Received: 11 July 2018 | Accepted: 16 August 2018

DOI: 10.1111/rda.13318

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

# Combined treatment with oestradiol benzoate, d-cloprostenol and oxytocin permits cervical dilation and nonsurgical embryo recovery in ewes

Jeferson Ferreira da Fonseca[1] | Fabiana Nunes Zambrini[2] | José Domingos Guimarães[2] | Marcio Roberto Silva[3] | Maria Emilia Franco Oliveira[4] | Felipe Zandonadi Brandão[5] | Pawel Mieczyslaw Bartlewski[6] | Joanna Maria Gonçalves Souza-Fabjan[5]

[1]Embrapa Caprinos e Ovinos, Sobral, Brazil

[2]Universidade Federal de Viçosa, Viçosa, Brazil

[3]Embrapa Gado de Leite, Juiz de Fora, Brazil

[4]Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista, Jaboticabal, Brazil

[5]Universidade Federal Fluminense, Niterói, Brazil

[6]University of Guelph, Guelph, Ontario, Canada

**Correspondence**
Jeferson Ferreira da Fonseca, Embrapa Caprinos e Ovinos, Coronel Pacheco, Brasil.
Email: jeferson.fonseca@embrapa.br

**Funding information**
Embrapa Goats and Sheep, Grant/Award Number: 03.12.01.031.00.00; Fapemig, Grant/Award Number: PPM 00042-14; CNPQ, Grant/Award Number: 310166/2012-8

## Contents

This study examined the feasibility of transcervical embryo recovery after the hormonal treatment to induce cervical dilation, following the 7-day oestrous synchronization protocol in multiparous Santa Inês ewes. A total of 23 cyclic ewes received two doses of 37.5 μg of d-cloprostenol by latero-vulvar route 7 days apart. After the second injection of d-cloprostenol, the ewes were checked for oestrus (every 12 hr) and then mated by fertile rams throughout the oestrous period. All ewes received 37.5 μg of d-cloprostenol (latero-vulvar) and 1 mg of oestradiol benzoate by either intramuscular (EBim group; $n$ = 12) or intravaginal (EBivg group; $n$ = 11) route 16 hr before embryo flushing. Twenty minutes before the flushing, 50 IU of oxytocin were administered intravenously. The oestrous response (i.e., the percentage of ewes that showed signs of oestrous behaviour after the second d-cloprostenol injection) was 91.3% (21/23). The proportion of successfully penetrated ewes (81.8% compared with 80.0%), the mean duration of embryo flushing (24.7 ± 2.0 min compared 26.2 ± 1.9 min), the flushing fluid recovery rate (94.8 ± 1.3% compared with 91.0 ± 2.9%) and the average number of structures recovered per ewe (0.5 ± 0.4 compared with 0.8 ± 0.4) did not vary ($p$ > 0.05) between the EBim and EBivg groups. Viable embryos were recovered from 41.2% (7/17) of successfully penetrated ewes. It can be concluded that nonsurgical (i.e., transcervical) embryo collection can be performed in oestrous-synchronized Santa Inês ewes pretreated with d-cloprostenol, oxytocin and oestradiol benzoate, with the latter hormone administered by either the intramuscular or intravaginal route.

KEYWORDS

cervical dilation, cloprostenol, oestradiol benzoate, oxytocin, sheep, transcervical embryo collection

  

## 1 | INTRODUCTION

In vivo embryo production (IVP) continues to be the primary method to produce ovine embryos for commercial embryo transfer in Brazil. Surgical embryo recovery from donor ewes is the technique of choice worldwide, even though repeated invasive procedures may have severe adverse effects such as adhesions, postoperative trauma and stress (Candappa & Bartlewski, 2011; Fonseca et al., 2016). Nonsurgical (i.e., transcervical) embryo recovery is feasible and efficient, but this approach still needs to be refined and tested in different breeds of sheep (Candappa & Bartlewski, 2011, 2014). The ease with which the uterine cervix of the ewe can be penetrated depends on several intrinsic and extrinsic factors. Cervical anatomy, namely the number, inner diameter and distribution of cervical rings, is a main determinant of cervical penetrability in sheep. While some animals have naturally greater anatomical predisposition for transcervical penetration (Kershaw et al., 2005), the application of various hormones can induce cervical dilation and facilitate the procedure.

The mechanism of cervical relaxation in cyclic ewes is complex. Moderate cervical dilation precedes luteolysis during the dioestrous phase of the interovulatory period (Candappa & Bartlewski, 2011). Towards the end of the luteal phase, oxytocin (OT) is synthesized and stored in the luteal cells (Wathes & Lamming, 1995). During and after luteolysis in ruminant species, ovarian steroidogenesis changes form progesterone- to oestrogen-dominated; this shift in ovarian steroid production marks the onset of proestrus and oestrogen secretion continues to rise during the oestrous phase and up until ovulation. Follicular oestradiol enhances the responsiveness of the uterus to OT by stimulating OT receptor synthesis and expression on endometrial cells. Prior exposure to progesterone ($P_4$) promotes uterine accumulation of arachidonic acid, prostaglandin endoperoxide synthase and other substances needed for synthesis of prostaglandin F2alpha ($PGF_2\alpha$). Collectively, these effects exerted by luteal $P_4$ stimulate $PGF_2\alpha$ synthesis at the most appropriate time to induce luteal regression (Silvia, Lewis, McCracken, Thatcher, & Wilson, 1991). In the absence of developing embryos and interferon-$\tau$ secretion (Thatcher et al., 1997), declining $P_4$ release and elevated systemic concentrations of OT (of the pituitary origin) initiate episodic secretion of $PGF_2\alpha$ from the uterus. This increase in $PGF_2\alpha$ synthesis leads to the degranulation of luteal OT stores, which further increases uterine $PGF_2\alpha$ synthesis and release into the uterine veins that are linked anatomically with the ovarian arteries (via the counter-current exchange system; Einer-Jensen & Hunter, 2005) and ultimately triggers the luteolytic cascade (Wathes & Lamming, 1995). One of the consequences of these changes in the preovulatory hormonal milieu is cervical opening occurring at oestrus.

