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Non-surgical embryo transfer in goats and sheep: the Brazilian experience

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Abstract. Brazil has presented tremendous progress in non-surgical embryo transfer (NSET) in sheep and goats. New instruments and techniques for non-surgical embryo recovery (NSER) and NSET in small ruminants were implemented. Recent improvements include refinement of the protocols for cervical relaxation combining oestradiol–oxytocin–cloprostenol treatment at specific times before NSER in sheep; recipient goats do not require any hormonal drugs to induce cervical dilation and direct embryo transfer by the cervical route yields excellent results. Transrectal ovarian ultrasonography (B-mode but especially colour Doppler) have proven to be accurate methods to localise and enumerate corpora lutea and luteinised unovulated follicles in recipient and donor does and ewes. An array of new criteria for selecting superior animals for NSER and NSET (e.g. cervical mapping) have been developed by Brazilian researchers. Extensive studies on both technologies were initially conducted in commercial breeds of goats and sheep but have been gradually extended to some native breeds of sheep (germplasm conservation) and dairy goat operations. It is speculated that, in future, NSER and NSET may become methods of choice for caprine and ovine embryo recovery and transfer in Brazil, and then globally. Due primarily to the efficiency of NSET in goats, a novel interspecies (e.g. bovine) IVP method may soon be developed on a large scale. The Brazilian experience is an invaluable source of information and know-how promoting the replacement of conventional surgical assisted reproductive technologies with non-surgical procedures and hence supporting the rapid development of the embryo transfer industry in small ruminants.

Additional keywords: cervical route, *in vivo* embryo production, multiple ovulation and embryo transfer (MOET), small ruminants, transcervical embryo collection.

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Introduction

Sheep and goats are not native to Brazil, but have been brought into Brazilian territory over a few centuries. After the discovery of Brazil in 1500, small ruminants had not been very extensively used, but in the 20th century they became important for commercial production of sheep wool in a southern region and goat milk in a south-eastern region of the country. Over the past four decades, the main goal of the Brazilian sheep industry has become the production of meat, and thus large numbers of Dorper embryos were imported and transferred. In addition, the developing sheep production systems required intensive *in vivo* embryo production using domestic sheep breeds such as the Santa Inês. Similar to the implementation of ovine embryo transfer (ET), the acquisition of South African goat breeds, including Boer, Savana and Kalahari breeds, paved the way for the development of goat ET. In the past two decades, the establishment of CapraGene (Facó *et al.* 2011), the Brazilian progeny test for dairy goats, solidified Brazil's position as one of the global leaders in dairy goat production (Lôbo *et al.* 2017). During the same period, the demand for sheep milk products stimulated the expansion of the Brazilian dairy sheep industry, which primarily involves the Lacaune breed.

Although the first successful transfers of ovine and caprine embryos in Brazil were performed nearly 40 years ago (sheep: Selaive and Mies Filho 1979; goats: Jaume and Bruschi 1985), no significant improvement in embryo recovery or transfer rates using surgical procedures has been achieved to date. At present, the continued demand for dairy and meat sheep and goat products in Brazil is a major driving force behind the frequent use of multiple ovulation and embryo transfer (MOET) programs in these species. Intensive research aimed at the development and adaptation of improved MOET techniques generated a significant knowledge base in this field. Most recently, the retrieval and transfer of embryos in animals raised in tropical and subtropical conditions using non-surgical methods, first in goats and then in sheep, has attracted considerable attention (Fonseca *et al.* 2014). Many efforts have been devoted to ameliorating non-surgical embryo recovery in sheep, including the development of techniques for transcervical penetration and embryo flushing, selection screening of donor ewes (Fonseca 2017) and protocols for cervical relaxation (Fonseca *et al.* 2016). The purpose of this review is to present the most salient and relevant stages of the development of non-surgical ET techniques accomplished in Brazil that could potentially replace surgical procedures in sheep and goat MOET programs worldwide.

In vivo embryo production in sheep and goats

Data from the International Embryo Transfer Society summarising global trends in sheep and goat embryo production are presented in Fig. 1. The total number of embryos produced remains relatively small, which is probably explained by MOET remaining the primary assisted reproductive technology used for sheep and goat embryos. Second, commercial production of sheep and goat embryos is fairly irregular, but after 1998 the production of ovine embryos has been consistently greater than that of caprine embryos. Thus, more attention should be given to developing strategies to ameliorate and simplify *in vivo* sheep embryo production by superovulation and ensuing transfer. Such strategies ought to include increased donor responsiveness to gonadotrophin stimulation, simplification and development of repeatable and accurate donor evaluation methods (e.g. predicting superovulatory yields) and more efficient embryo recovery and transfer techniques. Both non-surgical methods of embryo recovery (NSER) and non-surgical ET (NSET) studies exemplify and entail those research objectives.

The relatively low number of *in vivo*-produced sheep and goat embryos also indicates that due to a lower number of researchers or technicians involved in this area of reproductive and agricultural research around the world, the progress in small ruminant reproductive technologies is likely to be slower than that in other species, particularly bovine (Candappa and Bartlewski 2011). Conversely, countries such as Brazil, probably because of the absence of official databases and communication difficulties, have not been included in those statistics. Thus, the data for small ruminants collated in Fig. 1 may be somewhat of an underestimation.

