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Successful transcervical uterine flushing can be performed without or reduced dose of oestradiol benzoate in cervical relaxation protocol in Dorper ewes

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Abstract

This study assessed the efficiency of cervical relaxation protocol using none, half or full dose (1.0 mg) of oestradiol benzoate in Dorper ewes subjected to non-surgical embryo recovery (NSER). Thirty-six pluriparous ewes received progestogen sponge (60 mg) for 9 days plus eCG administration (300 IU i.m.) 24 hr before sponge removal. Ewes were not mated and were randomly assigned to receive at 16 hr before NSER 37.5 μg d-cloprostenol i.m. and different doses of oestradiol benzoate: 0.0 mg (0EB group; n = 12); 0.5 mg (0.5EB group; n = 12) or 1.0 mg of oestradiol (1.0EB group, n = 12). All ewes received oxytocin (50 IU) i.v. 20 min before NSER, which was performed 8 days after sponge removal. Corpora lutea were counted by transrectal ultrasonography 24 hr before NSER. After procedure, the ewes were kept in natural breeding period to check their post-NSER fertility. NSER was performed in 91.7% (33/36) of the animals with overall fluid recovery efficiency over 97% ($p > .05$). The cervical transposing with Hegar dilator was longer ($p < .05$) in 0EB (4.2 ± 0.3 min) compared to 0.5EB (1.7 ± 0.3 min) and 1.0EB group (1.5 ± 0.3 min). The cervical transposing with mandrel/catheter was longer ($p < .05$) in 0EB (2.4 ± 0.5 min) than 1.0EB group (1.3 ± 0.5 min). Overall duration of uterine flushing was 25.4 min with structure recovery rate of 43.5%, with no difference among groups ($p > .05$). The post-NSER fertility was higher ($p < .05$) in 0.0EB (90%) than 0.5EB group (36.4%). In conclusion, NSER can be successfully performed in Dorper ewes by using a cervical relaxation protocol without oestradiol benzoate.

KEYWORDS
cervical dilation, NSER, post-NSER fertility, sheep, transcervical embryo recovery

1 | INTRODUCTION

Successful non-surgical embryo recovery (NSER) has been proposed as an efficient choice for in vivo embryo production in goats and sheep (Fonseca, Oliveira et al., 2019; Fonseca et al., 2016) The success of NSER depends in first instance on the efficiency of cervical transposing (Candappa & Bartlewski, 2012). Although goats cervix can be transposed during dioestrus with only cloprostenol (Fonseca et al., 2013; Pereiraet, Sohnrey, & Holtz, 1998) or without drugs (Fonseca et al., 2014), in sheep a more complex combination

of hormones are needed for allowing relaxation and transposing of the cervix, which is a narrow, long and tortuous organ (Candappa & Bartlewski, 2012). There is also great variability of types of cervical os (Kershaw et al., 2005) and number of cervical rings between breeds (Kaabi et al., 2006) that may affect successful NSER rates. Some strategies, as a pre-selection of animals using a score of transposing difficulty with Hegar dilator (Fonseca, Oliveira et al., 2019, Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al.,2019) or a cervical map for guiding the mandrel/catheter transposing (Fonseca, Oliveira et al., 2019), appear to be helpful and easy tools during the procedure which can increase NSER successful rates.

Important advances related to successful cervical transposing and uterine flushing in dioestrus phase were obtained in Santa Inês ewes, testing different combination of drugs (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019; Leite et al., 2018), different routes of oestradiol (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al.,2019) and oxytocin (Prellwitz et al., 2019) administration as well as strategies to select and classify ewes according to degree of difficulty for cervical transposing (Fonseca et al., 2019a). Results of those studies showed near to 80% of cervical transposing in Santa Inês ewes using oestradiol benzoate-d-cloprostenol-oxytocin-based protocol to NSER. Interestingly, the same protocol has showed greater success (near to 100%) in Lacaune ewes (Fig ueira et al., 2020). Cervical transposing was possible in almost 95% in Dorper ewes using misoprostol (prostaglandin-E analogue) as cervical dilator (Gusmão et al., 2009) but there are no data in this breed using others mentioned protocols.

