Received: 2 May 2019 | Revised: 2 June 2019 | Accepted: 21 June 2019

DOI: 10.1111/rda.13499

ORIGINAL ARTICLE

Reproduction in Domestic Animals WILEY

Comparison of the intravenous and intravaginal route of oxytocin administration for cervical dilation protocol and non-surgical embryo recovery in oestrous-induced Santa Inês ewes

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Funding information
Embrapa Goats and Sheep, Grant/Award Number: 02.13.06.026.00.04; Fapemig, Grant/Award Number: CVZ-PPM 00042-14; CNPq

Abstract

This study compared the effects of intravaginal and intravenous routes of oxytocin (OT) administration in 46 oestrous-induced Santa Inês ewes (6-day treatment with progestin-releasing intravaginal sponges and a single injection of 200 IU of eCG at the time of sponge removal) that underwent transcervical embryo recovery 6–7 days after oestrous onset and mating. All ewes received 37.5 μg of d-cloprostenol via latero-vulvar route, and 1 mg of oestradiol benzoate i.m. 16 hr before and 50 IU of OT 20 min before non-surgical embryo recovery (NSER), with OT being administered intravenously (n = 21) or intravaginally (n = 21). An overall oestrous response was 95.6% (44/46), and adequate cervical retraction could be accomplished in 78.6% (33/42) of ewes. The percentage of successful NSER procedures was 57% (24/42) or 72.7% (24/33) of animals with sufficient cervical retraction. The duration of NSER procedure averaged 28 min (range: 17–40 min) and ~96% of flushing fluid could be recovered (range: 85%–100%). Out of 18 ewes that could not undergo NSER, 12 (66.6%) presented various anatomical barriers, whilst the other 33.4% did not present these barriers and still could not be traversed. Excluding the ewes with those anatomical features, the overall success rate of NSER was 80% (24/30). The route of OT administration had no effect on NSER efficiency or the ease with which transcervical embryo flushing was performed. Both routes of OT administration can be used for cervical dilation protocol. Discarding ewes with anatomical features precluding cervical penetration is highly recommended to increase the efficacy of NSER in sheep.

KEYWORDS
cervical relaxation, sheep, transcervical embryo collection, uterine flushing

1 | INTRODUCTION

Surgical embryo recovery and transfer procedures using laparotomy or laparoscopy possess several disadvantages such as high cost of materials, post-operative adhesions and undue stress endured by poorly responding animals (Candappa & Bartlewski, 2014). But since in vivo embryo production is the primary method to obtain high-quality ovine embryos (Fonseca, Oliveira, et al., 2019), there is an increasing demand for development of alternative non-surgical techniques of similar or greater efficiency.

In sheep, non-surgical embryo recovery (NSER) procedures are particularly challenging due mainly to unique features of cervical anatomy. The factors that mostly limit cervical penetration in sheep are the number and distribution of cervical folds and small inner diameter of the cervical lumen (Kaabi et al., 2006). Multiple attempts have been made to improve NSER in ewes, including the development and refinement of techniques for transcervical penetration and embryo flushing, screening and selection of donor ewes (Santos et al., 2019), and advances in understanding the mechanisms of cervical ripening that paved the way to various hormonal protocols for cervical dilation (Candappa & Bartlewski, 2014; Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski, et al. 2019).

The role of oxytocin (OT) in triggering uterine contractility and cervical dilation during parturition (Khalifa, Sayre, & Lewis, 1992) as well as in stimulating endometrial $PGF_{2\alpha}$ secretion to promote luteolysis in ruminants (Falchi & Scaramuzzi, 2015) is commonly known. In cows (Fuchs, Ivell, Fields, Chang, & Fields, 1996) and ewes (Ayad, Leung, Parkinson, & Wathes, 2004), elevated oestradiol concentrations during the follicular phase of the oestrous cycle lead to an increase in oxytocin receptors (OTR) concentration in the epithelial cells of the cervical mucosa layer. Systemic and luteal OT binding to these receptors promotes the release of arachidonic acid from the phospholipid membranes of the endometrial tissue, which is then converted by cyclooxygenase 2 into prostaglandin H, the precursor for PGE_2 synthesis (Falchi & Scaramuzzi, 2015). PGE_2 acts on the adjacent connective tissue and smooth muscle cells to induce cervical dilation and facilitate sperm transport through the cervical canal during the periconceptional period (Fuchs, Graddy, Kowalski, & Fields, 2002).

