24)	
	2013		100.0 (4/4)	
Season	Winter	159	36.9[b] (24/65)	0.0094
	Spring		64.2[a] (27/42)	
	Summer		54.1[a] (13/24)	
	Autumn		32.1[b] (9/28)	

Different letters indicate significant differences (a, b: Chi-square test).

Significant differences were found between seasons. The highest fertility was find in spring and summer when compared to autumn and winter (Table 1).

Intrinsic factors

The fertility variation according to the age of the sheep is shown in Figure 2. Although no significant differences were seen in the Chi-square test (P = 0.3308), the highest fertility occurred in sheep between 25 and 36 months of age (7 pregnant in 12 inseminated sheep) and fertility abruptly declined in sheep over 49 months of age (3 pregnant in 13 inseminated sheep).

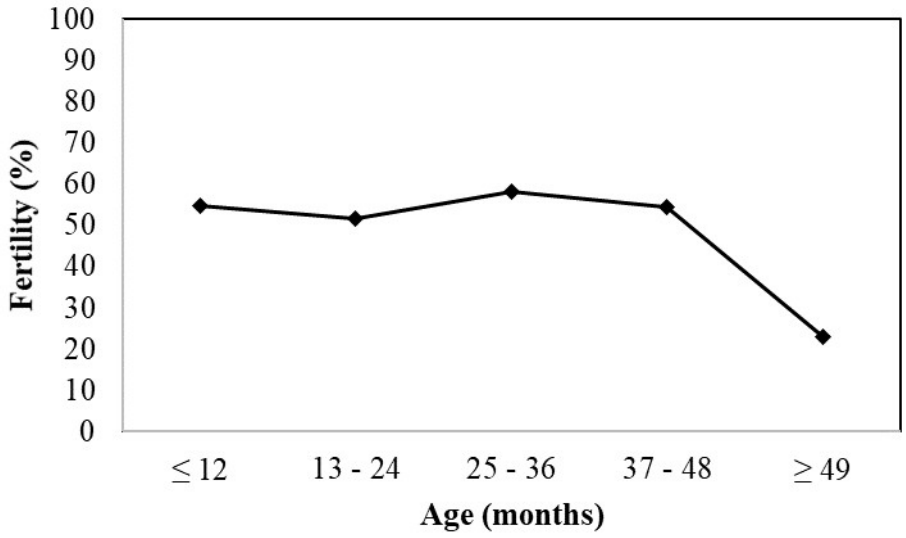

Figure 2- Fertility after artificial insemination according to sheep age.

Of the intrinsic factors studied, the sheep breed was the only one that showed significant difference (P = 0.0078) in the fertility after AI (Figure 3). Dorper sheep had the highest gestation rate (58.02%, 47 pregnant in 81 inseminated sheep) when compared to White dorper (25%, 4 pregnant in 16 inseminated sheep) and Cross-breed sheep (30.23%, 13 pregnant in 43 inseminated sheep). The fertility (47,37%, 9 pregnant in 19 inseminated sheep) of the Texel breed did not differ from the other breeds evaluated.

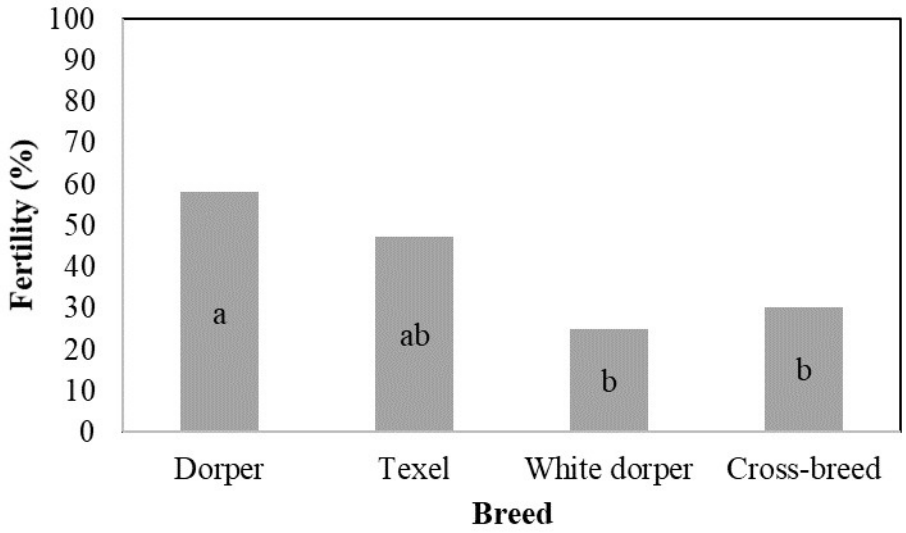

Figure 3- Fertility after artificial insemination according to the breed of the sheep. Different letters indicate significant differences (a, ab, b: Chi-square test).