In order to apply the NSER worldwide, it is important to evaluate the efficiency and adapt this technique in other commercial breeds of sheep. Dorper sheep has a great economic importance in ovine meat production system. In addition, the hormonal combination used for cervical dilation should consider the use of drugs available worldwide. In Europe and Oceania, the sale and use of oestrogen analogues are restricted or prohibited by environmental waste and residues in animal meat (Directive 81/602/EEC) once that data available confirmed oestradiol-17β as a carcinogen agent (European Commission, 2017). In sheep, the use of oestrogen in the dioestrus period did not affect embryonic implantation and pregnancy rate (Lewis, 2010). Its effect on embryos in vitro has not been evaluated yet but there are some successful data on lambing rate in animals undergoing embryo transfer after NSER procedure (Figueira et al., 2020). Even though in South America there is still no restriction on the use of this drug, it is possible that in the future it will be replaced by potentially less harmful hormones.

Taking into account that in goats the cervical relaxation for NSER is done only with d-cloprostenol (Fonseca et al., 2013) and that there was a high degree of facility of transposition in Lacaune ewes submitted to the standard protocol (Figueira et al., 2020), we hypothesized that in sheep it would be possible to perform the NSER without or with reduced oestrogen dose. Thus, the aim of this study was to evaluate the efficiency of cervical relaxation protocol using none, half or full dose of oestrogen analogue for NSER success in Dorper ewes.

2 | MATERIAL AND METHODS

2.1 | Animals

This study was conducted after the approval of the Ethics in Animal Care Committee of the Embrapa Dairy Cattle (process 6365271119). The experiment was carried out from August to September at a commercial farm, located in Juiz de Fora city, Minas Gerais state, Brazil (latitude 45′51″ S, longitude 43°20′59″W). A total of 36 pluriparous Dorper ewes, with average of 4.3 ± 0.2 years old (mean ± SEM), 46.9 ± 1.1 kg of body weight and 2.9 ± 0.1 of body condition score (scale from 1–5, when 1 = emaciated and 5 = obese; Villaquiran et al., 2007) and with 100–120 days post-partum (weaning at 80–90 days), were used. The animals were kept in semi-confinement system, maintained in native pasture (*Brachiaria decubens*) and supplemented with corn silage according to demands (NRC, 2007), and had ad libitum access to freshwater and mineral salt.

2.2 | Experimental design

All ewes received an intravaginal sponge containing 60 mg of medroxyprogesterone acetate (MAP, PROGESPON®, Zoetis) maintained for 9 days, plus an administration of 300 IU of eCG (Novormon®, Zoetis) i.m. 24 hr before sponge removal. Ewes in oestrus were not mated and were randomly assigned to receive at 16 hr before NSER intramuscularly d-cloprostenol (37.5 μg; Prolise®, Agener União Saúde Animal) and different doses of oestradiol benzoate: 0.0 mg (0EB group; *n* = 12); 0.5 mg (0.5EB group; *n* = 12) or 1.0 mg (1.0EB

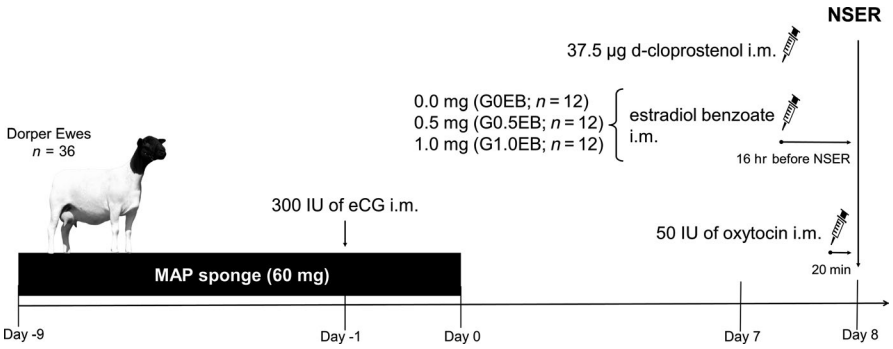

FIGURE 1 Schematic representation of the experimental procedures used to assess in Dorper ewes the effect of cervical relaxation protocols containing none (0.0), half (0.5) or full dose (1.0) of oestradiol benzoate in oestrous-induced ewes subjected to non-surgical embryo recovery (NSER). MAP, medroxyprogesterone acetate; eCG, equine chorionic gonadotropin; i.m., intramuscular

group, *n* = 12) of oestradiol benzoate (RIC-BE®, Agener União). All ewes received oxytocin 50 IU (Ocitocina Forte UCB®), i.v. 20 min before NSER. The experimental design is shown in Figure 1.