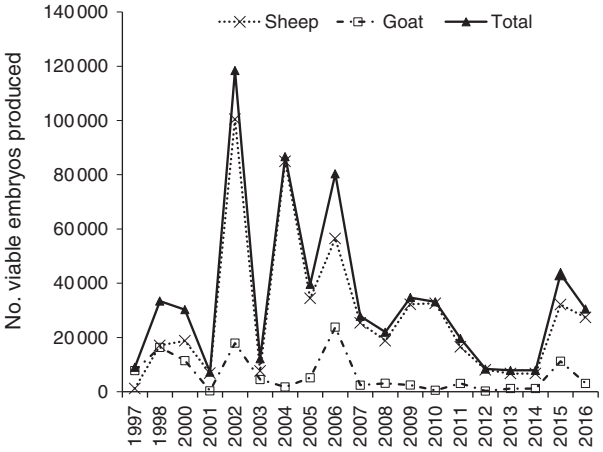

Fig. 1. Data for global *in vivo* embryo production in sheep and goats. Data were sourced from International Embryo Transfer Society (IETS) newsletter from 1998 to 2016 (http://www.iets.org/comm_data.asp, accessed 30 July 2018).

Strategies to select donors for NSET and associated techniques

Several criteria should be considered before attempting non-surgical penetration of the uterine cervix in sheep and goats. Because of the relative ease with which NSET can be performed in goats (i.e. no need for induction of cervical relaxation; Fonseca *et al.* 2014), the following section focuses on the selection of donor ewes, as proposed recently (Fonseca 2017).

Reproductive history and physiological status

Nulliparous ewes are not generally recommended for NSER or NSET because of the small diameter of their cervical lumen (Naqvi *et al.* 2005) and thus increased level of difficulty of cervical penetration compared with multiparous animals (Kaabi *et al.* 2006). Considering the body condition score (BCS) on a scale from 1 (very thin) to 5 (obese), intermediate conditions (BCS 2.5–4.0) are preferred. Ewes should be at least 100 days into lactation or be non-lactating. For the latter subset of animals, a longer period of time (>1 year) from lambing to superovulation is, in our experience, less desirable due to a greater degree of cervical constriction.

Determinants of successful cervical penetration and embryo recovery in certain sheep breeds

It is well known that performing cervical penetration for AI in ewes is strongly affected by the breed (Kaabi *et al.* 2006). Thus, although different breeds were not directly compared in a single study using NSET, similar breed-related differences are expected for uterine flushing and embryo recovery. In Brazil, non-surgical cervical penetration has been attempted in Texel (Fonseca 2006), Santa Inês (Gusmão *et al.* 2007), Dorper (Gusmão *et al.* 2009), Morada Nova (Fonseca *et al.* 2015*a*) and Crioula (Gastal *et al.* 2012) ewes; the efficiency achieved (i.e. the percentage of animals from which embryos were successfully collected) varied between 61.2% and 100% using cervical relaxation protocols. The Embrapa protocol for cervical

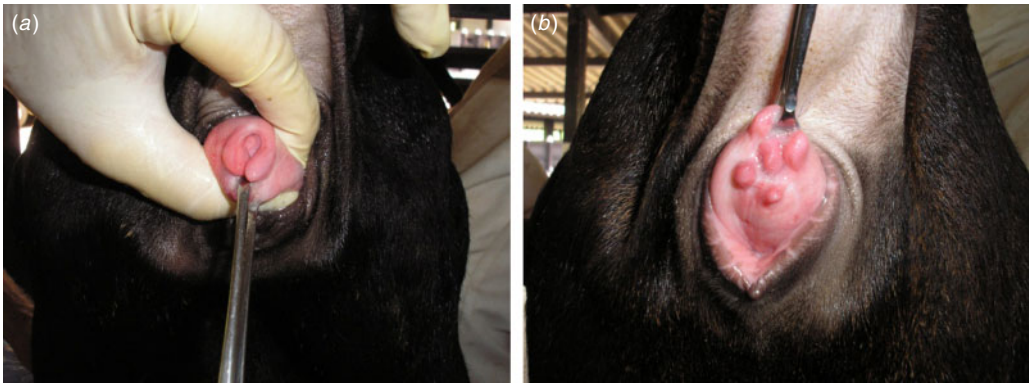

Fig. 2. Appearance of the caudal ostium of the ovine cervix in oestrus. Note variations in cervical anatomy between the two cervices clipped ventrally (*a*) or dorsally (*b*) with the 26-cm Allis forceps.

relaxation, developed primarily in Santa Inês ewes (Fonseca *et al.* 2016), could also be effectively performed in Morada Nova ewes (Fonseca *et al.* 2015*a*), and initial testing of the procedure in Lacaune sheep was encouraging (Figueira *et al.* 2018*a*, 2018*b*; Souza-Fabjan *et al.* 2018); with this method, transcervical embryo flushing could be done in 91 to 100% of ewes.

