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Review

Transcervical uterine flushing and embryo transfer in sheep: Morphophysiological basis for approaches currently used, major challenges, potential improvements, and new directions (alas, including some old ideas)

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ABSTRACT

At present, the success of non-surgical embryo recovery (NSER) and transfer (NSET) hinges upon the cervical passage of catheters, but penetration of the uterine cervix in ewes is problematic due to its anatomical structure (i.e., long and narrow cervical lumen with misaligned folds and rings). It is a major obstacle limiting the widespread application of NSER and NSET in sheep. While initial attempts to traverse the uterine cervix focused on adapting or re-designing insemination catheters, more recent studies demonstrated that cervical relaxation protocols were instrumental for transcervical penetration in the ewe. An application of such protocols more than tripled cervical penetration rates (currently at 90–95 %) in sheep of different breeds (e.g., Dorper, Lacaune, Santa Inês, crossbred, and indigenous Brazilian breeds) and ages/parity. There is now sufficient evidence to suggest that even repeatedly performed cervical passages do not adversely affect overall health and reproductive function of ewes. Despite these improvements, appropriate selection of donors and recipients remains one of the most important requirements for maintaining high success rates of NSER and NSET, respectively. Non-surgical ovine embryo recovery has gradually become a commercially viable method as even though the procedure still cannot be performed by untrained individuals, it is inexpensive, yields satisfactory results, and complies with current public expectations of animal welfare standards. This article reviews critical morphophysiological aspects of transcervical embryo flushing and transfer, and the prospect of both techniques to replace surgical methods for multiple ovulation and embryo transfer (MOET) programs in sheep. We have also discussed some potential pharmacological and technical developments in the field of non-invasive embryo recovery and deposition.

1. Introduction

Transcervical access to the uterine lumen is central to successful non-surgical embryo recovery and transfer. It replaces surgical procedures, laparotomy and laparoscopy, which are more costly and time consuming, and require considerable expertize. Cervical penetration is a part of routine reproductive management of large ruminants. The size of sheep precludes transrectal cervical immobilization and misalignment of multiple cervical rings decreases the ease with which catheters can be passed through the cervix [1]. Therefore, most assisted reproduction

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technologies in sheep use surgical approaches [2], albeit the alternative methods have been tested and successfully performed [3]. Non-surgical embryo recovery (NSER) has frequently been used in multiple ovulation and embryo transfer (MOET) programs in goats [4].

Utilizing a transcervical route for assisted reproductive techniques eliminates the need for fasting and general anesthesia as well as a risk of potential post-operative complications such as abdominal adhesions [4,5]. Transcervical manipulations are routinely performed after light sedation, an epidural block and local (cervical) anesthesia in animals restrained in a standing position [4]. Recent studies have shown that the total time to perform uterine flushing is less using NSER compared with surgical techniques, while the embryo recovery rate does not differ between the two approaches and fluid recovery appears to be greater with NSER [6,7]. This improved efficiency of NSER in ewes is due mainly to a development of new hormonal protocols for inducing cervical dilation [8] that have been tested in different breeds of sheep (Santa Inês [9,10], Lacaune [11], Dorper [12], Morada Nova [8] and crossbred ewes [13]). Transcervical techniques in small ruminants can be used in conjunction with MOET protocols but also as an integral part of conservation programs [5,8,14]. Finally, transcervical AI, NSER and NSET are all minimally invasive techniques and their frequent use can be a desirable option for implementing reproductive biotechnologies that comply with maintaining animal well-being.

Despite these advantages, both NSER and NSET have yet to become commercially used methods in small ruminant husbandry. Inadequate knowledge of these procedures, difficulty traversing the uterine cervix, and a lack of qualified veterinary technicians to perform transcervical manipulations also limit the use of NSER/NSET in reproductive research. Therefore, the primary aim of this review was to collate pertinent information and updates on the principles of NSER/NSET, and to indicate potential improvements to this technique that can accelerate its widespread application in sheep reproductive management.

2. Anatomical barriers to cervical penetration in sheep

The uterine cervix is a posterior entrance to the uterus and a passageway for the transport of semen and expulsion of fetus. The ovine cervix is a tubular organ between 4 and 7 cm long [15,16], with a narrow lumen and several annular folds [1]. The opening of the cervix in the vagina (a.k.a. the external os) is covered by the mucosal and fibrous tissue folds [17]. The specific arrangement and shape of the folds (i.e., flap, duckbill, smooth, spiral, rosette, or papilla) vary among breeds and even among individual animals of the same genotype [1,15]. Based on the author's experience, a "smoother" (i.e., flap type) opening is typically associated with a difficult cervical clamping, which progresses faster with a duckbill or rosette type of the os cervix. However, the type of cervical opening did not affect the overall success rate of NSER in Dorper ewes (Fig. 1; [12] What hampers the success of NSER is the occurrence of anatomical abnormalities such as vestibulo-vaginal stenosis, completely preventing the immobilization and retraction of the cervix (Fig. 1A; [5,18]. Prellwitz et al. [9] reported that the main reasons precluding transcervical penetration in ewes included the following anatomical alterations or features: vestibulo-vaginal stenosis (25 %), inadequate cervical distension (50 %) or extremely long and tortuous cervical canal (25 % of animals).

Cervical rings are multiple circular projections with a corkscrew-like arrangement [1]. The diameter, height, and spacing of ovine cervical rings are highly variable [15,19]. The second posterior cervical ring, which tends to be more misaligned than all the other rings, is a major physical barrier to the passage of catheters. The inner diameter of cervical rings is greater at the uterine end of the cervix [16]. Asymmetrical cervical folds, or sacks, separate individual rings [19]. As the cervical luminal shape is inherent [20], it is possible that anatomical barriers to transcervical penetration in ewes can be mitigated, at least partly, through the selective breeding of ewes. It is worth considering since impossible or difficult cervical passage is a major factor negatively impacting embryo recovery rates at NSER [21].

3. Histological structure of the cervical wall

In mammalian species, the cervical wall consists of three distinctive layers easily distinguishable in the transverse histological sections: the mucosa layer composed of an inner luminal epithelium and subepithelial stroma, the smooth muscle layer containing circular, oblique, and longitudinal bundles of smooth muscles, and the outer serosal layer covered by squamous epithelial cells (mesothelium; [19,22]. The surface

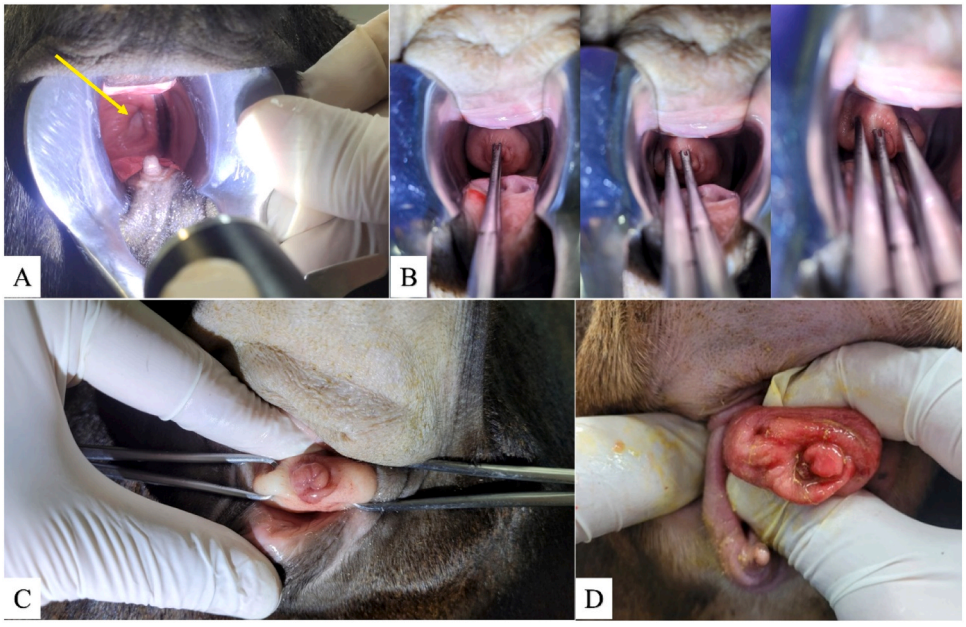

Fig. 1. Vaginoscopy in a ewe showing insertion of the vaginal speculum and clamping/retraction of the cervical os. (A) A ewe with the vestibule-vaginal stenosis (hymenal fold); the arrow indicates the small opening of the vagina where it is difficult or impossible to insert the speculum and it is not possible to visualize the cervical os for clamping and retraction; (B) immobilization of the os cervix; (C) cervical retraction; and (D) post-NSER appearance of the cervical os.

of the cervix is lined with non-keratinized epithelium that is composed of stratified squamous cells at the external (Fig. 2A) and columnar epithelial cells in the remaining segments of the cervix (Fig. 2B). In addition to ciliated and non-ciliated epithelial cells, the cervical lining contains wedge-shaped peg cells and convex-shaped secretory cells, functionally resembling the goblet cells described by [23]. Approximately 1–2 days before the onset of estrus (follicular phase), the secretory cells differentiate and reorganize, initiating the synthesis and dispersion of abundant mucus. After estrus, this population of cells undergoes atrophy and returns to the amorphous state characteristic of the luteal phase [24]. These consecutive changes are mediated by estradiol-17β (E_2) and progesterone (P_4), respectively, binding to their epithelial cell receptors. Estradiol increases the proliferation of secretory cells and modulates the expression of proteins linked to the "relaxation" of tight junctions, promoting cell rearrangement for mucus excretion [25]. Progesterone progressively modulates cellular function, causing changes in the composition and organization of the extracellular matrix (ECM) [26].