2.3 | Cervical manipulation and uterine flushing

Cervical penetration and uterine flushing were attempted 8 days after the sponge removal. Animals in a standing position were restrained in a cart and received acepromazine maleate (0.1 mg/kg; Acepromazin®; Vencofarma) i.m. and 10 ml (5 ml i.v. and 5 ml i.m.) of dipyrone and n-butyl hyoscine bromide solution (Buscofin Composto®, Agener União), both at 20 min before procedure. A lidocaine epidural block (S5-C1) using 2 ml of 2% lidocaine (BLOC®, J.A. Saúde Animal) was performed immediately before insertion of a vaginal speculum. The cervical penetration and uterine flushing approach used in this study were identical to that previously described for Lacaune ewes (Figueira et al., 2020). After vaginal speculum insertion, cervical os was classified according to Kershaw et al. (2005) modificated and anatomical features of the uterine cervices (i.e. number and relative position of consecutive cervical rings, depth of penetration) were recorded for each donor animal to create the 'cervical map' (Fonseca, Oliveira et al., 2019). The time taken to traverse the cervix with Hegar dilator and mandrel/catheter and the duration of flushing were recorded. All ewes were classified based on time required for cervical penetration and successful penetration rates into the five following categories or grades: Grade 1 (very easy; cervical penetration achieved in less than 1 min; Grade 2 (easy; between 1 and 3 min); Grade 3 (moderate difficulty; between 3 and 7 min); Grade 4 (difficult; between 7 and 10 min); and Grade 5 (impossible to penetrate the cervix or a lack of complete cervical passage) as previously described (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al.,2019). All recovered structures were classified according to the International Embryo Society Transfer manual (Stringfellow & Seidel, 1999).

2.4 | Ultrasound assessment

Ultrasound evaluation was performed 24 hr before NSER in all ewes by B-mode Ultrasound (Mindray M5Vet®) equipped with a 8.0 MHz transrectal linear probe, fitted with a rigid protractor. Corpora lutea (CL) were counted, and animals presenting absence of CL were not considered for structure recovery rate.

2.5 | Post-NSER fertility

Seminal evaluation 1 week before breeding revealed that two rams used reached adequate seminal levels (≥80% seminal progressive linear motility and ≤20% seminal pathology) suggested by Brazilian College of Animal Reproduction (2013). The ram´s fertility in the previous breeding period was ≥65%. Ten days after NSER, all ewes received 37.5 µg d-cloprostenol i.m. They were equally assigned to two rams (1:18 ram: ewe ratio) and kept in continuous natural breeding for 7 weeks. Pregnancy rates were assessed 60 days after the NSER by the same ultrasound.

2.6 | Statistical analyses

The following data were recorded for all ewes studied: mean number of corpora lutea per ewe; type of cervical os; Hegar transposing success rate (number of ewes successful transposed with Hegar/total number of ewes of the group × 100); number of cervical rings; duration of Hegar transposing (in minutes, time from Hegar dilator insertion to removal); degree of difficulty performing Hegar penetration procedure; duration of mandrel/catheter transposing (in minutes, time from mandrel/catheter insertion to removal); degree of difficulty performing mandrel/catheter penetration procedure; duration of flushing (in minutes, time from the first 5 Ml injection of PBS solution to flushing catheter removal); NSER success rate (number of ewes successfully flushed/total number of ewes × 100); fluid recovery efficiency rate (percentage of fluid recovered post-infusion: volume retrieved/400 Ml × 100); number and type of structures recovered (unfertilized eggs); ewes with at least one structure recovered rate (number of ewes with one or more structures recovered/number of ewes successfully flushed × 100); average structures recovered (mean number of structures recovered); structure recovery rate (considering animals presenting at least one corpora lutea: number of structures recovered/number of corpora lutea × 100); post-flushing fertility rate (number of pregnant ewes/number of successfully flushed ewes × 100).

