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Review: Non-surgical artificial insemination and embryo recovery as safe tools for genetic preservation in small ruminants

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ABSTRACT

Artificial insemination (**AI**) and *in vivo* embryo production (or multiple ovulation and embryo transfer, **MOET**) programs are both instrumental in accelerating the propagation of genetically and economically superior goats and sheep. The aim of this review was to present the current gestalt of non-surgical AI and embryo recovery (**NSER**) procedures in small ruminants. Small body size, precluding rectal palpation, and highly limited penetrability of the uterine cervix in ewes are the major reasons for the scarce use of non-surgical assisted reproduction techniques in this species. As a result, AI and embryo recovery techniques in sheep mainly involve laparoscopy or laparotomy (**LAP**). In does, however, the Embrapa method of AI allows for successful intrauterine deposition of semen, resulting in pregnancy rates from 50 to 80% under field conditions (>3 000 goats inseminated) when frozen-thawed semen is used. After the administration of prostaglandin $F_{2\alpha}$ (**$PGF_{2\alpha}$**), non-surgical (transcervical) embryo recovery is also feasible in goats, with the cervical penetration rate approaching 100%. There is a paucity of information on the efficacy of non-surgical AI using frozen semen in sheep, but the results are satisfactory with fresh, cooled, or chilled ram semen. An application of the NSER technique in ewes has greatly improved over the last decade, and cervical penetration rates of ~90% can be achieved when a hormonal cervical dilation protocol using $PGF_{2\alpha}$, oxytocin, and/or estradiol ester (e.g., estradiol benzoate) is applied. In some genotypes of sheep, sufficient cervical dilation can be induced without estradiol ester included in the protocol. Several studies indicated that recovery of transferable quality ovine embryos using NSER is comparable to that employing a ventral midline laparotomy, and NSER is evidently a method of choice when animal welfare is concerned. Considering both the number of retrievable embryos and animal well-being, the NSER is a viable alternative for surgical procedures. With further developments, it has the makings of a primary, if not exclusive, embryo recovery technique in small ruminants worldwide.

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Implications

Assisted reproductive technologies such as artificial insemination and *in vivo* embryo production are of paramount importance for small ruminant producers. The most useful assisted reproductive technologies should be efficient, affordable, safe (low invasiveness), and relatively easy to perform in commercial settings. This review addresses currently available options and requirements for effectively using non-surgical artificial insemination and embryo recovery techniques in goats and sheep. Prospective developments in the simple and rapid transcervical transfer of sheep and goat embryos are also discussed.

Introduction

Artificial insemination (**AI**) and multiple ovulation and embryo transfer (**MOET**) programs are high-impact reproductive technologies that are pivotal for the propagation of animals with high genetic and economic merit. Both technologies are important for facilitating international trade, by mitigating the risks associated with live animal transit, and significantly contribute to salvaging endangered animal populations (species or breeds). While AI is primarily focused on preserving and spreading male genetics, the MOET program contributes to the propagation of both male and

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female genetic resources. Moreover, the application of AI effectively prevents the spread of sexually transmitted diseases. Lastly, semen cryopreservation and AI allow for postmortem reproduction of males or for utilizing male genetic material after semen donors have aged or suffered from fertility-limiting injuries (Fonseca et al., 2019a; Souza-Fabjan et al., 2021a).

The acquisition and cryopreservation of high-quality semen and embryos are the most efficient strategies for germplasm biorepositories. Although embryo production is more complex and expensive than semen collection, the former is a method of choice in a process of breed/species reconstitution (Boettcher et al., 2005). The MOET-derived embryos are more cryotolerant than their *in vitro*-produced counterparts and hence are generally preferred for establishing and operating biobanks (Arrais et al., 2021; Fonseca et al., 2021). For that reason, the MOET programs also remain the primary method used for commercial ovine and caprine embryo production (Souza-Fabjan et al., 2021a).

Since the small body size of the ewe limits the rectal palpation to digital exams and the uterine cervix is difficult to penetrate with most insemination/flushing catheters, the use of assisted reproductive technologies (**ARTs**) in sheep is significantly less widespread compared with that in goats. Sheep have a narrow, long, rigid, and tortuous uterine cervix with a series of 4–8 eccentric, funnel-shaped folds (Halbert et al., 1990a and 1990b), which prevent an easy penetration of the cervical canal (transcervical uterine access) even in the ewes during the receptive-to-mating, estrogen-dominant phase of the estrous cycle (Fig. 1A). Although does also have a small uterine cervix, it is relatively short and possesses fewer cervical rings that are symmetrically aligned, and hence easier to traverse compared with those in sheep (Santoyo, 1990; Fig. 1B). These anatomical differences determine the type of AI and approaches to performing embryo recovery/transfer in both species.

Regarding AI, pregnancy rates are generally lower when frozen-thawed (**F/T**) rather than fresh ram semen is deposited in the cervical os (approximately 30 vs 75%; Salamon and Maxwell, 2000). The exact cause of impaired F/T sperm cervical transit leading to this difference in conception rates remains unclear. There is a great deal of evidence to suggest that various cryopreservation-induced alterations in seminal plasma molecules (including proteins, lipids, and RNAs) might be responsible for this impairment (Reviewed by Fair et al., 2019; Warr et al., 2022). Interestingly, some Norwegian breeds appear to be a notable exception to this rule, as their sperm retains the ability to traverse the uterine cervix in relatively high numbers, resulting in satisfactory pregnancy rates after cervical AI using F/T semen (Paulenz et al., 2004; Fair et al., 2005; Abril-Parreño et al., 2021). It can be expected that studies focusing on semen proteomics and transcriptomics will improve our understanding of the interactions among sperm, seminal plasma, and cervical structures in sheep (reviewed by Fair et al., 2019; Warr et al., 2022), providing solutions to boost F/T AI efficacy in all genotypes of sheep.

