

## Cervical penetration rates and efficiency of non-surgical embryo recovery in estrous-synchronized Santa Inês ewes after administration of estradiol ester (benzoate or cypionate) in combination with d-cloprostenol and oxytocin



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### ABSTRACT

The effects of estradiol esters, d-cloprostenol and oxytocin on induction of cervical dilation prior to non-surgical embryo recovery in Santa Inês ewes (Days 6–7 estrous cycle) were assessed in this study. In Trial 1, transcervical embryo flushing was performed in estrous-induced ewes administered 37.5 µg of d-cloprostenol i.m. 10 h before and 50 IU of oxytocin i.v. 20 min before uterine flushing with (EB-PGF-OT; *n* = 13) or without (PGF-OT; *n* = 11) 1 mg of estradiol benzoate i.m. administered concurrently with d-cloprostenol injection. In Trial 2, the estrous-synchronized animals were treated with 1 mg of estradiol benzoate (EB-PGF-OT; *n* = 12) or estradiol cypionate (EC-PGF-OT; *n* = 12) i.m. along with 37.5 µg of d-cloprostenol i.m. 16 h before and 50 IU of oxytocin i.v. 20 min before uterine flushing. In Trial 1, uterine flushing could be accomplished in 38% of ewes in the EB-PGF-OT and 27% those in the PGF-OT (*P* > 0.05) group. Flushing fluid recovery averaged 90% and there were 1.0 ± 1.1 embryos/ewe collected with mean duration of the flushing procedure being ~36 min. In Trial 2, uterine flushing was accomplished in 78% of ewes in the EB-PGF-OT and 44% of those in the EC-PGF-OT group (*P* > 0.05) with mean flushing fluid recovery rate being 88% and time elapsing to complete flushing being ~33 min. Within the subsets of animals treated with EB, the percentages of successful transcervical penetrations were 38% compared with 78% in Trials 1 and 2, respectively (i.e., with EB administered 10 h compared with 16 h before uterine flushing: *P* < 0.05). The interval from EB administration to the beginning of transcervical penetration can affect the efficacy of embryo recovery procedures utilizing a combined EB/d-cloprostenol/oxytocin pre-treatment.

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## 1. Introduction

Embryo transfer technology has been widely used to provide a rapid increase in the genetic quality of livestock and in conservation programs (Betteridge, 2006; Candappa and Bartlewski, 2011; Bartlewski et al., 2016). Safe and efficient embryo recovery techniques are essential for the success of embryo transfer. Laparotomy and laparoscopy are the most frequently used methods of embryo recovery in ewes (Bari et al., 2001). Several disadvantages of surgical approaches, including the relatively greater cost compared to non-surgical collections of equipment, stress endured by animals, and the formation of adhesions, significantly limit the frequency and number of such procedures that can be successfully conducted (Fonseca et al., 2016). Thus, repeated laparotomy and laparoscopic embryo flushing remain a concern when considering animal welfare implications when there is use of these procedures (Walker et al., 2011).

There is increasing evidence that non-surgical embryo recovery is a viable alternative to the surgical methods in small ruminants (Candappa and Bartlewski, 2011; Fonseca et al., 2016). While transcervical manipulations and embryo flushing in goats do not normally require the induction of cervical dilation, the hormonal treatments promoting moderate cervical dilation greatly facilitate transcervical embryo recovery in ewes (Candappa and Bartlewski, 2011; Fonseca et al., 2016). Several compounds and combinations of compounds have successfully been applied to dilate uterine cervix for transcervical artificial insemination (Khalifa et al., 1992; Candappa and Bartlewski, 2011), but the attempts to stimulate cervical dilation during the mid-luteal phase of the ewe's estrous cycle/early pregnancy have produced variable results (Candappa and Bartlewski, 2014). An application of misoprostol (prostaglandin E<sub>1</sub> analogue) in estrous cyclic Santa Inês ewes resulted in nearly 95% embryo flushing efficiency (Gusmão et al., 2009); however, the use of misoprostol in veterinary practice is currently prohibited in Brazil. The use of prostaglandin F<sub>2α</sub> alone has also increased the ease with which transcervical embryo recovery could be performed in goats (Pereira et al., 1998) and ewes (Gusmão et al., 2007). Transcervical embryo recovery in ewes following the treatment with estradiol-17β and oxytocin was nearly as successful as laparoscopic embryo recovery (Wulster-Radcliffe et al., 1999). There have been reports that a combined treatment with estradiol benzoate, n-cloprostenol (both administered 16 h before embryo flushing) and oxytocin (20 min prior to cervical penetration attempt) provides for an efficient means of cervical dilation prior to embryo recovery in Santa Inês ewes (Fonseca et al., 2019a).