Due to its physiological functions, OT is often used in cervical dilation protocols when it is typically administrated intravenously (Candappa & Bartlewski, 2014; Fonseca, Oliveira, et al., 2019). Since OT is also produced and acts locally in the female reproductive tract, we hypothesized that the intravaginal route of administration could improve efficiency of cervical dilation protocols compared with the intravenous injection. Therefore, the specific aim of this study was to determine and compare the efficacy of transcervical embryo recovery in oestrous-induced Santa Inês ewes following the hormonal cervical dilation protocol using the intravenous or intravaginal route of drug administration.

2 | MATERIALS AND METHODS

2.1 | General experimental conditions

The present experiment study was conducted with the approval of the Ethics in Animal Care Committee of the Embrapa Dairy Cattle (process 2512100516). It was performed during the month of September (non-breeding season) in São Carlos, southeast Brazil (latitude 22°01′S, longitude 47°54′W, altitude 850 m).

Forty-six clinically healthy, multiparous Santa Inês ewes were kept in a semi-intensive management system. The animals were kept in *Megathyrsus maximus* paddocks and received daily rations of corn silage, according to the animals' nutritional demands (National Research Council, 2007). The ewes also had freshwater and mineralized salt licks offered *ad libitum*.

2.2 | Experimental animals and treatments

Oestrus was induced and synchronized with the insertion of intravaginal sponges containing 60 mg of medroxyprogesterone acetate (MAP; Progespon®, Syntex) on Day 0 (random day of the anovulatory period), which was kept in place for 6 days (removal on Day 6). All ewes received 200 IU of eCG i.m. (Novormon 5000®, Syntex) and 37.5 μg of d-cloprostenol (Prolise®, ARSA S.R.L.) via a latero-vulvar injection 24 hr before sponge removal (Day 5). Oestrus was monitored twice daily after sponge removal, and the ewes were mated naturally by previously tested fertile rams throughout the period of behavioural oestrus.

Subsequently, the ewes were equally allocated according to interval to oestrus, body condition score (BCS; scale 1-5 with 1 being very thin and 5 being obese), age (4–5 years) and parity (3–4) into two groups: OTivs group (n=21; BCS: 3.6 ± 0.09) receiving an i.v. injection of OT and OTivg group (n=21; BCS: 3.5 ± 0.08) with the intravaginal OT administration. The cervical dilation protocol consisted of a latero-vulvar injection of 37.5 μg of d-cloprostenol and an i.m. dose of 1 mg of oestradiol benzoate (Estrogin®, Biofarm) given 16 hr before embryo recovery followed by 50 IU of oxytocin (5 ml; Ocitocina Forte UCB®) administered 20 min before NSER. For the intravaginal administration of OT, the ewes were placed in an anterior bipedal position and OT was deposited in the vaginal fornix with the aid of a sponge applicator, inserted and removed in <30 s and animals returned to a standing position.

2.3 | Cervical assessment, penetration and uterine flushing procedures

The NSER procedure was attempted by cervical route 6–7 days after the onset of behavioural oestrus by the same experienced technician. Animals were restrained in a standing position and received mild sedation with i.m. acepromazine maleate 20 min before the NSER and an epidural block (2 ml of 2% lidocaine, S5-C1) immediately before cervical penetration, as previously described (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski, et al. 2019).