Despite cervical remodelling during the oestrous phase, cervical penetration for artificial insemination procedures in ewes remains problematic (Candappa & Bartlewski, 2011; Fonseca et al., 2016). Therefore, the use of 200-400-600 USP of exogenous OT was implemented to facilitate cervical dilation, which permitted complete cervical penetration in 74-75-83% of ewes, respectively, as opposed to 0% of saline-treated animals (Khalifa, Sayre, & Lewis, 1992). In addition, administration of 100 or 200 μg of oestradiol 12 hr prior to OT injection further increased cervical penetration rates (Khalifa et al., 1992), resulting in >80% of transcervically inseminated ewes. Gusmão et al. (2007) reported that cervical penetration was not possible in Santa Inês ewes without any drug administration, but 50 μg of cloprostenol given intramuscularly 12 hr before embryo recovery allowed for the completion of transcervical embryo flushing in 59% of ewes. Thus, we hypothesized that a combined treatment with oestrogen, $PGF_2\alpha$ and OT would promote sufficient cervical relaxation and uterine access in dioestrous sheep.

Intravaginal route has been used for prostaglandin $E_1$ ($PGE_1$) administration in Santa Inês (Gusmão et al., 2007) and Dorper ewes (Gusmão, Silva, Bittencourt, Martins, & Barbosa, 2009). In those earlier studies, no treatment, pretreatment with $PGF_2\alpha$ at 12 hr or with intravaginal $PGE_1$ at 5 hr before embryo recovery resulted in 0%, 58.8% or 63.2% of successful transcervical passages in Santa Inês ewes respectively (Gusmão et al., 2007). Oestrogen was not tested in those trials nor was its administration by intravaginal route evaluated in ewes. Furthermore, $PGE_1$ was diluted in saline solution (Gusmão et al., 2007, 2009). $PGF_2\alpha$ analogues are also typically dissolved in aqueous solution, which allows for their rapid absorption, whereas commercial oestradiol preparations are prepared in organic solvents (e.g., oil), which results in the prolonged absorption time and action. Because the effects of oestradiol on cervical relaxation are exerted locally, like those produced by $PGE_1$ (Gusmão et al., 2007), we resolved to test and compare its effects after intramuscular and intravaginal administration.

To recapitulate, the main purpose of this study was to examine if a combination of oestradiol benzoate, cloprostenol and OT would induce cervical relaxation permitting uterine flushing and embryo recovery in dioestrous ewes. Additionally, we examined if changing the route of oestradiol administration from intramuscular to intravaginal would ameliorate the uterine access and the ease with which uterine flushing by cervical route can be performed in oestrous-synchronized Santa Inês ewes.

## 2 | MATERIALS AND METHODS

### 2.1 | General experimental conditions

The present research proposal was reviewed and approved by the Animal Care Committee of Embrapa Dairy Cattle (protocol 15/2014). This study was conducted during the breeding season (April to June) at the Experimental Campus of Embrapa Dairy Cattle, in the rural area of Coronel Pacheco, Brazil (latitude 21°35′S, longitude 43°15′W and altitude of 435 m a.s.l.). A total of 23 multiparous (2–3 parity), nonlactating Santa Inês ewes were kept in an intensive management system and were fed corn silage and *Pennisetum purpureum* as forage, with a balanced concentrate offered according to animals' nutritional demand (National Research Council, 2007). Mineralized salt licks and drinking water were available ad libitum.

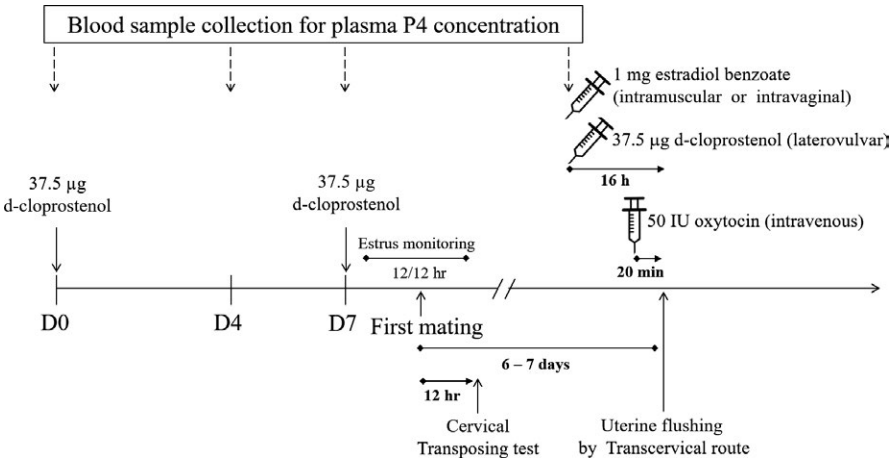


**FIGURE 1** Schematic representation of the experiment undertaken to test different routes of oestradiol benzoate administration for cervical dilation prior to nonsurgical embryo recovery attempt in oestrous-synchronized, multiparous Santa Inês ewes

## 2.2 | Experimental animals and treatments

All ewes received two doses of 37.5 µg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil)/by latero-vulvar route 7 days apart (Fonseca et al., 2017). After the second cloprostenol injection, the ewes were checked for oestrus every 12 hr and ewes in oestrus were mated with fertile rams (1:4 ram to ewes ratio) throughout the duration of the oestrous period (two to three times according to mounting acceptance). Ewes were allocated, based on their body weight (BW) and body condition score (BCS: range 1–5 with 0.25 steps) to one of the two treatment groups. All ewes received a latero-vulvar injection of 37.5 µg of d-cloprostenol 16 hr and 50 IU of oxytocin (Ocitocina forte®, UCB, São Paulo, Brazil) given intravenously 20 min before embryo flushing with oestradiol benzoate (EB; Estrogin®, Farmavet, São Paulo, Brazil) administered intramuscularly (1 ml of oil containing 1 mg of oestradiol benzoate; EBim group, $n$ = 12, BW: 56.4 ± 3.3 kg, BCS: 4.3 ± 0.1) or intravaginally (EBivg group, $n$ = 11, BW: 60.0 ± 1.4 kg, BCS: 4.2 ± 0.1). An insulin syringe (1 ml volume) without needle was introduced through the vulva and vestibule to administer oestradiol benzoate into vagina. The experimental design is summarized in Figure 1.