An ideal hormonal treatment to induce cervical dilation should not result in any adverse maternal or embryonic effects (Candappa and Bartlewski, 2011). The administration of estrogens and cloprostenol-induced luteolysis, however, may have deleterious effects on embryo viability. Maternal exposure to estrogens can induce long-term adverse changes in the preimplantation embryo (Flöter et al., 2018). The duration of the period from the time of exogenous estrogen administration to embryo recovery in ewes should be minimized or if possible the practice of administering estrogens prior to uterine flushing should be eliminated. The effects of shortening the interval from estradiol injections to the time of uterine flushing on the duration and feasibility of the procedure, however, have not been studied. In addition, different estradiol esters with similar potency can have dissimilar pharmacokinetic properties. For example, the administration of estradiol cypionate results in a lower amplitude peak in concentrations of estradiol and estrone compared with when there is administration of estradiol valerate or benzoate (Oriowo et al., 1980). Thus, the potential for inclusion of various estradiol esters in cervical dilation protocols needs to be evaluated.

Considering all of the previous findings with research involving non-surgical embryo recovery in ewes, there was design of two separate trials to determine: 1) the efficiency of a combined cloprostenol-oxytocin priming with or without estradiol benzoate administered 10 h before transcervical embryo recovery (i.e., 6 h later compared with the previously used protocols); and 2) the effect of two estradiol esters (estradiol benzoate and cypionate) on cervical dilation and transcervical penetration in estrous cycling (mid-luteal phase) Santa Inês ewes.