Factors related to AI procedure

The cumulative number of AIs per ewe and the estrus synchronization protocol did not have a significant effect on sheep fertility after AI. However, fertility was higher (P=0.0488) in sheep inseminated with fresh semen when compared to sheep inseminated with frozen semen (Table 3).

Table 3 - Fertility percentage, number of pregnant ewes, number of ewes inseminated, total number of ewes in the group and P value according cumulative artificial insemination per ewe, synchronization protocol and sperm preservation method.

Group	Category	*n*	% Fertility (Pregnant/Inseminated)	P value
Cumulative AI per ewe	1	116	52.3 (45/86)	0.4807
	2		60.0 (12/20)	
	3		28.6 (2/7)	
	4		33.3 (1/3)	
Protocol	Short	159	60.0 (9/15)	0.2499
	Long		44.4 (64/144)	
Sperm	Frozen	159	38.3^{b} (31/81)	0.0488
	Fresh		53.8^{a} (42/78)	

Different letters indicate significant differences (a, b: Chi-square test).

DISCUSSION

To increase productivity after an AI program in sheep, factors contributing to reproductive failure should be minimized. In this study we identified the intrinsic, extrinsic and process-related factors that are related to fertility after laparoscopic AI in sheep.

Among the extrinsic factors studied, the year had no significant effect on fertility, similar results have been reported by other authors in goats (ARRÉBOLA *et al.*, 2016). However, the season influenced the fertility rate after AI. The highest gestation rates were in spring and summer and was an unexpected result since this is the non-reproductive season of sheep (PALACÍN et al., 2012). This result differs from that reported by other authors after cervical or transcervical AI in sheep (ANEL *et al.*, 2005; BUCKRELL *et al.*, 1994). According to Anel et al. (2005) the season is more significant when vaginal AI is performed as compared to AI by laparoscopy, probably due to variations in the quality of cervical mucus and consequent deficient seminal transport. Studies have reported that the protocols using progestogens and eCG are efficient in inducing ovulation of sheep in a period of anestrus (Neves, et al., 1994; Palacín et al., 2012). Arrébola *et al.* (2012) also identified a reduced probability of pregnancy when goats were inseminated in autumn and winter and associated this result with temperature. These same authors have identified, in another study, that low winter temperatures are risk factors for fertility reduction after AI in goats from Florida, USA(ARRÉBOLA *et al.*, 2016). Another hypothesis is that AI performed at the time of reduced food supply (winter and autumn), impacts gestation development because of food shortage and this factor may have influenced fertility (MACHADO E SIMPLÍCIO, 1998; ROSA et al., 2003; VIVIEN-ROELS E PÉVET, 1983).

The age of the ewes did not produce a significant difference in our study, however there was a trend to decreased fertility from 36 months of age, and sheep 49 months of age or older showed an abrupt drop in gestation rate. Aged ewes have increased risks of reproductive disorders and decreased quality of oocytes compared with young ewes(PALACÍN et al., 2012). Anel et al. (2005) reported a decrease in the lambing rate of 2.07% per year from 1.5 years of age in Churra ewes inseminated by laparoscopy. The highest gestation rate in this study was in ewes aged between 25 and 36 months, this result may be related to the fact that in this category the nutritional requirement is lower than that of younger sheep, since mature weight has already been reached.