Cervical anatomy

The number of cervical rings in the uterine cervix varies in sheep (Moura *et al.* 2011), as does the appearance of the caudal orifice (Fig. 2), and the uterine cervix can be classified into several categories on the basis of these anatomical features (for reviews, see Kershaw *et al.* 2005; Candappa and Bartlewski 2011). However, the degree of difficulty encountered during cervical penetration has not been compared among cervices with different types of caudal ostium of the cervix. Once again, the breed may be a determining factor because the anatomy and degree of cervical penetration at AI differ among various genotypes of sheep (Kaabi *et al.* 2006).

Success rates of cervical penetration in sheep

The records of cervical penetration attempts in ewes can provide additional information on successful NSER in a particular breed, or even in individual animals, and so it is of paramount importance to update data from transcervical AIs. High cervical penetration and intrauterine deposition rates of semen may be indicative of potentially high embryo collection via the transcervical route. Therefore, sheep undergoing oestrus induction or synchronisation can be tested for their suitability as prospective embryo donors using NSER (Bruno-Galarraga *et al.* 2014). Approximately 10–12 h after the onset of oestrus, an attempt to penetrate the cervix of the ewe can be made similar to a procedure used during embryo collection but using only the Hegar dilator. Our group evaluated the cervical transposition method as a tool to select ewes for embryo collection by the transcervical route (Santos *et al.* 2018). Adult Santa Inês ewes ($n = 50$) received superovulatory treatment. The cervix transposition test was performed at both oestrus and embryo collection. The latter was preceded by the hormonal cervical dilation proposed by Leite *et al.* (2018). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the cervix transposition test were 85.7%, 66.6%, 85.7%, 66.6% and 80.0% respectively. The kappa index yielded a moderate score ($\kappa = 0.52$). The high sensitivity and accuracy indicated that the cervical transposition test was a screening option to select ewes for embryo collection by the transcervical route.

Degree of difficulty of visualisation, clamping and retraction of the cervix

As described for goats and sheep (Fonseca *et al.* 2016), the uterine cervix should be visualised, clipped and retracted before attempting penetration. In some animals, the presence of vestibulovaginal stenosis (hymen) can hinder or even completely prevent insertion of the speculum. In some cases, the use of a speculum with a smaller diameter may facilitate the passage of the larger speculum at a subsequent attempt. However, if this initial physical barrier cannot be overcome, the animal is removed from the MOET program based on embryo collection by the transcervical route. It is imperative that the animal undergoing transcervical manipulations in a standing position is adequately and comfortably restrained (Fig. 3), and that appropriate sedation and analgesia are used (Fonseca *et al.* 2016).

If the speculum can be advanced beyond the hymeneal fold, but sufficient vaginal dilation and efficient cervical clamping cannot be performed, the animals should be excluded from NSET. Occasionally, the cervix can be visualised and effectively clamped, but adequate cervical retraction cannot be achieved; this typically results in inadequate access and cervical manipulation facilitating cervical penetration. Consequently, if the uterine cervix cannot be efficiently retracted, the animals should only be conditionally recommended for NSER.

In addition to cervical clipping and retraction, a few other criteria must be considered in assessing the potential degree of difficulty during NSER and NSET, including the topographic arrangement and number of cervical rings. The three distribution patterns of the five ovine cervical rings are shown in Fig. 4. This kind of ‘cervical map’ can and should be recorded during the first cervical penetration attempt in the ewe and can act as a

Fig. 3. A sheep restrained in a cart with attached padding in preparation for the transcervical penetration and uterine flushing procedures. Reproduced with permission from Fonseca (2017).

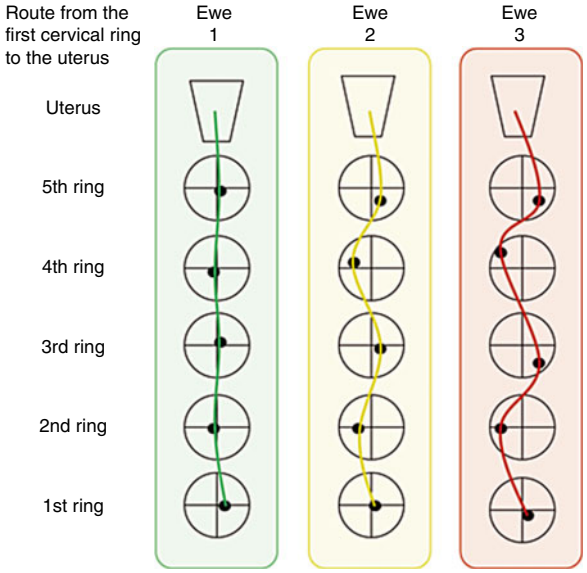

Fig. 4. The 'cervical map' for achieving transcervical uterine access in sheep. The location of the ostium of each ring (●) is recorded according to its position on the 'clock' and distance from the central axis.

'guide' during ensuing attempts to perform transcervical AI, embryo harvesting or deposition. In the goat, due to the greater regularity and linearity of cervical ring arrangements, this approach is not of significant importance, but can also be performed if so desired. In general, the idea is to sketch a schematic sequence of 'clocks' that, at each cervical ring passage, denote the location of its opening. Depending on the duration of transcervical manipulation and physiological status of the animal, these locations can change, but if proper animal restraint and cervical retraction are performed, the changes in the positioning of the rings tend to be minimal (Fonseca *et al.* 2013, 2016).