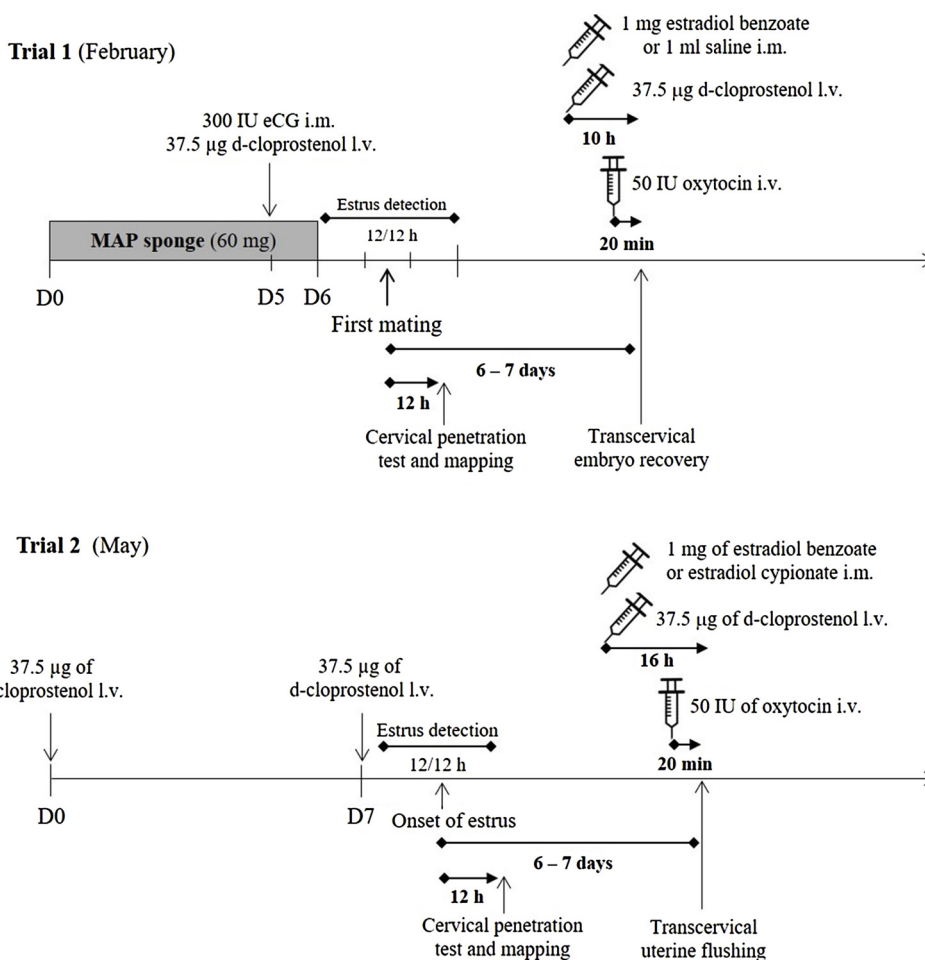
## 2. Materials and methods

### 2.1. General experimental conditions and animals

The present research project followed the guidelines of the National Council for Control of Animal Experimentation (CONCEA) and had been approved by the Ethics Committee on Animal Use of the Embrapa Dairy Cattle Corporate (CEUA/EGL) (protocol number CEUA 2,512,100,516; 06/30/2016). This study was conducted from February to May in Brazil (latitude 21°35'S, longitude 43°15'W, and altitude of 435 m.a.s.l.). Multiparous Santa Inês ( $n = 24$ ) ewes selected for the present experiments (aged 4 years, 12 to 14 months after last lambing) underwent clinical evaluation including vaginoscopy and transrectal ultrasonography of the reproductive tract using the 8.0-MHz probe and the M5VET device (Mindray®; Shenzhen, Guangdong, China). The ewes were maintained in an intensive production system and received corn silage and *Pennisetum purpureum* as forage, with a balanced concentrate offered according to their nutritional demands (National Research Council–NRC, 2007). Mineralized salt licks and drinking water were available *ad libitum*.

### 2.2. Experimental design

Two trials (Fig. 1) with the starting dates 12 weeks apart were performed during the first half of the local breeding season (Balara et al., 2014). To ensure there would be an adequate number of animals in estrus at the beginning of the study, all ewes in Trial 1 received intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon®, Syntex, Buenos Aires, Argentina) for 6 d plus 300 IU of eCG (Novormon 5000®, Syntex, Buenos Aires, Argentina) i.m. and 37.5 µg of n-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) via the laterovulvar route 24 h before sponge removal. In Trial 2, time of estrus was synchronized in the same ewes



**Fig. 1.** Schematic representation of the experimental procedures used in two trials designed to assess different hormonal protocols for cervical dilation and uterine flushing at 6 or 7 days after the onset of estrus in multiparous Santa Inês ewes; MAP: medroxyprogesterone acetate; eCG: equine chorionic gonadotropin; i.m. intramuscular; i.v. intravenous; l.v.: laterovulvar.

using two laterovulvar injections of 37.5 µg of D-cloprostenol 7 d apart. After sponge removal, or after the second dose of D-cloprostenol, estrus was detected with “teaser” rams that were placed in the pen with ewes twice a day for 30 min, and the ewes with expressions of behavioral estrous signs mated with fertile rams (Trial 1) with a ram:ewe ratio of approximately 1:6 whereas in Trial 2 non-pregnant ewes in the mid-luteal phase of their estrous cycle were used. In both trials, the ewes were then grouped according to similar body weight (BW) and condition score (BCS: range 1-very thin to 5-obese) into two treatment groups. In Trial 1, the ewes in the EB-PGF-OT group ( $n = 13$ , BW:  $62.7 \pm 7.2$  kg, BCS:  $4.3 \pm 0.4$ ) was administered 1 mg of estradiol benzoate i.m. (EB; Estrogen<sup>®</sup>, Farmavet, São Paulo, Brazil) and 37.5 µg d-cloprostenol (Prolise<sup>®</sup>; Tecnopec, São Paulo, Brazil) by the laterovulvar route and 50 IU of oxytocin i.v. (OT; Ocitocina forte<sup>®</sup>, UCB, São Paulo, Brazil) approximately 10 h and during a 20 min period before uterine flushing on Day 7, respectively (Day 0 = onset of behavioral estrus). The second group of ewes (PGF-OT group;  $n = 11$ , BW:  $63.4 \pm 13.2$  kg, BCS:  $4.3 \pm 0.3$ ) was administered an i.m. injection of saline. In Trial 2, the ewes were allocated to the EB-PGF-OT group ( $n = 12$ , BW:  $63.0 \pm 7.2$  kg, BCS:  $4.2 \pm 0.3$ ) and the EC-PGF-OT group ( $n = 12$ , BW:  $62.4 \pm 6.9$  kg, BCS:  $4.1 \pm 0.2$ ) with the latter group being administered 1 mg of estradiol cypionate (EC; ECP<sup>®</sup>, Zoetis, Brazil). In both groups, hormonal treatments were administered 16 h before (EB, EC and D-cloprostenol injections) and 50 IU of oxytocin i.v. was administered 20 min before the uterine flushing that was conducted 7 d after the onset of estrus. All embryos recovered in Trial 1 were cryopreserved as previously described by Fonseca et al. (2019a).