In sheep, embryo collection usually involves laparotomy (**LAP**), which yields satisfactory embryo recovery rates. This method, however, is still labor-intensive and costly, and associated with the risk of postsurgical complications (e.g., adhesions; Torres and Sevellec, 1987; FAO, 2012; Pinto et al., 2020) that may compromise the future fertility of embryo donors and limit the number and frequency of collections (Pinto et al., 2020). A previous edition of the report on "Animal Production and Health/Cryopreservation of Animal Genetics Resources", published 11 years ago by the Food and Agriculture Organization (**FAO**), endorsed non-surgical AI, but it did not recommend non-surgical embryo recovery (**NSER**) for use in small ruminants. The authors concluded that the degree of difficulty associated with NSER was "4" in goats and "5" in sheep, while in cattle, it was graded "1" (on an ascending difficulty scale from 1 to 5; FAO, 2012). In the last decade, however, several studies have been dedicated to making NSER procedures in small ruminants more easily performable. Most importantly, there is a great deal of evidence to suggest that NSER is equally efficient as LAP in terms of embryo recovery rates (Oliveira et al., 2018a; Santos et al., 2020; Souza-Fabjan et al., 2022), and it is eminently more suitable and preferable considering animal welfare regulations (Fig. 2). The most recent FAO report published this year recommends the NSER for application in goats, but the LAP technique continues to be the primary recommendation for sheep (Blesbois et al., 2023).

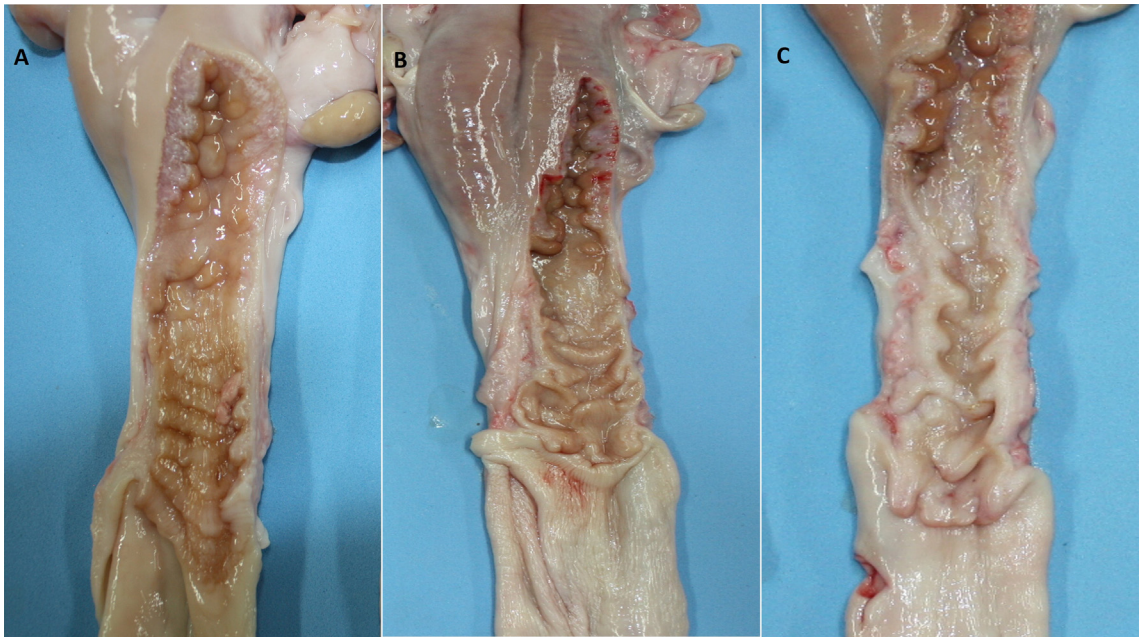

Fig. 1. Reproductive tracts of a goat (A) and sheep (B, C) collected after slaughter and depicting the features of cervical anatomy, namely an arrangement of cervical folds and rings.

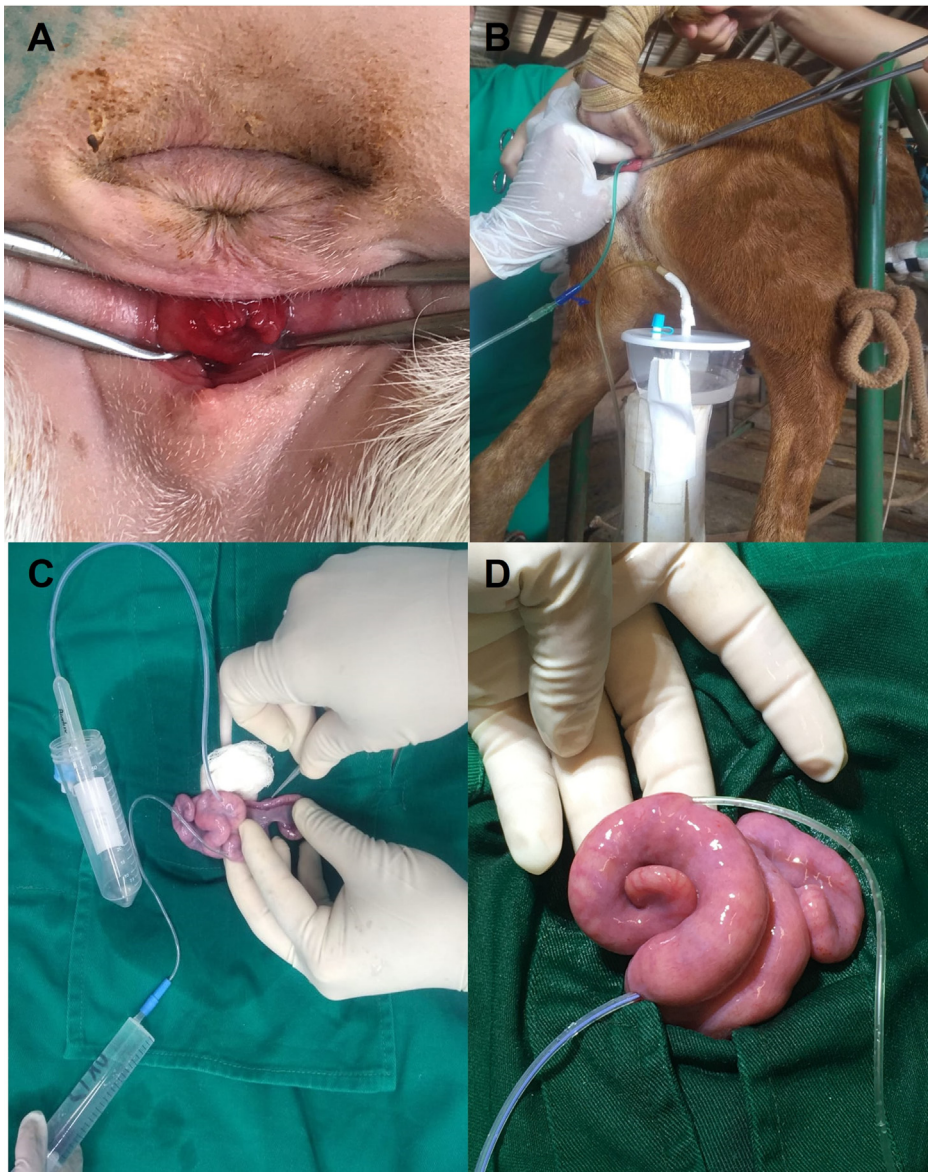

Fig. 2. (A) Lateral clipping of the cervical os using two pairs of Pozzi forceps; (B) general view of the transcervical uterine flushing in sheep; (C) surgical (laparotomy) embryo collection in sheep; and (D) exteriorized ovine reproductive tract.

