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Resynchronization of estrus in sheep using two or three artificial inseminations with frozen semen

Ressincronização de estro em ovinos utilizando duas ou três inseminações artificiais com sêmen congelado

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Abstract: This study aimed to evaluate the efficiency of two or three estrus synchronizations followed by artificial insemination (AI) by laparoscopy with frozen semen in sheep. Santa Inês sheep (n=147) were randomly distributed into two groups: Re-sync – 2FTAI (n=72) and Doppler – 3FTAI (n=75), synchronized with a short protocol associated with GnRH and AI by laparoscopy with frozen semen 50 h after removing the P4 device (D0). Two AIs were performed in the Re-sync group, with the start of the resynchronization protocol on D23 and gestational diagnosis on D30; the non-pregnant females were inseminated again. The sheep were resynchronized (D10) in the Doppler group and the gestation diagnosis was made early at 17 days using Doppler ultrasound. The open females were inseminated again, totaling three inseminations in 42 days. Early gestation diagnoses were confirmed at 30 days. Re-sync (29.16%) and Doppler (21.33%) were similar to each other in terms of cumulative gestation rate (P>0.05). The indicators of the non-gestation diagnosis technique at 17 days were sensitivity of 100%, specificity of 28%, positive predictive value of 21.3%, negative predictive value of 100%, and accuracy of 39.8%. Early diagnosis allowed sheep to be inseminated two to three times in the same period and was effective in diagnosing truly non-pregnant females. Therefore, estrus resynchronization associated with AI by laparoscopy using frozen semen can be applied in sheep, but a higher gestation rate could not be achieved with two or three inseminations at the end of the breeding season.

Keywords: FTAI; Doppler ultrasound; diagnosis of early gestation; laparoscopy; animal reproduction

Resumo: Objetivou-se avaliar a eficiência de duas ou três sincronizações de estro seguida da inseminação artificial (IA) por laparoscopia com sêmen congelado em ovinos. As ovelhas

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Santa Inês (n=147) foram distribuídas aleatoriamente em 2 grupos: Re-sync – 2IATF (n=72) e Doppler - 3IATF (n=75), sincronizadas com protocolo curto associado ao GnRH e IA por laparoscopia com sêmen congelado 50 horas após a retirada do dispositivo P4 (D0). No grupo Re-sync foram realizadas duas IA com início do protocolo de ressincronização em D23 e diagnóstico gestacional em D30, as fêmeas não gestantes eram inseminadas novamente. No grupo Doppler as ovelhas foram ressincronizadas (D10) e o diagnóstico de gestação realizado precocemente aos 17 dias, com auxílio da ultrassonografia Doppler. As fêmeas vazias eram inseminadas novamente, totalizando três inseminações em 42 dias. Os diagnósticos precoces de gestação foram confirmados com 30 dias. Re-sync (29,16%) e Doppler (21,33%), foram semelhantes entre si na taxa de prenhez acumulativa (P>0,05). Os indicadores da técnica de diagnóstico de não-gestação aos 17 dias foram: sensibilidade 100%, especificidade 28%, valor preditivo positivo 21,3%, valor preditivo negativo 100% e acurácia 39,8%. Com o auxílio do diagnóstico precoce as ovelhas puderam ser inseminadas de duas a três vezes no mesmo período e mostrou efetividade em diagnosticar as fêmeas verdadeiramente não gestante. Conclui-se que a ressincronização de estro associada a IA por laparoscopia utilizando sêmen congelado pode ser aplicada em ovinos, entretanto não foi possível alcançar maior taxa de prenhez com duas ou três inseminações ao final da estação de monta.

Palavras-chave: IATF; ultrassonografia doppler; diagnóstico de gestação precoce; laparoscopia; reprodução animal

1. Introduction

Synchronization of estrus in sheep is a reproductive tool that allows births to be concentrated at desirable times and facilitates the use of AI by reducing the period of estrus control and even using FTAI[1]. Improving estrus synchronization protocols is crucial to improving reproductive indices and increasing the use of AI. Therefore, estrus induction and ovulation synchronization protocols need to be improved to accurately determine the best time for AI and obtain more functional CL, with better progesterone production to maintain gestation[2].

