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

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ABSTRACT

There has been development of an antiretrograde flow device (DARIO), for sheep cervical artificial insemination (CAI). There, however, needs to be optimization of sperm volume and concentration of insemination doses when the DARIO is used for CAI. Objectives were to compare fertility rates (proportion of ewes lambing as a result of CAI) when there was use of the DARIO for CAI: two sperm volumes containing equal numbers of spermatozoa: 0.25 mL of $1,600 \times 10^6$ spermatozoa/mL and 0.50 mL of 800×10^6 spermatozoa/mL (Test 1 group), and two sperm volumes with a different number of spermatozoa/AI dose: 0.25 mL and 0.50 mL of $1,600 \times 10^6$ spermatozoa/mL (Test 2 group). There were 335 ewes from seven farms assigned to 60 batches (equally divided into a Control and Test 1 group). For the Test 2 group, 462 ewes from nine farms were assigned to 88 batches (equally proportioned into Control group and Test 2 groups). For the Test 1 group, proportion of ewes lambing as a result of CAI were 0.701 ± 0.2679 and 0.595 ± 0.2393 for the Control and Test 1 groups, respectively ($P = 0.163$). For the Test 2 group, proportions of ewes lambing were 0.550 ± 0.2598 and 0.658 ± 0.2412 for the Control and Test 2 group, respectively ($P = 0.041$). An inclusion of a larger number of spermatozoa per insemination in a 0.50 mL dose volume resulted improved proportion of ewes lambing as a result of CAI when there was used of the DARIO.

1. Introduction

Artificial insemination (AI) is a fundamental procedure for the development of sheep improvement programs (Anel et al., 2005). Inseminate volume and number of motile spermatozoa vary depending on the AI procedure (vaginal, cervical, trans-cervical intrauterine, laparoscopic intrauterine) and procedure for semen preservation (fresh, liquid, frozen). The closer the site semen is deposited to the site of fertilization, the smaller the volume of semen required for AI. Furthermore, the number of total motile spermatozoa usually needs to be greater when using frozen-thawed, as compared with cooled, semen for AI of ewes. For vaginal AI, the volume and the number of motile spermatozoa needs to be in the range of 0.3 to 0.5 mL and 300 to 400×10^6, respectively; whereas, for intra-cervical AI, smaller doses needs to be used (0.05-0.2 mL; 100-180 $\times 10^6$ motile spermatozoa) if there are to be acceptable pregnancy rates resulting from AI (Evans and Maxwell, 1987). Cervical AI (CAI) using cooled semen is the most frequently

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used technique; for cooled semen, with the recommended AI volume and number of motile spermatozoa being 0.2 mL and 400 × 10^6, respectively (Cseh et al., 2012). Trans-cervical intrauterine AI uses of 0.1 to 0.5 mL and 60 × 10^6 motile spermatozoa (liquid semen) and for laparoscopic intrauterine AI, 0.05 to 0.10 mL per uterine horn with 20 × 10^6 motile spermatozoa need to be used if there are to acceptable pregnancy rates resulting from AI (Shipley et al., 2007).

With CAI, the sperm concentration per insemination dose must be large compared to when there is use of fresh semen, and dose volume is determined by both cervix capacity for retaining semen and minimum semen volume that can be effectively utilized for AI (Salamon and Maxwell, 2000). The semen is deposited at the cervix entrance. The complex cervical structure results from numerous, irregularly-distributed, eccentric folds (Halbert et al., 1990). These typical anatomical characteristics makes access to the cervix difficult (Kershaw et al., 2005; Kaabi et al., 2006) and are significant barriers when attempting to deposit the semen within the cervix or uterine horns, which generally leads to lesser pregnancy rates when there is AI of ewes at these locations in the reproductive tract (Eppleston et al., 1994; Richardson et al., 2012).

The main limiting factors of CAI is the large variability in fertility results and the specific application problems when there is use of this technique for AI of ewes (Álvarez et al., 2019). To address these problems and further develop the CAI procedure as an effective approach for AI of ewes, there was development of a new anti-retrograde flow device for sheep insemination (DARIO; patent ES 2556215 A1; Rebollar et al., 2016). The DARIO allows for the deposition of semen into the cervix without any modification of the conventional procedure for cervical insemination of ewes and with use of the DARIO there are resulting greater fertility and fecundity rates (Macías et al., 2017).