## 2.3 | Cervical penetration and uterine flushing

Cervical penetration and cervical embryo flushing were attempted 6–7 days after the onset of behavioural oestrus (e.g., first acceptance of mating) in individual ewes. Animals in a standing position were restrained in a cart and received acepromazine maleate (1 mg/kg; Aceproven®; Vencofarma, Londrina, Paraná, Brazil) i.m. 10 min before and 2 ml of 2% lidocaine epidural block (S5-C1) (Lidovet®, Bravet, Rio de Janeiro, Brazil) immediately before insertion of a vaginal speculum.

The present approach to cervical penetration was identical to that previously described for goats (Fonseca et al., 2013). Briefly, after washing the perineal area of donor ewes with clean water and detergent, a Collin speculum (No. 1–3) previously lubricated with gel was slowly inserted into the vagina. The speculum was manipulated and positioned to visualize the cervical opening and then custom-made, 25-cm forceps (Pinça Embrapa® for cervical immobilization and traction in small ruminants; Embrapa, Brasília, Brazil) were inserted into and under the cervical os. Following the immobilization of the uterine cervix, sterile gauze soaked with 5 ml of 2% lidocaine without vasoconstrictors was gently placed ventrally to the cervical opening using the Allis forceps (26 cm). The cervical os was gently retracted to facilitate first the passage of Hegar dilator and after a catheter (No. 08; Sonda Embrapa® for goat/sheep embryo recovery; Embrapa, Brasília, Brazil) with a metal mandrel is used to traverse the cervical rings. After insertion of the catheter into the cervical opening, it was rotated and gently advanced along the inner cervical canal. The outermost caudal rings were traversed with aid of a thumb and index finger inserted under and above of the retracted cervix respectively (Figure 2a). To traverse the last cervical rings, the middle finger was inserted per rectum to allow cervical manipulation (Figure 2b). Prior to uterine flushing, the mandrel was removed, and the flushing catheter placed in the desired uterine horn using transrectal digital manipulation. After successfully penetrating the uterine cervix, a wooden box (50 cm long × 40 cm wide and 15 cm high) was used to elevate the ewes' hindquarters to avoid the flow of flushing fluid through the cervical canal outside of the catheter (Figure 2c). The catheter was then connected to a perfusion system (Circuito Embrapa® for goat/sheep embryo recovery; Embrapa, Brasília, Brazil) including a 60-ml syringe (Figure 2c) that was used to inject flushing liquid into each uterine horn, usually in fractions of ~10 ml. During each washing procedure, the part of a circuit attached to the filter was temporarily closed by a valve and part attached to the catheter remained open such that all flushing medium could be injected into each uterine horn. Subsequently, the part attached to the filter was opened allowing the flushing medium to be evacuated from the uterus. A total of 180 ml of medium was used to flush each uterine horn. The last step included careful removal of the gauge and forceps. Cervical penetration at oestrus and prior to embryo recovery was performed by the same experienced technician. All embryos collected were cryopreserved. The depth of the Hegar dilator insertion and the time taken to traverse the uterine cervix 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 <1 min; Grade 2 (easy;