In their literature review on the advances in reproductive biotechnologies in dairy cattle, Moore and Hasler (2017) remarked that communication between the public and scientists remained one of the major challenges for the implementation of ARTs. Members of the general community are frequently voicing their opinions on the importance of animal welfare in food production, and this trend may impinge on the use of MOET procedures in small ruminant production as well. The authors of the present review attempted to present the current state of non-surgical AI and NSER in small ruminant species to other members of the scientific community, who can potentially use this information in various extension programs.

Artificial insemination techniques in small ruminants

Uterine semen deposition in small ruminants can be accomplished by a laparotomic (Lopyrin and Loginova, 1958; Salamon and Lightfoot, 1967; Silla et al., 2021), laparoscopic (Killen and Caffery, 1982; Maxwell et al., 1984; Rocha et al., 2022), transcervical (Salamon and Lightfoot, 1967; Andersen et al., 1973; Fonseca et al., 2019a), or pericervical/vaginal (Lopyrin and Loginova, 1958; Salamon and Lightfoot, 1967; Menchaca et al., 2005) technique/route. Transcervical AI may utilize either cervical fixation (a.k.a. cervical clipping) or cervical traction techniques. Cervical fixation, typically used in goats, comprises cervical immobilization with a pair of Allis forceps (Fonseca et al., 2017a; Bonato et al., 2019), whereas cervical retraction, popular in ewes, involves the clipping of the uterine cervix with two pairs of forceps (Allis/Pozzi) and its gentle repositioning to the location where it can be transrectally palpated with fingers (Halbert et al., 1990a and 1990b; Casali et al., 2017). Even though no anesthesia is required during cervical manipulations, a few studies have suggested that cervical retraction may not be innocuous to the female (Halbert et al., 1990a) and histological examinations revealed the occurrence of cervical lesions associated with cervical clipping or retraction, raising some ethical concerns (Campbell et al., 1996).

An optimal sperm concentration in inseminate doses depends on the distance between the semen deposition and fertilization

sites (oviductal ampulla), but also on the type of semen used (fresh, cooled/chilled, or F/T). In sheep and goats alike, approximately 100 total million sperm (Reviewed by Gibbons et al., 2019) and 160–200 million motile sperm are required (Lightfoot and Salamon, 1970; Windsor et al., 1994) when performing pericervical/vaginal AI with fresh or F/T semen, respectively. For intrauterine insemination by transcervical route, 100–160 million F/T sperm are sufficient (Salamon and Lightfoot, 1970; Andersen et al., 1973; Corteel et al., 1988; Fonseca et al., 2017a) and 20–25 million motile sperm are usually used for laparoscopic AI with either fresh or F/T semen (Evans and Maxwell, 1987; Salamon and Maxwell, 2000).

Due mainly to the complex cervical anatomy (Kershaw et al., 2005; Fig. 1A), the most widespread AI techniques in sheep are

Table 1
Examples of studies conducted in sheep and aimed at increasing the penetrability of the uterine cervix during transcervical artificial insemination procedures.

Breed/Parity	Strategy/Hormonal protocol	Efficiency (%)				Overall outcomes	Reference
		Intrauterine penetration		Pregnancy			
		Treatment*	Control*	Treatment*	Control*		
Suffolk, Dorset, Cheviot, Suffolk crossbred, Leicester, Clun Forest, and Hampshire cross/multiparous	Guelph system of AI (different specula, forceps, instruments; dorsal recumbency restraint + cervical traction)	82 (73/89)	ND	ND	ND	High uterine penetration by using cervical traction	Halbert et al. (1990a)
Suffolk and white-faced/ diverse	Guelph system of AI	54 (49/90)	ND	80 (72/90)[A,B]	ND	Higher pregnancy when AI was intrauterine, compared to mid cervical (88 × 57%)	Halbert et al. (1990b)
Rambouillet and crossbreds/ Multiparous	Flexible catheter (designed to allow semen deposition in the uterine horn) and retroload AI gun	ND	ND	5 (5/99)[B]	ND	When depositing small numbers of sperm, it reduced pregnancy compared to LAI	Wulster-Radcliffe et al. (2004)
Rasa Aragonesa/multiparous	Antiretrograde flow device for sheep cervical AI (DARIO)	ND	ND	59 (390/662)[B]	50 (316/637)[B]	DARIO avoided visual cervix injuries, decreased retrograde flow de visu, and increased fertility rate	Macias et al. (2017)
Sarda/Multiparous	Surgical incision of cervical folds	90 (35/39)	ND	72 (28/39) [A]	ND	Facilitated the transcervical passage and intrauterine semen deposition, resulting in pregnancy rate similar to LAI (72 vs 70%)	Pau et al. (2020)
Dorset, Rambouillet, Hampshire, and Suffolk/diverse	200–600 USP OT (i.v.)	77 (33/43)	0 (0/15)	ND	ND	OT allowed a greater cervical penetration, but fertility was not assessed	Khalifa et al. (1992)
Crossbred/multiparous	200 USP OT (i.v.)	ND	ND	51 (28/55)[A] with LAI	66 (36/55)[A] with LAI	OT and cervical manipulation both decreased fertilization rate (47 vs 59%) and the former affected fertility after LAI	Stellflug et al. (2001)
Welsh Mountain, Île-de-France, Vendéenne, Romanov and Sarda/multiparous	2 mg oFSH (i.c.) 2 mg misoprostol, PGE1 (i.c.) 2 mg oFSH + 300 IU OT (i.c.) Ram effect	ND ND	ND ND	ND	ND	Cervical relaxation was enhanced by the presence of a ram but not by any drug used	Falchi et al. (2012)
Rideau Arcott × Polled Dorset/ Multiparous	Guelph system of AI + controlled slow-release vaginal inserts of PGE2 (Cervidil®)	90 (36/40)	75 (30/40)	3 (1/40)[A]	8 (3/40)[A]	Reduced the time to penetrate the cervix in the reproductive season, but reached similar pregnancy rates	Bartlewski and Candappa (2015)
Welsh Mountain/multiparous	2 mg oFSH (i.c.) 1 mg misoprostol, PGE1 (i.c.)	100 (9/9) 100 (9/9)	ND	ND	ND	Either oFSH or misoprostol facilitated cervical penetration, but their combination had no benefit	Leethongdee et al. (2007)
Rideau Arcott, Rideau Arcott × Suffolk/multiparous	5 μg human interleukin-α 8 (vaginal suppository)	40 (2/5)	0 (0/2)	ND	ND	Not sufficient to relax the cervix	Croy et al. (1999)
Kivircik/Diverse	0.5 mg carazolol (i.m.)	0 (0/150)	0 (0/150)	63 (95/150)[A,B]	57 (85/150)[A,B]	Increased the rate of ewes in which deep penetration of cervix was achieved (48 vs 33%), but did not affect lambing rate	Gündüz et al. (2010)