In recent years, alternatives have been sought in terms of estrus synchronization protocols to reduce the period of identifying and synchronizing non-pregnant ewes again and allowing them to be inserted again into reproductive programs. Thus, estrus resynchronization in sheep seeks to reduce the period between inseminations. Miranda et al.[3] started another protocol with a P4 device without knowing the gestational condition, carrying out two artificial inseminations at an interval of 14 days in Texel sheep in the south of Brazil with the effect of seasonality, and obtained a 55.4% gestation rate. Another study also used a progesterone device 12 days after AI without knowing the gestational condition and observed no effect on gestation in the first service[4].

The use of early gestation diagnosis at 17 days is a possibility to identify open females early, facilitating decision-making regarding management or even resynchronization of females to be subjected to an additional service[5].

The association of the two techniques, that is, estrus resynchronization and predictive diagnosis of gestation with Doppler ultrasound at 17 days, has the main objective of reducing the interval between inseminations and also the stress caused in females, being implemented in breeding programs to increase reproductive efficiency and reduce costs. Thus, estrus resynchronization opens up opportunities to be included in hormonal resynchronization protocols in different categories of females, such as primiparous and multiparous females, achieving similar results between them in the first service rate[6].

Estrus resynchronization has been used in sheep in association with artificial insemination with fresh semen[3,4,6]. However, cryopreserved sheep semen offers advantages such as the use of rams with high genetic merit, an accelerated genetic gain in the herd in a much shorter period, convenient semen transportation, and indefinite conservation time. Considering the advantages of using frozen semen for animal genetic breeding programs, this study aimed to evaluate the efficiency of using frozen semen in two or three FTAI in estrus resynchronization protocols in sheep during a breeding season.

2. Material and methods

The experiment was conducted from January to April, the reproductive season, at the Experimental Field Sector of the Fazenda Sucupira (SCEFS) of Embrapa Genetic Resources and Biotechnology – Cenargen, located in Brasília, DF, Brazil. A total of 147 ewes and two rams of the Santa Inês breed, aged between two and five years, with an average body score of 2.5 (on a scale from 1 to 5, where 1 means very thin and 5 obese), were used. The sheep were previously evaluated for their general clinical, health, and reproductive statuses. This experiment was approved by the Animal Use Ethics Committee of Embrapa Genetic Resources and Biotechnology under approval no. CEUA/Cenargen 03/2019.

The animals were maintained on Mombaça grass (*Panicum maximum*) pasture and supplemented with a concentrate of 180 g/kg of crude protein (100 g/animal, twice a day) that started 15 days before the experiment and was maintained until its end. Mineral salt and water were available *ad libitum*.

The females were randomly separated into two experimental groups, as shown in Figure 1. Both groups, Re-sync (n=72) and Doppler-AI (n=75), were synchronized with the short hormonal protocol in which a progesterone device (P4) (Primer® Tecnopec, 36 g of progesterone) was inserted on D9 and removed seven days later (D-2). Additionally, 300 IU of eCG (Sincro eCG® Ourofino, 6,000 IU of equine chorionic gonadotropin) and 0.275 mg of cloprostenol sodium (Cioton® JA Saúde Animal, 26.3 mg of cloprostenol sodium) were applied. After removing the device (36 h, D1), 25 µg of buserelin acetate (Sincroforte® Ourofino, 0.042 mg of buserelin acetate) was applied. Artificial insemination with frozen semen was performed by laparoscopy 50 h after removing the device (D0).

The animals in the Re-sync group underwent two FTAIs over a period of 62 days, starting a new estrus synchronization at 23 days (D23) after the first FTAI, with the placement of the P4 device and the diagnosis of gestation occurring at 30 days (D30). The second FTAI occurred on D32, in open animals, with gestation diagnosis being performed on D62 (Figure 1).

