Technical Note: A new device for cervical insemination of sheep – design and field test[1]

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ABSTRACT: Deep semen deposition, avoiding retrograde flow, lesions and stress, has proved to be very important in the success of sheep AI. The objective of the present study was to develop a new, suitable anti-retrograde flow device for sheep cervical AI (DARIO) that enables deep deposition of semen into the cervix without any modifications to the procedures currently used, and to compare the fertility, fecundity, and prolificacy rates between DARIO and a traditional catheter. Field tests were performed on 16 farms actively participating in the non-profit National Association of Rasa Aragonesa Breeders´ genetic selection scheme and where sheep management was similar. A total of 242 AI lots were considered, including 1,299 ewes; 126 lots (662 ewes) were inseminated using DARIO, and 116 lots (637 ewes) using a traditional commercially-available catheter (control group). Several factors affecting AI results were included in the model for mean comparison between DARIO and control groups (farm and ram as random factors; catheter, year and photoperiod as fixed effects; catheter × photoperiod interaction). The type of catheter had a significant effect on fertility ($P < 0.01$) and fecundity rates ($P < 0.01$) but no significant effect was detected on the prolificacy rate ($P = 0.45$). For fertility rate (percentage of ewes lambing after AI), means ± SE for DARIO and control groups were 59.44 ± 2.13% and 49.60 ± 2.48%, respectively; for fecundity rates, means ± SE for DARIO and control groups were 0.99 ± 0.04 and 0.82 ± 0.05 lambs/inseminated ewe, respectively, and, for prolificacy rates, means ± SE for DARIO and control groups were 1.68 ± 0.04 and 1.63 ± 0.04 lambs/ewe lambing, respectively. Fertility rate was greater in the decreasing photoperiod ($P = 0.01$). Significant effects were found for both year ($P < 0.05$) and farm ($P < 0.01$) on fertility, fecundity, and prolificacy rates. Neither ram nor catheter × photoperiod showed any significant effects on the variables investigated ($P > 0.05$). Overall, the use of DARIO instead of the traditional commercially-available catheter increased both fertility and fecundity rates; the marginal mean differences were 9.05 pregnant ewes per 100 inseminated and 0.15 lambs per inseminated ewe, respectively.

Key words: artificial insemination, cervix, fecundity, fertility, prolificacy, ovine

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INTRODUCTION

Technical difficulties, general management, low value per animal (Foote, 2002), and irregular fertility (Anel et al., 2006) explain why AI is not a common technique in sheep production (FAO Assessments, 2015). Widespread demand for laparoscopic intrauterine AI (**IUAI**) is limited due to welfare concerns, high economic cost, and the need for experienced technicians (Evans and Maxwell, 1987); however, fertility and lambing rates compared to those of natural service are only achieved by this technique (Salamon and Maxwell,

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1995). In transcervical AI (**TCAI**), the catheter is passed across the cervix to deposit the semen into the uterus; the conception rate averages between 40 and 60% (Anel et al., 2005). The ewe's cervix is a fibrous and tubular structure with several folds that cause the catheter to be misdirected from the central lumen, and inserted no more than 1 cm into the cervical canal (Kershaw et al., 2005). Low fertility rates for TCAI were associated with lesions in the uterus and cervix (Campbell et al., 1996); therefore, several curved tips were developed (Anel et al., 2006), and treatments for relaxing the cervix were used (Robinson et al., 2011). However, this approach makes the AI procedure slower, creates discomfort and generates stress with the consequent liberation of cortisol and other corticosteroids negatively affecting reproduction (Charmandari et al., 2005). In cervical AI (**CAI**), the semen is deposited at the deepest point into the first fold without using force to penetrate the cervical canal (King et al., 2004). Preventing retrograde flow and deposition of semen as deep as possible were associated with greater fertility rates (Leethongdee, 2010). Our objective was to develop a new, suitable anti-retrograde flow device for CAI in sheep, that enables deep deposition of semen into the cervix without any modifications of the procedures currently used, and to compare fertility, fecundity, and prolificacy rates between the new and traditional CAI devices.