The volume and concentration of sperm have been the same for CAI of ewes when there was utilization of the DARIO and with the conventional CAI procedure (0.25 mL, 1,600 × 10^6 spermatozoa/mL). Because of the more desirable fertility results with use of the DARIO than the conventional procedure for ewe CAI, adjustments to the sperm volume and concentration should be evaluated with use of mini straws for semen storage. Semen doses for CAI can be obtained from the Agrifood Transfer Center (CTA, Movera, Spain) of the Government of Aragon which has the capacity to supply double-volume mini straws (0.50 mL).

The objective of this study was twofold: (1) to compare the fertility rates when there was use DARIO for CAI at two sperm volumes with the same number of final spermatozoa being 0.25 mL of 1,600 × 10^6 and 0.50 mL of 800 × 10^6 spermatozoa/mL (Test 1 group), and (2) to compare the fertility rates when there was use of DARIO for CAI at two sperm volumes with different numbers of spermatozoa in the semen dose: 0.25 mL of 1,600 × 10^6 and 0.50 mL of 1,600 × 10^6 spermatozoa/ mL (Test 2 group).

2. Materials and methods

2.1. Ethical declaration

The methods used in the present study complied with ARRIVE guidelines (Kilkeny et al., 2010) and were conducted using approaches consistent 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 complies with the Spanish legislation for animal protection in experimentation and other scientific purposes, including teaching (Real Decreto 53/2013). Because this study was conducted on commercial farms during the usual routine of insemination period, approval of the Animal Care and Use Committee was not necessary. Informed consent, however, 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; with personnel involved in the caring and handling of animals being licensed veterinarians.

2.2. Animals and experimental design

This field study was conducted from May 2016 to July 2019 in farms located in the Aragon Autonomous Community (Northeastern Spain). The experimental animals 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, developed for meat production. As a rustic sheep breed, ewes have lesser reproductive functions during the spring (March to May) and pregnancy rates are only 33% as a result of natural mating during this period, while pregnancy rates are maximal (73%) in the autumn/winter season (Sanz, 2019).

The estrous synchronization treatment regimen that was used consisted of the intravaginal application of polyurethane polyester sponges (Chronogest CR® 20 mg, MSD Animal Health, Madrid, Spain) impregnated in flugestone acetate for 12 to 14 days, followed by an intramuscular administration (480 U.I.) of equine chorionic gonadotropin (PMSG; FOLIGON 6000 U.I., MSD Animal Health, Madrid, Spain) administrated at the time of sponge withdrawal.

Batches of ewes used for CAI were the experimental units. For these batches, there were homogenous uses of AI technicians, management, flushing, body condition, and age; no health or production problems occurring with any ewe included in the experimental groups. Management was comprised of two categories (grazing and housing) and also there was or was not flushing (no/yes). Body condition was subjectively scored on a 5-point-scale (Russel et al., 1969; Calavas et al., 1998).

For the Test 1 group, there were 335 ewes assigned to 60 insemination batches on seven farms (mean size = 5.58 individuals/batch; SD = 2.976 ewes/batch). Paired batches on each farm, season and year, management type, flushing, body condition, age and ram were randomly assigned to the Control and Test 1 group (30 batches/group). For the Test 2 group, data from 462 ewes, distributed among 88 insemination batches and from nine farms were analyzed. The mean size of batches was 5.25 individuals/batch (SD = 1.456 ewes/batch). Paired batches for farm, season and year, management, flushing, body condition, age and ram were

Table 1
Characteristics of the farms and batches of ewes where Test1 1 was conducted; *n*: number of batches of ewes used for cervical artificial insemination (CAI) using the DARIO

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

randomly assigned to the control group and Test 2 group (44 batches/group). In Tables 1 and 2 the characteristics of the ewes in these 148 batches are described. For Farms 1, 6, and 8, there were ewes included in both Test 1 and 2 groups, but paired batches were different for Test 1 and 2 groups on these farms.

