Original research paper.

Cervical artificial insemination in sheep: sperm volume and concentration using a new antiretrograde flow device

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ABSTRACT

We developed an antiretrograde flow device for the cervical artificial insemination of sheep (DARIO), which previously demonstrated suitability with refrigerated semen; an adjustment to the sperm volume and the concentration of insemination doses was needed. Our first objective was to compare the fertility rate obtained with two volumes of the same sperm concentration (volume comparison): 0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} (control group) vs. 0.50 mL, 1,600 x 10^6 spermatozoa x mL^{-1} (test 1 group). Once the sperm volume was adjusted, the second objective was to check the fertility rates when using larger volumes at lower concentrations (sperm concentration comparison): 0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} (control group) vs. 0.50 mL, 800 x 10^6 spermatozoa x mL^{-1} (test 2 group). Paired lots for farm, season and year, management, flushing, body condition, and age were randomly assigned to the control and test groups. For volume comparisons, we analyzed 462 ewes belonging to nine farms and distributed among 88 lots (equally split into the control and test 1 groups). For the sperm concentration comparison, we considered 335 ewes from eight farms distributed among 60 lots (equally divided into the control and test 2 groups). Fertility increased by using a 0.50 mL sperm doses instead of the standard 25 mL ($P = 0.041$). No significant differences ($P = 0.163$) were found when using 0.50 mL sperm doses at lower concentrations (800 x 10^6 spermatozoa x mL^{-1}). A higher number of spermatozoa/insemination, accompanied by an adequate sperm volume, improved fertility.

Keywords: Cervical artificial insemination; Antiretrograde flow device; Sheep; Fertility rate; Sperm volume; Sperm concentration.

1. Introduction

Artificial insemination (AI) is a fundamental tool for the development of sheep improvement programs (Anel et al., 2005). Cervical AI (CAI) using refrigerated semen is the most usual technique. For refrigerated semen, recommended inseminate volume and number of motile spermatozoa are 0.2 mL and 400 x 10^6, respectively (Cseh et al., 2012). Sperm concentration per insemination dose must be high compared to fresh semen, and dose volume is determined by both cervix capacity for retaining semen and minimum volume enabling suitable management (Salamon and Maxwell, 2000). The semen is deposited at the cervix entrance. The cervix complex structure is composed of numerous, irregularly-distributed, eccentric folds (Halbert et al., 1990); these typical anatomical characteristics of ewes makes both access to cervix (Kershaw et al., 2005; Kaabi et al., 2006) and a deeper deposit of the semen difficult, which results in a lower fertility (Eppleston et al., 1994; Richardson et al., 2012).

The main limiting factors of CAI are the high variability of fertility results and the specific application problems (Álvarez et al., 2019). In order to solve these problems and promote CAI, we developed a new anti-retrograde flow device for sheep insemination (DARIO; patent ES 2556215 A1; Rebollar et al., 2016). DARIO allows the deep deposit of semen into the cervix without any modification of the standard procedure and results in increased fertility and fecundity rates (Macías et al., 2017). Initially, sperm volume and concentration were similar for both DARIO and previous protocol (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1}); adjusting sperm volume and concentration of insemination doses using DARIO is needed.

Therefore, the first objective of the present study was to compare the fertility rates obtained using DARIO for CAI at two sperm volumes with the same concentration (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} vs. 0.50 mL, 1,600 x 10^6 spermatozoa x mL^{-}

[1]). Once sperm volume was adjusted, the second objective was to check fertility rates when using larger volumes at lower concentrations (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} vs. 0.50 mL, 800 x 10^6 spermatozoa x mL^{-1}). In accordance with these two objectives, the present study was comprised of two stages: the first stage (volume comparison) and the second stage (sperm concentration comparison).

2. Materials and methods

2.1. Ethical declaration

The present study complied with ARRIVE guidelines (Kilkeny et al., 2010) and was carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. This study also fulfills Spanish legislation for animal protection in experimentation and other scientific purposes, including teaching (Real Decreto 53/2013). Since this study was carried out in commercial farms during the usual routine of insemination, approval of the Animal Care and Use Committee was not necessary. Informed consent was obtained from every farm owner. The authors declare that all procedures in the experiment were conducted in ways consistent with the precepts of animal welfare; personnel involved in the caring and handle of animals were expert veterinarians.