MATERIALS AND METHODS

This study was conducted on commercial farms and the personnel involved were veterinarians with relevant expertise in the caring and handling of animals. All the farmers volunteered to participate in the field test. Approval of the Animal care and use committee was not necessary for this study. This study fulfills Spanish legislation for animal protection in experimentation and other scientific purposes, including teaching (Real Decreto 53/2013; Real Decreto, 2013).

Device Design

The new catheter would allow a slight penetration and block the cervix os. The first prototype was designed by high-definition 3D. A post mortem check was performed on the cervix of the Rasa Aragonesa ewes before in vivo tests. This new catheter tapers conically toward a blunt, soft tip, where one central hole and 2 lateral holes open; the lateral holes enable semen deposition even when the central hole makes contact with the cervical folds and becomes ineffective. The hemispherical body shows a flattening or lateral recess, which makes it easier for the technician to see the cervix; also, this hemispherical body blocks the cervical os so as to decrease retrograde flow (Fig. 1). The interior canal allows for the mounting of the catheter currently used for sheep CAI. The connection is strong enough to avoid the accidental detachment of the device. This new device is disposable and is made of Forprene 6NM901D40 (SO.F.TER. SPA, Forlì, Italy), a flexible polymer with a very soft touch finish, to prevent injuries and enable a better adaptation to the entrance of the cervix. The device is placed onto the current catheter top. The industrial property of this new device has been protected by patent ES 2556215 A1 (Rebollar et al., 2016). This Anti-Retrograde flow Device for Sheep Insemination (**DARIO**: Dispositivo Anti Reflujo para Inseminación Ovina in Spanish) is currently marketed under the trade name DARIO by Consorcio Mercantil de Huesca, S.L. (Huesca, Spain). The use of DARIO increases the cost of AI materials by only 4.31% per ewe (cost estimated from our own data, based on current market prices of hormonal treatments, applicators, batteries, lubricant, alcohol, antibiotics, disinfectant, and DARIO).

Field Test

Field tests were performed from 2013 to 2016 on 16 farms actively participating in the non-profit National Association of Rasa Aragonesa Breeders (**ANGRA**) genetic selection scheme and where sheep management was similar. For this study, we chose homogenous lots (experimental units) comprising approximately 5 Rasa Aragonesa ewes per lot, 2 to 5 yr old, with adequate body condition, without any health or production flaws, and whose lambing-AI interval was > 60 d. A total of 242 AI lots were considered, including 1,299 ewes; 126 lots (662 ewes) were inseminated using DARIO (DARIO group), and 116 lots (637 ewes) using a traditional and commercially-available catheter (control group). A randomized-block approach was used. Therefore, in each farm the number of lots in the DARIO group was almost equal to the number of lots in the control group.

Three AI technicians participated in this research; most AIs were performed by Technician 1 (190/242 lots; 78.51%), while Technicians 2 and 3 inseminated only 32/242 and 20/242 lots (13.22% and 8.26%), respectively. The randomized-block approach ensured that half of the lots assigned to each technician were inseminated using DARIO. These technicians were ANGRA veterinarians with many years of appropriate experience in sheep AI who had obtained high levels of reproductive success using a commercially available catheter. The ANGRA provided access to ovine AI using DARIO and the control catheter. Given that ANGRA does not commercialize any AI instruments and salaries of these technicians are independent of the results of this study, any bias can be disregarded.