Abbreviations: i.v. = intravenous; i.c. = intracervical; i.m. = intramuscular; ND = not determined; AI = artificial insemination; LAI = laparoscopic AI; OT = oxytocin; oFSH = ovine follicle-stimulating hormone; PGE = prostaglandin E; USP = units of oxytocin.

* Treatment: strategy/hormones applied to enhance cervical penetrability; Control: respective controls for the treatment applied; () number of animals.

[A] Frozen-thawed semen.

[B] Chilled semen.

the laparoscopic (intrauterine) technique (when F/T semen is used) and vaginal insemination (with fresh/cooled/chilled semen; reviewed by Gibbons et al., 2019). Several strategies have been proposed to increase the ease of intrauterine semen deposition by the transcervical route in ewes (Table 1). Even though achieving acceptable pregnancy rates is feasible after applying invasive interventions (e.g., surgical incision of cervical folds; Pau et al., 2020), such improvements have not been typically seen for cervical AI using F/T semen in sheep. With the aid of specialized instruments and/or restraint and positioning of animals (e.g., Guelph system for transcervical AI; Halbert et al., 1990a), transcervical (intrauterine) AI may result in similar pregnancy rates as laparoscopic AI [32 vs 48%, respectively, using F/T semen in Merino ewes (Windsor et al., 1994) or 42 vs 50%, respectively, using fresh semen in Corriedale ewes (Casali et al., 2017)]. Interestingly, supplementing F/T semen with seminal plasma increased the ability of spermatozoa to penetrate cervical mucus and boosted pregnancy rates after cervical AI in Merino ewes (51 vs 28% for seminal plasma-supplemented vs non-supplemented semen, respectively; Maxwell et al., 1999). The same group later demonstrated that the addition of seminal plasma to epididymal sperm improved its cervical transport, suggesting that the process might be influenced by an unknown component(s) of seminal plasma (Rickard et al., 2014). Although there are few studies reporting satisfactory outcomes after transcervical AI with F/T semen in ewes, overall fertility is low and results remain inconsistent (Candappa and Bartlewski, 2011).

The uterine cervix in does is shorter than in ewes and contains fewer cervical rings that are symmetrically aligned (Santoyo, 1990; Fig. 1B). Therefore, the most used insemination technique in goats is the anterior bipedal transcervical AI (a.k.a. the French technique), although laparoscopic AI is still practiced. The former has some drawbacks including incomplete intrauterine semen deposition (semen is frequently placed between the cervical rings) and discomfort to animals. The "Embrapa technique" of transcervical AI by cervical immobilization in goats, with the female restrained in a standing (quadrupedal) position, was designed about a decade ago (Fonseca et al., 2011) and reported internationally a few years later (Fonseca et al., 2017a). This method permits a high rate of intrauterine semen deposition (≥90%), takes ~30 s per doe to complete, is much better tolerated by the inseminated animal, and has proven feasible in field conditions (Fonseca et al., 2019a), yielding high pregnancy rates [62.5% (Fonseca et al., 2017a); 66–80% (Bonato et al., 2019); and 68% (Carvalho-de-Paula et al., 2020)] with F/T semen.

Principles of non-surgical embryo recovery

Donor selection

The primary criteria for selecting donor ewes and goats are their genetic/economical value and responsiveness to hormonal superovulation (Pinto et al., 2018). When using NSER, however, the anatomical features and penetrability of the uterine cervix are both equally important criteria. We have recently shown that testing for cervical penetrability with a Hegar dilator during estrus is a useful predictor of the ewes' suitability for NSER; the sensitivity and accuracy of the prediction were 85.7% and 80.0%, respectively (Santos et al., 2019). The results of the test were regarded as "positive" if all cervical rings could be traversed after a maximum of three attempts (Santos et al., 2019). Transrectal ultrasonographic evaluation of the cervical rings can also aid in selecting the ewes eligible for NSER (Fig. 3). Ultrasonographic scans performed either 12 h after the onset of estrus or immediately before NSER allowed to classify ewes into the three categories (with rectilinear, intermediate, or highly asymmetrical cervical rings), and cervical retraction and passage were not feasible only in the ewes allocated to the last category (Figueira et al., 2020a). Nulliparous ewes that have a very small cervical diameter and short distance between cervical rings are usually not recommended for NSER (Fonseca et al., 2019b). As with other transcervical manipulations in small ruminants,