2.2. Animals

This field test was conducted from May 2016 to July 2019 in farms located in the Aragon Autonomous Community (Northeastern Spain). The studied individuals were Rasa Aragonesa ewes inscribed in the breed genealogical book, managed by ANGRA (National Association of Rasa Aragonesa Breeders). Rasa Aragonesa is an autochthonous sheep breed, characterized by its meat aptitude. As a rustic sheep breed,

ewes show decreased sexual activity in spring (March to May), causing a 33% real fertility; maximal fertility (73%) is reached in fall-winter season (Sanz, 2019).

Estrus synchronization consisted of the intravaginal application of polyurethane polyester sponges (Chronogest CR® 20 mg, MSD Animal Health, Madrid, Spain) impregnated in flugestone acetate for 12–4 days, followed by an intramuscular dose (480 U.I.) of equine chorionic gonadotropin (PMSG; FOLIGON 6000 U.I., MSD Animal Health, Madrid, Spain) administrated at sponge withdrawal.

Lots for CAI were the experimental units. These lots were homogenous for AI technicians, management, flushing, body condition, and age; no health or production flaws occurred in any included individual. Management was comprised of two categories (grazing and housing) and also did flushing (no/yes). Body condition was subjectively scored on a 5-point-scale (Russel et al., 1969; Calavas et al., 1998).

In the first stage of this study (volume comparison), data from 462 ewes, distributed among 88 insemination lots and belonging to nine farms were analyzed. The mean size of lots was 5.25 individuals/lot (SD = 1.456 ewes/lot). Paired lots for farm, season and year, management, flushing, body condition, and age were randomly assigned to the control and test 1 group (44 lots/group). In the second stage (sperm concentration comparison), 335 ewes, distributed in 60 insemination lots and belonging to eight farms were considered (mean size = 5.58 individuals/lot; SD = 2.976 ewes/lot). Also, paired lots for season and year, management, flushing, body condition, and age were randomly assigned to the control and test 2 group (30 lots/group). Tables 1 and 2 show the characteristics of these 148 lots; as can be seen, farms 1, 6, and 8 took part in both stages of the present study but the season and year were different. Other characteristics also differed.

2.3. Sperm collection and doses elaboration

Semen doses for AI were obtained by the Agrifood Transfer Center (CTA, Movera, Spain) of the Government of Aragon. These doses came from 23 Rasa Aragonesa rams that are part of the ANGRA´s genetic selection scheme. Semen collection was carried out by using an artificial vagina with petroleum jelly as lubricant at 35–40ºC. Semen concentration was directly estimated by spectrophotometry after dilution (1:400) in a saline solution with glutaraldehyde (AccRead de IMV Technologies, HUMECO, Huesca, Spain). A graduated collector tube (mL) was used for measuring volume. Individual sperm motility was evaluated by observation with the ISAS system (Integrated semen analysis, Proiser, Paterna, Valencia, Spain). Mass sperm motility was ascertained by direct observation in optical microscope (10 x) and scored 0–5.

Packaging of semen doses differed in volume and sperm concentration for the considered study groups. For control groups (both stages of the study), semen was put into mini straws for sheeps and goats (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1}, IMV™, Instruments de Médecine Vétérinaire, L'Aigle, Francia); both volume and sperm concentration were standards for the ANGRA AI program. For test 1group (first stage of the study), volume and concentration were 0.50 mL and 1,600 x 10^6 spermatozoa x mL^{-1}, respectively. Volume was 0.50 mL and concentration was 800 x 10^6 spermatozoa x mL^{-1} for test 2 group (second stage of the study). After chilling, semen doses were stored at 15°C until insemination.

2.4. Field test

In every case, cervical AI (CAI) was carried out using an anti-retrograde flow devise for sheep insemination (DARIO, HUMECO S.L., Huesca, Spain). Both the DARIO description and use protocol were described elsewhere (Macías et al., 2017).

Only two experts and qualified technicians performed all CAI at 53 ± 1h after sponge withdrawal using a 0.25 mL AI gun for control groups and a 0.50 mL AI gun for both groups 1 and 2 (IMV^{TM}, Instruments de Médecine Vétérinaire, L'Aigle, Francia). Semen doses from every ram were distributed equally between paired groups (control and test 1 or test 2 groups). CAI data was recorded in specific files and lambing data was registered in the farms by production controls.