Sheep subjected to transcervical AI with cervical clipping and retraction typically exhibit one of those three cervical structures or patterns. It can be expected that cervical penetration at oestrus will be easier in Ewes 1 and 2 and that it would be more difficult or even impossible to perform in Ewe 3. Moreover, several ewes can exhibit a sinusoidal pattern of cervical ring distribution that is a combination of any two or all three of those patterns. Cervical retraction and stretching cause conformational changes in the cervix resulting in the alignment of consecutive rings and their easier traversal. This cervical distension may also increase the total length of the uterine cervix. As a result, the dimensions and configuration of the retracted ovine cervix may be quite different from those described in the reproductive tracts collected after slaughter (Kershaw *et al.* 2005).

Degree of difficulty of cervical penetration between Days 6 and 7 of the oestrous cycle

Day 6 or 7 of the oestrous cycle or after AI is when surgical embryo harvesting is usually conducted in sheep. When the ewe is in pro-oestrus or oestrus, several substances act to stimulate the relaxation and opening of the cervical canal so that spermatozoa can have access to the uterus and be transported to the site of fertilisation in the isthmus of the uterine tube. This relaxation allows for cervical penetration with the insemination gun or semen applicator. Pro-oestrus and oestrus collectively constitute the follicular phase of the interovulatory period, during which oestrogens are the steroids with preponderant activity. Alternatively, when embryo collection is performed on Day 6 or 7 of the oestrous cycle, the dominating steroid hormone is luteal progesterone (P4). During the luteal phase of the oestrous cycle, the uterine cervix is closed because the endocrine mechanisms that induce its relaxation and opening are not adequately active; this level of cervical constriction is necessary for the establishment and maintenance of early pregnancy.

During the luteal phase, the uterus of the goat can be accessed for ET by the non-surgical method without the use of any hormonal cervical dilators (Fonseca *et al.* 2014). This is also feasible in sheep (Fonseca 2006), but embryo collection in sheep can hardly be accomplished without the use of cervical-dilating agents. FSH, oestrogen, hyaluronic acid, prostaglandins and oxytocin are the main substances that induce cervical relaxation and opening (Lewis 2010; Candappa and Bartlewski 2011, 2014). Thus, the administration of these agents alone (Gusmão *et al.* 2007) or in combination (Fonseca *et al.* 2016) is necessary for successful embryo collection by the transcervical route in sheep. In a series of studies, the optimal timing and dosage of various drugs was tested, which ultimately resulted in the development of cervical penetration and uterine washing techniques with an efficiency of approximately ≥80% (a.k.a. the Embrapa protocol for cervical relaxation in sheep), which has been successfully used for embryo recovery in both oestrus-synchronized or -induced and superovulated ewes (Fonseca *et al.* 2016).

Duration of cervical penetration

The time required to complete cervical penetration is another indicator of the difficulty of this procedure. Obviously the time taken to traverse the uterine cervix depends on the combination of several aforementioned factors and can result in the withdrawal of an animal from NSER or NSET. A time-related classification on sheep undergoing cervical penetration procedures is proposed in Table 1.

Table 1. Cervical transposition scores proposed for ewes in oestrus or subjected to cervical relaxation protocols
Adapted, with permission, from Fonseca 2017

Score	Time from onset to completion of cervical penetration (min)	Relative difficulty
1	≤1	Very easy
2	>1 and ≤5	Easy
3	>5 and ≤10	Moderate
4	>10	Difficult
5	Not transposed	Impossible

Technical skills

As with many other assisted reproductive techniques, the success of cervical penetration in sheep depends heavily on the qualifications and experience of the technician who performs it. The technician and his/her assistants should be properly trained in all aspects of the procedure. The use of specialised equipment and cervical manipulations should be conducted smoothly without any sudden and/or large movements. Previous training and experience in AI greatly improves the confidence and efficiency of the NSER and NSET technician, leading to satisfactory success rates in transcervical embryo collection and transfer in sheep. Improper handling of the cervix during cervical penetration can result in intracervical bleeding due to organ rupture or perforation, which will certainly prevent the immediate collection of embryos. Cervical and uterine infections, or even adhesions, caused by cervical penetration attempts cannot be completely disregarded because their residual effects can compromise future attempts and negatively affect fertility (Candappa and Bartlewski 2014). For all these reasons, the importance of and requirement for adequate training of the technician performing cervical penetration cannot be overemphasised.

Methods to assess superovulatory responses in donor animals

Despite improved control of many extrinsic and intrinsic factors, the results of hormonal ovarian superstimulation in small ruminants are highly variable (Bartlewski *et al.* 2016). Identification of the superovulatory response is important to help predict the female embryo recovery rate, especially in those animals in which uterine flushing is performed by the transcervical route.