After washing the perineal area of donor ewes with clean water and detergent, a Collin speculum (no. 1–3) lubricated with sterile hydrosoluble gel was introduced into the vagina and positioned to visualize the cervical opening. A custom-made, 25-cm forceps (Pinça Embrapa® for cervical immobilization and retraction in small ruminants; Embrapa) was then inserted into the cervical os. A sterile gauze soaked with 5 ml of 2% lidocaine without vasoconstrictors was gently placed ventrally to the cervical opening using Allis forceps (26 cm). The cervical os was gently retracted to facilitate the passage of the Hegar dilator, and during this procedure, the cervix was classified as normal, long (i.e., >10 cm) or extremely tortuous (Fonseca, Oliveira, et al., 2019). Each animal received a score from 1 to 5 according to the degree of difficulty in traversing the uterine cervix with the Hegar dilator: Grade 1 (very easy; cervical penetration achieved in <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) (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al., 2019). Anatomical barriers such as the vestibule-vaginal stenosis (hymen constriction did not allow cervical clipping) or poor cervical distension (when cervix was clipped but could not be retracted) were also recorded at this stage. Immediately after successfully traversing the cervical canal with the Hegar dilator, a catheter (no. 08; Sonda Embrapa® for goat/sheep embryo recovery; Embrapa) with a metal mandrel was used to traverse the cervical rings, following the method previously described by Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão, et al. (2019. If the cervix was successfully traversed with the catheter, the uterine flushing technique, described by the same authors, was performed using a custom-designed circuit (Circuito Embrapa® for goat/sheep embryo recovery; Embrapa) connected to the catheter positioned firstly in the right uterine horn and afterwards left (Fonseca et al., 2016). A total of 180 ml of sterile PBS medium at 37°C was used to flush each uterine horn, in addition to 20 ml at the beginning and at the end of the flushing procedure, for the filter lubrication and system washing, respectively. All recovered embryos were classified based on the morphological criteria detailed by the International Embryo Transfer Society (Stringfellow & Seidel, 1999).

2.4 | Data recorded and statistical analyses

The following data were recorded for all ewes studied: oestrous response (number of ewes in oestrus/number of treated ewes × 100%); time of oestrous onset (relative to the time of sponge removal); duration of oestrus; degree of difficulty performing transcervical penetration procedure; percentage of ewes successfully flushed (ewes flushed/total ewes that manifested oestrus × 100); percentage of ewes with successful embryo recovery (number of ewes in which uterine flushing had at least one structure recovered/number of ewes flushed × 100); duration of the cervical penetration procedure (in minutes, time from Hegar dilator insertion to removal); duration of the uterine flushing procedure (in minutes, time from flushing catheter insertion to removal); duration of NSER procedure (in minutes, time from insertion of the Hegar dilator to flushing catheter removal); fluid recovery efficiency (percentage of fluid recovered post-infusion: volume retrieved/400 ml × 100%); and number and type of structures recovered (i.e., embryos and/or unfertilized eggs).

Statistical analyses were performed using R core team 3.5.5 version (R Core Team, 2019). A Fisher exact test was used for non-parametric analyses, whereas a one-way analysis of variance (ANOVA) was used for parametric data. All results were expressed as mean±-standard error of mean (*SEM*). The analysis of proportions utilized a chi-square test (Brandt and Snedecor formula for analysis of proportions: Snedecor & Cochran, 1989). All results with p value < 0.05 were considered statistically significant.

3 | RESULTS

There were no differences ($p > 0.05$) between the two subsets of ewes (OTivs vs OTivg) for any of the end points analysed in this study. Regardless the treatment groups, an overall oestrous response was 95.6% (44/46 synchronized ewes). An average time from MAP sponge removal to the onset of oestrus was 47.2 ± 1.6 hr, and the oestrous duration was 32.7 ± 1.8 hr. Two ewes showed signs of oestrus later (3 days) than all other animals and were withdrawn from the present experiment.