The subepithelial stroma of loose connective tissue houses fibroblasts, macrophages, and mast cells (Fig. 2B). While macrophages and

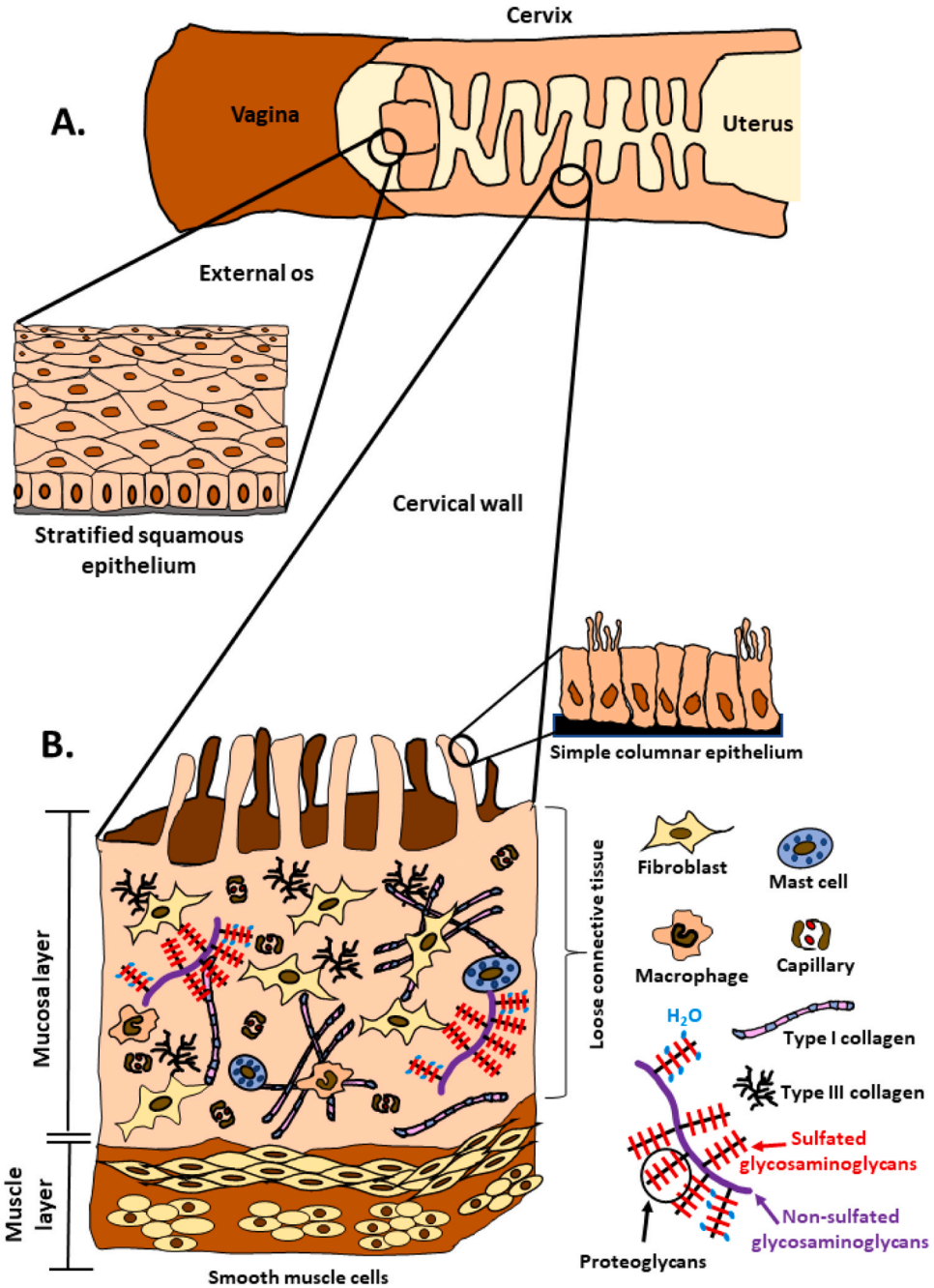

Fig. 2. (A) Diagrammatic representation of the uterine cervix. Cervical rings are present along the entire length of the cervix. The outer layer is lined with non-keratinized stratified squamous epithelium. (B) The cervical canal in turn is lined with simple columnar epithelium containing cylindrical ciliated cells, non-ciliated secretory cells (mucinogenous), and wedge-shaped peg cells. The cervical wall contains the mucosa and muscle layers. The mucosal layer covered by columnar epithelium contains stroma of loose connective tissue with capillaries, fibroblasts, macrophages, mast cells, and extracellular matrix components such as reticular (type-III collagen) fibrils, collagen (type-I collagen) fibrils, sulfated glycosaminoglycans (dermatan sulfate, chondroitin sulfate, heparan sulfate, and keratan sulfate), non-sulfated glycosaminoglycans (hyaluronan) and proteoglycans. Water molecules are bound to glycosaminoglycans.

mast cells provide immunological protection, fibroblasts produce extracellular matrix components, including reticular (type-III collagen) fibrils, collagen (type-I collagen) fibrils organized in bundles and fibers, and amorphous substances, including glycosaminoglycans, proteoglycans and glycoproteins (Fig. 2B). Collagens are the main component of the cervical structure responsible for its rigidity and elasticity [27]. The maintenance of tension generated by collagen fibrils is due to the action of glycosaminoglycans [28]. There are two classes of glycosaminoglycans: non-sulfated glycosaminoglycans such as hyaluronan, and sulfated glycosaminoglycans, which are composed of dermatan sulfate, chondroitin sulfate, heparan sulfate, and keratan sulfate. Sulfated glycosaminoglycans congregate to form proteoglycans, which act as an extracellular matrix component binding amino acid residues present in collagen as well as fibroblast, mast cells, and multiple growth factors [29]. Proteoglycans are essential for the "hydration" of the extracellular matrix as glycosaminoglycans facilitate the retention of water [30]. Fibronectin and laminin, the most abundant glycoproteins in the extracellular matrix, are responsible for adhesion, proliferation, migration, and differentiation of matrix cells [29]. Finally, the presence of interleukins and proteinases was observed in the extracellular matrix and is thought to be associated with the processes of matrix remodeling through collagen degeneration [31,32]. Collectively, all these components play an important role in the maintenance of cervical conformation and hence are involved in cervical ripening or relaxation [15].

Smooth muscle cells and elastic fibers embedded into the connective tissue comprise the muscle layer located below the cervical mucosa. Muscle cells are arranged both in the circular and longitudinal orientation (relative to the long axis of the ewe's cervix). The smooth muscle layer of the cervix is continuous with that of the vagina. This cervical layer is involved in the formation of cervical rings; smooth muscle bundles embedded in collagen and reticular fibers run parallel to the central part of each ring [19]. The outer layer of the cervical wall is the serous membrane, consisting of the loose connective tissue and the mesothelium [1]. As in other internal organs, this layer functions to minimize friction, provide resistance to infections, promote cellular transport of fluids and particles, and facilitate the migration of leukocytes [33].