On the other hand, the animals in the Doppler-AI group underwent three FTAIs over a period of 68 days. A new synchronization began 10 days (D10) after the first FTAI; an early diagnosis of non-pregnancy was made at 17 days (D17); and the ewes with a negative diagnosis continued with the estrus synchronization protocol and the second artificial insemination (D19). The ewes were subjected to a new estrus synchronization 10 days after the second FTAI (D29) and early gestation diagnosis protocol (D36). Non-pregnant females were subjected to a new AI. All animals had their gestation confirmed 30 days after AI (Figure 1).

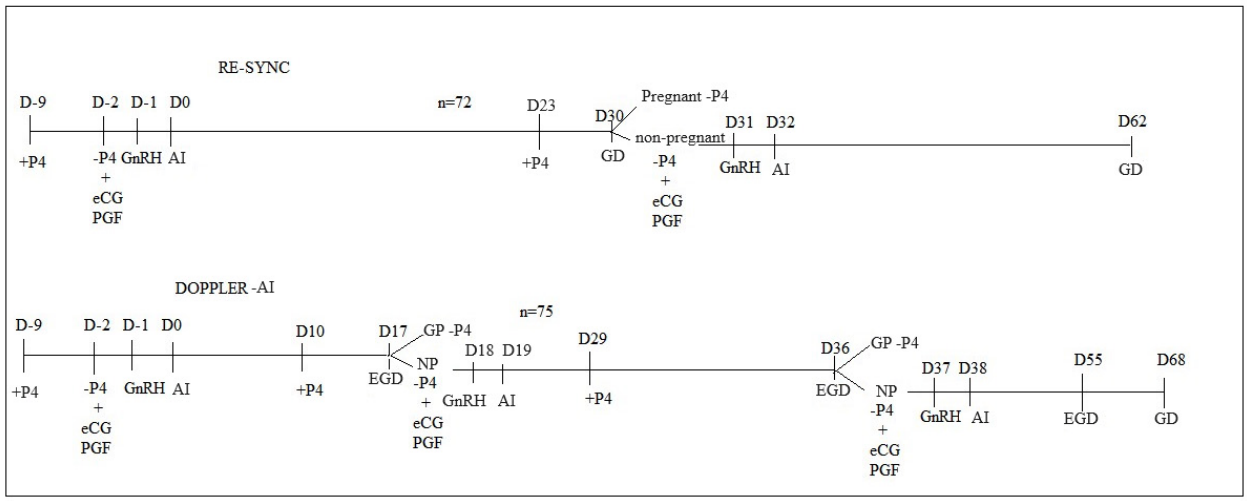

Figure 1 Scheme of experimental groups using a short estrus synchronization/resynchronization protocol: Re-sync with two artificial inseminations and Doppler-AI with three artificial inseminations. Legend: GD: gestation diagnosis; EGD: early gestation diagnosis; GP: gestation prediction; NP: non-pregnant.

Two rams were selected for freezing the semen used in FTAIs after a general clinical and andrological examination. Semen was collected using an artificial vagina and all semen samples were freshly evaluated for sperm motility, vigor, pathology, and concentration, according to the Andrological Examination Manual[7]. The semen was diluted in a medium based on Tris, citric acid, glucose, egg yolk, and glycerol, according to Evans and Maxwell[8] at a concentration of 100×10^6 spermatozoa per 0.25-ml straw, placed on top of a styrofoam and frozen using the classic method with the cooling curve at ±5 °C for two hours. Subsequently, the semen was exposed to nitrogen vapor for 20 min and the straws were dropped for total immersion in nitrogen to later be packed in racks and stored in nitrogen cylinders. The semen was thawed in a water bath at 37 °C for 30 s and was assessed both during the thawing process and through a thermoresistance test conducted two hours post-thawing, during which it was kept in the water bath at 37 °C. At both times, the samples were evaluated for sperm motility, progressive motility, and curvilinear velocity (CLV) by CASA (Computer-Assisted Semen Analyses) and plasma membrane integrity, using 6-carboxyfluorescein diacetate and propidium iodide, and acrosome integrity using a conjugation of fluorescein isothiocyanate with peanut lecithin by fluorescence microscopy. The samples were selected according to the following minimum values: sperm motility ≥ 70%, progressive motility ≥ 40%, CLV (curvilinear velocity) of motile sperms ≥ 167, IMIA/IM (intact membrane in both fluorescence techniques and intact acrosome) ≥ 30%.