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 program. Ram ages ranged from 1 to 6 years. Semen collection was conducted by using an artificial vagina with petroleum jelly as lubricant at 35 to 40 °C. Once per month, the quality of semen based on sperm motility was evaluated using the CASA system (Computer Assisted Sperm Analysis System, Integrated Semen Analysis, ISAS, version 1.0.16, Proiser, Paterna, Valencia Spain). Negative phase contrast was configured to analyze at 25 frames per second, 10x Nikon scale. Parameter settings were as following: 3 to 70μm^2 particle area; slow spermatozoa: > 10 μm/s; intermediate spermatozoa: > 45μm/s; fast spermatozoa > 75μm/s; progressive spermatozoa: straightness (STR): 80%; amplitude of lateral head displacement (ALH): 10. A graduated collector tube (mL) was used for measuring volume. Semen concentration was directly estimated using spectrophotometry procedures after dilution (1:400) in a saline solution with glutaraldehyde (AccRead de IMV Technologies, HUMECO, Huesca, Spain). Mass sperm motility was ascertained by direct observation in an optical microscope (10 x) and scored from 0 to 5. The semen from every ram had values that were equal to or greater than the minimum values for semen characteristics: volume ≥ 0.5 mL, sperm concentration ≥ 3×10^9 spermatozoa/mL and sperm motility ≥ 80%. Supplementary File 1 contains the data for mean, SE, minimum and maximum for the available variables of sperm quality, measured monthly in the 23 rams for a 12 month experimental period: Volume (cc), Masal Motility (0-5), Concentration (x10^3 spermatozoa/mL), Static spermatozoa (%),Non-progressive mobile spermatozoa (%), Progressive mobile spermatozoa (%), Total motile spermatozoa (%), Fast spermatozoa (%), Intermediate spermatozoa (%) and Slow spermatozoa (%).

The extender used to process the semen was INRA 96 (IMV, Technologies, L´Aigle, France); it contains the purified fraction of milk cellular proteins, antibiotics (penicillin and gentamicin) and fungicide (amphotericin B). The efficacy of this extender for cooling ram semen was ascertained by O´Hara et al. (2010). Packaging of semen doses differed in volume and sperm concentration for the experimental groups. For Control groups (both stages of the study), semen was placed in mini straws used for storage of ram and goat semen (0.25 mL, $1{,}600 \times 10^6$ spermatozoa/mL, IMV^{TM}, Instruments de Médecine Vétérinaire, L'Aigle, France); both volume and sperm concentration were those routinely used for the ANGRA AI program. For the Test 1 group, volume and concentration were 0.50 mL and 800×10^6 spermatozoa/mL, respectively. Volume was 0.50 mL and the concentration was $1{,}600 \times 10^6$ spermatozoa/mL for the Test 2 group. After cooling, semen doses were stored at 15 °C until insemination (3-5 hours after dose preparation).

Table 2
Characteristics of the farms and batches where Test 2 was conducted; *n*: number of batches of ewes used for cervical artificial insemination (CAI) using the DARIO

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	14	Grazing	Yes	> 3.5	2-4
3	Spring-summer 2016	12	Housing	No	2.5-3.5	2-4
8	Spring-summer 2016	6	Housing	No	2.5-3.5	2-4
9	Spring-summer 2016	6	Housing	No	2.5-3.5	4-6
10	Autumn-winter 2017	8	Grazing	No	2.5-3.5	4-6
11	Spring-summer 2016	10	Grazing	No	2.5-3.5	2-4
12	Autumn-winter 2017	8	Housing	Yes	> 3.5	4-6
13	Spring-summer 2016	16	Housing	No	2.5-3.5	4-6
Total		88				

2.4. DARIO field test using different insemination volumes and sperm concentrations

For all insemination procedures, cervical AI (CAI) was conducted using the DARIO, an anti-retrograde flow device for sheep insemination (HUMECO S.L., Huesca, Spain). The DARIO has a tip for the CAI catheter that is in the shape of a hemispherical body with a blunt tip, provided with three holes for semen deposition in the cervix and a canal for the conventional catheter used for CAI. The hemispherical body blocks the cervical os and the lateral recess makes it easier for the visual detection of the cervix by the technician. Only two experts and qualified technicians performed all CAI at 53 ± 1 h after sponge withdrawal using a 0.25 mL AI device for insemination of ewes of the Control groups and a 0.50 mL AI device for insemination of ewes of both Test 1 and 2 groups (IMV^{TM}, Instruments de Médecine Vétérinaire, L'Aigle, France). With a slight pressure, the DARIO was placed onto the catheter top. For CAI, the ewe must be gently restrained with the ewes hindquarters elevated over a bar. To locate the cervix a speculum with light was used, the device was then inserted as far as possible into the reproductive tract until there was no more cranial insertion progression to avoid causing injuries to the ewe. The DARIO was placed in contact with the cervix on the outside by light pressure, to block the cervix and avoid semen reflux. The semen was subsequently deposited slowly into the reproductive tract. A detailed description and a protocol for proper use the DARIO can be found in Macias et al. (2017).