4. Morphophysiological changes in matrix components during cervical relaxation

The relative amount and chemical composition of the cervical matrix changes under the influence of cyclically secreted sex steroids [22], resulting in the state of cervical "relaxation" during estrus and periovulatory period. Estrogens increase the number of estradiol receptors, while progesterone down-regulates the expression of estradiol receptors in the female reproductive organs [34]. Therefore, an increase in plasma E_2 concentrations triggers biochemical reactions in the extracellular matrix culminating in cervical remodeling. Follicular E_2 biosynthesis rises during the proestrus and estrus due to the action of pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), on ovarian cells (Fig. 3A; [35]. Estradiol-17β binds primarily to its ERα receptors in the stromal and epithelial cells of cervical mucosa [34]. Earlier studies revealed the presence of FSH and LH receptors in both the stroma and smooth muscle cells of the ovine cervix during behavioral estrus [36,37], indicating that gonadotropic hormones may exert direct influence on cervical structure and function (Fig. 3B). According to Kershaw-Young et al. [38], gonadotropins play a role in the regulation of cervical prostaglandin (PG) receptor expression by an unknown mechanism. Prostaglandin E_2 has been shown to increase the expression of cyclooxygenase-2 (COX-2) mRNA and gonadotropin receptors, suggesting the existence of a positive feedback loop between PGs and gonadotropins during cervical relaxation [37].

Estradiol-17β, FSH and LH can all increase the expression of oxytocin receptors (OTR) in the cervical mucosa [39,40]. Oxytocin binding to its G protein-coupled receptor activates phospholipase C (PLC), which in turn converts phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). While IP_3 promotes intracellular calcium (Ca^{2+}) influx by opening Ca^{2+} channels, DAG activates the protein kinase C. High levels of intracellular Ca^{2+} lead to the activation and phosphorylation of phospholipase A_2 hydrolyzing membrane phospholipids to release arachidonic acid [41], which is a substrate for the synthesis of PGH_2 by cyclooxygenase-2 (COX-2). Subsequently, prostaglandin-endoperoxide synthase 2 (PTGS2) converts COX-2-derived PGH_2 to other PGs including PGE_2 (Fig. 3 [42–44]. In sheep, cervical COX-2 mRNA is upregulated by E_2 and OT, with the highest expression observed in the muscle layer and fibroblasts [38,44].

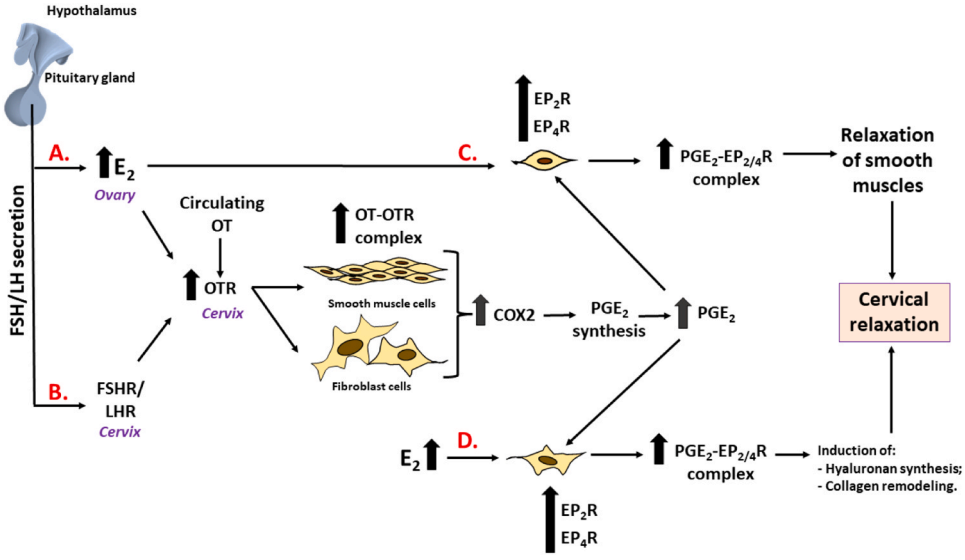

Fig. 3. Summary of the hormonal effects triggered by pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and culminating in cervical softening during the periovulatory period of the estrous cycle. (A) Follicular estradiol-17β (E_2) stimulates the expression of oxytocin receptors (OTR) in fibroblasts and smooth muscle cells. (B) FSH and LH bind to their receptors (FSHR/LHR) further stimulating OTR expression. Circulating oxytocin (OT) from the pituitary gland stimulates the production of cyclooxygenase-2 (COX-2) and the synthesis of prostaglandin E_2 (PGE_2). Dilatation effects of PGE_2 in the uterine cervix are mediated by EP_2R and EP_4R, and include (C) smooth muscle relaxation as well as (D) increased hyaluronan synthesis and collagen remodeling.

Kershaw et al. [38], described the presence of PTGS2 mRNA mainly in the inner portion of the smooth muscle layer of the ovine cervix, like the local increase in PTGS2 during cervical relaxation in cows [45].

During the estrus in sheep, there is an increase in the expression of COX-2 mRNA, which triggers the synthesis of PGE_2 and remodeling of the extracellular matrix [46,47]. Periovulatory production of PGE_2 induced by E_2 and gonadotropins promotes cervical relaxation by a complex mechanism involving several cervical components. In the smooth muscle layer, PGE_2 binds to EP_2 and EP_4 receptors, and induces muscle relaxation (Fig. 2C). Interestingly, PGE_2 can also bind to EP_1 and EP_3 receptors causing contraction of cervical smooth muscle in ewes [46]. Although cervical EP_2 and EP_4 receptors are expressed throughout the entire estrous cycle in ewes, the expression of EP_2 mRNA increases just prior to the preovulatory LH surge. The ligand-receptor interaction between PGE_2 and EP_4 in fibroblasts causes an increase in the synthesis of hyaluronan [48] and interleukin 8 [49], leading to a further reduction in smooth muscle tone and collagen dispersion [50]. There is a greater proportion of collagen in the cervical stroma before the LH surge due to the separation of collagen bundles and fibers [47].

The process of stroma remodeling is initiated by an increase in the concentration of sulfate glycosaminoglycans [51] and biosynthesis of hyaluronan, a high-molecular-mass polysaccharide that attracts water to the extracellular matrix [52,53]. Interleukin-1 and prostaglandins stimulate the production of hyaluronic acid by fibroblasts [54]. With an increase in hyaluronic acid accumulation in the cervix, water molecules are recruited, and hydration of the cervical matrix increases, promoting the dispersion of collagen fibers and decreasing/increasing cervical tension/flexibility [55]. The more the fibers are dispersed, the greater the accumulation of water [56]. In addition to high affinity for water, hyaluronic acid also increases the chemotactic response of neutrophils and eosinophils [54,56–58]. According to Ludmir and Sehdev [54], hyaluronic acid synthesis is stimulated by interleukin-8 and it also spontaneously increases interleukin-8 levels. By increasing the production of inflammatory interleukins, hyaluronan enhances the migration of inflammatory cells to the cervical epithelium. It has been demonstrated that when administered vaginally, interleukin-8 promotes an increase in leukocyte infiltration [59,60]. Interleukin-4 and interleukin-10, in turn, are involved in stimulating the release of PGs [61]. Although lymphocytes, monocytes, and macrophages are a primary source of inflammatory interleukins, these proteins can also be produced by fibroblasts and smooth muscle cells [62].

Matrix metalloproteinases (MMPs) are a class of zinc-dependent endoproteinases that degrade various substrates in the extracellular matrix (ECM; [63] Collagen degradation in the matrix depends on the activity of matrix metalloproteinases, which act by cleaving types I, III, and IV collagen [64,65]. Type I and IV collagen regulate organ elasticity, while type III collagen is associated with a high content of glycoproteins and proteoglycans [29]. Degradation of cervical ECM increases during the follicular phase of the estrous cycle due to high pre-ovulatory levels of E_2 increasing collagen degradation [66]. In large domestic ruminants, a dispersion of collagen bonds in the cervix occurs during parturition; this is due to the high content of hyaluronan, which attracts water molecules, increasing the hydration of collagen fibers [61]. Another glycosaminoglycan present in the uterine cervix is dermatan sulfate [31]. Its synthesis is stimulated by PGE_2 binding to EP_4 receptors [48]. Dermatan sulfate forms cross-links between collagen fibers and strengthens collagen bundles [46]. Therefore, a low concentration of dermatan sulfate seen in estrus may be responsible for the "weakening" of collagen bundles contributing to cervical relaxation [46].