Adequate cervical clipping and retraction was achieved in 78.6% (33/42; OTivs = 16 and OTivg = 17) of the ewes. Transcervical uterine flushing was successfully performed in 24 ewes (57% of all experimental animals and 72.7% of ewes with adequate cervical retraction; OTivs = 12 and OTivg = 12). The NSER procedure could be performed in 12 ewes from each treatment group (57.1%). There were no significant differences in any aspect of the NSER procedure or its efficiency ($p > 0.05$) between the two subsets of ewes (Table 1). Out of the remaining 18 ewes in which NSER could not be performed, 12 (66.6%; OTivs = 7 and OTivg = 5) animals had anatomical problems such as the vestibule-vaginal stenosis (25%; 3/12), poor cervical distension (50%; 6/12) or extremely long or tortuous cervical canal (25%; 3/12). In addition, the hormonal relaxation protocol was not effective in 6/18 (33.3%; OTivs = 2 and OTivg = 4) ewes that were unable to undergo NSER. Excluding the ewes with anatomical complications, the overall NSER success rate was 80.0% (24/30). A total of 10 embryos (one early blastocyst with the blastocoel less than half of the volume of the embryo, two blastocysts with a blastocoel completely filling the embryo and six expanded blastocysts with a blastocoel volume larger than that of the early embryo, with a thinning zona) and an unfertilized egg were recovered from 58% (10/17) of successfully penetrated ewes. All collected embryos were classified as grade one blastocysts. The NSER success rates recorded in animals varying in the degree of difficulty traversing the uterine cervix with the Hegar dilator just prior to NSER (scale 1-5) are shown in Figure 1. Two out of 42 ewes could not be penetrated with the Hegar dilator (Grade 5). The proportions of ewes that underwent NSER were greater ($p < 0.05$) for the subsets of animals that received scores 1-3 compared with the ewes classified as Grade 4.

TABLE 1 The responses (mean ± *SEM*) recorded oestrous-induced in Santa Inês ewes after the intravenous (OTivs group) or intravaginal (OTivg group) administration of 50 IU of oxytocin 20 min before non-surgical embryo recovery (NSER) combined with 37.5 μg of d-cloprostenol (latero-vulvar) and 1 mg of oestradiol benzoate i.m. given 16 hr before NSER procedure performed 6–7 days after the onset of behavioural oestrus

End points	OTivs	OTivg
Number of animals	21	21
% of successful NSER	57.14% (12/21)	57.14% (12/21)
Duration of NSER procedure (min)	29.4 ± 2.3	26.5 ± 2.1
Duration of cervical penetration (min)	5.9 ± 0.8	5.4 ± 0.8
Duration of uterine flushing procedure (min)	24.5 ± 1.7	23.2 ± 1.5
Fluid recovery efficiency (%)	96.2 ± 1.2	95.4 ± 2.1

4 | DISCUSSION

The aim of the present study was to evaluate and compare the intravaginal and intravenous route of administration of oxytocin (OT) in oestrous-synchronized Santa Inês ewes undergoing the NSER procedure. We assumed that the intravaginal route and direct deposition of OT in the proximity to the uterine cervix could improve the success of cervical penetrability at the time of NSER. For the purpose of cervical ripening at the time of labour in women, PGE_2 analogues are administered as vaginal inserts (Candappa & Bartlewski, 2011) and OT in a glycerine formulation was successfully absorbed through the vaginal mucosa in milking cows (Netto et al., 2019). Studies examining the feasibility of transcervical artificial insemination in ewes have also used the local routes (intracervical or intravaginal) of drug administration (hyluronan: Perry, Haresign, Whates & Khalid., 2010; FSH and misoprostol: Leethongdee et al., 2007; FSH, misoprostol and OT: Falchi, Taema, La Clanche & Scaramuzzi, 2012; dinoprostone: Bartlewski & Candappa, 2015). The best results were obtained after an application of FSH and misoprostol (a 100% penetration rate in the treatment groups inseminated 54 hr after sponge removal). Similar studies conducted during the luteal phase of the oestrous cycle and using NSER also reported an increase in cervical penetration rates after a local (intravaginal) administration of misoprostol (Gusmão et al., 2007) or controlled slow-release dinoprostone-containing vaginal inserts (Candappa & Bartlewski, 2014). In a study by Gusmão et al. (2007), cervical penetration was not possible in control (untreated) animals but it could be performed in 63% of ewes after vaginal deposition of 200 μg of misoprostol 5 hr before the procedure. Candappa and Bartlewski (2014) reported that complete transcervical penetration could be achieved in 55% of dinoprostone-treated 24–48 hr and 9% of control Rideau Arcott ewes. In the present experiment, however, there was no substantial improvement in the ability to traverse the uterine cervix after intravaginal administration when compared with an i.v. injection of OT. Since a similar degree of cervical dilation can be obtained with OT given by a generally more convenient and less time-consuming i.v. route (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al., 2019), the latter approach may be preferred in practical settings.