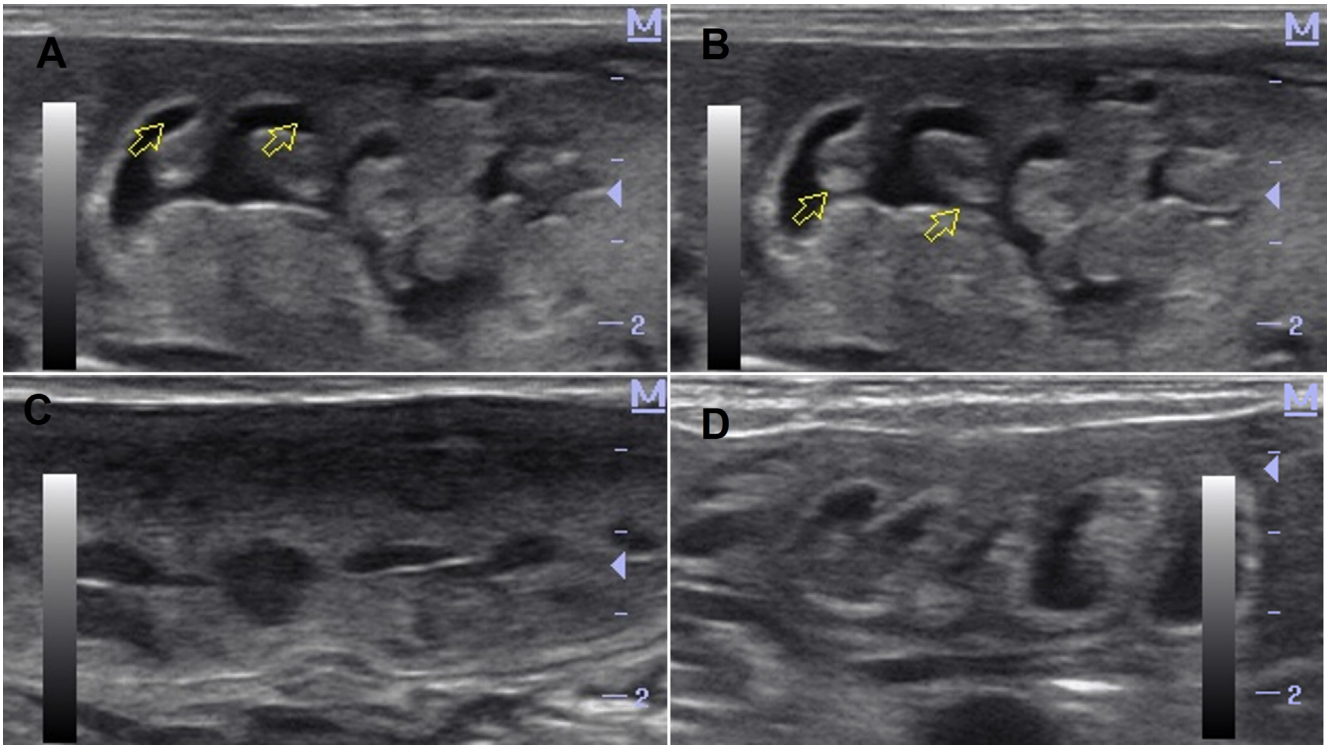

Fig. 3. Ultrasonographic evaluation of the sheep cervix. In the upper panels, yellow arrows indicate the convolutions of (A) and passage between cervical rings (B). Different types of cervical ring arrangements: (A, B) highly asymmetrical, (C) typical of the rectilinear cervix, and (D) intermediate.

the optimal body condition score of NSER females should be intermediate (not too thin but not obese; Fonseca et al., 2019b). The success of cervical penetration varies between different breeds of sheep (e.g., it is more difficult to traverse the uterine cervix in Churra and Assaf, and the ease of cervical penetration is greater in Merino and Castellana ewes; Kaabi et al., 2006), although there exists a tremendous individual variability among females of the same breed, age, and reproductive history. Based on our experience, postpartum ewes more than 100 days but less than five months after lambing are best suited for NSER procedures (Fonseca et al., 2019b; Fonseca et al., 2021).

Non-invasive methods to assess superovulatory responses

Ideally, embryo collection from each individual doe or ewe should be presaged by the determination of their response to the superovulatory treatment. The ability to determine the outcomes of hormonal ovarian superstimulation is extremely beneficial in terms of both ethical and practical senses (Oliveira et al., 2014). Identifying poor responders could spare the cost and undue stress of animals undergoing unnecessary procedures to recover the embryos. Such an evaluation is typically accomplished by counting corpora lutea (**CL**) in the ovaries of hormonally superstimulated donors and the primary technique used is laparoscopy or video endoscopy (Bruno-Galarraga et al., 2015; Pinto et al., 2020; Santos et al., 2020). However, the development and widespread use of non-invasive techniques to enumerate luteal structures in superovulated does and ewes would be an asset.

Since the first studies published in the 1980s, real-time B-mode ultrasonography has permitted the monitoring of reproductive events in small ruminants in a minimally invasive manner (Ginther, 2014). Doppler ultrasonography (Fig. 4) permits the detection of blood flow in internal organs and tissues (Ginther, 2014). Both imaging modalities have recently been applied to assess the response to superovulatory treatments in small ruminants (Oliveira et al., 2014). There is a strong positive correlation between the numbers of CL determined using ultrasonography and laparoscopy, although the accuracy of ultrasonographic detection of luteal structures decreased (0.89 vs 0.67) when more than four CL were present in each ovary of sheep (Pinto et al., 2018). The use of color Doppler ultrasonography has been associated with a greater accuracy of CL detection and enumeration compared with B-mode scans (Oliveira et al., 2018b). Thus, transrectal ultrasonography of ovaries and especially color Doppler sonography can be used to identify and remove the poorly responding ewes from *in vivo* embryo production programs.

Strategies to induce cervical dilation for non-surgical embryo recovery

The need for cervical dilation before embryo recovery is one of the major limitations in popularizing the use of NSER in small ruminants, particularly in sheep (Fonseca et al., 2019b and 2021; Dias et al., 2023). The hormonal protocols designed to induce cervical dilation were originally based on the complex physiological mechanisms of cervical ripening that occur naturally during estrus and, to a significantly greater extent, before parturition. As reviewed by Candappa and Bartlewski (2011), the changes in the release pattern of several hormones, including estradiol and prostaglandin E_2 (**PGE_2**), induce luteolysis and cervical relaxation. The latter is mediated by remodeling of the extracellular cervical matrix, and it proceeds the activation of uterine oxytocin (**OT**) receptors that trigger uterine contractions. In addition to the expected physiological variations among species (Wagner et al., 2017), significant differences in the cervical gene expression or transcriptome profile linked to smooth muscle contraction, extracellular matrix development, and immune responses were also identified between prolific and non-prolific breeds of sheep (Abril-Parreño et al., 2021).