Artificial inseminations by laparoscopy occurred 50 h after removal of the P4 devices. The sheep were previously prepared with a 24-h food and 12-h water fast. For inseminations, the sheep were positioned on a stretcher in the Trendelenburg position at a 45° angle with their heads down to facilitate visualization of the abdominal cavity. Shaving, antisepsis, and local anesthesia with 2% lidocaine hydrochloride were performed in the area where the trocar was positioned to access the abdominal cavity. A light source was inserted on the left side for visualization and on the right side for the AI sheath. The abdominal cavity was inflated using a nebulizer. The semen was thawed in a water bath at 37 °C for 30 s, the straw dose was fractioned and divided into two AI sheaths so that each uterine horn received half an inseminating dose. The AI sheath was positioned perpendicular to the uterine wall in the greater curvature of the uterus, where the needle was inserted, and the semen was deposited in each uterine horn. After the procedure, the trocars were removed, the abdominal cavity was deflated, and the incisions were bandaged.

The diagnosis of early gestation in the Doppler-AI group occurred 17 days after AI using an ultrasound equipped with color Doppler (MyLabvet 30 VetGold, Esaote, Genova, Italy) by a rectal probe with a 7.5 MHz frequency and 75% gain. The rectum was lubricated with mucilage before the beginning of assessments. The ovaries were visualized and the corpus luteum was identified and evaluated with Doppler, being subjectively classified according to the degree of irrigation: degree 1 (0 to 25% of the percentage of CL irrigation), 2 (25 to 50%), 3 (50 to 75%), or 4 (75 to 100%). Sheep were considered non-pregnant when they showed a percentage of CL vascularization classified as degree 1. Degrees 2, 3, and 4 were considered predictive of gestation[2]. The degrees were divided that way to determine the percentages of irrigations to predict gestation at 17 days. Gestation diagnosis at 30 days after AI occurred in both groups using a B-mode ultrasound equipped with a rectal probe at a 7.5 MHz frequency (Mindary, DP10 Vet Power, China), based on visualization of the embryonic vesicle and heartbeat.

The following parameters were used: true positive (TP) – females pregnant in the two gestation diagnoses; true negative (TN) – females non-pregnant in the two gestation diagnoses; false positive (FP) – considered predictive of gestation in the early gestation diagnosis and not pregnant at 30 days; and false negative (FN) – considered not pregnant in the early gestation diagnosis and pregnant at 30 days.

The chi-square test was used to analyze the cumulative gestation rate. The gestation rate of early diagnosis at 17 days with Doppler ultrasound was compared with the gestation rate of confirmatory diagnosis at 30 days with B-mode ultrasound using the chi-square test. The degrees of irrigation of the corpus luteum were evaluated in the early diagnosis of different inseminations and tested by the Student's T-test.

3. Results

Table 1 shows the final gestation rates of the groups. Re-sync and Doppler-AI were similar to each other (P>0.05). No difference was observed between the assessments of the predictive diagnosis of early gestation at 17 days and the gestation diagnosis reassessed at 30 days (Table 2). The indicators of the early gestation diagnosis technique at 17 days using

Doppler ultrasound were sensitivity of 100%, specificity of 28%, positive predictive value of 21.3%, negative predictive value of 100%, and accuracy of 39.8%.

Table 1 Gestation rates at 30 days after artificial insemination by laparoscopy with frozen semen, determined with B-mode ultrasound evaluated in Santa Inês sheep subjected to estrus resynchronization protocols during a breeding season

	Re-sync	Doppler-AI
1st AI	18.05% (13/72)[a]	12% (9/75)[a]
2nd AI	13.55% (8/59)[b]	12.5% (5/40)[b]
3rd AI	-	7.40% (2/27)
Cumulative rate	29.16% (21/72)[c]	21.33% (16/75)[c]

Means followed by the same letter in the row do not differ from each other (P>0.05).