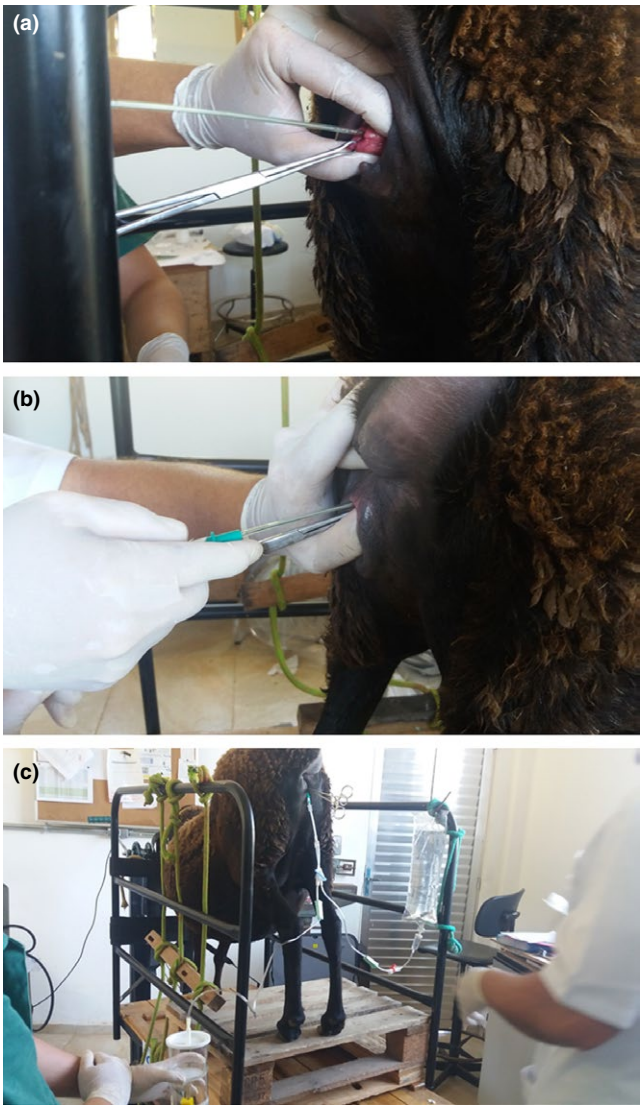


**FIGURE 2** Sequential steps of cervical penetration procedure: an ewe is restrained in an elevated cart, the uterine cervix is located, clipped and exteriorized with the aid of custom-made forceps (Embrapa® forceps for cervical immobilization and retraction). Initially, the first cervical rings are traversed using the thumb and index finger inserted, under and above the prolapsed cervical os, respectively (a), and the middle finger is inserted into the rectum to facilitate the penetration of the last cervical rings (b); after complete cervical penetration, the mandrel is removed, and the catheter is connected to the flushing circuit. A wooden box (50 cm × 40 cm and 15 cm high) is used to elevate the ewe's hindquarters to avoiding the loss of flushing fluid (c)

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).

## 2.4 | Blood sample collection and progesterone assays

Blood samples were collected from all ewes by jugular venipuncture into heparinized tubes on Day 0 (D0, just before the first administration of d-cloprostenol), D4, D7 (just before the second administration of d-cloprostenol) and D14 (immediately before the administration of EB). Blood plasma was separated by centrifugation at 1,500 × *g* for 15 min and stored at −20°C for hormone assays at a later date. The measurement of plasma progesterone ($P_4$) concentrations was done using solid-phase radioimmunoassay kits (Beckman Coulter; Immunotech, Marseille, France). The assay sensitivity and intra-assay coefficients of variation were 0.05 ng/ml and 12%, respectively.

## 2.5 | Data recorded and statistical analyses

The following data were recorded for both groups of ewes: (a) oestrous response (number of ewes in oestrous/number of treated ewes × 100%); (b) time of the oestrous onset (relative to second injection of cloprostenol); (c) maximum penetration depth at oestrus and during embryo flushing (measurement in cm obtained with the Hegar uterine dilator); (d) degree of difficulty of transcervical penetration in oestrous ewes and at the time of embryo flushing (Grades 1–5); (e) percentage of ewes with successful embryo collections; (f) duration of the flushing procedure (in minutes, from insertion of a vaginal speculum to clamp removal); (g) flushing fluid recovery rates (percentage of fluid recovered postinfusion); (h) number of recovered embryos/eggs; and (i) plasma $P_4$ concentration (ng/ml).

Statistical analyses were performed using the SAEG software (Ribeiro, 2001). A Fisher Exact test was used for nonparametric analyses, whereas one-way analysis of variance (ANOVA) was used for parametric data. All results were expressed as mean ± standard error (*SE*). Pearson correlation analyses were also performed. *p* value <0.05 was considered statistically significant.

# 3 | RESULTS

Following the second injection of cloprostenol, 91.3% (21/23) of ewes showed signs of behavioural oestrus that started, on average, 41.2 ± 8.1 hr after the second dose of cloprostenol. All ewes had $P_4$ concentration >1.5 ng/ml at least once during the study period. Plasma $P_4$ concentrations in ewes that were in oestrus after the synchronization protocol are shown in Table 1.

Transcervical uterine flushing was successfully performed in 17 females (81%) (Table 2). Five of 17 successfully penetrated ewes (two in EBim and three in EBivg group) had $P_4$ concentrations <1 ng/ml on Day 14 and embryos were recovered in only one of them (20.0%) while 12 ewes had $P_4$ concentrations >1 ng/ml on Day 14 and embryos were recovered in six of them (50%). Thus, the average percentage of successful uterine flushings that resulted in embryo recovery was 41.2% (7/17). A total of 11 structures were recovered from these seven ewes; 72.7% were nonviable (six unfertilized oocytes and two 4–8 cells embryos) and 27.3% were viable blastocysts. One ewe with no embryos recovered was diagnosed with uterine infection characterized by the presence of turbid uterine discharge. Times spent for cervical transposing at oestrus and embryo flushing

**TABLE 1** Mean (±*SEM*) plasma progesterone ($P_4$) concentrations (ng/ml) in Santa Inês ewes in which oestrus was synchronized* and nonsurgical embryo recovery (6–7 days after the onset of oestrus; on Day 14) was attempted following the application of cervical dilation protocols** with oestradiol benzoate (EB) administered by intramuscular (EBim) or intravaginal routes (EBivg)

| Day of sampling | EBim | EBivg |
|---|---|---|
| Day 0 | 2.9 ± 0.4 | 4.3 ± 1.3 |
| Day 4 | 1.9 ± 0.6 | 1.0 ± 0.2 |
| Day 7 | 3.5 ± 0.5 | 2.5 ± 0.5 |
| Day 14*** | 3.3 ± 0.9 | 2.7 ± 0.7 |

*Note*. $p > 0.05$. *Protocol: two injections of 37.5 μg of d-cloprostenol (Day 0 and Day 7); **Protocols: 37.5 μg of d-cloprostenol (latero-vulvar) and 1 mg of EB (intramuscular or intravaginal) were given 16 hr, and 50 IU of oxytocin (intravenous) were given 20 min before uterine procedure; ***Immediately before EB administration.