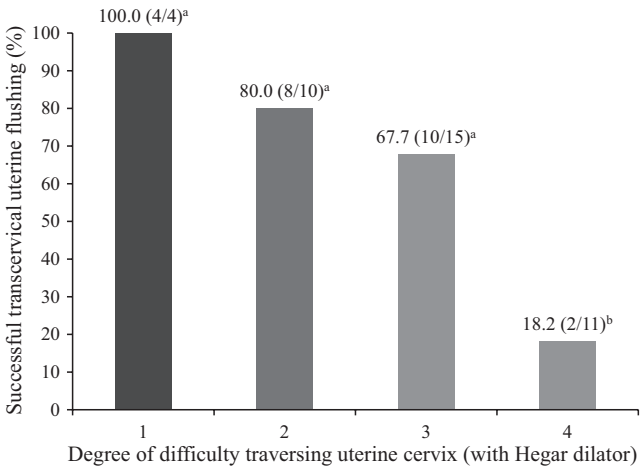

FIGURE 1 Percentages of Santa Inês ewes in which non-surgical embryo recovery (NSER) procedures using a customized flushing catheter were successfully completed (y-axis). The x-axis represents the level of difficulty encountered during the attempt to traverse the uterine cervix with the Hegar dilator immediately before the flushing procedure. Scale 1–5:1: very easy, cervical penetration achieved in <1 min; 2: easy, between 1 and 3 min; 3: moderate difficulty, between 3 and 7 min; 4: difficult; between 7 and 10 min; and five (impossible to penetrate the cervix with dilator or a lack of complete cervical passage animals removed from statistical analyses). $^{ab}p < 0.05$

Successful transcervical embryo recovery was performed in nearly 60% of all animals and in 80% of the animals excluding those that had various anatomical barriers precluding cervical penetration. All animals used in this study were randomly selected from an experimental herd without any previous information on their cervical conformation. It has been well established that there exists tremendous inter- and intra-breed variation in the complexity of cervical anatomy (Halbert, Dobson, Walton, & Buckrell, 1990; Kaabi et al., 2006). The Santa Inês is a synthetic breed derived from crosses of the Morada Nova, Bergamasca and native Brazilian coarse-wooled sheep (Gusmão et al., 2007), which may have contributed to the observed high variability in the shape and length of the uterine cervix in individual animals. Moreover, there is still a paucity of information on the outcome of NSER procedures in sheep and particularly in Santa Inês ewes. Gusmão et al. (2007), reported the 63% NSER success rate in Santa Inês ewes and 95% of cervical penetrability in Dorper ewes (Gusmão et al., 2009), suggesting that the attempts to

penetrate the cervix in the Santa Inês breed are rather challenging. In the present study, we achieved approximately 96% of fluid recovery rates and the mean duration of an NSER procedure averaged 28 min; both of those values are similar to the results previously reported by Gusmão et al. (2007) for Santa Inês ewes (95% of uterine fluid recovery and the average duration of NSER of ~32 min).