Data analysis was performed using the libraries of the 'car', 'stats', 'agricolae' and 'emmeans' packages of the R software (version 3.6.1, The R foundation for Statistical Computing). To assess the normality of the residues, the Shapiro–Wilk test was used, and the Levene test for the assumption of homogeneity of variance of the treatments. The Box-Cox transformation was performed when necessary, and the data were returned to the original condition for presentation of the results. The Kruskal–Wallis test and Fisher's exact test were used for non-parametric analyses, depending on the variable. Parametric data were analysed by analysis of variance (ANOVA) followed by Tukey's test, for mean comparisons. Values are presented as mean ± standard error. The level of significance used for all analyses was 5%.

3 | RESULTS

Regardless of the treatment, the efficiency of NSER according to cervical os presentation is described in Figure 2 ($p > .05$). Overall Hegar transposing success and uterine flushing success were reached in 97.2% (35/36) and 94.3% (33/35) of ewes, respectively. Overall, the NSER could not be performed in 8.3% of ewes (3/36; 0.0EB = 2 and 0.5EB = 1), due to anatomical problems.

The average number of cervical rings was 7.4 ± 0.18 (5–9 range). The total procedure duration averaged 25.4 min (range: 14–33 min). A total of 27 structures were recovered and classified as unfertilized eggs. The overall structure recovery rate was 43.5% (27/62), considering only ewes successful flushed. One ewe successfully flushed from 0.5EB group presented no CL and was not considered for data related to structures recovered. Data regarding to the efficiency of transcervical embryo recovery according to treatment groups are presented in Table 1.

The degree of difficulty of cervical transposing with Hegar dilator and mandrel/catheter is presented in Figure 3 a and b, respectively.

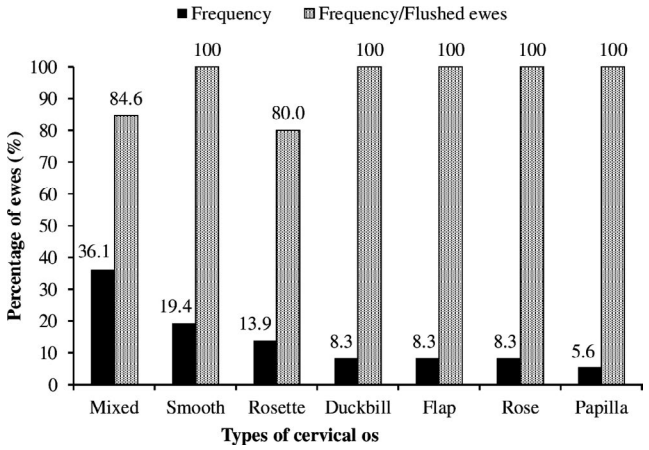

FIGURE 2 Frequency of different types of cervical os observed in Dorper ewes at the time of non-surgical embryo recovery (NSER) and their influence on the efficiency of the technique ($p > .05$)

4 | DISCUSSION

Considering that the objective of the present study was to evaluate cervical relaxation protocols using different doses of oestrogen, it is possible to infer that NSER can be efficiently performed with no or reduced oestrogen dose without affecting the feasibility of the whole technique. Our results showed a transposition rate with Hegar dilator close to 100%, regardless of the protocol used, and a success rate of NSER with a linear, but not significant, increase when using doses of 0.0 (83.0%), 0.5 (91.7%) and 1.0 (100.0%) mg of oestradiol benzoate in Dorper ewes subjected to cervical relaxation. Moreover, it should be highlighted that the three ewes not flushed had anatomical problems detected at the time of the procedure. Applying screening tests to identify and avoid ewes with such problems (Prellwitz et al., 2019) may increase the NSER success in Dorper ewes. In fact, the design used in the present study can also be an efficient, field applied and no expensive way to test NSER success in any breed before superovulation.