Although ovarian structures can primarily be visualised and accurately quantified using invasive (exploratory laparotomy) or semi-invasive (laparoscopy) techniques (Oliveira 2011), the usefulness of B-mode and colour Doppler sonography for detecting luteal structures in superovulated ewes has recently been demonstrated (Oliveira *et al.* 2018*a*; Pinto *et al.* 2018). Strong positive correlations were recorded between the total number of corpora lutea (CL) detected by B-mode or colour Doppler ultrasonography (Oliveira *et al.* 2018*a*; Pinto *et al.* 2018) on the day of embryo recovery and those observed with videolaparoscopy (the gold standard). The use of colour Doppler imaging was associated with increased accuracy of CL counting compared with grey scale ultrasonography (~82.3% vs 73.6% for colour Doppler and B-mode ultrasonography respectively), due primarily to easier detection of luteal structure boundaries in colour Doppler ultrasonograms (Oliveira *et al.* 2018*a*). Even though Pinto *et al.* (2018) showed that sensitivity of the colour Doppler technique decreased when more than four CL were present, the aforementioned studies support the notion that both ultrasonographic imaging modalities are useful and efficient diagnostic tools for estimating ovarian responses (quantifying CL and luteinised unovulated follicles) in superovulated donor ewes. In an ultrasonographic B-mode study using 12 superovulated goats (Nascimento *et al.* 2012), 108 structures (embryos or unfertilised oocytes) were recovered from all animals in which 110 luteal structures were detected. In six goats, the number of recovered eggs/embryos was identical to that of detected luteal structures (9/9, 9/9, 8/8, 7/7, 5/5 and 5/5); in two goats there were more structures recovered than luteal glands counted (21/19 and 23/20), and in three goats the number of ultrasonographically detected luteal structures was greater than that of oocytes/embryos recovered (14/10, 9/8 and 5/3).

The colour Doppler technique allows detection of dynamic changes in the vascularisation of luteal tissue and may thus potentially be used to monitor luteal regression (Figueira *et al.* 2015). However, in our recent study, it was not possible to visually identify vascular differences between the normal and inadequate CL (Oliveira *et al.* 2018*a*). This warrants further investigation using computer-assisted image analyses of luteal colour Doppler images from superovulated animals.

Measurements of circulating P4 concentrations on the day of embryo collection can be used to identify ewes with a high superovulatory response (Amiridis *et al.* 2002); however, the accuracy of this method can be affected by the breed, season, exogenous gonadotrophin used and the incidence of precocious regression of CL or luteinised unovulated follicles (Oliveira *et al.* 2018*a*). Serum P4 concentrations were correlated with the number of fully functional, healthy CL and, although there was a negative correlation with the number of prematurely regressing CL, the accuracy of predicting the number of short-lived CL with serum P4 concentrations in superovulated ewes was very poor (Oliveira *et al.* 2018*a*). In addition, the practicality of hormone assays using samples obtained on the day of embryo recovery to predict the superovulatory yields remains highly limited.

Approaches to inducing cervical dilation in goats and sheep

Pereira et al. (1998) reported the use of prostaglandin (PG) $F_{2\alpha}$ analogue with or without oxytocin to induce cervical dilation before non-surgical cervical penetration in the goat. That study provided the basis for Brazilian investigations into the development of other methods to ameliorate NSER in goats. Although the first Brazilian studies reported NSER success rates of <75% (Andrioli *et al.* 1999; Lima-Verde *et al.* 2003), it was possible to traverse the uterine cervix in 100% of superovulated goats pretreated with cloprostenol between 24 h (Amorim *et al.* 2011; Fonseca *et al.* 2013) and 16 h (Fonseca *et al.* 2018*a*) before the NSER attempt; a single dose of cloprostenol 16 h before NSER is now recommended for donor goats. Any

Table 2. Efficiency of uterine flushing through the cervical route in Lacaune sheep

Reference	Number donors	% Donors in oestrus (*n*/*N*)	% Successful collections (*n*/*N*)	Number of structures recovered	Number of viable embryos recovered
Figueira *et al.* (2018*b*)[A]	28	87 (24/28)	92 (22/24)	0.6–1.6	0.4–1.2
Figueira *et al.* (2018*a*)[B]	46	74 (34/46)	91 (31/34)	4–5.6	1.4–2.8
Souza-Fabjan *et al.* (2018)[B]	25	88 (22/25)	100 (22/22)	–	0.2–5.2

[A]Oestrus induced ewes.
[B]Superovulatated ewes.

deleterious effect on embryo viability due to an apparent inadequate environment evoked by cloprostenol appeared to be not significant for pregnancy establishment in recipient goats receiving embryos by laparotomy (Fonseca *et al.* 2018*a*). Obviously, for recipient goats, $PGF_{2\alpha}$ analogue pretreatment is not allowed. However, cervical penetration was feasible without any drug priming (Fonseca *et al.* 2014; Esteves *et al.* 2015).