Several cervical dilation protocols using prostaglandin analogs, mainly prostaglandin E_1 (**PGE_1**), PGE_2, or $PGF_{2\alpha}$, with or without OT and estradiol esters, have been tested in small ruminants. PGE_1 (Gusmão et al., 2009; Leite et al., 2018) and PGE_2 (Candappa and Bartlewski, 2014) analogs were deposited directly into the vagina from 48 to 24 h before cervical penetration. Misoprostol (PGE_1 analog) used alone (Gusmão et al., 2009) or in combination with estradiol benzoate (**EB**) and OT has successfully been used for cervical dilation in sheep (cervical penetration rate between 83% and 95%; Leite et al., 2018). This drug, however, is not commercially available in Brazil so over the last two decades, $PGF_{2\alpha}$ has been used for inducing cervical dilatation in sheep and goats. For uterine flushing by the transcervical route in goats (Table 2), the administration of $PGF_{2\alpha}$ at 24, 16, 8, or 0 h before the NSER, with or without OT, has been tested (Pereira et al., 1998; Lima-Verde et al., 2003; Amorim et al., 2011; Fonseca et al., 2013 and 2018) and the best results were achieved with a single dose of cloprostenol given 12–16 h before NSER (Fonseca et al., 2013; 2019b; Maia et al., 2020). In sheep, a combined treatment with $PGF_{2\alpha}$ and EB given 16 h before and OT administered 20 min prior to NSER has recently been identified as the most effective hormonal induction of cervical dilation (Fonseca et al., 2019b and 2019c; Table 3). The development of these protocols was preceded by a series of studies using different estradiol esters (EB or estradiol cypionate; Fonseca et al., 2019c), times of $PGF_{2\alpha}$ administration (18, 16, 12 or 10 h before NSER; Fonseca et al., 2019b, 2019c and 2019d; Santos et al., 2021) as well as routes of EB (intramuscular or intravaginal; Fonseca et al., 2019d) and OT administration (intravenous and intravaginal; Prellwitz et al., 2019). Further, the recommended cervical dilation protocol has been tested and proven effective in different breeds of sheep varying in size and prolificacy, including Lacaune (Figueira et al., 2020b and 2020c), Santa Inês (Oliveira et al., 2020; Fonseca et al., 2021), Somalis Brasileira (Fonseca et al., 2021), and Morada Nova (Arrais

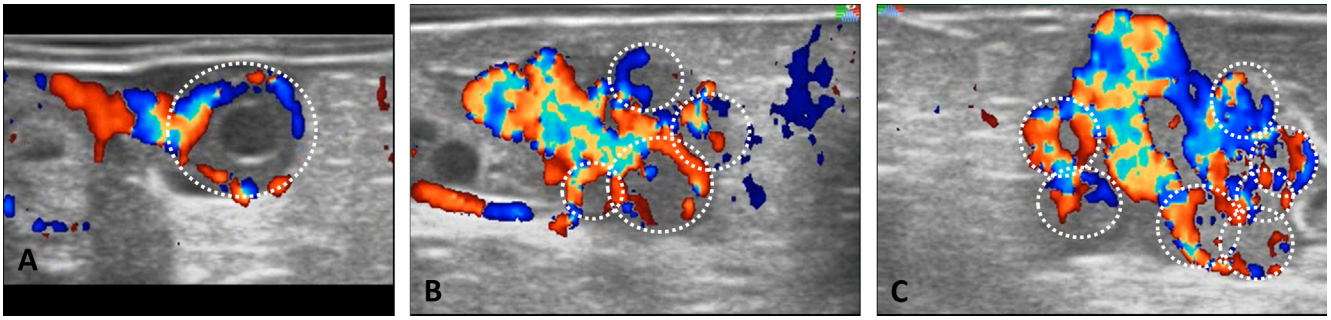

Fig. 4. Ultrasonographic evaluation of the ovaries in superovulated ewes, approximately six days after ovulation. White dotted lines delineate corpora lutea (CLs). The three response patterns are presented: (A) low response, only one CL; (B) moderate response, four CLs; and (C) satisfactory superovulatory response, seven CLs.

Table 2
Summary of studies conducted in sheep that were subjected to hormonal cervical dilation protocols prior to non-surgical embryo recovery.

Breed	Parity	Hormonal Protocol	Efficiency (%)		Reference
			Cervical penetration*	Embryo recovery**	
Crossbreed (Lacaune × Santa Inês)	Diverse	1 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	64 (9/14)	83 (80/97)	Dias et al. (2023)
		0.5 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	83 (10/12)	107 (89/83)	
		0.0 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	90 (9/10)	60 (55/91)	
Morada Nova	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	86 (25/29)	71 (176/248)	Oliveira et al. (2022)
Morada Nova	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and	94 (15/16)	99 (135/136)	Fonseca et al. (2021)
Santa Inês	Multiparous	50 IU OT i.v. (−20 min to NSER)	90 (17/19)	83 (209/252)	
Somalis	Multiparous		94 (17/18)	57 (101/178)	
Morada Nova	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	94 (16/17)	60 (95/159)	Arrais et al. (2021)
Santa Inês	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	84 (26/31)	ND	Oliveira et al. (2020)
Lacaune	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−10 h) and 50 IU OT i.v. (−20 min to NSER)	94 (31/33)	61 (187/306)	Figueira et al. (2020c)
Lacaune	Diverse	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	89 (32/36)	62 (143/232)	Figueira et al. (2020b)
Dorper	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	100 (12/12)	40 (8/20)	Dias et al. (2020)
		0.5 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	92 (11/12)	39 (9/23)	
		0.0 mg EB i.m. + 37.5 μg d-cloprostenol i.m. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	83 (10/12)	53 (10/19)	
Santa Inês	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	57 (12/21)	ND	Prellwitz et al. (2019)
		1 mg EB i.m. + 37.5 μg d-cloprostenol i.v. (−16 h) and 50 IU OT i.v.g. (−20 min to NSER)	57 (12/21)	ND	
Santa Inês	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	82 (9/11)	ND	Fonseca et al. (2019d)
		1 mg EB i.v.g. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	80 (8/10)	ND	
Santa Inês	Multiparous	1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	78 (7/9)	ND	Fonseca et al. (2019c)
		1 mg EC i.m. + 37.5 μg d-cloprostenol l.v. (−16 h) and 50 IU OT i.v. (−20 min to NSER)	44 (4/9)	ND	
		1 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−10 h) and 50 IU OT i.v. (−20 min to NSER)	39 (5/13)	ND	
		0.0 mg EB i.m. + 37.5 μg d-cloprostenol l.v. (−10 h) and 50 IU OT i.v. (−20 min to NSER)	27 (3/11)	ND	
Santa Inês	Multiparous	200 μg misoprostol i.v.g. (PGE1 analog) (−5 h to NSER)	67 (20/30)	ND	Leite et al. (2018)
		100 IU OT i.v. (−15 min) + 100 μg EB i.v.	90 (27/30)	ND	
		200 μg misoprostol i.v.g. + 100 IU OT i.v. (−15 min) and 100 μg EB i.v. (−12 h to NSER)	83 (25/30)	ND	
Dorper	Multiparous	200 μg misoprostol i.v.g. (−5 h to NSER)	95 (55/58)	ND	Gusmão et al. (2009)