Semen doses from every ram were distributed equally between paired groups (Control, Test 1 group or Test 2 group). The CAI data were recorded in specific files and lambing data were recorded at the farms as part of the production records.

2.5. Statistical analysis

Fertility rates per batch were estimated using the proportion of ewes lambing in each batch as a result of CAI. Statistical analyses 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, and Test 2 groups). In addition, the mean and SD for each farm was calculated globally, without considering the treatment groups. The Shapiro –Wilk test was applied to detect departures for normality of fertility rates. Significant departures for normality ($P < 0.05$) were detected for 2/14 farm x treatment group cells in the Test 1 and 3/18 farm x treatment group cells in Test 2 groups. The ANOVA is considered a robust test for assessing the normality assumption. In the few cases where there were departures from normality of data distribution in the present study the efficacy of use of the ANOVA provided for further validation of the use of these techniques for this purpose. A two-way, mixed ANOVA (analysis of variance) was performed with consideration of between-subject factor (farm, fixed effect) and a within-subject factor as having related groups (paired treatment groups: Control compared with Test 1 group; Control compared with Test 2 group). There was homogeneity of variances and covariances ($P > 0.05$) as assessed using the Levene´s test of homogeneity of variances and Box´s M test, respectively. When there were P-values < 0.050, there were considered to be mean differences. When significant effects were detected among farms, pairwise comparisons with the Bonferroni's correction were applied.

3. Results

The data for comparisons of the DARIO in Control and Test 1 groups are included in Table 3. There was no interaction between the farm and the treatment group regarding fertility rates ($F(6, 23) = 0.487$, $P = 0.811$). There were no differences for proportion of ewes lambing as a result of CAI using the DARIO between the Control and Test 1 groups [$F(1, 23) = 2.080$, $P = 0.163$] or among farms [$F(6, 23) = 1.939$, $P = 0.117$].

Data for comparisons of the results from the Control and Test 2 groups are provided in Table 4. There was no interaction between the farm and the treatment group on proportion of ewes lambing as a result of CAI using the DARIO [$F(8, 35) = 1.723$, $P = 0.128$]. There was a group effect for fertility rate when these comparisons were made [$F(1, 35) = 4.485$, $P = 0.041$]; mean proportion of ewes lambing as a result of CAI using the DARIO was greater in the ewes of the Test 2 as compared with the Control group. There was also an effect of farm on fertility rates [$F(8, 35) = 3.683$, $P = 0.003$]; with data included in Table 4 indicating in detail these differences.

Table 3
Proportions of ewes lambing of those for which cervical artificial insemination (CAI) was imposed using the DARIO per batch, per farm and total number of ewes in the Control group (0.25 mL, 1,600 × 10^6 spermatozoa/mL) and Test 1 group (0.50 mL and 800 × 10^6 spermatozoa/ mL); SD: standard deviation; *n*: number of batches of ewes for cervical artificial insemination (CAI).

Farm	Control group			Test 1 group			Total/farm		
	n	Mean	SD	*n*	Mean	SD	*N*	Mean	SD
1	4	0.768	0.1808	4	0.626	0.1149	8	0.697	0.0846
2	6	0.609	0.2724	6	0.505	0.1858	12	0.557	0.0691
3	3	0.667	0.5773	3	0.300	0.2646	6	0.483	0.0977
4	2	0.616	0.3409	2	0.729	0.1473	4	0.673	0.1196
5	5	0.836	0.2509	5	0.796	0.1883	10	0.816	0.0757
6	5	0.661	0.2483	5	0.487	0.2012	10	0.574	0.0757
7	5	0.720	0.1891	5	0.710	0.2770	10	0.715	0.0757
Total	30	0.701	0.2679	30	0.595	0.2393			