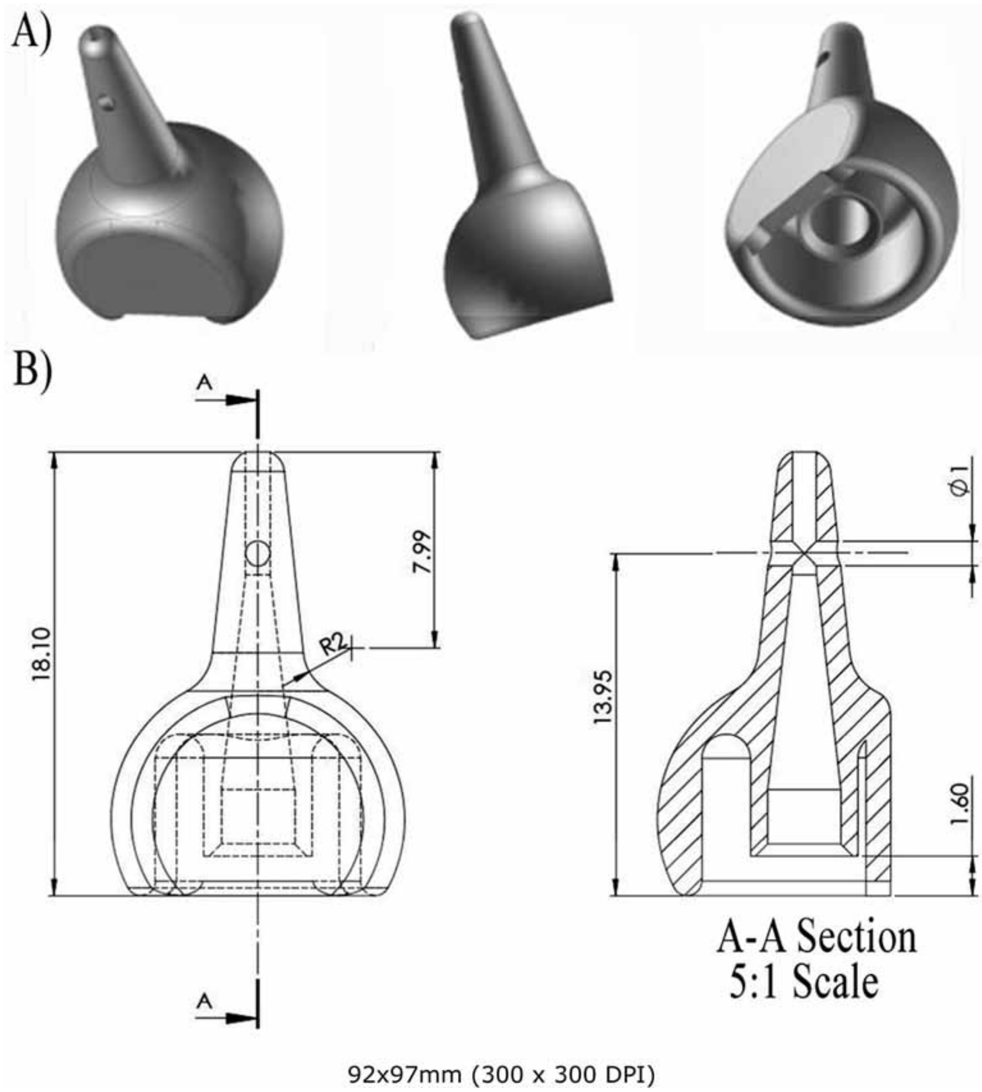

Figure 1. Description of the anti-retrograde flow device for sheep insemination (DARIO) made of Forprene 6NM901D40 (SO.F.TER. SPA, Forlì, Italy. A: Digital image. B: Design with dimensions (mm; 5:1 scale).R: radius; ø: diameter.

In 2013, 2014, 2015, and 2016, the study involved 36, 76, 102, and 28 lots of ewes, respectively. DARIO and control lot numbers were approximately equal each year. In the increasing photoperiod, 115 lots were used (56 as DARIO group and 59 as control group) while in the decreasing photoperiod 127 lots were used (70 as DARIO group and 57 as control group).