In sheep, 200 μg misoprostol administered deep into the vagina 5 h before NSER promoted no extra benefits when compared with oxytocin and oestradiol alone for performing cervical penetration (Leite *et al.* 2018) and uterine flushing in Santa Inês (Gusmão *et al.* 2007), Dorper (Gusmão *et al.* 2009) and Crioula (Oliveira *et al.* 2018*b*) ewes. From 2014 to 2015, our group conducted a series of experiments in Santa Inês ewes, with or without the use of different oestradiol esters, various times and routes of oestradiol administration (relative to NSER; Fonseca *et al.* 2018*c*) and a combination of oestradiol benzoate–cloprostenol–oxytocin pretreatment (Zambrini *et al.* 2014). As a result, the Embrapa protocol for cervical relaxation in sheep was developed (Fonseca *et al.* 2016) and successfully used for NSER in Santa Inês ewes (Zambrini *et al.* 2015). Data from Santa Inês ewes have been summarised previously (Fonseca *et al.* 2016). This protocol consists of injection of 1 mg, i.m., oestradiol benzoate and 37.5 μg cloprostenol (laterovulvar), both 16 h prior to NSER, and 50 IU, i.v., oxytocin 20 min before the NSER attempt. Since 2015, the Embrapa protocol has been successfully used in other breeds. A preliminary study in Morada Nova sheep (Fonseca *et al.* 2015*a*) encouraged present studies in this breed, which have shown close to 90% successful transcervical embryo collection (J.F. Fonseca, unpubl. data). Because both Santa Inês and Morada Nova sheep are breeds of Brazilian interest, we tried to use the Embrapa protocol for cervical relaxation in Lacaune sheep, probably the main dairy sheep breed in the world. The initial results, summarised in Table 2, are possibly the first successful NSER results in this breed. Laparotomy transfer of non-surgically recovered embryos after the freeze–thawing procedure as described for goats (Fonseca *et al.* 2018*a*) resulted in a 40% pregnancy rate (J.F. Fonseca, unpubl. data).

Assessment of animal well-being after non-surgical uterine flushing in goats and sheep

The animal welfare precepts defined by international legislation (Universal Declaration of Animal Rights adopted by UNESCO on 15 October 1978 (https://constitutii.files.wordpress.com/2016/06/file-id-607.pdf. Access in 07/30/2018) and the Resolution of the European Parliament of 20 July 1998 (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31998L0058&from=EN, accessed 21 August 2018) are important limitations to the application and widespread use of *in vivo* embryo production. In a recent literature review on the development of reproductive biotechnologies in dairy cattle over the past 100 years, Moore and Hasler (2017) recommended improvements in communication and understanding between the public, animal rights activists and scientists who conduct animal research. It is becoming clear that the general public is increasingly aware of and alert to the principles of animal welfare in food production, and before long this certainly should be expected to extend into procedures used for embryo production in small ruminants.

Thus, it is imperative to develop *in vivo* embryo production protocols that effectively control animal pain and stress at all stages of this technology. Evidently the surgical collection of embryos generates the greatest amount of stress and painful stimuli for the animals. A comparison among the three techniques is presented in Table 3.

Techniques for non-surgical uterine flushing in goats and sheep

The NSER techniques currently used in goats and sheep may use different devices (e.g. injection circuit and catheters), forceps and specula (for details, see Fonseca *et al.* 2016). However, successful NSER depends primarily on successful cervical clipping, retraction and exteriorisation. The very first NSER technique used two Allis forceps in goats (Pereira *et al.* 1998) or two Pozzi forceps in goats (Amorim *et al.* 2011) and sheep (Gusmão *et al.* 2007, 2009). After insertion of the Collin speculum into the vagina and visualisation of the cervical os with a flashlight (Fig. 5*a*), the Pozzi forceps are inserted laterally to the cervical os in both sheep (Fig. 5*b*) and goats (Fig. 5*c*), and the cervix is retracted to allow easier manipulation per vagina or rectum. The modality of this NSER technique uses custom-developed forceps inserted into and under the cervical os; this less traumatic technique was termed the 'Embrapa technique for non-surgical embryo recovery in goats and sheep' and has been successfully used to recover embryos in superovulated Santa Inês ewes (Zambrini *et al.* 2015). Cervical retraction using two Pozzi forceps in the ewe is shown in Fig. 5*b*, and in the goat in Fig. 5*c*; a retraction with a single set of the Allis forceps in the ewe is shown in Fig. 5*d*. After cervical

Table 3. Comparisons among embryo recovery techniques in goats and sheep in relation to the main steps related to techniques

Procedure	Laparotomy	Laparoscopy	Transcervical
Animal fasting	>24 h	>24 h	Not needed
Sedation	Deep	Deep	Superficial
Animal position during the procedure	Dorsal recumbence	Dorsal recumbence	Four leg station
Anaesthesia	General	General	Epidural or local
Laparoscopic equipment	Needed	Needed	Not needed
Ultrasound equipment	Not needed	Not needed	Recommended for CL count
Embryo recovery efficiency	Precise	Precise	Imprecise without CL count
Fluid recovery efficiency	High (>90%)	–	High (>90%)
Animal return to basal physiological conditions	Long (6 h)	Long (6 h)	Low (minutes)
Sequelae in the reproductive tract	High	Intermediate	Low
Minimal time between successive flushings	>30 days	>30 days	1 week
Effective number of flushings per donor	3	–	6
Animal welfare	Low	Intermediate	High
Technician skill needed	Intermediate	High	High
Total cost	High	High	Low