Table 2 Gestation rate of the Doppler-AI group comparing early diagnosis assessments by Doppler ultrasound at 17 days, with diagnosis by B-mode ultrasound at 30 days after artificial insemination in Santa Inês sheep

Doppler	D17	D30
1st AI	46.66% (35/75)[a]	12% (9/75)[a]
2nd AI	30% (12/40)[b]	12.5% (5/40)[b]
3rd AI	18.51% (5/27)[c]	7.40% (2/27)[c]
Final rate	69.33% (52/75)[d]	21.33% (16/75)[d]

Means followed by the same letter in the row do not differ from each other (P>0.05).

The frequency of degrees of irrigation of the corpus luteum in the early gestation diagnosis at 17 days was similar (P>0.05) and is shown in Figure 2. Twenty-four semen samples were frozen but only 12 reached the standards established for selection. Table 3 shows the results of the evaluation of the selected samples.

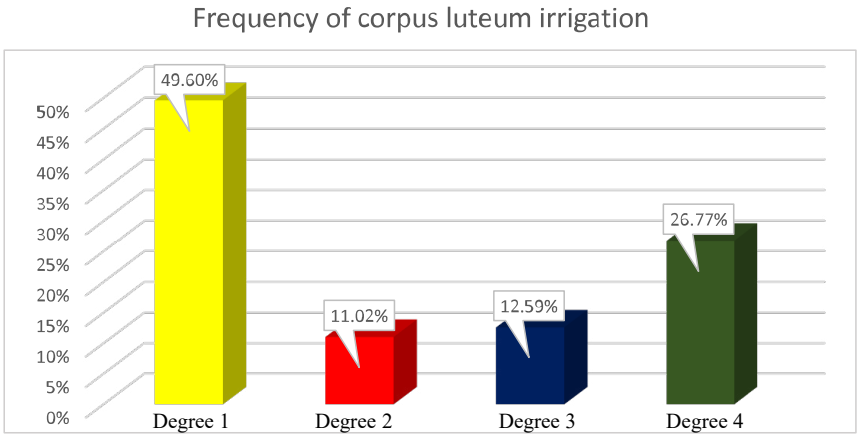

Figure 2 Frequency of degrees of corpus luteum irrigation in the diagnosis of early pregnancy using Doppler ultrasound at 17 days, in which degree 1 (0 to 25% of irrigation) indicates an empty diagnosis and degrees 2 (26 to 50%), 3 (51 to 75%), and 4 (76 to 100%) are predictive of pregnancy in Santa Ines ewes (P>0.05).

Table 3 List of selected samples of frozen semen from rams used for artificial insemination in estrus synchronization and resynchronization protocols in Santa Ines sheep. The samples were selected according to the following minimum values at TRT 0 h: sperm motility ≥ 70%, progressive motility ≥ 40%, CLV ≥ 167, IMIA/IM ≥ 30%

Sample	TRT 0 h					FLUORESCENCE 0 h				TRT 2 h					FLUORESCENCE 2 h			
	CASA			CFDA		FITC				CASA			CFDA		FITC			
	Mot	Progressive	CLV	IM	DM	IMIA	DMRA	DMIA	IMRA	Mot	Progressive	CLV	IM	DM	IMIA	DMRA	DMIA	IMRA
P5	77	45	167	43	57	42	8	49	0	63	21	90	33	66	37	12	51	0
P7	72	45	127	49	51	50	5	44	0	59	35	109	46	53	45	11	41	2
P9	71	52	183	40	60	44	6	50	0	44	33	111	37	62	39	16	42	2.5
P6	77	59	167	45	55	43	2.5	54	0	63	28	104	45	55	50	10	39	0
P14	89.5	61.8	169.82	85	15	80	2	12	6	89.3	63.3	146.5	65	35	58	0	28	14
P16	87.3	76.9	148.76	80	20	75	1	21	3	82.6	52.3	105.8	70	30	60	2	8	30
P17	81.1	63.6	116.24	67	33	57	6	33	4	57.4	29.6	110.2	57	43	47	0	25	28
P19	70.3	61.9	172.43	78	22	83	1	11	5	84.8	70.3	112.4	50	50	43	1	21	35
P20	82.4	51.3	196.98	77	23	67	1	28	4	86.4	52.8	130.5	60	40	55	3	7	35
P21	85.4	52.6	115.65	65	35	55	1	26	18	65.4	22	93.12	60	40	50	11	18	21
P22	86	66.5	140.66	50	50	47	5	35	13	76.3	51	85.53	30	70	35	1	10	50
P23	83.8	62.7	149.36	89	11	82	1	9	8	73.2	35.5	70.05	38	62	39	1	29	31