Estrus synchronization was performed with fluorogestone acetate sponges (SYNCRO-PART sponges, Ceva Santé Animale, Libourne, France) containing 30 mg of fluorogestone and applied for 12 to 14 d. At sponge withdrawal, the ewes were treated intramuscularly with 480 IU of eCG (equine chorionic gonadotropin, SYNCRO-PART PMSG 6000 UI, Ceva Santé Animale, Libourne, France). Semen was obtained from 29 Rasa Aragonesa rams belonging to ANGRA's genetic selection scheme. Semen for AI was obtained from 2 semen testing centers, both semen suppliers to ANGRA (Centro de Transferencia Agroalimentaria, Gobierno de Aragón, Zaragoza, Spain and Centro de Inseminación Artificial Ovina "El Chantre," Diputación de Teruel, Teruel, Spain). After natural ejaculation, semen was collected with an artificial vagina at 35 to 40°C lubricated with petroleum jelly. The concentration (1:400 dilution in saline solution plus glutaraldehyde) was evaluated by spectrophotometry (AccRead, IMV Technologies, HUMECO, Huesca, Spain). The volume was measured using a collector tube graduated in milliliters. Mass motility was estimated by optical microscopy at 10x and scored 0 to 5; individual motility was measured by ISAS (Integrated Semen Analysis System, Proiser, Paterna, Valencia, Spain). Semen was put into French mini straws (0.25 mL) at a concentration of 300 × 10^6 spermatozoa × ml^{-1} (standard dose for the ANGRA program). We estimated that 0.01 mL of semen dose (4%) remains in the device after AI. Semen doses were chilled and stored at 15°C for 2–4 h until insemination, depending on the distance from the semen testing center to the farms, thus ensuring that this time was the same for both DARIO and control groups on the same farm. The AI procedure was performed at 54 ± 1 h after sponge withdrawal, using an ovine AI gun (IMV, Instruments de Medicine Veterinaire, L'Aigle, France) for 0.25 mL French mini straws. Semen doses from every ram were distributed equally between DARIO and control groups.

For the DARIO group, the protocol was as follows (See Supplemental Material on online version of journal; Fig. 1): After mounting the French mini straw and the disposable sheath on the ovine AI catheter, DARIO was placed onto the catheter tip using slight pressure. Then, the speculum was introduced into the vagina to find the cervical opening. The hemispherical shape of DARIO allowed easy observation of the external cervical os and penetration without force. We put DARIO in the best position for tamponade; then, we applied a light pressure to the fold without going in-depth, and finally, we carefully deposited the semen, trying to decrease retrograde flow. Measuring retrograde flow was not possible and we only could carry out an estimation de visu; therefore, the comparison between this new device and a commercially available catheter was based on measurable items (fertility, prolificacy, and fecundity rates). The control group was inseminated in the same way, excepting DARIO use. The AI data were recorded in suitable lists and births were reported on farms through the production control. No early pregnancy diagnosis was done; therefore, fertility rate was defined as the percentage of ewes lambing after AI. Data on lambing were taken from farm production controls. Prolificacy rate was the average number of lambs born per lambing ewe. Fecundity rate was estimated as the average number of lambs born per inseminated ewe. All these rates were calculated on a lot basis because this was the experimental unit; we calculated the rates for each lot of approximately 5 ewes, and then the 242 observations became the data used for statistical evaluation.

Statistical Methods

Statistical analysis was performed using the IBM SPSS Statistics v. 22 software (IBM, Armonk, NY). Means and SE were calculated. Bivariate correlations for fertility, fecundity, and prolificacy rates were estimated. The GLM procedure was used to compare means between DARIO and control groups; $P < 0.05$ were considered statistically significant. This procedure can incorporate fixed and random factors, and interactions; in this way, several factors affecting AI results were incorporated in the model (farm and ram as random factors; catheter, year and photoperiod as fixed effects; catheter × photoperiod interaction). When significant effects were detected, the size effect was estimated by partial η^2 (percentage of the total variance in a dependent variable that is associated with the membership of different groups defined by an independent variable, the effects of other independent variables and interactions being cancelled out). Additionally, pairwise comparisons with Bonferroni correction were applied, and the significant marginal mean differences (**MMD**) with a 95% CI were estimated (Petrie and Watson, 1999). The *r* for fertility, fecundity, and prolificacy rates was also estimated.