2.5. Statistical analysis

Fertility rates per lot were estimated using the percentage of ewes lambing in each lot after CAI. Statistical analyzes were performed using IBM SPSS statistics version 26 (2019) software (IBM, Armonk, NY, USA). Means and SD (standard deviation) were calculated for every farm and treatment group (control, test 1 group, and test 2 group). A two-way, mixed ANOVA (analysis of variance) was run; it considered a between-subject factor (farm) and a within-subject factor as having related groups (paired treatment groups: control vs. test 1 group in the first stage of the study; control vs. test 2 group in second stage of the study). There was homogeneity of variances and covariances ($P > 0.05$) as assessed by Levene´s test of homogeneity of variances and Box´s M test, respectively. P-values < 0.050 were considered to be statistically significant. When significant effects were detected among farms, pairwise comparisons with Bonferroni's correction were applied.

3. Results

Table 3 shows the results from the first stage of this study. There was no statistically significant interaction between the treatment group and the farm on fertility rate ($F(8, 35) = 1.723$, $P = 0.128$). The main effect of the treatment group showed a statistically significant difference in mean fertility rate ($F(1, 35) = 4.485$, $P = 0.041$); mean fertility rate was higher for test 1 group. The main effect of the farm showed that

174 there was a statistically significant difference in mean fertility rates among farms (F (8,
175 35) = 3.683, *P* = 0.003); mean fertility rates from farm 6 were significantly higher than
176 on farms 2 and 9 (*P* = 0.007 and *P* = 0.029, respectively), and mean fertility rate from
177 farm 7 was significantly higher than on farm 2 (*P* = 0.040).

178 The results from the second stage of this study are shown in Table 4. There was
179 no statistically significant interaction between the treatment group and farm regarding
180 fertility rates (F (6, 23) = 0.487, *P* = 0.811). No significant differences for mean fertility
181 rates were found between control and test 2 group (F (1, 23) = 2.080, *P* = 0.163) or
182 among farms (F (6, 23) = 1.939, *P* = 0.117).

183 **4. Discussion**

184 Factors, such as the farm, inseminating ram, season, managing, flushing, body
185 condition, and age can affect fertility of ewes (Tejedor et al., 2017; Munoz et al., 2019).
186 Therefore, a paired data design was applied to guarantee control and test groups to be as
187 similar as possible, except for the characteristic to compare. In addition, when lots are
188 matched within the farms (see Tables 1 and 2), any differences due to these factors
189 could be considered to be included in the differences among the farms.

190 The ram ejaculate usually ranges 0.8- 1.5 mL with 3,000–7,000 x 106
191 spermatozoa x mL-1 (Abecia and Forcada, 2010); the range of the spermatozoa number
192 per ejaculate is 2,400–10,500 x 106. Spermatozoa number per AI could influence an
193 ewes´ fertility. Langford et al. (1982) found that fertility after insemination of 400 or
194 200 x 106 spermatozoa was similar to observed after natural service, while fertility
195 greatly declined when using ≤ 100 x 106 spermatozoa per insemination. Also, Naim et
196 al. (2009) obtained higher fertility rates with 300 x 10^6 spermatozoa per insemination
197 than with 150 x 10^6 spermatozoa per insemination. In the first stage of this study, 400 x
198 10^6 and 800 x 10^6 spermatozoa per insemination were used in the control and test 1

group, respectively, while in the second stage, spermatozoa number per insemination was 400 x 10^6 in both the control and test 2 groups. Therefore, the considered number of spermatozoa per insemination was always higher than the limit set by these authors for severe decrease in fertility.

In a previous work in Rasa Aragonesa (Macías et al., 2017), we found that the fertility rate for traditional CAI with chilled semen was 0.496 ± 0.2784, while the fertility rate using DARIO for CAI was as high as 0.594 ± 0.2294 (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} in both cases). In the present work, fertility rates obtained from control groups (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1}) also reached high values for this breed, similar to the results previously described using DARIO.