TABLE 1 Summary (mean ± *SEM* or %) of the cervical penetration attempts and embryo recovery procedures in pluriparous Dorper ewes who received different concentrations of oestradiol benzoate 16 hr before non-surgical embryo recovery (NSER) in a cervical relaxation induction protocol

	Oestradiol benzoate dose (mg)		
End Points	**0.0**	**0.5**	**1.0**
Number of animals	12	12	12
Mean number of corpora lutea/ewe*	2.0 ± 0.3 (19)	2.1 ± 0.3 (21)	1.7 ± 0.2 (20)
Hegar transposing successful (%)	100.0 (12/12)	91.7 (11/12)	100.0 (12/12)
Duration of Hegar dilator transposing (min)	4.2 ± 0.3[a]	1.7 ± 0.3[b]	1.6 ± 0.3[b]
Number of cervical rings transposed	7.3 ± 0.3	7.4 ± 0.3	7.7 ± 0.4
Duration of mandrel/catheter transposing (min)	2.4 ± 0.5[a]	1.6 ± 0.4[ab]	1.3 ± 0.5[b]
Duration of flushing (min)	19.2 ± 1.2	21.4 ± 1.4	18.6 ± 1.1
Total duration of procedure (min)	25.4 ± 1.6	24.0 ± 1.6	21.6 ± 1.2
NSER success (%)	83.3 (10/12)	91.7 (11/12)	100.0 (12/12)
Fluid recovery efficiency (%)	98.0 ± 2.0	99.0 ± 1.0	97.0 ± 1.6
Ewes with at least one structure recovered (%)*	70.0 (7/10)	60.0 (6/10)	58.3 (7/12)
Average structures recovered*	1.0 ± 0.3	0.9 ± 0.3	0.7 ± 0.2
Structure recovery (%)	52.6 (10/19)	39.1 (9/23)	40.0 (8/20)
Post-flushing fertility (%)	90.0 (9/10)[A]	36.4 (4/11)[B]	58.3 (7/12)[AB]

a,b Means with different superscripts within rows differed (Tukey's test; $p < .05$).

A,B Means with different superscripts within rows differed (Fisher's exact test; $p < .05$).

( ) The values into the parenthesis indicate number of corpora lutea, animals or structures.

*Only ewes successfully flushed and with at least one CL counted.

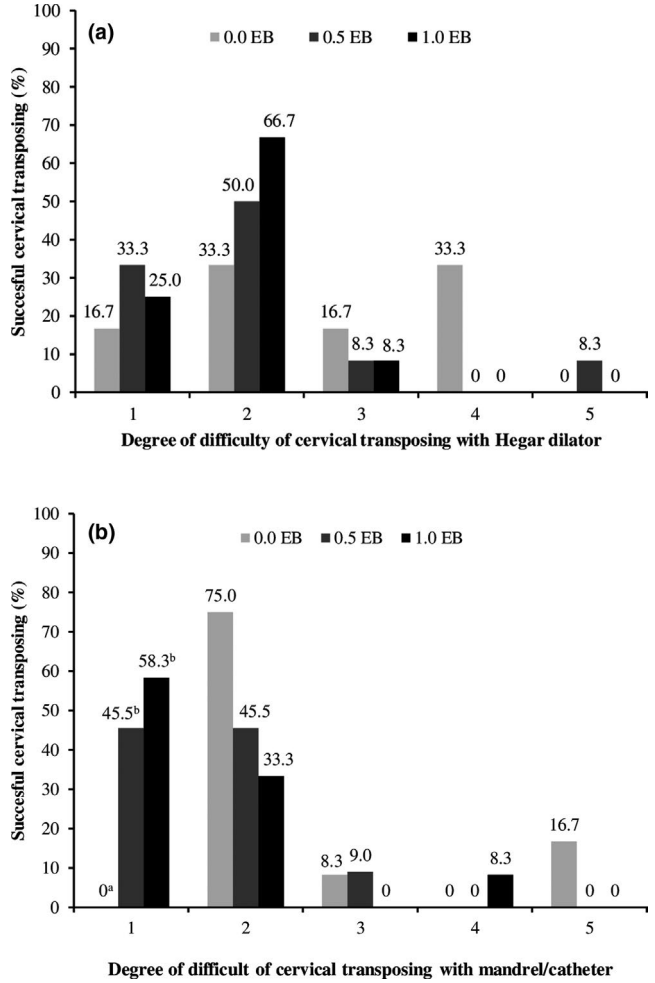

FIGURE 3 Number of Dorper ewes successfully submitted to non-surgical embryo recovery (NSER; y-axis). The x-axis means the degree of difficulty in transposing the cervix with Hegar dilator (3A) and mandrel and catheter equipment (3B). Scales 1–5, where (a) very easy, cervical penetration achieved in < 1 min; (b) easy, between 1 and 3 min; (c) moderate difficulty, between 3 and 7 min; (d) difficult; between 7 and 10 min; and (e) impossible to penetrate the cervix with dilator or a lack of complete cervical passage. [a,b]Values with different letters differ between grades (Fisher's exact test; $p < .05$)