Despite the relatively low embryo recovery, it must be highlighted that the main objective of this study was to verify in the first instance the success of transcervical uterine access and flushing. Due to expenses and mainly in function of unpredictability of successful on cervical traversing, at this stage we did not use superovulation or superovulatory doses of gonadotropins. Thus, it was used a successful protocol to induce synchronous oestrus in Santa Inês sheep using low gonadotropin stimulatory doses (200 IU eCG). It was assumed that superovulation would be the next step after establishing an efficient protocol of cervical relaxation. In fact, the synchronous oestrous induction protocol used in this study was efficient to support the proposed methodology, not compromising the data investigated. Perhaps reduced dose of eCG used in this study resulted in less ovulation and embryo yield when compared to average embryos recovered (1.0 ± 1.1 embryos) by transcervical route in Santa Inês ewes receiving 300 IU eCG (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski, et al., 2019), but similar to those not receiving gonadotropin stimuli (Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão et al., 2019).

Three main anatomical conditions that typically hamper the NSER procedure were observed in some individual ewes of the present study, namely the vaginal-vestibule stenosis, poor cervical distension and elongated/tortuous cervices (Fonseca, Oliveira, et al., 2019). These conditions cannot be easily overcome by the hormonal treatment aimed to dilate the uterine cervix. Using vaginoscopy to locate the os cervix, an NSER technician may be able to detect stenosis (i.e., inability to locate the cervical opening), but the difficulty clamping and retracting the uterine cervix cannot be determined with the visual inspection of the vagina. Similarly, the length and internal structure of the cervical canal cannot be determined with vaginoscopy. Therefore, the grading system was proposed by Fonseca, Zambrini, Guimarães, Silva, Oliveira, Bartlewski, et al. (2019, Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão, et al. (2019 and devised to establish the difficulty with which the uterine cervix can be traversed using the Hegar dilator appears to be critical for proper selection of individual animals that are suitable to undergo NSER; in the present experiment, most animals were classified as Grade four and all Grade five animals (cervical penetration times from 7 to 10 min or impenetrable cervices, respectively) could not be used for NSER. This is similar to the earlier results by Fonseca, Zambrini, Guimarães, Silva, Oliveira, Brandão, et al. (2019, where 75% of ewes that received Grade four during the traversing attempt at oestrus could not undergo NSER 6-7 days later. A proper selection method of donor ewes for NSER procedures including vaginoscopy and a screening system including an attempt to clamp/retract the uterine cervix and traverse the cervical rings with the Hegar dilator may substantially reduce the waste of time and resources on inapt animals.

5 | CONCLUSIONS

The route of oxytocin administration (intravaginal or intravenous) during the application of the cervical dilation protocol (a combined treatment with d-cloprostenol and oestradiol benzoate) did not affect the efficacy of NSER procedures in oestrous-induced Santa Inês ewes.

ACKNOWLEDGEMENTS

This work was supported by Embrapa Goats and Sheep [grant number: 02.13.06.026.00.04]; Fapemig [grant number CVZ-PPM 00042-14]. J.F.F and M.E.F.O. are fellows of CNPq. J.M.G.S-F. is a fellow of FAPERJ.

CONFLICTS 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 and collected the data from the animals. LP and JFF analysed the data and wrote the first version of the manuscript. FNZ and JMGS-F collected the data from the animals. ARG and SNE assisted with the animals and helped collect and analyse the data. JDG, MEFO and JMGS-F discussed the design of the experiment and analysed the data. MAPS and PMB elaborated and worked on the statistics. PMB revised it critically, and JFF, PMB, MEFO and JMGS-F 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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How to cite this article: Prellwitz L, Zambrini FN, Guimarães JD, et al. Comparison of the intravenous and intravaginal route of oxytocin administration for cervical dilation protocol and non-surgical embryo recovery in oestrous-induced Santa Inês ewes. *Reprod Dom Anim*. 2019;00:1–6. https://doi.org/10.1111/rda.13499