**TABLE 2** Treatment responses in Santa Inês ewes (mean ± *SEM*) subjected to an oestrous synchronization protocol with two doses of d-cloprostenol (Day 0 and Day 7) and nonsurgical embryo flushing (6–7 days after the onset of oestrus) performed after cervical relaxation protocols* using oestradiol benzoate (EB) administered by intramuscular (EMim) or intravaginal (EBivg) route

| End points | EBim | EBivg |
|---|---|---|
| Oestrous-synchronized response (%) | 91.6 (11/12) | 90.9 (10/11) |
| Ewe successfully penetrated at oestrus (%) | 100 (11/11) | 100 (10/10) |
| Depth of cervical penetration at oestrus (cm) | 9.7 ± 0.5 | 9.0 ± 0.6 |
| Time of cervical penetration at oestrus (min) | 3.9 ± 0.8 | 3.1 ± 0.8 |
| Time of cervical penetration at embryo flushing (min) | 4.0 ± 0.6 | 4.4 ± 1.0 |
| Ewes successfully collected (%) | 81.8 (9/11) | 80.0 (8/10) |
| Total duration of flushing procedure (min) | 24.7 ± 2.0 | 26.2 ± 1.9 |
| Fluid recovery efficiency (%) | 94.8 ± 1.3 | 90.1 ± 2.9 |
| Total structures recovered per ewe | 0.5 ± 0.4 | 0.8 ± 0.4 |
| Viable structures recovered per ewe | 0.1 ± 0.1 | 0.3 ± 0.1 |
| Viable embryos (%) | 20.0 (1/5) | 33.3 (2/6) |

*Note*. $p > 0.05$. *Protocols: 37.5 μg of d-cloprostenol (latero-vulvar) and 1 mg of EB (intramuscular or intravaginal) were given 16 hr and 50 IU of oxytocin (intravenous) 20 min before transcervical embryo recovery.

did not differ significantly ($p > 0.05$) within and between the two groups and were positively correlated ($r = 0.42$; $p < 0.05$).

Both cervical relaxation treatments produced similar ($p > 0.05$) results (Table 2). The mean time required to penetrate the cervix did not differ ($p > 0.05$) between the two treatment groups. The degree of difficulty at cervical penetration is presented in Table 3. From three out of four ewes that received Grade 4 at oestrus it was not possible to transverse the uterine cervix for embryo flushing.

## 4 | DISCUSSION

The main objective of the present trial-to perform transcervical embryo recovery from oestrous synchronized ewes-was achieved in 17 of 21 animals. Approximately 20% of ewes in the breeding season may still require surgical procedures to recover embryos. As stated earlier, the consistency and repeatability of the "Embrapa protocol for cervical relaxation and uterine flushing" in other breeds of sheep ewes remains to be determined (Fonseca et al., 2016).

In the present treatment groups (with intramuscular or intravaginal administration of oestradiol benzoate), the success rate of cervical penetration and embryo flushing was 80%–82%. The application of different cervical relaxation treatments may strongly influence the overall success of cervical penetration (Szabados, Gergatz, Vitinger, Zi, & Gyoker, 2005). However, there are very few studies on cervical relaxation during the luteal phase of the oestrous cycle in sheep (Candappa & Bartlewski, 2014; Fonseca et al., 2016; Robinson et al., 2011). In terms of cervical softening and penetrability, very promising results were obtained with intravaginal controlled release inserts containing $PGE_2$ (55% of cervical penetration rate; Candappa & Bartlewski, 2011), but this approach has not yet been tested in Santa Inês ewes raised in a subtropical or tropical climate. Pretreatment of ewes with $PGF_2\alpha$ (administered in the submucosa of the vaginal vestibule) at 12 hr or intravaginal $PGE_1$ at 5 hr before embryo recovery resulted in 58.8% and 63.2% of successful transcervical passages in Santa Inês ewes, respectively (Gusmão et al., 2007); that study utilized only those animals that showed signs of oestrus, as in the present study. Because of the success of uterine flushing achieved after intravaginal administration of cervical relaxing agents (Gusmão et al., 2007, 2009), we tried to use the same route of oestradiol benzoate administration in hope to further facilitate the cervical penetration, but no additional benefits were obtained by using intravaginal oestradiol benzoate administration. The duration of embryo flushing approached to 25 min in both treatment groups. This time is similar to 30 min reported in Dorper sheep, also receiving epidural anaesthesia (Gusmão et al., 2009), or cervical (26 min) and laparoscopic (30 min) embryo recovery in Crioula wool sheep (Oliveira et al., 2018). In addition, the overall flushing fluid recovery (~93%) appeared to be satisfactory, and it was similar to the 95% recovery rate reported by Gusmão et al. (2007), and slightly greater than 84% reported by Barry, Van Niekerk, Rust, and Van Der Walt (1990). It also should be emphasized that in the present study, a less invasive technique was used. While Gusmão et al. (2007, 2009) used two perforating Pozzi forceps, the present study used the Embrapa forceps, which provided sufficient cervical immobilization and retraction. Thus, the technique currently applied significantly reduces the number of forceps and hence potential vaginal injuries or abrasions.

Santa Inês sheep have a relatively low ovulation rate (1–1.3 ovulation per oestrous-induced ewe; Cavalcanti, Brandão, Nogueira, &

**TABLE 3** Number of Santa Inês ewes' responses (Grades 1–5) for degree of difficulty to transverse the uterine cervix at oestrus and prior to embryo flushing (6–7 days after the onset of oestrus). Embryo recovery was attempted after an application of the cervical dilation protocols* using oestradiol benzoate (EB) administered by intramuscular (EBim) or intravaginal (EBivg) route

| | EBim | | EBivg | |
|---|---|---|---|---|
| Grades | Oestrus | Embryo flushing | Oestrus | Embryo flushing |
| Total no. of ewes | 11 | 11 | 10 | 10 |
| 1 (<1 min) | 2 | 0 | 2 | 2 |
| 2 (between 1 and 3 min) | 3 | 2 | 6 | 1 |
| 3 (between 3 and 7 min) | 3 | 6 | 1 | 2 |
| 4 (between 7 and 10 min) | 3 | 1 | 1 | 3 |
| 5 (incomplete penetration after 10 min) | 0 | 2 | 0 | 2 |

*Note*. $p > 0.05$. *Protocols: 37.5 μg of d-cloprostenol (latero-vulvar) and 1 mg of EB (intramuscular or intravaginal) were given 16 hr and 50 IU of oxytocin (intravenous) 20 min before transcervical embryo recovery.