Abbreviations: NSER = non-surgical embryo recovery; ND = not determined; EB = estradiol benzoate; EC = estradiol cypionate; OT = oxytocin; PGE = prostaglandin E; i.m. = intramuscular; i.v. = intravenous; i.v.g. = intravaginal; l.v. = inlaterovulvar.
* Donors successfully penetrated and flushed × 100/donors with corpora lutea at NSER (%).
** Number of total structures recovered × 100/ number of corpora lutea per flushed female (%).

Table 3
Examples of studies conducted in goats that were subjected to hormonal cervical dilation protocols prior to non-surgical embryo recovery.

Breed	Category	Hormonal Protocol	Efficiency (%)		Reference
			Cervical penetration*	Embryo recovery**	
Canindé	Multiparous	37.5 μg d-cloprostenol i.m. (−16 h to NSER)	100 (11/11)	35 (37/106)	Fonseca et al. (2021)
Moxotó	Multiparous		100 (12/12)	53 (88/167)	
Saanen	Multiparous	37.5 μg d-cloprostenol i.m. (−12 h to NSER)	100 (21/21)	71 (121/170)	Maia et al. (2020)
Saanen	Multiparous	30 μg d-cloprostenol i.m. (−12 h to NSER)	100 (10/10)	ND	Fonseca et al. (2013)
Toggenburg	Multiparous	125 μg cloprostenol i.m. (−24 h to NSER)	100 (18/18)	ND	Amorim et al. (2011)
Saanen	Multiparous	50 μg cloprostenol i.m. (−24 h to NSER)	62 (8/13)	53 (50/94)	Lima-Verde et al. (2003)
Boer	Multiparous	5 mg dinoprost i.m. (−16 h) + 1 IU OT i.v. (−0 h)	100 (7/7)	91 (82/90)	Pereira et al. (1998)
		5 mg dinoprost i.m. (−8 h) + 1 IU OT i.v. (−0 h)	100 (6/6)	91 (48/53)	
		5 mg dinoprost i.m. (−0 h) + 1 IU OT i.v. (−0 h to NSER)	100 (6/6)	52 (28/54)	

Abbreviations: NSER = non-surgical embryo recovery; ND = not determined; OT = oxytocin; i.m. = intramuscular; i.v. = intravenous.
* Donors successfully penetrated and flushed × 100/donors with corpora lutea at NSER.
** Number of total structures recovered × 100/number of corpora lutea per flushed female.

et al., 2021; Fonseca et al., 2021; Oliveira et al., 2022) ewes. Since the veterinary use of EB has been banned in some countries, and due to its potentially deleterious effects on preimplantation embryos (Santos et al., 2021), our group has recently examined the possibility of reducing the dose (1.0 vs 0.5 mg) or even eliminating the ester from the cervical dilation protocols. Results of two studies in Dorper ewes (Dias et al., 2020 and 2023) indicated that NSER can successfully be performed using a cervical dilation protocol without EB. This protocol still needs to be assessed in different breeds of sheep. Methodological details of the studies cited in this section are outlined in Tables 2 and 3.

General description and major advantages of the non-surgical embryo recovery technique

Several articles have previously detailed NSER procedures in goats and sheep (Fonseca et al., 2013; 2019b). Briefly, after applying the cervical dilation protocol, the female is sedated (hyoscine-N-butylbromide, sodium dipyrone and acepromazine maleate), restrained in an elevated cart to avoid lateral movements and treated with local anesthetics (pericervical gauze and epidural lidocaine treatment). The cervical os is then visualized after inserting a Collin speculum, clipped and retracted, and the uterine cervix is traversed with a Hegar dilator (size 3–4), which is kept in place for ~30 s, and then with a sterile catheter #8 stiffened with a metal mandrel. A mandrel is eventually removed before connecting the catheter to a flushing circuit, connecting the source of flushing media to the filter (Fig. 2).

The NSER technique can offer several advantages over surgical embryo recovery procedures. Firstly, it does not require previous fasting of donor animals. It only entails a relatively simple anesthesia/analgesia protocol that can be easily completed in field conditions (Fonseca et al., 2019b, 2019c and 2019d; Maia et al., 2020; Fonseca et al., 2021). During the NSER procedure, donor animals continue to maintain visual, auditory and olfactory contact with their herd mates, if any, and once the collection is finished, the females can immediately be returned to their pens or paddocks (Figueira et al., 2020a,b; Santos et al., 2020; Arrais et al., 2021). The average duration of an NSER procedure performed by an experienced operator is ~20 min (Maia et al., 2020) and ~25–30 min (Santos et al., 2020; Arrais et al., 2021; Dias et al., 2023) in goats and sheep, respectively.

Transcervical embryo recovery is less costly than surgical techniques, and it can be performed in both superovulated and estrus-induced (non-superovulated) females (Dias et al., 2020; Figueira et al., 2020b; Arrais et al., 2021). The latter can potentially be used when superovulation is not feasible (e.g., in wild animals) or for maximizing the genetic gain or diversity at the relatively low cost (i.e., without hormonal treatments). Cervical penetration and embryo recovery rates after NSER in sheep and goats subjected to different hormonal protocols for cervical dilation are summarized in Tables 2 and 3, respectively.

In a few studies comparing the efficiency of NSER and LAP in goats (Souza-Fabjan et al., 2022) and sheep (Oliveira et al., 2018a; Santos et al., 2020), the surgical approach was never superior in terms of any major efficiency metrics. In fact, the duration of embryo collection has consistently been less (~25 min vs 32 min) and fluid recovery has been greater (99 vs 92%) with NSER compared with LAP, while the total number of structures recovered per animal did not vary significantly between the two techniques (4.2 vs 3.0 for NSER and LAP, respectively; Santos et al., 2020).