Cervical mucus containing multiple matrix metalloproteinases is also a vital component of the cervical dilation process [67,68]. Moreover, mucus secretion and its properties can affect cervical penetration. In humans, cervical mucus becomes more hydrated during the follicular/secretory phase of the menstrual cycle [69], due to the actions of estrogens increasing the volume and permeability of cervical cells [70]. Intracellular protein O-glycosylation, or the addition of the simple sugar O-linked N acetylglucosamine (O-GlcNAc) to serine/threonine residues, is a recently identified post- translational modification [69,71]. Abrill Parreno et al. [71] characterized the composition of O glycan in the cervical mucus during the follicular phase of six breeds of European sheep. In sheep, different O-glycosylated proteins, called O glucans or mucins, are major constituents of mucus glycoproteins; one hundred and twenty-four mucins have been identified in sheep cervical secretions [72]. Interestingly, the secretion of core 4 glycans (GlcNAcβ1–3 [GlcNAcβ1–6]GalNAc) that are more abundant in the low-fertility sheep genotypes compared with their more prolific counterparts, is negatively correlated with mucus viscosity [73]. Secretion of less viscous cervical mucus lubricating the entire cervical epithelium facilitates cervical penetration during insemination, embryo collection, and embryo transfer procedures [3,12,13].

5. Cervical relaxation protocols and transcervical uterine access

"For the purposes of artificial insemination (AI) and embryo transfer (ET), an ideal pharmacologic agent to induce cervical ripening in the non-pregnant ewe must be cost and time effective, engineered for easy delivery, and cause minimal discomfort to recipient ewes. Furthermore, such an agent should not yield adverse maternal or embryonic effects, or detrimental consequences to terminal follicular development, ovulation, and oocyte quality, as well as sperm viability and transport. Therefore, it should not induce undesirable uterine effects such as atony or hyperstimulation; both conditions may contribute to the retrograde flow of semen deposited during AI, thus greatly decreasing the chances of fertilization. Similarly, they could interfere with the ability of a transferred embryo to successfully implant in the endometrial layer. Lastly, the agent should have the ability to induce dilation during a variety of reproductive states regardless of fluctuations in the concentrations of endogenous hormones" [74].

Knowledge surrounding the endocrine regulation of cervical dilation was fundamental for designing and revamping hormonal cervical relaxation protocols for transcervical AI and embryo recovery/deposition in ewes (Table 1). Firstly, the development of cervical relaxation protocols reduced the need for using modified catheters [75]. Second, prior to the application of hormonal priming, the cervical penetration rate was highly variable (9.6–66.7 %; [76,77], which greatly limited the use of this technique in commercial settings. Finally, although transcervical manipulations do not typically induce a generalized inflammatory response [78], repeated attempts to traverse the narrow cervical canal can cause epithelial damage in the most sensitive (i.e., narrowest and most convoluted) parts of the cervix [75]. Physical damage to the epithelium can trigger repair mechanisms including local inflammation, cell proliferation, and increased synthesis of ECM constituents [79]. The healing process begins with tissue granulation and reorganization of proliferating collagen fibers [80]. Subsequently, these microinjuries undergo tissue repair, which commonly results in the formation of scars partially obstructing the cervical lumen.

Earlier cervical dilation protocols (Table 1) used oxytocin [81], FSH alone or with oxytocin [82], interleukin-8 [83], α/β-adrenoreceptor antagonist [84], hyaluronan [85], prostaglandin E_1 (PGE_1; misoprostol; [37], prostaglandin E_2 (PGE2; dinoprostone; [3] or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analog (d-cloprostenol), with [5] or without E_2 [86]. The average cervical penetration depth of 5.8 cm and a complete penetration rate of 77 % were obtained after a single intramuscular (i.m.) injection of 200–600 IU of oxytocin at the time of AI, immediately before the cervical penetration attempt [81]. Falchi et al. [82] used 300 IU of oxytocin (intracervical administration) 30 h after removing the progestin-releasing sponge; however, they observed that oxytocin alone did not induce sufficient cervical dilation for consistent uterine access. The mechanism whereby oxytocin stimulates cervical softening involves upregulation of PGE_2 release. However, the expression of oxytocin receptors (OTR) is very low in the luteal phase of the estrous cycle, with an increase observed only just prior to luteolysis [39,82] and

Table 1
Summary of cervical relaxation protocols used in conjunction with non-surgical embryo recovery or transfer (NSER/NSET) in ewes.

Breed	Drugs used for cervical relaxation protocol	Time of drug application in relation to the beginning of cervical penetration	Cervical penetration rate (%)	Duration of NSER/NSET (min)	Reference
Xbred	OT (200 USP units i.v.)	0 h	(74.0) 14/19	7.0 *	Khalifa et al. (1992) [81]a+
Xbred	OT (400 USP units i.v.)	0 h	(75.0) 9/12	7.0 *	Khalifa et al. (1992) [81]a+
Xbred	OT (600 USP units i.v.)	0 h	(83.0) 10/12	6.2 *	Khalifa et al. (1992) [81]a+
Xbred	E_2 (200 μg i.v) + OT (400 USP units i.v.)	E_2 (−6 h); OT (0 h)	(50.0) 3/6	6.8 *	Khalifa et al. (1992) [81]a+
Xbred	E_2 (200 μg i.v) + OT (400 USP units i.v.)	E_2 (−12 h); OT (0 h)	(83.0) 5/6	6.2 *	Khalifa et al. (1992) [81]a+
SI	$PGF_{2\alpha}$ (50 μg vaginal submucosa)	− 12 h	(58.8) 10/17	27.30+	Gusmão et al. (2007) [86]b*
SI	PGE_1 (200 μg intravaginal)	− 5 h	(63.0) 12/19	32.75+	Gusmão et al. (2007) [86]b*
DP	PGE_1 (200 μg intravaginal)	− 5 h	(94.8) 55/58	-	Gusmão et al. (2009) [90]b*
RA x PD	PGE_2 (10 mg intravaginal)	− 12 h	(40.0) 2/5	2.8 *	Candappa; Bartlewski (2014) [3]a&
RA x PD	PGE_2 (10 mg intravaginal)	− 24 h	(67.0) 4/6	1.8 *	Candappa; Bartlewski (2014) [3]a&
MN	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−18 h); OT (−20 min)	(100) 4/4	5.0+	Fonseca et al. (2015) [135]a*
LA	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(85.7) 24/28	25.15+	Figueira et al. (2018a) [136]b*
LA	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(91.0) 31/34	29.25+	Figueira et al. (2018b) [137]a*
SI	EB (100 μg i.v.) + OT (100 IU i.v.)	PGE_1 + EB (−12 h); OT (−15 min)	(90.0) 27/30	-	Leite et al. (2020) [89]a+
SI	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (100 μg i.v.)	$PGF_{2\alpha}$ + EB (−12 h); OT (−15 min)	(83.3) 25/30	-	Leite et al. (2020) [89]a+
	+ OT (100 IU i.v.)				
LA	$PGF_{2\alpha}$ (37.5 μg i.m) + EB (1 mg i.m) + OT (50 IU i.v)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(100.0) 17/17	30.0+	Souza-Fabjan et al. (2018) [138]b*
LA	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(88.9) 8/9	3.4 *	Figueira et al. (2020) [11]a*
SI	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	77.8 (7/9)	35.6+	Fonseca et al. (2019) [5]a*
SI	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−10 h); OT (−20 min)	38.5 (5/13)	35.2+	Fonseca et al. (2019) [5]a*
SI	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(57.14) 12/21	5.9 *	Prellwitz et al. (2019) [9]a*
SI	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1 mg i.m.) + OT (50 IU intravaginal)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(57.14) 12/21	5.4 *	Prellwitz et al. (2019) [9]a*
SI	EB (100 mg i.v.) + OT (100 IU i.v)	EB (−12 h); OT (−15 min)	(88.0) 22/25	-	Santos et al. (2020) [10]a*
SI	OT (100 IU i.v.)	OT (−15 min)	(84.0) 21/25	-	Santos et al. (2020) [10]a*
SI	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	86.7 (13/15)	6.4 *	Oliveira et al. (2020) [139]b*
MN	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1.0 mg i.m.) + OT (50 IU i.v)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	82.3 (14/17)	33.5+	Arrais et al. (2021) [8]a*
MN	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1.0 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	94.1 (16/17)	31.9+	Arrais et al. (2021) [8]b*
DP	$PGF_{2\alpha}$ (37.5 μg i.m.) + OT (50 IU i.v,)	$PGF_{2\alpha}$ (−16 h); OT (−20 min)	(83.3) 10/12	25.4+	Dias et al. (2020) [12]a*
DP	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (0.5 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(91.7) 11/12	24.0+	Dias et al. (2020) [12]a*