The NSER success (92–100%) in Dorper ewes of the present study using an association of prostaglandin F2α and oxytocin (PGF-OT) and oestradiol benzoate protocol for cervical relaxation showed superior results than those reported in Santa Inês ewes (range 44%–81.8%) (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al.,2019; Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019; Prellwitz et al., 2019). Interestingly, the same misoprostol (PGE2)-based protocol for cervical relaxation revealed successful transcervical uterine flushing rates of 94.8% (55/58) in pluriparous Dorper ewes (Gusmão et al., 2009) and 63.1% (12/19) in pluriparous Santa Inês ewes (Gusmão et al., 2007). These findings suggest that the same protocol could reach variable efficiencies when using different sheep breeds.

The NSER success ranged from 83% to 92% in the protocols using oestradiol benzoate dose reduction combined with PGF-OT and was superior than another report that used the same protocol in Santa Inês ewes (27.3%; Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019). Our data show that the removal of oestrogen in the relaxation protocol does not affect the overall success of the technique, as it does not affect successful NSER rate, fluid recovery rate and structure recovery rate, as already demonstrated in other studies (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019). It means that in the study conditions the use oestradiol benzoate in protocols for cervical relaxation and NSER can not only be reduced but also abolished in Dorper ewes and possibly in other sheep breeds. Therefore, the results of this study can directly affect the wide dissemination of the transcervical embryo recovery technique, since there are countries where the use of oestrogen is not allowed. In addition, the present results can also promote a reduction in the amount of hormones needed in protocols for the embryo recovery in sheep and decreasing hormonal residues in the environment as proposed by Martin and Kadokawa (2006).

Our results showed that only one animal from 0.5EB group could not be transposed with Hegar dilator, while two animals from 0.0EB could not be successfully flushed (mandrel/catheter transposition). This result is better than that obtained in Santa Inês ewes using oestradiol benzoate (Prellwitz et al., 2019) and shows that NSER can be spread in different breeds. As we know, each ewe has an individual cervical characteristic of os, cervix alignment and number or thickness of cervical rings (Kershaw et al., 2005), which may vary according to age, breed and lambing (Kaabi et al., 2006). In the present study, it was not observed significant difference among groups related to the type of cervical os or interference of the type of cervical os and successful flushing. Fonseca, Oliveira et al. (2019) suggest a 'cervical map', which is a sketch of cervical alignment made during cervical transposition with Hegar dilator, and serves as a guide for AI or NSER. However, transposition is not always possible with the mandrel/catheter as they are more flexible compared to Hegar, which justifies the difference found between the two transpositions in these studies. It is also worth remembering that the animals in this study were not submitted to previous evaluation of cervical anatomy when they were selected to this study.

Despite cervical passage time was longer in 0.0EB group compared to the 1.0EB group, it did not affect the duration of flushing or the fluid recovery efficiency and structure recovery parameters, corroborating that it is possible to successfully perform NSER using a PGF-OT based protocol in Dorper ewes.

The reduction or non-use of oestradiol benzoate in sheep for cervical relaxation was not detrimental to the safety and/or the level of difficulty of the procedure, nor to the comfort of the animal. In addition, our data on the duration of transposition and the total duration of the procedure were even lower than those found in other studies (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019; Prellwitz et al., 2019), but the breed factor can interfere with the duration of the procedure (Kaabi et al., 2006), explaining

this difference. The mechanism of oestradiol on cervical relaxation is through the remodelling of the cervical extracellular matrix, acting by increasing the action of hyaluronan on collagen fibres (Robinson et al., 2011). This consequent softening that occurs allows a deeper penetration, facilitating intrauterine access through the cervix, which is desired when it comes to transcervical AI or embryo recovery and justifies the faster penetration of Hegar and mandril dilators in the 1.0EB treated group of this study.