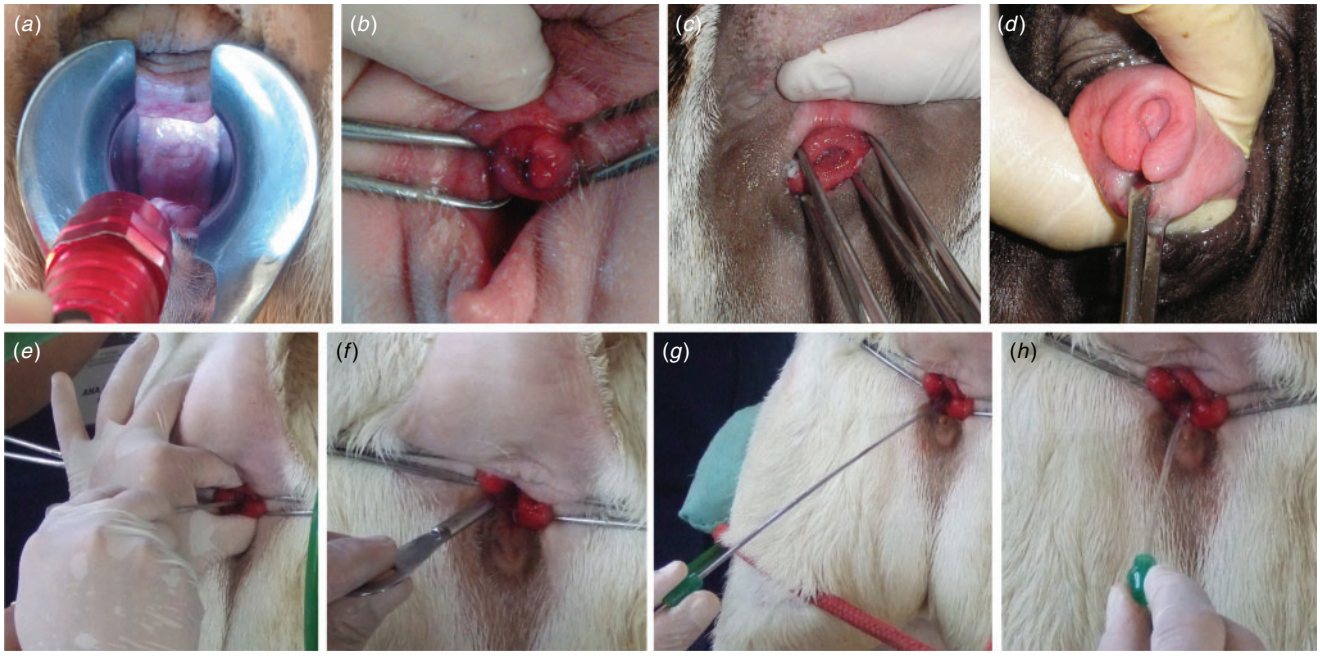

Fig. 5. Sequential steps to traverse the uterine cervix of sheep and goats before non-surgical embryo recovery: A – Cervical os view after Collin specula introduction into vagina; B – Lateral clipping of cervical os by two Pozzi forceps in sheep (B) and goat (C); D – Cervical os clipping by Embrapa forceps in sheep; Initial (E) and total (F) cervical transposition by Hegar dilator; G - Cervical transposition by catheter with mandrel inside; and H – Total cervical transposition and removal of mandrel.

retraction, a Number 3 Hegar dilator is introduced into the cervical os and fingers are inserted under and above the prolapsed cervix to help traverse the first cervical rings (Fig. 5*e*). Sometimes, placing the thumb under the cervix is needed to introduce a middle finger into the rectum for manipulating the last cervical rings. After complete penetration of the cervix (Fig. 5*f*), the Hegar dilator is replaced with a catheter equipped with a mandrel and the cervical penetration is done again with this new tool (Fig. 3*g*). Finally, the catheter is directed to the desired uterine horn (normally ipsilateral to the ovary with a greater CL count) and the mandrel is gradually removed as the catheter is slowly advanced (Fig. 3*h*). The catheter is then connected to the flushing circuit as described previously (Fonseca *et al.* 2013, 2016).

Non-surgical ET

The success of ET is primarily dependent upon the synchrony between donor and recipient animals, as well as on the recipients' ability to carry conceptuses to full-term. Normally, five to 10 recipients are synchronised per superovulated donor, if no embryos are intended for cryopreservation. ET is the last step of the entire MOET procedure. There are a few studies describing the use of non-surgical ET in small ruminants; the first live births following the procedure in small ruminants were reported by Otsuki and Soma (1964) in goats and by Fonseca (2006) in sheep. There are several advantages of using the non-surgical technique, such as increased safety, reduced invasiveness and no need for complex anaesthesia of recipient animals (Fonseca *et al.* 2014). Clearly the repeated use of this technique poses a concern regarding animal welfare (Candappa and Bartlewski 2014). Throughout the ET procedure, the recipient goats do not show any behavioural signs of pain, such as vocalisation or postural discomfort (Fonseca *et al.* 2014, 2016). In fact, ET via the cervical route can be performed in goats in a similar manner as AI. As with AI procedures, both the age and parity of the doe can affect the penetrability of the cervical canal (Kaabi *et al.* 2006). Obviously, the technique is substantially easier in goats than in sheep due to the differences in cervical anatomy between the two species. NSETs in recipient goats without any drug pretreatment to induce cervical relaxation resulted in 46–50% pregnancy and 39–50% of parturition rates (Fonseca *et al.* 2014; Esteves *et al.* 2015).