Fonseca, 2012; Teixeira et al., 2016; Venturi et al., 2016) and lamb productivity (1.3 lambs born per ewe; Mexia et al., 2004). Overall, the average egg/embryo recovery rate of 41.2% (7/17) obtained in this study appeared to be low. The most reasonable explanation of this fact is that in the nonstimulated (nonsuperovulated) ewes of the present study, we attempted either a single- or two-embryo recoveries. The other reason could be the occurrence of short-lived corpora lutea (CL) frequently associated with an oestrous synchronization protocol employed in the present study. In this experiment, 24% (5/21) of ewes had inadequate CL as indicated by plasma progesterone concentrations <1 ng/ml on the day of oestradiol administration (16 hr before embryo flushing). It has been well established that luteal progesterone affects early embryo development and self-regulates CL lifespan. Prolonged exposure to progesterone promotes uterine accumulation of arachidonic acid, prostaglandin endoperoxide synthase and other substances necessary for the synthesis of luteolysin-$PGF_2\alpha$. Progesterone also exerts a suppressive effect on $PGF_2\alpha$ secretion, which wanes later in the dioestrous phase (Silvia et al., 1991). Therefore, it is feasible that animals exhibiting abnormally short luteal phases did not have appropriate endocrine milieu to support cervical softening in response to a hormonal "cocktail" used in the present study. Low circulating progesterone concentrations could have also impinged negatively on embryo quality and survivability. Clearly, more research is needed on the influence of oestrous synchronization protocols and CL health status on the efficiency of embryo recovery in nonsuperstimulated ewes. In the ewes of the present study, only 27.3% of retrieved structures were viable. Menchaca et al. (2004), using the same oestrous synchronization protocol to that in the present study combined with the timed AI in sheep, reported conception rates ranging from 22% to 37%, while the same protocol used in goats resulted in mean conception rates of 55% to 85% (Maia et al., 2017). The reasons for dissimilar responses of sheep and goats to the same oestrous synchronization protocol remain unknown. One possible explanation of this phenomenon is the existence of differences in ovarian antral follicular dynamics between the two species. Two or three follicular waves are typically observed during the 17-day oestrous cycle of ewes (Bartlewski, Baby, & Giffin, 2011; Evans, Duffy, Hynes, & Boland, 2000) whilst three to four follicular waves and 21 days of oestrous cycle is reported in goats (Ginther & Kot, 1994; Simões et al., 2006). Some of the ovarian follicles that ovulated after the short-term oestrous synchronization protocol (7 days) in sheep could be inadequately mature and shed oocytes that were not fertilized or gave rise to poor-quality embryos after fertilization, which would explain the low number of viable structures recovered in the present study.

It is unlikely that a combination of hormonal drugs used in this study exerted negative effects on embryo viability although their direct influence on embryo health remains to be tested. Earlier studies have shown that treatment of goats with $PGF_2\alpha$ (Fonseca et al., 2014), $PGF_2\alpha$+OT (Pereira, Sohnrey, & Holtz, 1998) or ewes with EB+OT (Wulster-Radcliffe, Costine, & Lewis, 1999) prior to embryo recovery did not adversely affect embryo quality. Transfer of embryos recovered from donor goats pretreated with cloprostenol for 16 hr prior to embryo recovery resulted in nearly 60% pregnancy rate when both the fresh or frozen-thawed, good and excellent quality embryos were used (Fonseca et al., 2018). It will be interesting to evaluate the quality of the ovine embryos generated in this experiment with ensuing transfers or in vitro trials.

Time required for cervical penetration at oestrus was directly related to the time of complete cervical penetration prior to embryo flushing. In addition, in 75% of ewes that received Grade 4 (difficult cervical penetration) at oestrus, the attempt to traverse the uterine cervix at the time of embryo recovery was unsuccessful. Kershaw et al. (2005) described the three major types of cervical anatomy based on the alignment of cervical rings, namely the rectilinear, intermediate and highly asymmetrical. Although we could not visualize the details of cervical anatomy in live animals in the present study, we can speculate that according to the presently proposed classification criteria, Grades 1 and 2 probably correspond to the rectilinear

cervix, Grade 3 to the intermediated cervix and Grades 4 and 5 to the highly asymmetrical cervix. Thus, the scores obtained during attempt to penetrate the uterine cervix at oestrus are reliable predictors of a cervical penetration success rate during the nonsurgical embryo recovery in ewes.

Finally, the degree of difficulty at cervical penetration, the high percentage of successfully completed embryo flushing procedures (80%), the time required to complete transcervical embryo recovery (<30 min) and the rate of flushing media recovery (>90%) in the present dioestrous ewes, all promise to achieve similar results in superovulated donors. The feasibility of cervical access and manipulation (i.e., immobilization, retraction and penetrability) should be tested in individual donor ewes before any embryo transfer attempt. A method of embryo recovery described in this study is quick, efficient and safe. It certainly is a valid alternative to surgical techniques of embryo flushing presently used in small ruminants.

In summary, results of the present study indicate that nonsurgical (transcervical) uterine flushing can successfully be performed in oestrous-synchronized Santa Inês ewes receiving d-cloprostenol, oxytocin and oestradiol benzoate for cervical dilation, regardless of the route of oestradiol benzoate administration (intramuscular vs. intravaginal). This suitability of this protocol for embryo recovery in other breeds of sheep, at various times of the year and in superovulated ewes as well as the possible influence of this combination of cervical relaxing drugs on embryo viability remain to be assessed.