RESULTS AND DISCUSSION

For CAI with chilled semen, the fertility rate for our control group (49.60 ± 2.48%) was greater than the values in the Sarde (45%; Sanna et al., 1995), Churra (31%; Abrough, 2000), Assaf (40.2%; Kaabi et al., 2006), Castellana (45.5%; Kaabi et al., 2006), and even in Rasa Aragonesa breeds, according to a recent study (45%; Abecia et al., 2016); a similar value was obtained in the Manchega breed (50%; Montoro et al., 2002). For DARIO group, the fertility rate (59.4 ± 2.13%) was slightly lower than the values in the Lacaune (62%; Aguer et al., 1992) and Merino breeds (61%; Shackell et al., 1990). These differences among breeds could be due to differences in the anatomy of the cervix; such anatomical differences were described by Halbert et al. (1990). Rates from our control group were greater in the Rasa Aragonesa breed than in other breeds, but results improved significantly when DARIO was used. For fecundity rates, means ± SE for DARIO and control groups were 0.99 ± 0.04 and 0.82 ± 0.05 lambs/inseminated ewe, respectively, and, for prolificacy rates, means ± SE for DARIO and control groups were 1.68 ± 0.04 and 1.63 ± 0.04 lambs/ewe lambing, respectively.

Table 1 shows the results from the GLM procedure used to compare means for fertility, prolificacy and fecundity rates between DARIO and control groups (*P* values, η^2, MMD, and 95% CI). Neither ram nor catheter × photoperiod showed significant effects on the variables studied ($P > 0.05$). Photoperiod only had a significant effect on the fertility rate ($P = 0.01$), which was greater in the decreasing photoperiod. Significant effects on the fertility, fecundity, and prolificacy rates were found for both year ($P < 0.05$) and farm ($P < 0.01$). Year was enclosed in GLM as a confounding variable to be controlled; therefore, MMD and 95% CI for year are not shown. On the other hand, MMD and 95% CI for farm were not calculated because farm was a random factor. The type of catheter (DARIO vs. control) had a significant effect on the fertility and fecundity rates but no significant effect was detected on the prolificacy rate. As shown in Table 1, farm, year, and type of catheter affect significantly both fertility and fecundity, therefore improving fertility rates would be accompanied by increased fecundity rates.

Table 1. Results from the General Linear Model (GLM) used to compare means for fertility, prolificacy and fecundity rates between DARIO[1] and control groups (fixed effects: catheter type, year and photoperiod; random factors: farm and ram; interaction: catheter type × photoperiod)

	Fertility rate, %				Prolificacy rate, lambs/ewe lambing		Fecundity rate, lambs/inseminated ewe			
Item	*P*-value	Partial η^2, % [2]	MMD [3]	95% CI[4]	*P*-value	Partial η^2, %	*P*-value	Partial η^2, %	MMD	95% CI
Catheter	< 0.01	4.80	9.05	3.27 ÷ 14.84	0.45	–	< 0.01	4.00	0.15	0.05 ÷ 0.26
Year	< 0.01	6.30	–	–	< 0.05	4.30	0.02	5.20	–	–
Photoperiod	0.01	3.20	32.27	6.85 ÷ 57.69	0.44	–	0.12	–	–	–
Farm	< 0.01	17.60	–	–	< 0.01	20.20	< 0.01	22.00	–	–
Ram	0.15	–	–	–	0.53	–	0.07	–	–	–
Catheter × photoperiod	0.29	–	–	–	0.68	–	0.19	–	–	–

[1]DARIO = suitable anti-retrograde flow device for sheep cervical AI that enables deep deposition of semen into the cervix without any modifications to the procedures currently used; control = traditional and commercially available catheter. *P* values < 0.05 were considered statistically significant.

[2]Partial η^2 = Percentage of the total variance in fertility, prolificacy and fecundity rates that is asociated with the membership of different groups defined by items, only calculated for significant effects.