In both trials, an initial attempt of transcervical penetration was performed ~12 h after the onset of estrus. As in a previous experiment (Fonseca et al., 2019a), this attempt occurred to determine if transcervical passage of a catheter in estrous ewes would be predictive of cervical penetration rates 6 or 7 d later. All ewes were restrained in a sheep cart in a standing position and were treated using a chemical restraint protocol immediately before the insertion of a vaginal speculum (Table 1).

**Table 1**

Chemical restraint protocol used for embryo donor sheep before cervical clipping attempt both at estrus and at non-surgical uterine flushing.

Drug	Dose	Route	Time before uterine flushing
Acepromazine maleate 1%*	0.1 mg / kg BW	Intramuscular	20 min
Lidocaine 2%**	2 mL / animal	Epidural (S5-C1)	5 min
Lidocaine 2%	3 to 5 mL / animal	Intravaginal	3 min
Dipyron and n-butyl hyoscine bromide solution***	10 mL	Intravenous (5 mL) Intramuscular (5 mL)	20 min

\* Aceproven® Vencofarma, Londrina, Paraná, Brazil.

\*\* Lidovet®, Bravet, Rio de Janeiro, Brazil.

\*\*\* Bucofin Composto®, Agener União, Taboão da Serra, Brazil.

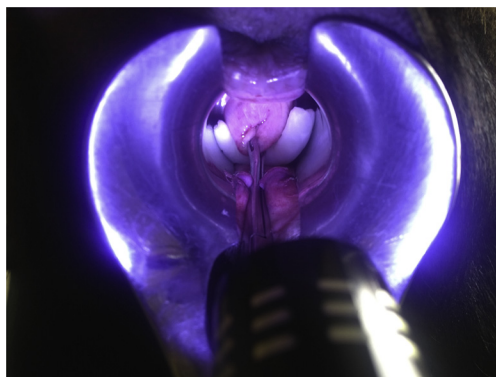
### 2.3. Cervical manipulations and uterine flushing

The perineal area of donor ewes was washed with clean water and detergent. A Collin speculum (size 2) lubricated with sterile, hydrosoluble gel was slowly inserted into the vagina. A light source was used to facilitate maintenance of the speculum in a central position where there could be visualization of the cervical opening. A custom-made, 25-cm forceps (Pinça Embrapa® for cervical immobilization and traction in small ruminants; Embrapa, Brasília, Brazil) with the tips inserted into and under the cervical os was used to immobilize the uterine cervix. After cervical immobilization, a sterile gauze soaked with 5 mL of 2% lidocaine without vasoconstrictors (Table 1) was gently placed ventrally to the cervical opening with the aid of an Allis forceps (26 cm) and left in place for the duration of the procedure (Fig. 2). The cervical os was retreated and a number 3 Hegar uterine dilator (at estrus) or a number 8 catheter (Sonda Embrapa® for goat/sheep embryo recovery; Embrapa, Brasília, Brazil) equipped with a metal mandrel (at uterine flushing) was inserted into the cervical opening. A gentle rotation and forward movement were needed to slowly penetrate through the cervix between the cervical rings. The first caudal rings were traversed using the thumb and index finger inserted under and above of the prolapsed cervix, respectively. 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 at estrus to create the “cervical map”, as previously described (Fonseca, 2017; Fonseca et al., 2019b). The cervical map was then used to help position the flushing catheter during the cervical passage prior to embryo recovery. The uterine flushing technique used in the present study has previously been described (Fonseca et al., 2019a). Briefly, after traversing the cervical rings, the catheter was positioned in the desired uterine horn which was confirmed by rectal palpation (only fingers), and the mandrel was slowly removed as there was cranial traversing with the catheter. A custom-designed circuit was then connected to the catheter (Circuito Embrapa® for goat/sheep embryo recovery; Embrapa, Brasília, Brazil). A total of 180 mL of medium was used to flush each uterine horn, usually in fractions of ~10 to 20 mL. After flushing, the gauge and forceps were gently removed.