## ACKNOWLEDGEMENTS

The authors wish to thank staff members of the Embrapa Dairy Cattle for excellent technical assistance; the Embrapa Goats and Sheep (Project 03.12.01.031.00.00), Fapemig (PPM 00042-14), and The National Council for Scientific and Technological Development (CNPq; Project 310166/2012-8) for financial support. F.N. Zambrini was supported by CAPES-Embrapa Program. M.E.F. Oliveira and F.Z. Brandão are fellows of CNPq.

## CONFLICT OF INTEREST

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.

## AUTHOR CONTRIBUTIONS

JFF elaborated the hypothesis, discussed the experimental design, collected the data from the animals, analysed the data and wrote the first version of the manuscript. FNZ and JMGS-F collected the data from the animals. JDG, MEFO and JMGS-F discussed the design of the experiment and analysed the data. FZB performed the hormonal analyses and analysed the data. MRS elaborated and worked on the statistics. PMB revised it critically and JFF, PMB, MEFO and JMGS-F approved the final version of the manuscript.

## ORCID

*Jeferson Ferreira Fonseca* http://orcid.org/0000-0001-8028-3984

*Maria Emilia Franco Oliveira* http://orcid.org/0000-0002-7730-290X

## REFERENCES

Barry, D. M., Van Niekerk, C. H., Rust, J., & Van Der Walt, T. (1990). Cervical embryo collection in sheep after ripening of the cervix with prostaglandin $E_2$ and oestradiol. *Theriogenology*, *33*, 190. https://doi.org/10.1016/0093-691X(90)90614-Y

Bartlewski, P. M., Baby, T. E., & Giffin, J. L. (2011). Reproductive cycles in sheep. *Animal Reproduction Science*, *124*, 259–268. https://doi.org/10.1016/j.anireprosci.2011.02.024

Candappa, I. B. R., & Bartlewski, P. M. (2011). Review of advances in artificial insemination (AI) and embryo transfer (ET) in sheep, with the special reference to hormonal induction of cervical dilation and its implications for controlled animal reproduction and surgical techniques. *The Open Reproductive Science Journal*, *3*, 162–175. https://doi.org/10.2174/1874255601103010162

Candappa, I. B. R., & Bartlewski, P. M. (2014). Induction of cervical dilation for transcervical embryo transfer in ewes. *Reproductive Biology and Endocrinology*, *12*, 8. https://doi.org/10.1186/1477-7827-12-8

Cavalcanti, A. S., Brandão, F. Z., Nogueira, L. A. G., & Fonseca, J. F. (2012). Effects of GnRH administration on ovulation and fertility in ewes subjected to estrous synchronization. *Revista Brasileira de Zootecnia*, *41*, 1412–1418. https://doi.org/10.1590/S1516-35982012000600014

Einer-Jensen, N., & Hunter, R. (2005). Counter-current transfer in reproductive biology. *Reproduction*, *129*, 9–18. https://doi.org/10.1530/rep.1.00278

Evans, A. C., Duffy, P., Hynes, N., & Boland, M. P. (2000). Waves of follicle development during the estrous cycle in sheep. *Theriogenology*, *53*, 699–715. https://doi.org/10.1016/S0093-691X(99)00268-X

Fonseca, J. F., Batista, R. I. T. P., Souza-Fabjan, J. M. G., Oliveira, M. E. F., Brandão, F. Z., & Viana, J. H. M. (2018). Freezing Goat Embryos at different developmental stages and quality using Ethylene Glycol and a Slow Cooling Rate. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *70*, 1489–1496.

Fonseca, J. F., Esteves, L. V., Zambrini, F. N., Brandão, F. Z., Peixoto, M. G. C. D., Verneque, R. S., … Viana, J. H. M. (2014). Viable offspring after successful nonsurgical embryo transfer in goats. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *66*, 613–616. https://doi.org/10.1590/1678-41626783

Fonseca, J. F., Souza-Fabjan, J. M. G., Oliveira, M. E. F., Cruz, R. C., Esteves, L. V., Matos de Paiva, M. P. S. L., … Mancio, A. B. (2017). Evaluation of cervical mucus and reproductive efficiency of seasonally anovular dairy goats after short-term progestagen-based estrous induction protocols with different gonadotropins. *Reproductive Biology*, *17*, 363–369. https://doi.org/10.1016/j.repbio.2017.10.002

Fonseca, J. F., Souza-Fabjan, J. M. G., Oliveira, M. E. F., Leite, C. R., Nascimento-Penido, P. M. P., Brandão, F. Z., & Lehloenya, K. C. (2016). Nonsurgical embryo recovery and transfer in sheep and goats. *Theriogenology*, *86*, 144–151. https://doi.org/10.1016/j.theriogenology.2016.04.025

Fonseca, J. F., Zambrini, F. N., Alvim, G. P., Peixoto, M. G. C. D., Verneque, R. S., & Viana, J. H. M. (2013). Embryo production and recovery in goats by non-surgical transcervical technique. *Small Ruminant Research*, *111*, 96–99. https://doi.org/10.1016/j.smallrumres.2012.08.007

Ginther, O. J., & Kot, K. (1994). Follicular dynamics during the ovulatory season in goats. *Theriogenology*, *42*, 987–1001. https://doi.org/10.1016/0093-691X(94)90121-X

Gusmão, A. L., Silva, J. C., Bittencourt, L. E. P., Martins, H. D. G., & Barbosa, L. P. (2009). Coleta transcervical de embriões em ovinos da raça Dorper no semiárido do Nordeste Brasileiro. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, *61*, 313–318. https://doi.org/10.1590/S0102-09352009000200005

Gusmão, A. L., Silva, J. C., Quintela, A., Moura, J. C. A., Resende, J., Gordiano, H., … Ribeiro Filho, A. L. (2007). Colheita transcervical de ovinos da raça Santa Inês no semi-árido nordestino. *Revista Brasileira de Saúde e Produção Animal*, *8*, 1–10.