(continued on next page)

Table 1 (*continued*)

Breed	Drugs used for cervical relaxation protocol	Time of drug application in relation to the beginning of cervical penetration	Cervical penetration rate (%)	Duration of NSER/ NSET (min)	Reference
DP	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1.0 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(100.0) 12/ 12	21.6^{+}	Dias et al. (2020) [12][a*]
MN	$PGF_{2\alpha}$ (37.5 μg i.v.) + EB (1.0 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(91.6) 11/ 12	31.4^{+}	Oliveira et al. (2022) [140][*b]
SI x LA	$PGF_{2\alpha}$ (37.5 μg i.m.) + OT (50 UI i.v)	$PGF_{2\alpha}$ (−16 h); OT (−20 min)	(90) 9/10	20.4^{+}	Dias et al. (2023) [13][b*]
SI x LA	$PGF_{2\alpha}$ (37.5 μg i.m) + EB (0.5 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(83.3) 10/ 12	25.9^{+}	Dias et al. (2023) [13][b*]
SI x LA	$PGF_{2\alpha}$ (37.5 μg i.m.) + EB (1.0 mg i.m.) + OT (50 IU i.v.)	$PGF_{2\alpha}$ + EB (−16 h); OT (−20 min)	(64.3) 9/14	22.2^{+}	Dias et al. (2023) [13][b*]

[a]Estrus-induced (non-superovulated) ewes. [b]Superovulated ewes. Duration of NSER (min) = $^{+}$Duration of the entire procedure (cervical penetration and uterine flushing); *Duration of the cervical penetration process only. Xbred: Dorset, Rambouillet, Hampshire, and Suffolk crossbred ewes; SI: Santa Inês; DP: Dorper; RA x PD: Rideau Arcott x Polled Dorset; MN: Morada Nova; LA: Lacaune; SI x LA: Santa Inês x Lacaune. Reference = *NSER; [&]NSET; $^{+}$Cervical transposition test.

conceptus-derived interferons further suppress the effects of oxytocin in the female reproductive tract [87,88]. Therefore, oxytocin doses higher than the dose used at the time of AI (>20 IU) and/or combining oxytocin with other dilating agents are necessary at the time of transcervical embryo recovery [4].

Prostaglandin E analogs, namely PGE_1 (misoprostol) and PGE_2 (dinoprostone, Cervidil®), were used for inducing cervical dilation, with an average depth of penetration of 2.8 to 3.6 cm [82,85] and the duration of cervical penetration procedure (using the Guelph method of AI) spanning 1–3 min [3]. Cervical penetration rates after intravaginal deposition of PGE_2 ranged from 63.0 to 94.8 % in Santa Inês [86,89], Dorper [90] and crossbred Rideau Arcott x Polled Dorset ewes [3,91] These prostaglandins are available in different forms (e.g., slow-release intravaginal inserts or gel), but in some countries, their availability is restricted because of their abortifacient effects. In Brazil for example, although allowed for research, the commercialization of misoprostol is currently prohibited. However, it was noted that endogenous and exogenous sources of PGE_2 stimulated endogenous $PGF_{2\alpha}$ production [92]. Therefore, $PGF_{2\alpha}$ analogs were tested for cervical relaxation protocols, and they showed similar efficiency to that of PGE_1 [86,89].

In 2016, Fonseca et al. [4] developed the EMBRAPA (Brazilian Agricultural Research Corporation) cervical relaxation protocol for non-surgical embryo recovery in ewes. Since then, it has then been tested in Santa Inês [93], Dorper [12], Lacaune [11], Morada Nova [8] and crossbred [13] Table 1). This specific protocol entails the administration of 37.5 μg of $PGF_{2\alpha}$ and 1 mg of estradiol benzoate (EB) i.m. approximately 16 h before, and of an intravenous (i.v.) dose of 50 IU of oxytocin 20 min before NSER. The timing of the first two hormonal treatments ($PGF_{2\alpha}$ and EB) was based on the estimated time required to increase the number of oxytocin receptors (from 12 to 16 h; [4,81]). Of note, due to its rapidly exerted effects, oxytocin used in cervical dilation protocols is typically administered intravenously [5,9]. Although the EMBRAPA protocol requires a lot of animal handling, it has consistently shown satisfactory success rates (77.8 %; [5]); (88.9 %; [11]); (91.7 %; [12]); (82.3 %; [8]) and (90 %; [18]).

6. Importance of donor selection for the success of transcervical uterine flushing

Donor selection as a prequel to NSER procedures is the prerequisite for its ultimate success. Here we present the guidelines and specific tests developed for NSER at EMBRAPA. To maximize the efficiency of the technique in MOET programs, it is necessary to select donors based on their reproductive history and genital anatomy. A set of criteria proposed by Fonseca et al. [5] includes a visual inspection of the vaginal vestibule to check for any potential anatomical abnormalities precluding the transcervical passage that cannot be alleviated by applying the cervical relaxation protocol [9]. The selection criteria should also include animals' lambing history and frequency/dates of previous transcervical AI/NSER/NSET procedures [13,94].

6.1. Cervical penetration test at estrus

Cervical penetration with the Hegar dilator number 3 during the estrus before embryo recovery was the first screening test to select the donors suitable for NSER [4,95]. A direct association exists between cervical penetration success with Hegar dilator at estrus and cervical penetration rates ~7 days later [96]; approximately (80 %; 17/21) of the ewes with the uterine cervix penetrable at estrus can then be successfully used for NSER [5]. Despite the useful information provided by the "Hegar dilator test", it should be noted that the Hegar dilator is a more rigid instrument than the catheter used for NSER, which may result in an occurrence of "false positives" determined with this method.

6.2. Cervical penetration "rank" according to the duration of cervical penetration

The time elapsed to complete the cervical penetration at estrus was another metric recorded during the selection of ewes for NSER [5,95]. Cervical penetration procedures were categorized as Class 1 (<1 min), Class 2 (>1 and ≤3 min), Class 3 (>3 and ≤7 min), Class 4 (> 7 to ≤10 min to complete), and Class 5 (incomplete cervical passage or impossible penetration). Only 25 % of Class 4 donor ewes examined at estrus could be used for NSER 7 days later [5]. Thus, the Class 4 sheep should be avoided whenever possible [12], and Class 5 animals are simply not suitable for NSER.

6.3. Cervical "mapping" using Hegar dilator or ultrasonography

The position of the orifice of each cervical ring was compared to the position of clock hands; thus, the informal term "cervical clock" was coined [5]. With this approach, the position of cervical rings is first determined with the Hegar. Afterward, the technician can attempt cervical passage using the information from the "cervical clock". Several field trials have confirmed that traversing the cervical canal with the mandrel/catheter after cervical mapping was faster compared with that during the first attempt using the Hegar dilator [5,8,12,95,97]. This approach is essentially based on "sketching" the cervical anatomy as described by Kershaw et al. [1], who categorized the arrangement of ovine cervical rings as Grade 1 (straight), 2 (intermediate), or 3 (highly asymmetric). Cervical mapping performed with the Hegar dilator was compared with that using cervical imaging with B-mode transrectal

ultrasonography (M5VET®, Mindray–8.0 MHz). There was a close agreement between the numbers of cervical rings counted during the Hegar insertion and those determined with ultrasound [98], suggesting that transrectal ultrasonography is a useful method of donor selection prior to AI/NSER/NSET in ewes.

6.4. Vaginoscopy and lambing history

The ovine cervix must be accessible to allow cervical clipping and retraction [5]. This can be ascertained with the exploratory vaginoscopy utilizing a Collin speculum [9,18]. Clearly, assessing the ease of introducing Collin's speculum during cervical penetration test is the most economical method of selecting sheep for all types of transcervical procedures [12,18,94].

Cervical penetration rates are higher in ewes within 4 months of lambing ((92.3 %) compared with all other females (82.45 %)) [99]. In the ewes of the Australian Merino breed, successful cervical penetration was directly related to the number of earlier lambings [76]. In sexually mature Rideau Arcott x Polled Dorset ewes during the breeding season, the duration of cervical penetration and uterine deposition of embryos was negatively correlated with the ewes' age, parity, lifetime lamb productivity, and the duration of the post-partum interval; only the ewes with no previous transcervical or laparoscopic artificial inseminations or NSER/NSET attempts, superovulatory treatments or reproductive tract surgeries were used in that study [3]. A greater ease of cervical penetration in ewes that lambed previously is attributed to the residual effects of cervical matrix remodeling, namely a partial dispersion of collagen bonds in the cervical extracellular matrix [61,100,101].