TRT – thermoresistance test at 0 h and 2 h after thawing. S – frozen semen sample. CASA – computer-assisted semen analysis. CLV – curvilinear velocity of motile spermatozoa. CFDA – 6-carboxyfluorescein diacetate and propidium iodide. FITC – fluorescein isothiocyanate conjugation with peanut lecithin. IM – intact membrane. DM – damaged membrane. IMIA – integral membrane and intact acrosome. DMRA – damaged membrane and reacted acrosome. DMIA – damaged membrane and intact acrosome. IMRA – intact membrane and reacted acrosome.

4. Discussion

This study sought to evaluate the efficiency of using frozen semen for estrus resynchronization with two or three estrus synchronizations followed by laparoscopic artificial insemination in sheep, whether or not using early diagnosis at 17 days with Doppler ultrasound. Estrus resynchronization in sheep has been an alternative to reduce the interval between inseminations in open females from the first service and increase the gestation rate at the end of the breeding season, enabling an increase in the use of artificial insemination with frozen semen. Studies found in the literature have used estrus resynchronization in sheep with superficial cervical artificial insemination with fresh semen, achieving results in cumulative gestation rates between 40 and 60%[3,5].

The groups of artificial insemination with frozen semen achieved cumulative gestation rates of 29.16% and 21.33%, respectively, in the Re-sync and Doppler-AI groups, which is below what is observed in studies using frozen semen. Rabassa et al.[9] obtained a 40% gestation rate in Corriedale females, while Cardoso[10] obtained a 61.7% rate in AI with frozen semen, demonstrating the variation in gestation rates using frozen semen in FTAI. This variation may be associated with several factors that can interfere with the results, such as season of the year, feed flushing, body condition, stressful factors, hormonal protocol, AI timing, quality of thawed semen, and individual response[11]. The low gestation rates obtained in the present study may be related to the interference of the hormonal protocol applied.

GnRH was applied 30 h after removing the P4 device to better synchronize the moment of ovulation and, therefore, optimize the gestation rate with frozen semen. The main purpose of using GnRH in estrus synchronization protocols is to synchronize ovulation, which can be advanced by 17 h compared to animals that did not receive GnRH, with ovulation occurring, on average, 57 h after removal of the P4 device[12]. Knowing the moment of ovulation is important to determine the best time for artificial insemination. In the present study, AI occurred 50 h after removal of the P4 device, suggesting that the moment of AI occurred a long time before ovulation, possibly decreasing sperm viability at the time of ovulation and, consequently, affecting the gestation rate.

Embryonic losses may have occurred during the evaluation interval, between 17 and 30 days. The occurrence of embryonic losses in sheep is approximately 17.2 to 37.7%, which can vary depending on the number of ovulations, and the causes can be the most diverse, affecting the maintenance of gestation after 12 days of mating, reaching up to 32 days[13,14]. Cosentino et al.[4] found a 20% gestation loss rate between 17 and 42 days in ewes that demonstrated estrus and were inseminated and diagnosed early with Doppler ultrasound.

In the present study, the estrus resynchronization groups received another progesterone device ten or 23 days after artificial insemination without knowing the gestational status. Similarly, Miranda et al.[3] demonstrated the possibility of estrus resynchronization and another AI without changing the gestation of the first AI. Therefore, two or three artificial inseminations were performed in the present study on non-pregnant females with estrus resynchronization starting even before gestation was diagnosed. This made it possible to carry out artificial inseminations within the same period, with the Doppler-AI group showing the possibility of three AIs within the same period as the Re-sync group (68 and 62 days, respectively), which had the possibility of two AIs.