### 2.4. Data recorded and statistical analyses

The following data were recorded for all animals in both trials: estrous response (number of ewes in estrus/number of estrous synchronized ewes × 100%); the number of cervical rings traversed and maximum penetration depth (measured with the Hegar uterine dilator from the cervical os opening towards the uterine lumen) at estrus and at the time of uterine embryo recovery; flushing fluid recovery (%) and the total number of structures recovered at embryo flushing.

Statistical analyses were performed using the SAEG software (SAEG Sistema para Análises Estatísticas, Versão 9.1: Fundação Arthur Bernardes, UFV, Viçosa, 2007). A Fisher Exact test was used for non-parametric analyses, whereas one-way analysis of variance (ANOVA) and Tukey test were used to analyze numeric data expressed as means ± standard deviations (SD). Proportions were analyzed using a  $\chi^2$ -square test (Brendt-Snedecor formula; Cochran and Cox, 1957). *P* value of < 0.05 was considered



**Fig. 2.** A photograph depicting the illuminated and partially retracted cervical os, and a swab soaked with 2 mL of lidocaine placed underneath the cervical opening.

**Table 2**

Summary (mean  $\pm$  SD or %) of the cervical penetration attempts and embryo recovery procedures in multiparous Santa Inês ewes that received 1 mg of estradiol benzoate (EB-PGF-OT group) or saline (PGF-OT group) i.m. and 37.5  $\mu$ g d-cloprostenol via the laterovulvar route 10 h before and 50 IU of oxytocin i.v. at 20 min before transcervical uterine flushing performed 6 or 7 d after estrous synchronization of ewes during the early portion of the breeding season.

End-points	Treatment		Overall
	EB-PGF-OT (n = 13)	PGF-OT (n = 11)	
% of ewes penetrated at estrus (%)	53.8 (7/13)	36.3 (4/11)	45.8 (11/24)
Number of cervical rings traversed at estrus	4.7 $\pm$ 1.4	4.0 $\pm$ 1.8	4.4 $\pm$ 1.6
Maximum depth of penetration at estrus (cm)	8.7 $\pm$ 4.1	5.9 $\pm$ 4.5	7.4 $\pm$ 4.5
Number of cervical rings traversed at uterine flushing	5.8 $\pm$ 1.3	6.0 $\pm$ 1.0	5.9 $\pm$ 1.1
Maximum depth of penetration at uterine flushing (cm)	10.0 $\pm$ 3.4	10.0 $\pm$ 6.1	10.0 $\pm$ 4.1
Ewes successfully collected (%)	38.5 (5/13)	27.3 (3/11)	33.3 (8/24)
Ewes penetrated at embryo collection/ewes penetrated at estrus (%)	71.4 (5/7)	75.0 (3/4)	72.7 (8/11)
Duration of uterine flushing (min)	35.2 $\pm$ 8.8	36.3 $\pm$ 14.3	35.6 $\pm$ 10.1
Fluid recovery (%)	90.4 $\pm$ 14.3	91.0 $\pm$ 8.3	90.1 $\pm$ 9.3
Number of embryos recovered	1.0 $\pm$ 1.2	1.0 $\pm$ 1.0	1.0 $\pm$ 1.1

statistically significant and  $P < 0.10$  was considered approaching significance or as a tendency.

### 3. Results

In Trial 1, all ewes expressed behavioral estrus within 72 h of the MAP sponge removal and were subsequently subjected to experimental procedures (i.e., cervical penetration attempt at estrus and embryo recovery 7 d after the onset of behavioral estrus). There were no differences ( $P > 0.05$ ) between the two cervical relaxation-promoting treatments (i.e., with or without estradiol benzoate being administered 10 h before embryo recovery) for any of the end points analyzed in this study (Table 2). At estrus, the average number of traversed cervical rings was  $3.3 \pm 1.6$  in the ewes with incomplete cervical penetration and  $5.9 \pm 1.1$  in the ewes with complete cervical passage. The average cervical penetration depth was greater by  $\sim 3$  cm at the time of uterine flushing compared with estrus. When data for all animals were pooled, the average duration of the uterine flushing procedure was  $35.6 \pm 10.1$  min (range: 27 to 52 min), the mean fluid recovery post-flushing was 90.1% (range: 70 to 100%), and the number of collected embryos was  $1.0 \pm 1.1$  (range: 0 to 3). No structures (i.e. embryos or unfertilized eggs) were recovered from two ewes in the EB-PGF-OT group and from one ewe in the PGF-OT group. In ewes of EB-PGF-OT group, five structures were recovered with four being viable embryos (80.0%) and one unfertilized oocyte. In ewes of the PGF-OT group, three structures were recovered with two being viable embryos (66.7%) and one unfertilized oocyte.