7. New approaches and perspectives

7.1. Pharmacological dilators of the uterine cervix

The use of estradiol esters (e.g., estradiol benzoate (EB)) in cervical relaxation protocols sparked controversy. The effects of the EMBRAPA protocol on luteal function and embryo quality in superovulated ewes have only recently been studied. Despite its efficiency as a cervical dilator, there were reports of adverse effects of EB on embryos recovered from superovulated sheep [102]. However, in a recent study, Batista et al. [103] found no deleterious effects of the presence of estradiol benzoate in the treatment of cervical relaxation for non-surgical embryo collection on embryonic morphological quality, cryosurvival and gene expression profile. A combination of EB (0.5 mg i.m.) + $PGF_{2\alpha}$ (37.5 μg i.m) 16 h before NSER + OT (50 IU i.v) 20 min before NSER decreased luteal tissue perfusion and circulating progesterone (P_4) concentration up to 12 h post-treatment compared with those after the administration of $PGF_{2\alpha}$ (37.5 μg i.m) 16 h before NSER + OT (50 IU i.v) 20 min before the procedure [13]. Inadequate P_4 levels can affect embryonic viability as luteal P_4 is essential for preimplantation embryo survival [104]. A study by Santos et al. [105], using a cervical relaxation protocol of $PGF_{2\alpha}$ (0.12 mg i.v) + EB (100 μg i.v) 12 h before NSER + OT (100 IU i.v) 15 min before the procedure, showed a reduced expression of the NANOG and OCT_4 genes in embryos, which are associated with embryonic differentiation. Although the protocol did not appear to affect the viability of ovine blastocysts harvested from superovulated ewes, more studies are needed to elucidate both the short- and long-term effects of EB on developing embryos. Moreover, estradiol residues accumulating in water can alter the population growth profile of fish [106]. Environmental leaching of hormones from animal production facilities can occur mainly through groundwater, which ends up affecting aquatic species. The agricultural use of EB has been restricted or even completely prohibited in some countries because consumption of meat and meat products derived from livestock treated with steroids can cause exposure of consumers to various levels of their residues [107]. The EB is not approved for use in animals in the USA and some European Union countries (Directive 2008/97/EC). This restriction also affects the countries in which EB is still used as it precludes the export of animal products obtained from EB-treated animals. Therefore, the development of alternative protocols is urgently needed.

In the countries where public policy challenged the use of steroid hormones in animal production, studies had been conducted to test the effectiveness of reduced EB doses or cervical dilation protocols without estrogens [81,86]. Three doses of EB (0.0 mg-vehicle, 0.5 mg or 1.0 mg i.m.) administered with $PGF_{2\alpha}$ (37.5 μg i.m.) 16 h before and OT (50 IU i.v.) 20 min before NSER were tested in estrus-induced Dorper ewes and there were no significant differences in the duration of NSER and overall penetration rates between protocols with different EB doses [12]. Similar results were seen in superovulated crossbreed and White Dorper ewes that received $PGF_{2\alpha}$ (37.5 μg i.m) 16 h before and OT (50 IU i.v) 20 min before NSER [18].

The application of phytoestrogens may be an alternative to EB use. It was proposed that phytoestrogens were a promising hormone replacement therapy that might provide post-menopausal women with relief for both their reproductive and asthma-related symptoms [108]. Besides their low toxicity, phytoestrogens have well-described anti-inflammatory and antioxidant properties, and they elicit mild to moderate estrogenic responses. The application of phytoestrogens in animal husbandry may have a promising future. However, there have been no reports of their suitability in cervical relaxation protocols.

Adrenergic drugs have been used in cervical dilation protocols even though their primary effects are on the contraction and relaxation of myometrium [109,110]. Both adrenergic receptor subclasses have been shown to mediate the contractile response of the uterus with the activation of α- and β-adrenergic receptors causing uterine contraction and relaxation, respectively [84,111]. Horta et al. [112] reported that vaginal administration of misoprostol and terbutaline sulfate (activator of β_2-adrenergic receptors) 6 h prior to AI did not affect the proportion of successful cervical inseminations, but significantly improved the fertility of inseminated Serra da Estrela ewes. Local administration of prazosin or tamsulosin alone (non-selective α_1-adrenergic receptor blockers with similar affinity to α_{1A}, $\alpha_{1B,}$ and α_{1D} receptors) was associated with an increase in cervical penetration rates, without any deleterious effects on the hemodynamics of the uterine artery or systemic blood pressure [110].

7.2. Expending NSER onto estrus-induced (non-superovulated) animals and bioconservation programs

The EMBRAPA protocol for cervical relaxation in sheep has recently been adapted as a preparation for uterine flushing in non-superovulated (i.e., estrus-synchronized) animals. This adaptation of NSER allows for the more widespread use of NSER/NSET in conservation programs [14]. Both techniques have all the necessary "ingredients" of a sufficient technique for embryo collection and ensuing biobanking in small ruminants [8] as it is equally efficient as the laparotomy and laparoscopy [5] and the use of less invasive approaches is preferred in animal conservation programs [8]. Earlier FAO guidelines [113] did not recommend NSER for use in sheep and goats; this was based on the studies indicating that NSER was less effective than surgical methods. However, most recently reviewed and updated FAO recommendations included NSER for use in goats [114]. Considering the latest advances in NSER in both non-superovulated and hormonally stimulated donor ewes [8,115] the NSER, and potentially NSET, could now be recommended for supporting embryo biobanking procedures in sheep [115].

7.3. The role of biomedical engineering

Conventional AI catheters used for other species, including goats, do not comply with the geometry of the ewe's uterine cervix. Moreover, manufacturing a customized catheter for sheep has proven to be a very challenging task [74]. Therefore, designing a gun-like device, which can be used to successfully inject or collect fluids via the transcervical route

in ewes and females of other small animal species, was consulted with biomechanical engineers in 2016. Throughout the consultation process, the finalized criteria and constraints for the detailed design were established (Table 2).

7.3.1. Detailed design and assembly instructions

A proposed device is comprised of a total of sixteen parts that make up four main components: the barrel, the handle, the cervical needle, and the air tank. All these components are anticipated to be machined out of Delrin, except for an air tank that can be purchased separately. Delrin is a tough and relatively creep-resistant material, which allows for durability with repeated use, cleaning, and disinfection (thermal or chemical) (http://plastics.dupont.com/plastics/pdflit/americas/delrin/H76836.pdf; last accessed May 2024).

The barrel consists of four parts (Fig. 4): the shell, the needle housing, a plug, and a camera. To assemble the barrel, a small hole must be drilled into the shell where desired. This will allow for the camera wires to connect to the LED screen. The camera can then be inserted to its appropriate location and the wires can be phished through the shell. Once the wires are in place, the plug can be permanently glued into the rear end of the shell. The last step is to dab one end of the needle housing with glue and insert it through the hole in the plug until it reaches the opposite end of the shell.

The handle is made of eight parts: the left handle, the right handle, the air tank clip, and the LED screen (not displayed). and the battery pack (not displayed). The handle is split into two parts (left and right) so that it can be manufactured by injection molding for the final product version. To assemble the handle, the five pins on the left piece can be glued and inserted into the five matching holes on the right piece. The air tank clip can then be inserted through the appropriate holes on the right side of the handle. The LED screen is to be placed on the top of the handle and the battery pack would be inserted into the cavity at the bottom of the handle. Lastly, there is a locking mechanism made of three pieces that is designed to hold the insemination needle in place. The lock can be inserted in between the two extrusions located at the top rear end of the handle (one on the left piece and one on the right). The lock pin can be inserted and secured in place by the safety pin. The position of the insemination needle can be further machined to make a customized locking section.

The insemination needle requires no assembly. It has a long and thin rod-like feature that is placed through the barrel. It also has a larger rod section that has two important characteristics. The first attribute is the centered chamber designed to adapt to a current inseminating syringe. A second chamber can be accessed through the extrusion on the rear end of the insemination needle. This extrusion provides an area to connect a hose that can then be connected to the air tank. These two chambers connect about 1 cm down the insemination needle, so once semen/fluid is injected into the first chamber, the valve on the air tank can be turned on to create an air flow that should push the semen/fluid through the rest of the needle and into the cervix. The insemination rod is inserted to the handle such that the air chamber is above the semen/fluid chamber; this is to prevent the backflow of the semen/fluid into the air chamber.