Kershaw, C. M., Khalid, M., Mcgowan, M. R., Ingram, K., Leethongdee, S., Wax, G., & Scaramuzzi, R. J. (2005). The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. *Theriogenology*, *64*, 1225–1235. https://doi.org/10.1016/j.theriogenology.2005.02.017

Khalifa, R. M. E., Sayre, B. L., & Lewis, G. S. (1992). Exogenous oxytocin dilates the cervix in ewes. *Journal of Animal Science*, *76*, 38–42.

Maia, A. L. R. S., Brandão, F. Z., Souza-Fabjan, J. M. G., Balaro, M. F. A., Oliveira, M. E. F., Facó, O., & Fonseca, J. F. (2017). Reproductive parameters of dairy goats after receiving two doses of d-cloprostenol at different intervals. *Animal Reproduction Science*, *181*, 16–23. https://doi.org/10.1016/j.anireprosci.2017.02.013

Menchaca, A., Miller, V., Gil, J., Pinczak, A., Laca, M., & Rubianes, E. (2004). Prostaglandin F2α treatment associated with timed artificial insemination in Ewes. *Reproduction in Domestic Animals*, *39*, 352–355. https://doi.org/10.1111/j.1439-0531.2004.00527.x

Mexia, A. A., Macedo, F. A. F., Alcalde Sakaguti, E. S., Martins, E. N., Yamamoto, M., & Macedo, R. M. G. (2004). Desempenho reprodutivo e produtivo de ovelhas Santa Inês suplementadas diferentes fases da gestação. *Revista Brasileira de Zootecnia*, *33*, 658–667. https://doi.org/10.1590/S1516-35982004000300014

National Research Council–NRC. (2007). Nutrient requirements of goats. Washington, DC, Ed. Natl. Acad. Science.

Oliveira, F. C., Haas, C. S., Ferreira, C. E. R., Goulartea, K. L., Pegoraro, L. M. C., Gasperina, B. G., … Vieira, A. D. (2018). Inflammatory markers in ewes submitted to surgical or transcervical embryo collection. *Small Ruminant Research*, *158*, 15–18. https://doi.org/10.1016/j.smallrumres.2017.11.012

Pereira, R. J. T. A., Sohnrey, B., & Holtz, W. (1998). Nonsurgical embryo collection in goats treated with prostaglandin F2α and oxytocin. *Journal of Animal Science*, *76*, 360–363. https://doi.org/10.2527/1998.762360x

Ribeiro, I. Jr (2001). *Análise estatísticas no SAEG* (p. 301). Viçosa, Brazil: Editora UFV.

Robinson, J. J., McKelvey, W. A. C., King, M. E., Mitchell, S. E., Mylne, M. J. A., McEvoy, T. G., … Williams, L. M., (2011). Traversing the ovine cervix: A challenge for cryopreserved semen and creative science. *Animal*, *5*, 1791–1804. https://doi.org/10.1017/S1751731111000978

Silvia, W. J., Lewis, G. S., McCracken, J. A., Thatcher, W. W., & Wilson, L. Jr (1991). Hormonal regulation of uterine secretion of prostaglandin F2, during luteolysis in ruminants. *Biology of Reproduction*, *45*, 655–663. https://doi.org/10.1095/biolreprod45.5.655

Simões, J., Almeida, J. C., Valentim, R., Baril, G., Azevedo, J., Fontes, P., & Mascarenhas, R. (2006). Follicular dynamics in Serrana goats. *Animal Reproduction Science*, *95*, 16–26. https://doi.org/10.1016/j.anireprosci.2005.09.005

Szabados, T., Gergatz, E., Vitinger, E., Zi, T., & Gyoker, E. (2005). Lambing rate as a function of artificial insemination depth in ewe lambs primiparous and multiparous ewes. *Acta Agraria Kaposvariensis*, *9*, 41–49.

Teixeira, T. A., Fonseca, J. F., Souza-Fabjan, J. M. G., Carvalheira, L. R., Fernandes, D. A. M., & Brandão, F. Z. (2016). Efficiency of different hormonal treatments for estrus synchronization in tropical Santa Inês sheep. *Tropical Animal Health and Production*, *48*, 545–551. https://doi.org/10.1007/s11250-015-0989-y

Thatcher, W. W., Binelli, M., Burke, J., Staples, C. R., Ambrose, J. D., & Coelho, S. (1997). Antiluteolytic signals between the conceptus and endometrium. *Theriogenology*, *47*, 131–147. https://doi.org/10.1016/S0093-691X(96)00347-0

Venturi, S. S., Fonseca, J. F., Ribeiro, A. C. S., Pinheiro Rogério, M. C., Oliveira, J., Araújo, A. R., … Brandão, F. Z. (2016). Energy supplementation in Santa Inês sheep subjected to estrus induction treatment. *Semina Ciências Agrárias*, *37*, 145–154. https://doi.org/10.5433/1679-0359.2016v37n1p145

Wathes, D. C., & Lamming, G. E. (1995). The oxytocin receptor, luteolysis and the maintenance of pregnancy. *Journal of Reproduction and Fertility Supplement*, *49*, 53–67.

Wulster-Radcliffe, M. C., Costine, B. A., & Lewis, G. S. (1999). Estradiol-17 beta - oxytocin- induced cervical dilation in sheep: Application to transcervical embryo transfer. *Journal of Animal Science*, *77*, 2587–2593. https://doi.org/10.2527/1999.77102587x

**How to cite this article:** da Fonseca JF, Zambrini FN, Guimarães JD, et al. Combined treatment with oestradiol benzoate, d-cloprostenol and oxytocin permits cervical dilation and nonsurgical embryo recovery in ewes. *Reprod Dom Anim*. 2019;54:118–125. https://doi.org/10.1111/rda.13318