In order to adjust sperm volume when using DARIO for CAI, we compared fertility rates obtained from 0.25 mL vs. 0.50 mL doses, both doses carrying 1,600 x 10^6 spermatozoa x mL^{-1}.

In natural service, semen moves through the ram penis, and upon arrival at its end, the vermiform appendix acts as a spray to place semen at the deep end of the ewe's vagina and cervix (Ferrer et al., 2012). Several works probed the positive effect of the deep semen deposit on fertility when CAI was carried out (Cameron et al., 1986; Eppleston et al., 1994; Richardson et al., 2012).

Moreover, sperm volume would be related to the ease of sperm to reach deeper regions of the female genital tract. As a fluid, the seminal dose tends to occupy the available space in the cervix, moving toward the uterus, so that the entrance hole to the cervix is blocked due to the spherical design of DARIO. In addition, the pressure exerted by the catheter and the sheep posture during insemination favors the progression of semen through the cervix. Since spermatozoa must overcome several anatomical and physiological barriers, only a reduced number of spermatozoa will reach the fertilization

point in the oviducts (Hawk, 1983). Using a larger volume of semen dose for CAI, avoiding reflux, could favor semen movement through the cervix and therefore cause an increased fertility rate (Leethongdee, 2010).

These facts could explain the higher mean fertility rate obtained in the test 1 group versus the control group. On the other hand, the spermatozoa number per insemination in the test 1 group was double (800 x 10^6 spermatozoa) that of the control group (400 x 10^6 spermatozoa), and this difference would also account for the higher fertility rate in the test 1 group.

The significant difference in mean fertility rate found among farms was due to high fertility rates in farms 6 and 7 for both the control and test 1 groups. These high values might be an explanation for the better body condition (> 3.5) and flushing in lots from both farms 6 and 7, as can be seen in Table 1.

Once it was established that a 0.50 mL dose worked better that a 0.25 mL dose, our second objective was to adjust the sperm concentration. It would be worth assessing fertility for larger sperm volumes at lower concentrations (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1} vs. 0.50 mL, 800 x 10^6 spermatozoa x mL^{-1}); perhaps less concentrated doses could be used to improve fertility rates with the consequent reduction in the dose cost.

During spermatozoa displacement through the ewe reproductive tract, several reservoirs are created in the cervix and uterine isthmus (Suarez, 1998). The gradient of spermatozoa concentration in these reservoirs seems important for reach adequate fertility; therefore, sperm doses with higher spermatozoa concentrations got better results for fertility rate (Paulenz et al., 2002). Lower spermatozoa concentrations would be expected to cause lower fertility; as shown in Table 4, mean fertility rate was lower in test 2 group, although no significant differences were found when comparing control

and the test 2 groups. The beneficial effect of a larger volume would have compensated for the possible negative effect of the lower concentration.

On the other hand, it is noteworthy that the numbers of sperm per insemination were identical in both the control and test 2 groups, and this fact would also explain for the non-detection of significant differences between both comparison groups.

5. Conclusions

When using DARIO for CAI, ewes' fertility increased by using 0.50 mL sperm doses instead of the standard 25 mL doses, both doses having 1,600 x 10^6 spermatozoa x mL^{-1}. However, no significant differences in fertility rate were found when using 0.50 mL sperm doses at lower concentrations (800 x 10^6 spermatozoa x mL^{-1}). Therefore, a higher number of spermatozoa per insemination, accompanied by an adequate sperm volume, seems to be a decisive factor for the improvement of the fertility rate; CAI by means of DARIO should use 0.5 mL sperm dose with 1,600 x 10^6 spermatozoa x mL^{-1} (800 x 10^6 spermatozoa per insemination).

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Author Contribution Statement

Angel Macías: Investigation, Data Curation, Conceptualization, Writing - Original Draft.

Elena Martín: Investigation, Writing - Review & Editing.

Adolfo Laviña: Investigation, Data Curation, Writing - Review & Editing.

Luis Miguel Ferrer: Methodology, Visualization, Writing - Review & Editing.

Iván Lidón : Methodology, Visualization, Funding acquisition, Writing - Review & Editing.

Rubén Rebollar: Methodology, Visualization, Funding acquisition, Writing - Review & Editing.

María Teresa Tejedor: Formal analysis, Writing - Original Draft, Supervision.