In Trial 2, cervical penetration was attempted only in the ewes that expressed signs of behavioral estrus within the 72-h period after estrous synchronization with d-cloprostenol (18/24 ewes; nine ewes from each treatment group). Complete cervical penetration was feasible in 11 of 18 estrous ewes (six ewes from the EB-PGF-OT group and five from the EC-PGF-OT group,  $P > 0.05$ ). From all the ewes in which cervical penetration could be performed at estrus, only in one ewe treated with EC could there not be cervical penetration at the time of embryo recovery, and one animal treated with EB in which transcervical penetration could not be performed at estrus was there successful uterine flushing 7 d after the onset of estrus. Collectively, in a total of 90.9% of ewes (10/11) in which there was transcervical penetration at estrus there was again transcervical penetration at the time when there was an attempt at embryo recovery (Table 3). No sequels or abrasions of the vaginal canal and cervical os were observed in Trial 2. There were no differences ( $P > 0.05$ ) between the two groups of ewes for any of the values for variables analyzed in this experiment.

**Table 3**

Results (mean  $\pm$  SD or %) of cervical penetration and uterine flushing procedures in estrus-synchronized Santa Inês ewes that received 1 mg of estradiol benzoate (EB) or cypionate (EC) i.m. 16 h before uterine flushing along with 37.5  $\mu$ g of d-cloprostenol (PGF, laterovulvar) administered at the same time of estradiol ester injections and oxytocin (OT, i.v.) administered 20 min before uterine flushing at 6 or 7 d after the onset of estrus.

End-points	Treatment		Overall
	EB-PGF-OT (n = 12)	EC-PGF-OT (n = 12)	
Estrous response (%)	75 (9/12)	75 (9/12)	75 (18/24)
Cervix surpassed at estrus (%)	66.7 (6/9)	55.5 (5/9)	61.1 (11/18)
Number of cervical rings traversed at estrus	4.4 $\pm$ 1.8	4.1 $\pm$ 2.0	4.2 $\pm$ 1.9
Maximum depth of penetration at estrus (cm)	7.3 $\pm$ 4.5	6.4 $\pm$ 5.1	6.9 $\pm$ 4.7
Number of cervical rings traversed at uterine flushing	5.7 $\pm$ 0.9	5.5 $\pm$ 1.3	5.6 $\pm$ 1.0
Maximum depth of penetration at uterine flushing (cm)	11.2 $\pm$ 3.6	10.2 $\pm$ 3.2	10.8 $\pm$ 3.3
Ewes successfully collected (%)	77.8 (7/9)	44.4 (4/9)	61.1 (11/18)
Ewes penetrated at embryo collection/ewes penetrated at estrus (%)	116.6 (7/6)	80.0 (4/5)	100.0 (11/11)
Duration of uterine flushing procedure (min)	35.6 $\pm$ 9.7	28.0 $\pm$ 10.4	32.8 $\pm$ 10.2
Fluid recovery efficiency (%)	87.2 $\pm$ 15.4	90.2 $\pm$ 11.4	88.3 $\pm$ 13.5

When considering only those animals that expressed signs of estrus regardless of the hormonal treatment(s) received, the overall rate of cervical penetration tended to be less ( $P = 0.07$ ) in Trial 1 (33.3% or 8/24) than in Trial 2 (61.1% or 11/18). Within the subsets of animals primed with estradiol benzoate, the difference in proportions of ewes in which there was successful transcervical penetration between the two trials (10 h compared with 16 h before uterine flushing) was even greater (38.5% or 5/13 compared with 78% or 7/9,  $P < 0.05$ ).