Table 4
Proportions of ewes lambing of those for which cervical artificial insemination (CAI) was imposed using the DARIO per batch, per farm and total in Control group (0.25 mL, 1,600 × 10^6 spermatozoa/mL) and test 2 group (0.50 mL and 1,600 × 10^6 spermatozoa/ mL); SD: standard deviation; *n*: number of batches of ewes for CAI; [a,b] Different letters in the same column indicate mean differences ($P < 0.050$); [A,B] Different letters in the same row indicate mean differences ($P < 0.050$)

Farm	Control group			Test 2 group			Total/farm		
	n	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
1	4	0.625	0.2567	4	0.584	0.0690	8	$0.605^{a,b}$	0.0785
2	7	0.717	0.2128	7	0.857	0.1420	14	0.787^{b}	0.0593
3	6	0.412	0.1665	6	0.736	0.1335	12	$0.574^{a,b}$	0.0641
8	3	0.361	0.2679	3	0.309	0.1326	6	0.335^{a}	0.0906
9	3	0.556	0.1924	3	0.439	0.2110	6	$0.497^{a,b}$	0.0906
10	4	0.562	0.1785	4	0.819	0.1675	8	0.690^{b}	0.0785
11	5	0.458	0.2259	5	0.743	0.2505	10	$0.600^{a,b}$	0.0702
12	4	0.650	0.1828	4	0.873	0.0881	8	0.761^{b}	0.0785
13	8	0.540	0.4063	8	0.437	0.1836	16	$0.489^{a,b}$	0.0555
Total	44	0.550^{A}	0.2598	44	0.658^{B}	0.2412			

4. Discussion

Factors, such as the farm, which rams semen was used for insemination, season, management of ewes, flushing, body condition, and age can affect fertility of ewes (Tejedor et al., 2016; Munoz et al., 2019). A paired data experimental design, therefore, was applied to ensure that the Control and Test groups were as similar as possible, except for the variable for which the study was conducted to compare. In addition, when batches were matched within the farms (see Tables 1 and 2), any differences due to these factors could be considered to be included in the differences among farms.

In a previous investigation in Rasa Aragonesa (Macías et al., 2017), proportion of ewes lambing when there was use of conventional procedures for CAI with cooled semen was 0.496 ± 0.2784, while the fertility rate using the DARIO for CAI was as great as 0.594 ± 0.2294 (0.25 mL, 1,600 × 10^6 spermatozoa/mL in both cases). In the present study, proportion of ewes lambing as a result of CAI using the DARIO in the Control groups (0.25 mL, 1,600 × 10^6 spermatozoa/mL) were also very acceptable for ewes of this breed, similar to the results previously reported when there was use of the DARIO for ewe cervical inseminations.

There were no differences in proportion of ewes lambing as a result of CAI using the DARIO between ewes of the Control and Test 1 group. Semen doses used to inseminate ewes of the Control and Test 1 groups contained the same total number of spermatozoa, however, differed in both volume (0.25 mL and 0.50 mL, respectively) and sperm concentration (1,600 × 10^9 and 800 × 10^9 spermatozoa/mL, respectively). The proportion of ewes lambing as a result of CAI using the DARIO was greater in ewes of the Test 2 group compared to Control group; where there was a larger number of total spermatozoa used in doses of semen used to inseminate ewes of the Test 2 group, with there being a larger volume (0.50 ml) and the same sperm concentration as that of the Control group (1,600 × 10^9 spermatozoa/mL). These results indicate there is an association between proportion of ewes lambing as a result of CAI using the DARIO and the total number of spermatozoa rather than with the sperm volume used for CAI. The most effective way to increase the number of spermatozoa per dose of semen used for CAI is using a larger volume of semen that has an adequate sperm concentration. The greater proportion of ewes lambing as a result of CAI using the DARIO of ewes in the Test 2 group might be explained by using both a suitable concentration and volume sperm in an AI semen dose.