Genetic composition can influence several productive (ELATI ET AL., 2018; KHALDARI E GHIASI, 2018; MCMANUS et al. 2016) and reproductive factors (ABECIA *et al.*, 2007; DENICOLO *et al.*, 2008) in sheep. In this study we identified that the breed

affects fertility after AI. Dorper breed had higher fertility when compared to the White Dorper and Cross-breeds. The significant difference in fertility between the Dorper and White Dorper breeds was unexpected, as these breeds come from the same origin (MILNE, 2000). According to Cloete et al. (2000) Dorper breed can be classified as intermediate with regard to its seasonal expression because Merino were found to be more seasonal, white Black-headed Persian were more aseasonal. The Dorper breed originated from the crossing of Blackhead Persian and Dorset horn breeds. Dorset horn was originated from the crossing of Leicester, Southdown and Merino breeds. The White Dorper breed originated with the crossing of Dorser Horn, Blackhead Persian and Merino in the proportion of ¼, ¼ and ½ respectively (MILNE, 2000).

The low number of White Dorper sheep used in the study may have influenced this result because it exacerbated individual variations. Another hypothesis is that because white dorper contains a greater proportion of Merino blood in their racial composition, they tend to be more seasonal when compared to dorpers, resulting in lower overall fertility. Donovan et al. (2004) also found significant fertility differences between breeds following insemination in sheep and hypothesized that the anatomy of the cervix and the timing of AI and ovulation would be the main factors associated with this difference. In our study the conformation of the cervix did not influence fertility since all inseminations were performed directly into the uterine horns. However, the timing of ovulation after implant withdrawal may have influenced the gestation rate, since the asynchrony between ovulation and AI was reported to be one of the most common causes of reproductive failure(JABBOUR E EVANS, 1991).

Studies report that sheep develop anti-eCG antibodies following the first application of this drug and that reproductive efficiency in subsequent protocols is compromised (BODIN et al., 1997; ROY et al., 1999). In this study, we did not identify a significant difference in fertility when the sheep received up to 4 hormonal protocols. Similar results were reported by Palacín et al. (2012) who associated the lack of significance of this factor with the low number of repetitions of the treatment.

The two hormonal protocols for synchronization and induction of ovulation used in this study produced similar fertility rates after AI. Similar results were reported by other authors using progesterone treatment intervals of 5 to 14 days in a multiple ovulation and embryo transfer program (MENCHACA et al., 2009) or 9 and 12 days in a laparoscopic AI program (EMSEN et al., 2011). Protocols requiring a longer period of progesterone treatment have greater synchronization of estrus and ovulation, however, the use of eCG at the time of progesterone implant withdrawal in short treatments seems

to decrease the variability at the time of ovulation (TAKADA et al., 2009). Probably the application of eCG in the two protocols used was efficient in synchronizing sheep ovulation at a time closer to the fixed time of AI 50 hours after the withdrawal of the progesterone implant.

Cryopreservation of semen causes damage to sperm cells increasing the population of non-viable sperm (JIMÉNEZ-RABADÁN et al., 2012). Although 40-60% of ram sperm remain motile after thawing, only 20-30% remains biologically intact (SALAMON e MAXWELL, 2000). Changes in the spermatozoa membranes may not affect motility but decreases cell survival and lessens or impairs fertilization potential (SALAMON e MAXWELL, 2000). In the present study the fertility of sheep after AI with frozen semen was lower ($P < 0.05$) when compared to fresh semen, it is possible that the inseminating dose of 200 x 10^6 spermatozoa was insufficient due to the reduction of sperm viability after thawing. EHLING *et al.* (2003) reported a significant difference in the rate of zygote production when sheep were inseminated by laparoscopy with an inseminating dose of 300 × 10^6 spermatozoa per ewe with fresh semen (64.7%) and frozen (52.8%) semen. In our previous studies (BERGSTEIN-GALAN et al., 2018) we did not find a significant difference in the number of embryos collected from donor ewes in a multiple ovulation and embryo transfer program when they were inseminated with fresh and frozen semen at a dose of 400 x 10^6 total spermatozoa per ewe. Based on these results we suggest that when frozen semen is used in an AI program in sheep the inseminating dose should be adjusted to at least 400 x 10^6 total spermatozoa or according to post-thaw evaluation higher than the inclusion criteria used in this study.

CONCLUSION

In conclusion, under the conditions of this study, sheep inseminated in spring and summer had a higher gestation rate. The dorper and texel sheep had higher fertility when compared to White dorper and Cross-breed sheep. The method of sperm preservation that produced highest fertility was the fresh semen. Year, age, cumulative AI procedures and synchronization protocol did not influence fertility after laparoscopic AI.

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