Declarations of interest

The Nacional Association of Rasa Aragonesa Breeders (ANGRA) is a non-profit organization that provided access to Rasa Aragonesa ewes .A. Macias, E. Martin and A. Laviña are members of the ANGRA veterinary staff and A. Macias and E. Martín carried every AIs in this study as a part of their current activities. The rest of authors disclose no potential or actual conflicts of interest related in the research presented in this manuscript.

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Table 1

Characteristics of the farms and lots where the stage 1 of the study was conducted. *n*: number of lots for cervical artificial insemination (CAI).

Farm	Season and year	*n*	Management	Flushing	Body condition	Age (yr)
1	Spring-summer 2016	8	Grazing	No	2.5-3.5	4 -6
2	Spring-summer 2016	6	Housing	No	2.5-3.5	2-4
3	Spring-summer 2016	6	Housing	No	2.5-3.5	4 -6
4	Autumn-winter 2017	8	Grazing	No	2.5-3.5	4 -6
5	Spring-summer 2016	10	Grazing	No	2.5-3.5	2-4
6	Spring-summer 2016	14	Grazing	Yes	> 3.5	2-4
7	Autumn-winter 2017	8	Housing	Yes	> 3.5	4 -6
8	Spring-summer 2016	12	Housing	No	2.5-3.5	2-4
9	Spring-summer 2016	16	Housing	No	2.5-3.5	4 -6
Total		88				

404

405 **Table 2**

406 Characteristics of the farms and lots where the stage 2 of the study was conducted. *n*:

407 number of lots for cervical artificial insemination (CAI).

408

Farm	Season and year	*n*	Management	Flushing	Body condition	Age (yr)
1	Spring-summer 2019	8	Grazing	Yes	2.5- 3.5	2-4
6	Autumn-winter 2017	12	Housing	Yes	> 3.5	4-6
8	Spring-summer 2019	6	Grazing	Yes	< 2.5	4-6
10	Spring-summer 2019	4	Grazing	No	< 2.5	4-6
11	Autumn-winter 2017	10	Housing	Yes	2.5- 3.5	2-4
12	Autumn-winter 2017	10	Grazing	No	2.5- 3.5	4-6
13	Spring-summer 2019	10	Grazing	Yes	2.5- 3.5	2-4
Total		60				

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Table 3

Fertility rate per lot and total in control group (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1}) and test 1 group (0.50 mL and 1,600 x 10^6 spermatozoa x mL^{-1}). Fertility rate: percentage of ewes lambing after CAI ; SD: standard deviation; *n*: number of lots for cervical artificial insemination (CAI).

Farm	Control group			Test 1 group		
	n	Mean	SD	*n*	Mean	SD
1	4	0.625	0.2567	4	0.584	0.0690
2	3	0.361	0.2679	3	0.309	0.1326
3	3	0.556	0.1924	3	0.439	0.2110
4	4	0.562	0.1785	4	0.819	0.1675
5	5	0.458	0.2259	5	0.743	0.2505
6	7	0.717	0.2128	7	0.857	0.1420
7	4	0.650	0.1828	4	0.873	0.0881
8	6	0.412	0.1665	6	0.736	0.1335
9	8	0.540	0.4063	8	0.437	0.1836
Total	44	0.550	0.2598	44	0.658	0.2412

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443 **Table 4**

444 Fertility rate per lot and total in control group (0.25 mL, 1,600 x 10^6 spermatozoa x mL^{-1})
445 and test 2 group (0.50 mL and 800 x 10^6 spermatozoa x mL^{-1}). Fertility rate:
446 percentage of ewes lambing after CAI ; SD: standard deviation; *n*: number of lots for
447 cervical artificial insemination (CAI).

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Farm	Control group			Test 2 group		
	n	Mean	SD	*n*	Mean	SD
1	4	0.768	0.1808	4	0.626	0.1149
6	6	0.609	0.2724	6	0.505	0.1858
8	3	0.667	0.5773	3	0.300	0.2646
10	2	0.616	0.3409	2	0.729	0.1473
11	5	0.836	0.2509	5	0.796	0.1883
12	5	0.661	0.2483	5	0.487	0.2012
13	5	0.720	0.1891	5	0.710	0.2770
Total	30	0.701	0.2679	30	0.595	0.2393

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