The ram ejaculate usually ranges from 0.8 to 1.5 mL with 3,000 to 7,000 × 10^6 spermatozoa/mL of semen (Abecia and Forcada, 2010); the range of the number of spermatozoa per ejaculate is 2,400 to 10,500 × 10^6. Greater total numbers of spermatozoa result when there are larger volumes of an ejaculate. The number of spermatozoa per AI can affect ewe fertility. Langford and Marcus (1982) reported that fertility after insemination of 400 or 200 × 10^6 spermatozoa was similar to that when there is natural mating, while fertility was markedly decreased when using ≤ 100 × 10^6 spermatozoa per insemination. With ewes in the Test 1 group, number of spermatozoa per insemination was 400 × 10^6 in both the Control and Test 1 groups while in with ewes of the Test 2 and the respective control group, there were 400 × 10^6 and 800 × 10^6 spermatozoa per insemination used for CAI of the ewes of the Control and Test 2 groups, respectively. The number of spermatozoa used per CAI, therefore, was always larger than the minimum that were considered to have a marked negative effect on ewe fertility. Naim et al. (2009) reported that there were greater fertility rates when there was insemination with 300 × 10^6 than 150 × 10^6 spermatozoa per insemination. In the present study, there was a greater proportion of ewes lambing as a result of CAI using the DARIO when there was a doubling of the number of spermatozoa in a 0.5 mL dose used for CAI.

With natural mating, semen is transported through the ram penis, and upon arrival at its end, the vermiform appendix functions to distribute the semen as a spraying motion so that there is deposition of the semen in the lumen at the cranial end of the vaginal and caudal end of the cervix (Ferrer et al., 2012). From several studies, it has been reported that there is a positive effect of semen deposition at the cranial vaginal-caudal cervical interface on fertility when CAI was conducted (Cameron et al., 1986; Eppleston et al., 1994; Richardson et al., 2012). Furthermore, sperm volume could be related to the capacity for transport of sperm to the site of fertilization in the oviduct. Because of the fluid consistency of semen, the seminal dose tends to occupy the available space in the cervix, with the direction of semen flow being toward the uterus. In addition, the pressure exerted by the catheter and the sheep

posture during insemination favors the transport of semen through the cervix. Because spermatozoa must be transported through several anatomical and physiological barriers that are present in the lumen of the reproductive tract from the site of semen deposition to the site where fertilization occurs, only a small number of spermatozoa reach the site of fertilization in the oviducts (Hawk, 1983). Using a larger volume semen dose for CAI, and avoiding the reflux of the semen that occurs with use of conventional AI procedures, could favor semen transport through the cervix and, therefore, result in a greater fertility rate (Leethongdee, 2010).

Furthermore, during sperm transport through the ewes' reproductive tract, several reservoirs are created in the cervix and uterine isthmus; the gradient of spermatozoa concentration in these reservoirs are apparently important for fertility at desirable rates (Suarez, 1998). Sperm doses with in which there are larger spermatozoa concentrations can help ensure that there are greater fertility rates when there is use of AI in sheep production enterprises (Paulenz et al., 2002).

At Farms 2 and 12, however, there were greater proportions of ewes lambing as a result of CAI using the DARIO for both the ewes of the Control and Test 2 groups when the mean rates for each farm were calculated globally, without considering the treatment groups. These greater values might be explained by flushing and a greater body condition (> 3.5) in batches of ewes from both Farms 2 and 12, as indicated by the data provided in Table 2. The benefits of flushing and body condition on pregnancy rates of ewes have been ascertained previously by Tejedor et al. (2016) and Munoz et al. (2019).

5. Conclusions

When using DARIO for CAI, there were no differences in proportion of ewes lambing as a result of CAI when using 0.50 mL sperm doses at the lesser sperm concentrations (800×10^6 spermatozoa/mL). The proportion of ewes lambing as a result of CAI using the DARIO, however, was greater when there was use of 0.50 mL sperm doses instead of the 0.25 mL doses conventionally used for AI of ewes, when both doses contained $1{,}600 \times 10^6$ spermatozoa/mL. A larger number of spermatozoa per insemination in a 0.50 ml dose volume apparently is an important factor for the improvement of the proportion of ewes lambing as a result of CAI using the DARIO. When there is CAI with use of the DARIO, therefore, there should use a 0.5 mL sperm dose with $1{,}600 \times 10^6$ spermatozoa/mL (800×10^6 spermatozoa per insemination).

CRediT authorship 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 competing 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. R. Rebollar, I. Lidón, L.M. Ferrer and A. Macías are the inventors of DARIO, whose patent is now owned by the Consorcio Mercantil de Huesca, Humeco, S.L.

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