#### 4. Discussion

Even though the percentage of ewes in which there was successful transcervical penetration of uterine cervixes after the EB-PGF-OT treatment was 10% greater than that in the PGF-OT group, the difference was only numerical and not statistically significant. The percentage of ewes in which uterine flushing could be accomplished in Trial 1 was 33%, which is less than that in primiparous cross Suffolk and mixed-parity Texel ewes (50% and 53%, respectively; Mylne et al., 1992). Interestingly, the transcervical passage the devices when ewes were in estrus (i.e., in animals without any cervical dilatation treatment) was possible in 53.8% of ewes in the EB-PGF-OT group and 36.3% of ewes in the PGF-OT group. Clearly, there exists tremendous individual variation in the ease with which the uterine cervix can be traversed with embryo collection instruments. A degree of cervical ring misalignment (i.e., anatomical characteristic of ewes that is not related to age or parity; Bartlewski and Candappa, 2015) may affect the extent to which cervical penetration can occur. Trial 1 was primarily designed to assess the effect of shortening the interval from estradiol benzoate administration to embryo recovery from 16 h (Fonseca et al., 2019a) to 10 h or eliminating estradiol benzoate injections from the cervical dilation protocol. Both regimens used in the present study appeared to be less effective compared with the use of estradiol benzoate-based protocols initiated 16 h before uterine flushing that resulted in the transcervical penetration rate of 82% in estrous cyclic Santa Inês ewes (Fonseca et al., 2019a). In Trial 2, the transcervical uterine flushing was performed in 61.1% of ewes, similarly to what was reported from an earlier study in Santa Inês sheep subjected to misoprostol-based cervical dilation pretreatment (Gusmão et al., 2007). Within the EB-PGF-OT group in the present study, uterine flushing was accomplished in 77.8% of ewes, confirming that a treatment with estradiol benzoate 16 h before transcervical embryo collection in Santa Inês ewes was consistent in yielding desirable outcomes for embryo recovery in ewes (Fonseca et al., 2019a).

Results of earlier studies indicate that the breed may affect the capacity for successful transcervical penetration in ewes (Kaabi et al., 2006; Bartlewski and Candappa, 2015). When the misoprostol-based cervical dilation protocol was initially assessed in Santa Inês ewes, there was a 61% cervical dilation success rate (Gusmão et al., 2007). This protocol was subsequently used in Dorper ewes (Gusmão et al., 2009) and transcervical penetration was possible in about 95% of pre-treated animals. Alternatively, the results with use of the estradiol benzoate-based cervical relaxation protocol initiated 16 h prior to embryo recovery were highly repeatable, with successful transcervical embryo recovery in 81% of estrous cyclic Santa Inês ewes (Fonseca et al., 2019a), 91% (estrous cyclic) to 100% (superovulated) Lacaune ewes (Figueira et al., 2018a, b; Souza-Fabjan et al., 2018), and 100% of estrous cyclic Morada Nova sheep (Fonseca et al., 2015). Those observations on the responsiveness to hormonal cervical dilation warrant further studies of the technique in different genotypes of ewes.

The average depth of transcervical penetration is usually greater in estrous ewes compared with ewes in the luteal phase of the estrous cycle possibly due to actions of ovarian follicular steroids exert on cervical structure and rigidity (Kershaw et al., 2005). In the present study, however, the maximum depth of transcervical penetration at the time of uterine flushing was ~3 cm greater than immediately after the onset of estrus. Hence, the administration of D-cloprostenol and estradiol esters as well as oxytocin infusion just prior to attempts at transcervical penetration appear to have more pronounced effects on cervical relaxation than physiological concentrations of estrogens. The average duration of transcervical uterine flushing was ~33 min, similarly to the 30 min reported in Dorper sheep by Gusmão et al. (2009). Further, the flushing fluid embryo recovery averaged 90% and it was comparable to the 95% embryo recovery rate reported by Gusmão et al. (2007) and Fonseca et al. (2019a), and slightly greater than the 84% rate reported by Barry et al. (1990) in superovulated Merino ewes. In Trial 1 of the present study, the embryo recovery rate was  $1.0 \pm 1.1$  and with this result being considered to be quite satisfactory because the Santa Inês sheep are a non-prolific breed with the mean ovulation rate of 1–1.3 (Mexia et al., 2004; Cavalcanti et al., 2012; Teixeira et al., 2016).

Morphological evaluation and percentage of viable embryos (67% to 80%) assessed after embryo recovery indicated there was no apparent detrimental effect of imposing the cervical relaxation protocol on embryo quality. Whether or not the “hormonal cocktail” administered to the ewes for uterine flushing and embryo collection in the present study has any detrimental effects on embryo quality remains to be elucidated after transferring an adequate number of embryos recovered from ewes previously subjected to cervical relaxation protocols. Results of previous studies have indicated that treatments with PGF2 $\alpha$  alone (Fonseca et al., 2014, 2018) and PGF2 $\alpha$  + OT (Pereira et al., 1998) in goats or EB + OT (Wulster-Radcliffe et al., 1999) prior to transcervical embryo recovery in ewes had no adverse effects on embryo morphology and viability. Similarly, administration of EB + OT 6 days after natural mating had no negative effect on the ensuing pregnancy rates of ewes (Lewis, 2010). It would still be justified and interesting to use embryos collected and cryopreserved in Trial 1 for additional post-thawing assessment and/or transfer trials to determine fertility outcomes.