Early diagnosis of non-gestation at 17 days resulted in a gestation rate in the 1st AI of 46.66%, the 2nd AI of 30%, and the 3rd AI of 18.51%, reaching a cumulative rate of 69.33%. However, there was a discrepancy in cumulative gestation rate when the diagnosis was confirmed by ultrasound at 30 days (21.33%), which was lower than the expected gestation rate. The accuracy of early gestation diagnosis in the present study was 39.8%, which is below the accuracy of the technique demonstrated in other studies, which reached around 87%[5,15]. Cosentino et al.[4] used estrus resynchronization with early diagnosis in sheep and obtained a gestation rate at the first AI of 46.7% (28/60), confirming the diagnosis at 42 days after AI, when a gestation rate of 58.3% (35/60) was recorded.

A 79% incidence of false positives can be observed during the evaluation of CL irrigation, which influenced the accuracy of the technique. Arashiro et al.[5] found an incidence of false positives of around 8 to 16% during the evaluation of CL irrigation with Doppler ultrasound. Corpora lutea exhibit higher volume and irrigation between the 6th and 13th day after ovulation in its young phase, and the CL volume begins to regress, and irrigation decreases from the 16th day onwards when luteolysis occurs[16]. Errors in assessing the percentage of CL irrigation may occur due to changes in the estrous cycle, such as longer estrous cycles, with CL showing good irrigation at the time of assessment. Thus, it is suggested that CLs,

when displaying good irrigation, were possibly in their young phase during the assessment at 17 days due to a long estrous cycle or late ovulation, thereby not predicting gestation and corroborating the hypothesis of inadequacy of the time of ovulation with the moment of AI.

Assessments of CL irrigation also showed a high frequency of degree one, that is, low irrigation compatible with CL in luteolysis. This observation revealed no false negative diagnosis, suggesting no errors in the interpretation of the technique in the early diagnosis of open females. It is important for the use of the estrus resynchronization protocol, as the animals will receive hormones that would lead to abortion if they were pregnant. Arashiro et al.[5] considered that the percentage of degree-one irrigation due to low or absent irrigation is more easily identified, but reported difficulty distinguishing degrees 2, 3, and 4 of CL irrigation. In the present study, we could identify the different percentages of CL irrigation, characterizing the four evaluation levels. However, no statistical difference was observed in the frequency of identification between them, with CL irrigation degrees 1 and 4 being the most easily identified.

Estrus resynchronization using FTAI by laparoscopy with frozen semen is a technique that may be applicable in sheep, but adjustments to estrus synchronization/resynchronization protocols are still necessary to obtain better gestation rates.

5. Conclusion

The use of frozen semen in estrus resynchronization did not achieve a higher percentage of gestation even using two or three artificial inseminations. However, the adopted protocols allowed sheep to be inseminated two to three times within a breeding season, which provides the opportunity to use superior animals through frozen semen. Adjustments are still needed regarding estrus synchronization/resynchronization protocols to use frozen semen, as estrus resynchronization in sheep with fresh semen increases the percentage of pregnant females. The diagnosis of early gestation in sheep was efficient in identifying effectively open females, allowing the continuation of estrus resynchronization protocols.

Declaration of conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Conceptualization: A.F. Ramos and B.D.M. Silva. *Data curation*: J. Drechmer and L.M.S. Basilio. *Formal analysis*: H.C.A. Teixeira and A.F. Ramos. *Acquisition of funding*: B.D.M. Silva. *Research*: J. Drechmer and L.M.S. Basilio. *Project administration*: J. Drechmer. *Methodology*: A.F. Ramos and B.D.M. Silva. *Resources*: A.F. Ramos and B.D.M. Silva. *Supervision*: A.F. Ramos and B.D.M. Silva. *Visualization*: J. Drechmer. *Writing (Original draft)*: J. Drechmer. *Writing (review and editing)*: J. Drechmer and B.D.M. Silva.

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