According to Fonseca, Oliveira et al. (2019), the successful NSER can be classified according to duration of cervical transposing, in a 1–5 score, where 1 is equivalent to very easy transposing and five is equivalent to impossible transposing. In our study, there was no statistical difference between the degrees of the Hegar dilator, but there was a difference between the degrees of the mandrel/catheter. This means that there is a preference for the cervical passage to become easier with the knowledge of the cervical map, since the number of animals classified as degrees three and four decreased in comparison to the two transpositions, although there is no significant difference. Even though it has already been observed in other experiments of our group, more studies need to be carried out to prove the efficiency of mapping the cervical alignment in order to facilitate the transposition. Besides that, scoring duration of Hegar or mandril penetration also can work as a selection method of donor ewes, since females classified as grade four during natural oestrus could not be traversed after 6–7 days for NSER (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al., 2019) or even when classified as grade four in the Hegar, the animal could not be transposed with mandrel/catheter during the same procedure, as observed in this study. The animal classified as grade five in this study for Hegar dilator could not be transposed, corroborating with Prellwitz et al. (2019) and Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski et al. (2019).

Even though it is a low cost and easily acquired hormone, oestrogen has had limited use in countries in Europe and Oceania due to residues in animal products and the environment, which becomes a barrier to the expansion of the NSER technique. Moreover, considering the need to recover optimal quality embryos to survive the embryo cryopreservation and transfer process, wouldn't it be possible that these high levels of oestradiol on early luteal phase cannot be affecting the embryo in terms of survival and implantation genes? Some studies in cows (Madsen et al., 2015) and sheep (Lewis, 2010) show that supraphysiological levels of oestradiol in the embryonic implantation period after ET or AI do not cause harmful effects to the embryo's survival in vivo. However, we found no studies showing these effects on embryo's survival in vitro.

Besides the success of uterine access, high fluid recovery rate and relatively little time required to perform NSER in Dorper ewes appear to be attractive. As well as reported for Lacaune ewes (Figueira et al., 2020), more than 50% of ewes flushed showed at least one structure and the structure recovery rates varied from 40% to 53% even considering low CL count. These findings suggest not only the efficiency but the relatively good precision of the NSER technique even when CL count is lower, opening the opportunity to apply NSER in ewes with low superovulatory response or in non-superovulated animals (Figueira et al., 2020).

From the best of our knowledge, pregnancy rates in ewes after embryo collection by any technique were not reported. In the present study, not only pregnancy after NSER was reported but fertility after this procedure was affected by treatment groups favouring 0.0EB group. Thus, NSER technique associated with no use of oestradiol benzoate apparently preserve ewe fertility, benefiting the production system by further maximizing the use of the animal. This finding must be confirmed in future and large-scale study.

Finally, despite the recent and significant increases in NSER success in sheep were done using Santa Inês sheep as model, the interest and dispersion of this breed are mostly restricted to Brazil and in some countries of South and Central America. Recently, NSER was encouraged to Lacaune sheep (Figueira et al., 2020), one of the most important dairy sheep breeds in the world. Results of the present study now encourage the use of NSER in Dorper, one of the most important breeds for lamb production in the world. In addition, NSER can be considered as more 'ethical' technique for embryo recovery than surgical procedures as proposed earlier (Martin, 1995; Martin & Kadokawa, 2006).

5 | CONCLUSIONS

Based on the data obtained in this study, the oestradiol benzoate can be either removed or reduced in protocols for cervical relaxation in Dorper ewes without any effect in the efficiency of the whole NSER process. Now tested efficiently in Dorper sheep, this design appears to be promising also in other breeds for NSER procedure, although it needs to be evaluated due to anatomical differences.

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CONFLICTS OF INTERESTS

All authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

AUTHORS CONTRIBUTIONS

JFF elaborated the hypothesis, discussed the experimental design. JHD collected the data from the animals. JHD analysed the data and wrote the first version of the manuscript. GSD, VLB, CJP and RITPB assisted with the animals and helped collecting and analysing the

data. JFF, RITPB, MEFO and JMGS-F discussed the design of the experiment and analysed the data. MAPS elaborated and worked on the statistics. JFF, MEFO and JMGSF approved the final version of the manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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