Potential drawbacks of non-surgical embryo recovery technique

The associated risk of postprocedural complications is minimal, and from all indications, the reproductive health and fertility of donor animals are not adversely affected by non-surgical embryo flushing (Dias et al., 2020; Fonseca et al., 2021). Successive NSER procedures at the intervals ranging from 30 to 60 days (Fonseca et al., 2013; Figueira et al., 2020b and 2020c; Oliveira et al., 2020) did not impair embryo yields, while a decline in embryo yields was reported in animals that underwent two or three successive LAP embryo collections at such intervals (Torres and Sevellec, 1987; Pinto et al., 2020). However, cervical manipulations can potentially cause trauma to the perineal and pelvic areas (DeRossi et al., 2009). Thus, our group tested different pharmacological approaches to induce analgesia and relaxation of the perineal region in females subjected to the NSER regimen. Even though epidural injections of ketamine alone or in combination with xylazine or morphine were not capable of inducing cervical dilation in Santa Inês sheep, they did promote satisfactory analgesia and muscular relaxation (Leite et al., 2018).

A major concern surrounding the cervical dilation protocol was that hormonal treatments applied just prior to embryo collection might be embryotoxic (Santos et al., 2021). In an experiment involving an application of the cervical dilation protocol in naturally mated ewes, estradiol and OT had no effect on pregnancy rates (Lewis, 2010). In another experiment, the ewes previously exposed to a cervical dilation protocol (EB, OT and cloprostenol) had fewer transcripts of NANOG and OCT4 (pluripotency) genes, but did not differ in the abundance of BAX, BCL2 (apoptosis), PRDX1 and HSP90 (cell stress) transcripts, compared to their control counterparts (Santos et al., 2021). The laparoscopic transfer of F/T embryos recovered using the NSER procedures resulted in the pregnancy rate of 52% in goats (Fonseca et al., 2018) and 39% in sheep (Figueira et al., 2019). Those rates are similar to that recently reported in ewes (51–52%) after the transfer of fresh embryos collected by laparotomy, without any cervical dilation treatment (King et al., 2022).

Recently, it has been reported that a rise in the expression of inflammatory markers (haptoglobin, total protein, and paraoxonase 1 activity) occurring after embryo collection was similar in ewes that underwent either LAP or NSER procedures (Oliveira et al., 2018a). In addition, we compared several parameters (physiological, endocrine, biochemical, and behavioral) between ewes subjected to LAP and NSER embryo recovery techniques, both during and after the collection procedure (Santos et al., 2020). The NSER ewes were sedated (0.1 mg/kg; acepromazine maleate i.v. and 0.3 mg/kg diazepam i.v.) and received an epidural anesthesia (ketamine; 2.0 mg/kg), while the LAP ewes received the same treatments plus general anesthesia, starting with an anesthetic induction using propofol (maximum dose of 4.0 mg/kg i.v.) and ketamine (6.0 mg/kg i.v.), and maintained with the 3% isoflurane (inhalation). The NSER group of ewes had a greater rectal temperature, heart rate, and elevated blood glucose levels immediately after embryo collection compared with their LAP counterparts. Alternatively, the heart rate (at 12 h and 24 h after embryo collection) and serum cortisol concentrations were greater in LAP than in NSER ewes immediately after embryo collection and 1 h later.

Non-surgical embryo transfer

Fonseca et al. (2014) have described the completion of non-surgical embryo transfer (**NSET**) in small ruminants. The insertion of the catheter is possible after cervical traction and embryos are typically deposited into the uterine horn ipsilateral to the CL previously detected with transrectal ultrasonography. The NSET is a valid alternative to the traditional laparoscopic embryo transfer due mainly to its lower cost (specialized equipment and general anesthesia required during laparoscopic surgery), shorter recovery period after procedure, and reduced stress level of a non-surgical technique. Even though the advantages of NSET may outweigh those of a laparoscopic approach, this method still has several lim-

itations like those associated with NSER, especially in ewes (Candappa and Bartlewski, 2011). The passage of the insemination catheter through the uterine cervix is problematic and requires the application of the cervical dilation protocol, and cervical/vaginal manipulations may cause trauma to the pelvic tissue (intracervical bleeding, uterine infections and/or adhesions; DeRossi et al., 2009), ultimately compromising the outcome of the embryo transfer.

Otsuki and Soma (1964) reported the first kid born after NSET, while Fonseca (2006) reported the birth of a first NSET lamb. In 2014, Candappa and Bartlewski reported the 55% transcervical embryo transfer rate and the 33% of early pregnancy detection rate, but no lamb was born. Conversely, studies in goats have reported satisfactory NSET pregnancy rates [32% (Morais et al., 2020), 39% (Flores-Foxworth et al., 1992), 43% (Agrawal and Bhattacharyya, 1982) or 50% (Fonseca et al., 2014)], which are comparable to those obtained with laparoscopy (Flores-Foxworth et al., 1992). Since then, no study has reported any significant refinement in NSET success rates in sheep. From all indications, the NSET has the makings of the method to be used in small ruminant embryo transfer programs.

Concluding remarks

The need to accelerate the progress in reproduction of small ruminants necessitates the incorporation of AI and MOET programs as a part of the modern animal breeding strategies. Continued improvements are required to guarantee that the application of these technologies is safe and maximizes the productivity outcomes in goats and sheep. The recent progress in AI has been rather slow and the techniques used are generally like those developed in the last several decades, except the implementation of the transcervical AI in goats and sheep (i.e., the "Embrapa technique"). Several improvements in the NSER technique were reported and the efficiency of the method is approaching to that of surgical/LAP embryo recovery techniques, which are more invasive but still predominantly used worldwide, possibly due to a lack of sufficient NSER training opportunities. Considering the well-being of animals, the NSER is a viable and appealing alternative to laparotomy and laparoscopy. The NSET still needs to be revamped, but it may also become a useful tool for embryo transfer in small ruminants.

Ethics approval

Not applicable.

Data and model availability statement

The original data from which the conclusions presented in this review were drawn are available from the corresponding author upon reasonable requests. None of the data were deposited in an official repository. No new datasets were created.

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Declaration 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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