Considering that all the procedures used in the present study were performed by the same experienced operator, it can be concluded that the omission of estradiol benzoate injections resulted in an impediment to transcervical penetration and a longer time post-injection (i.e., 16 h compared with 10 h) was associated with a significantly greater ease of transcervical instrument penetration in ewes. Furthermore, as indicated by results in Trial 2, even though there were no statistical differences in terms of transcervical penetrability among animals administered estradiol benzoate or cypionate 16 h before uterine flushing, the percentage of successful transcervical penetrations in ewes was 78% or 44% after the estradiol benzoate or estradiol cypionate treatment, respectively. It is

likely that increasing the number of animals in the trials would have been associated with a significant difference. Nevertheless, a combination of estradiol benzoate, D-cloprostenol (both 16 h before cervical penetration) and oxytocin (20 min before flushing) appears to be the most effective treatment to induce cervical dilation for transcervical embryo recovery in ewes.

As in previous studies (Fonseca, 2017; Fonseca et al., 2019a), the efficacy of transcervical penetrability of ewes during the period of behavioral estrus preceding uterine flushing was used to select ewes suitable for non-surgical embryo recovery and prepare cervical maps facilitating transcervical manipulations of catheters. The cervical map refers to a diagram or sketch marking the position of and/or distances between the ostia of each cervical ring in individual animals (Fonseca et al., 2019b). In the present experiments, a successful uterine flushing by cervical route was accomplished in 73% and 91% of ewes (Trial 1 and 2, respectively) in which transcervical penetration was accomplished at the time of estrus. Although there was not evaluation in the present study, it is also feasible that initial testing at estrus allowed for less time taken to perform uterine flushing and thus the reduction in likelihood for abrasions or injuries to the vaginal and cervical regions. In terms of reducing the potential stress endured by restrained animals undergoing transcervical embryo recovery, there were attempts to use relatively simple but efficient combinations of drugs and routes of administrations to avoid animal discomfort or pain before, during and immediately after performing of the transcervical penetration procedures. Potent systemic analgesics (dipyrone and n-butyl hyoscine bromide) administered i.v. (immediate action) and i.m. (maintaining analgesia) along with epidural (S5-C1) and local (cervical os) anesthesia, additional sedation (acepromazine maleate) and modified cervical clipping/retraction permitted safe and atraumatic procedures without ensuing lesions. Oliveira et al. (2018) reported that although transcervical passage is less invasive than laparotomy, both approaches may be associated with inflammatory responses. Additional preventive measures to reduce resultant inflammation in ewes (e.g., treatment with a non-steroidal anti-inflammatory drug tolfenamic acid; Schlapp et al., 2015) where there are attempts at embryo recovery should eventually be incorporated into the embryo flushing protocols.

## 5. Conclusions

To summarize, the present results of the present study indicate that the percentage of ewes in which cervical penetration was possible was nearly twice that when estradiol benzoate and D-cloprostenol were administered 16 h instead of 10 h before and oxytocin was administered 20 min before uterine flushing attempts. Thus, the timing of estradiol benzoate and cloprostenol administration relative to the beginning of embryo flushing on Days 6 or 7 (Day 0 = onset of estrus) is an important factor determining the success of embryo recovery. The results for average time elapsing to perform the procedure as well as flushing fluid and embryo recovery rates indicate that transcervical embryo flushing is a viable alternative to surgical embryo collection in ewes. In addition, the cervical mapping completed at the time of transcervical penetration attempts at the time of estrus (preceding the embryo recovery performed 6–7 days later) greatly facilitates the conducting of the embryo recovery procedure. The cervical penetrability at estrus appears to be a good indicator of the ease with which the cervical passage can be accomplished just prior to embryo flushing. Further studies are needed to delineate the effects of sheep genotype, age and reproductive season as well as an application of anti-inflammatory drugs post-treatment on the efficacy of the transcervical penetration procedures for embryo recovery in sheep. It is also necessary to perform confirmatory studies of the quality and developmental potential of sheep embryos collected after an application of the transcervical dilation protocol utilizing estradiol ester(s), D-cloprostenol and oxytocin.

## 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.

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