The air tank consists of three parts: the compressed air canister, a pressure valve, and an air hose. The first two parts can be purchased together from a company called RAP4 (Santa Clara, CA, USA). The compressed air canister is to be connected to the air tank clip in an inverted fashion (so the bottom of the canister faces the ceiling). Once it is in position, a simple nut and bolt can be placed through the air tank clip and tightened to hold the compressed air canister in place. The pressure valve is to allow for a controllable air flow and is installed on the opening of the compressed air tank. The air hose is then connected to the pressure valve. The final step of assembly is to screw on the barrel to the handle where it is threaded.

Table 2
The finalized criteria and constraints associated with the design of a new gun-like AI/NSER/NSET device for small ruminants (based on the expectations for the functionality and necessary features of the device).

Criteria	Constraints
• Mechanically safe for both the animal and operator when in use • The cartridge used to hold the semen should be easily accessible • Insert and remove from the device • LED screen to display information, operating mode, and camera view • Camera incorporated for improved visibility of the os cervix • Light source on camera head • Battery used to power the device for dexterity • Easy to use (i.e., little training required) • Compressed air canisters to reduce the number of pumps	• Device must comply with the International Embryo Technology Society regulations/ recommendations and national (e.g., EMBRAPA/Canadian Sheep Breeders Association) by-laws and rules of eligibility • Contains biocompatible materials only (stainless steel, Delrin, or certain plastics) • No corrosive materials can be used in production either (cleaning or chemical disinfection requirements) • All electrical components must be sealed off (necessitates replacement parts rather than repairs of electrical parts)

7.3.2. Design defense

This device has many advantages over the current insemination devices used in the small ruminant practice. The design of a gun-like apparatus allows for a non-invasive procedure with minimal penetration into the cervix of the ewe. There is only the initial insertion of the needle into the cervical opening. With no further penetration, there will be less stress and irritation on the spiral folds and crypts of the interior cervix. This device is designed to avoid cervical trauma and increase future reproduction success. This minimally invasive procedure also allows users with minimal expertize to perform the insemination procedure. Since the device is designed to have a camera attached at the end of the barrel it permits the user to be able to see the location of the needle permitting an accurate insertion of the needle into the cervix.

The material to be used in the manufacturing of this device is Delrin. Delrin is a highly useful engineering polymer. It is easily machined and has high mechanical strength and rigidity, resistance to repeated impact, long-term fatigue endurance as well as excellent resistance to moisture, and a large end-use temperature range. Lastly, the vaginal impedometer already used in sheep was referenced when determining the dimensions for the present device to ensure its optimal fit and safety [116].

7.3.3. Potential final version

In Fig. 4, numerous 3D renderings have been combined to show what a model of this device, equipped with air suction, would look like. The barrel would have to contain an extra tip designed to allow needle penetration and generate suction. Another future addition to be incorporated into the design is a flush to clean the camera lens. The vacuum suction at the end of the insertion rod will ensure that the device is attached to the cervix, creating a seal. This seal will provide a barrier, blocking anything from entering the area of the cervical opening. A camera flush would be beneficial as it will provide a way to clean the camera during the insemination/flushing procedure.

The next stages of the project include additional research on the force used in the device. Research into a pump and compressed air cylinders is to be conducted. Some experimental procedures to understand the amount of force and pressure needed to move liquid past the cervix of the ewe should be performed. Once appropriate research has been conducted, a full prototype of the device can be manufactured, and the testing stages of the device can begin.

7.4. Microrobotics

The study of medical microrobotics is an emerging multidisciplinary field that can revolutionize assisted reproduction technologies (ART; [117–120]). Microrobots are characterized by wireless motion, which makes them an attractive choice for non-invasive surgery and drug or

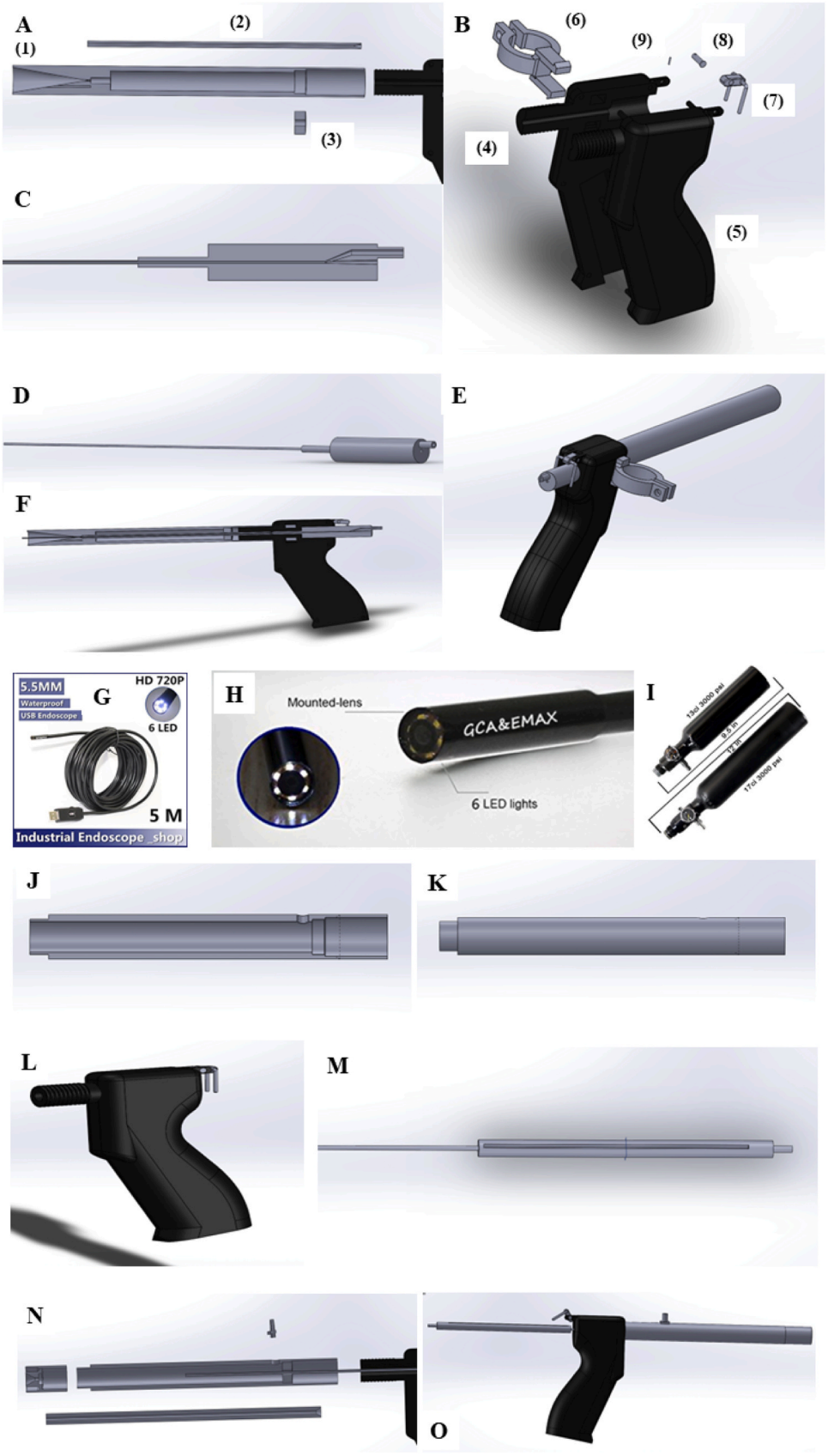

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Fig. 4. Three-dimensional renderings of the new AI/NSER/NSET gun designed for use in sheep. (A) The barrel consisting of the shell (1), needle housing (2), and plug (3); (B) handle assembly: left handle (4), right handle (5), air tank clip (6), lock (7), lock pin (8), and safety pin (9); (C) sliced view of the insemination needle to show how the two chambers connect; (D) 3D view of the insemination needle; (E and F) full assembly and a sliced view of the full assembly (without a needle); (G-I) external purchases: the camera to be implemented in this device is the GCA 5.5-mm diameter USB waterproof 6 LED endoscope (a close up of the camera and LEDs of the endoscope shown in the central panel) and the dimensions of air tanks equipped with pressure valves that can be purchased from the RAP4 company; (J-K) sliced and external view of the barrel; (K) handle with locking mechanism; (N) sliced view of a barrel assembly; and (O) complete assembly.

cell delivery [121]. Up to date, however, microrobotics have faced multiple obstacles in their clinical translation. Most of such obstacles entail technical issues (associated with the challenging and complex nature of the fabrication of microrobots, their composition, biocompatibility, powering, navigation, and real-time monitoring), but there exist other challenges and constraints such as safety and ethics of testing microrobots in clinical settings. As a result, the state-of-the-art in the field of ART microrobotics is limited to in vitro capture, transport, and release of motile [122,123] and immotile spermatozoa [124,125], and zygotes [126]. However, even from those preliminary indications, AI as well as in vivo ovum pick up and embryo recovery/transfer are all achievable goals for micro-roboticists ([127]; Fig. 5).