In sheep, very limited studies aiming to perform transcervical ET in ewes using the combination of 17β-oestradiol and oxytocin (Wulster-Radcliffe *et al.* 1999) or dinoprostone (PGE_2; Candappa and Bartlewski 2014) to achieve sufficient cervical relaxation reported embryo deposition in approximately 55% of animals. However, since the first lamb was born from ET by the cervical route (Fonseca 2006), no subsequent study has reported any significant advancement. Recently, we designed a preliminary study to test the effect of Embrapa's protocol for cervical relaxation without cloprostenol administration on pregnancy rate in oestrus-synchronised ewes (Arrais *et al.* 2018). After oestrus onset, females were allocated to receive either no drug, 1 mg oestradiol benzoate at 1600 hours on Day 6 plus 50 mg oxytocin at 0800 hours on Day 7 after oestrus onset or the same treatment plus300 IU human chorionic gonadotrophin (hCG) at 1600 hours on Day 7 after oestrus onset. Initial results suggest that the oestradiol–oxytocin combination could be deleterious to pregnancy establishment and that hCG administration possibly helped overcome this situation. This was probably by the action of hCG on accessory CL formation and/or progesterone elevation, as reported in Santa Inês ewes (Fonseca *et al.* 2018*b*). Some modified catheters have also been developed to improve traversal of the cervical canal in sheep, but the results remain inconsistent (Candappa and Bartlewski 2011). Further studies are needed to improve NSET and to use it on a large scale in small ruminants. We believe that this technique promises to become as commonplace for MOET in sheep and goats as it has been in cattle.

Novel applications of NSET techniques

During the preimplantation stage, mammalian embryos exhibit marked plasticity, which makes it possible for them to develop in a wide range of culture conditions. Due to these properties, it was possible to develop the technique of *in vitro* embryo production (IVP), which allows for greater use of mammalian oocytes for embryo production on a large scale. In recent years, IVP has contributed substantially to the genetic improvement of farm animals, increasing reproductive performance, contributing to the development of the animal industry and providing research subsidies (Blondin *et al.* 2012). However, the only ideal environment for the preimplantation development of the embryo is the uterine tube and uterus because these organs modify embryo function according to the phase of the oestrous cycle or early gestation and stage of embryogenesis (Buhi 2002) through the secretion of several factors for which specific receptors can be found in embryos, oviductal epithelial cells or the endometrium (Dadashpour Davachi *et al.* 2017). Deprivation of some of these maternal factors produced *in vivo* may be responsible for the impaired embryo development and viability of IVP-derived embryos (Rizos *et al.* 2002) and some pathological postnatal changes associated with this technique (Fernández-Gonzalez *et al.* 2007, 2008).

The use of techniques that amalgamate the advantages of *in vivo* and *in vitro* systems, such as the intrafollicular transfer of immature oocytes, have been proposed primarily to increase the quality of the embryos produced (Kassens *et al.* 2015). We demonstrated that the caprine uterus supports the nuclear maturation of bovine oocytes (*ex situ* maturation, 94.5% of recovered oocytes), in addition to reducing cytoplasmic lipid quantity compared with counterparts matured *in vitro* (Batista *et al.* 2017). Although further studies are required to improve the oocyte recovery rate (~50%), this is the first study that may link the use of NSET and NSER techniques for interspecies maturation of mammalian oocytes *in situ* (Batista *et al.* 2017). These results present an important alternative for oocyte improvement and thus possibly embryo quality.

Concluding remarks

Embryo recovery by the transcervical route is routinely used in cattle and goats. Transrectal ultrasonography (B-mode and colour Doppler) and serum P4 measurements on the day of embryo recovery can be used in small ruminants to evaluate the ovarian response to superovulatory treatments with similar accuracy to that of videolaparoscopy. In sheep, there has been considerable progress in the applications of various hormonal treatments (drug combinations, alternative routes and times of administration) to dilate the cervix and facilitate the passage of embryo recovery and transfer instruments. This progress fosters the use of assisted reproductive techniques that are non-invasive and hence widely acceptable considering constantly aired public concerns about animal welfare. It may imply that non-surgical methods may eventually replace surgical approaches to embryo flushing and transfer in small ruminants. The assessment and ranking of sheep for the degree of difficulty of performing transcervical penetration also suggest that surgical embryo recovery is not necessary in most multiparous females. Finally, the possibility of frequently performing transcervical penetration in small ruminants, especially in goats, can pave the way for the development of novel and highly efficacious methods of

interspecies (e.g. bovine) oocyte maturation and embryo production.

Conflicts of interest

The authors declare no conflict of interest.

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