[3]MMD = Marginal mean difference when applicable (DARIO vs. commercial catheter; increasing photoperiod vs. decreasing photoperiod).

[4]95% CI = 95% confidence interval for the marginal mean difference, when applicable.

Even in CAI, the technician has a significant effect on AI success (Anel et al., 2005). Because DARIO is obviously visible to the technicians during the AI procedure, strict blind testing is impossible. Unintentional AI technicians bias could therefore be a real issue, even if, as mentioned before, the result of the experiment was not relevant for them in terms of salary or professional progression. As it refers to ANGRA, bias can also be disregarded: if DARIO was not demonstrated to be a more efficient tool, ANGRA would simply continue using the traditional AI catheters.

Our results show a greater efficiency of DARIO when compared with current catheters; this situation could be explained by several factors. Fertility increases with insemination depth (Eppleston et al., 1994). However, Wulster-Radcliffe and Lewis (2002) concluded that fertilization or pregnancy rates increased when cervical manipulation associated with the AI catheter for TCAI or IUAI was minimized, preventing cervical injuries. Cervical damage could affect embryo implantation and hormonal balance, resulting in reduced fertility (Charmandari et al., 2005). Preventing the retrograde flow is an important factor for sheep AI success, and retrograde flow decreases with insemination depth (Álvarez et al., 1996). When semen is deposited less than 2 cm from the first and second cervical folds, a retrograde flow occurs, causing a decrease in AI efficacy (Álvarez et al., 2012). Without any attempt to traverse the cervix, the hemispherical body of DARIO blocks the external cervical os, minimizing retrograde flow de visu. As found by Álvarez et al. (1996), the best results were obtained when a 0.5 cm penetration without retrograde flow was achieved.

Álvarez et al. (2012) designed 2 new catheters for penetrating the ewe cervix in TCAI. One curved catheter showed significantly greater fertility rates than current catheters in both the Assaf (39.5% vs. 48.1%) and Churra breeds (29.0% vs. 39.0%). Another catheter, with 5 curvatures in a zigzag shape, had a lower fertility rate than other currently used catheters. The DARIO results for fertility rate in Rasa Aragonesa were greater than values for the best catheter tried by these authors. Differences in cervix anatomy among breeds and low risk for cervical injuries using DARIO could explain these results.

Correlation for fertility and fecundity rates showed a high, positive, and significant value ($r = 0.86$; $P < 0.01$); however, the correlation for fecundity and prolificacy rates was lower ($r = 0.54$; $P < 0.01$) and no significant correlation for fertility and prolificacy was found ($r = 0.03$; $P = 0.66$). These correlation values would explain why some factors affecting fertility and fecundity did not affect prolificacy. Fertility and fecundity rates refer to initiating and maintaining pregnancy; therefore, according to our results, the new device only had effects on these characteristics, whereas differences in prolificacy between DARIO and current catheters were not found.

The number of lambs born per lambing ewe would depend more on the characteristics and environmental factors of the ewe than on the AI circumstances (Abecia et al., 2015). When DARIO was used, we estimated an increase of 9.05 lambs per 100 inseminated ewes; assuming 1.6 lambs per lambing ewe (which is the mean prolificacy in the Rasa Aragonesa breed), approximately 15 more lambs would be obtained per 100 inseminated ewes when DARIO is utilized instead of the traditional AI catheter.

Conclusion

According to our experimental evidence based on 662 AI, the new device DARIO fits the cervix in Rasa

Aragonesa ewes, avoiding visually detectable cervix injuries and decreasing retrograde flow de visu. The AI procedures currently used do not need to be modified when using DARIO; adding the new device to the standard AI instrument is the only modification of usual tools. Compared with a traditional commercially-available CAI catheter, the use of DARIO increased both fertility and fecundity rates (MMD = 9.05 pregnant ewes per 100 inseminated ewes and MMD = 0.15 lambs per inseminated ewe, respectively).

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