Microrobots suitable for use in ART must be i. manufactured from soft, biocompatible, and programmable materials that can be remotely controlled (e.g., by magnetic fields); and ii. capable of converting the energy from the surrounding environment and/or embedded during their manufacturing for propulsion, shape-change, and generating signals detectable by medical imaging modalities (e.g., B-mode, enhanced-contrast ultrasonography [121,128]. At present, zwitterionic hydrogels appear to be the optimal candidate materials for ART microrobots [129, 130]. Zwitterionic hydrogels are a class of materials that contain both cationic and anionic groups, with their overall charge being neutral. Compared with PEG (polyethylene glycol) materials, zwitterionic materials have much stronger hydration, which is considered the most important factor for antifouling [131]. Their other properties such as stimuli-driven expansion/shrinkage in response to changes in pH, magnetic fields, acoustic waves, or secretory products present in the female's reproductive tract can be manipulated and/or controlled by copolymerization and addition of various nanoparticles [125,132]. Most importantly, however, the surface chemistry of newly synthesized hydrogels promotes the interactions between microrobots and eggs/embryos, permitting the "recognition" of these structures; any unwanted adhesion or non-specific interactions of hydrogels deposited in the female reproductive tract must be minimized or eliminated. An interaction between hydrogels and gametes/embryos is also fully reversible. The mechanical properties of microrobotic materials should be around 0.3 Nm^{-1} [133] to adjust their stiffness to gametes or embryos and prevent physical damage to germplasm. For the ovulated ovum pick up or NSER/NSET, the size and conformation of microrobotic carriers ("microgrippers") must match the size and shape of mammalian eggs and preimplantation embryos [134]. External navigation of microrobots can be accomplished with a single or combined external stimuli including magnetic fields, acoustic waves, light, and thermo- or chemotaxis.

8. Concluding remarks

The transcervical route of embryo recovery and transfer is widely used in cattle, goats, and horses, and recent results encourage the spread-out commercial application of this technique in different breeds of sheep. The progress in applying cervical relaxation protocols combined with appropriate donor selection greatly enhanced the usefulness of NSER in ewes. Since transcervical uterine flushing is a minimally invasive approach, it is an excellent alternative for surgical techniques both in commercial settings and conservation programs. Despite the considerable advancements in performing NSER, research on NSET in ewes appears to progress more slowly due to a variety of reasons. The complexity of inducing cervical dilation the time when endocrine milieu does not promote it (i.e., mid-luteal phase) can contribute to generally slower progress of NSET. Besides, the mechanism of cervical dilation has not been fully elucidated yet. However, recent studies resulted in the development of cervical relaxation protocols that are increasingly

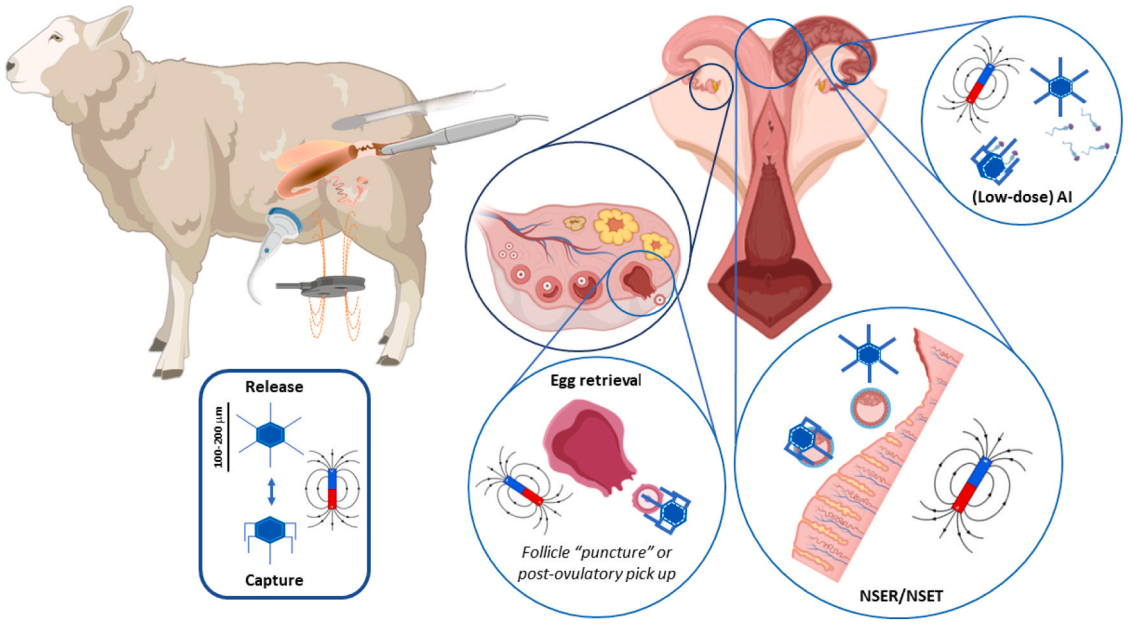

Fig. 5. General principles of and concepts for utilizing microrobots in ART. Biocompatible microrobots with a capture-release mechanism can be deposited in the female reproductive tract, navigated using external stimuli (e.g., electromagnetic field), and monitored with transabdominal, transvaginal or transrectal ultrasonography. Potential applications include, but are not limited to, intrauterine/intraoviductal AI, ovum pick up, and embryo recovery/transfer.

practical and effective, and do not appear to have adverse effects on ovine embryo viability. The use of adrenergic drugs and phytoestrogens promises to improve the ease with which NSER/NSET will be performed in the nearest future, even if some hormonal drugs are officially banned or severely restricted from being used in food producing animals due to public scrutiny, but adequate studies and field trials are lagging. Lastly, interdisciplinary approaches combining several disciplines such as reproductive biology and biotechnology, pharmacology, biomedical engineering, material science, and robotics may provide a new platform for further advances in ART applicable to small ruminants and other species of veterinary/agricultural interest. "One of these days" usually being "none of these days" simply cannot be a timeline for initiating new projects needed in this area.

CRediT authorship contribution statement

Joedson Dantas Gonçalves: Conceptualization, Research, Screening, Data Curation and original draft, writing review and editing. **Jenniffer Hauschildt Dias:** Conceptualization, Research, Screening, Data curation, Investigation, Writing. **Mariana Machado-Neves:** Figure drawing, Research, Screening, and Writing. **Gabriel Brun Vergani:** Writing and Review. **Bahareh Ahmadi:** Writing, review, and drawing of figures. **Ribrio Ivan Tavares Pereira Batista:** Writing, Review and Editing. **Joanna Maria Gonçalves Souza-Fabjan:** Writing, Review, Supervision, Data curation and Editing. **Maria Emília Franco Oliveira:** Writing, Review, Supervision, Data curation and Editing. **Pawel Mieczyslaw Bartlewski:** Writing, Proofreading, Supervision, Data curation, Editing, and Figure design. **Jeferson Ferreira da Fonseca:** Conceptualization, Writing, Review, Supervision, Data curation and Editing.

Declaration of Competing Interest

I, Jeferson Ferreira da Fonseca, declare that I do not have any personal or financial interests that could influence or bias my actions, decisions, or judgments in any professional capacity. I have no affiliations, investments, or relationships that could create conflicts of interest.

I acknowledge that conflicts of interest can compromise objectivity, fairness, and integrity, and I am committed to maintaining the highest level of ethical conduct in all my endeavors. I will disclose any potential conflicts of interest that may arise during the course of my work, and take appropriate steps to mitigate or address them transparently and in accordance with relevant laws, regulations, and organizational policies.

Furthermore, I understand that any actions or decisions I take in my professional role should be based on merit, relevant expertize, and the best interests of the individuals, organizations, or entities that I serve. I will act in a fair, impartial, and transparent manner, and avoid any actions that could compromise my ability to fulfill my professional duties objectively and with integrity.

I hereby affirm that the above declaration accurately reflects my current understanding and commitment to upholding high standards of integrity, transparency, and ethical conduct in all my professional endeavors.

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