The biological mechanisms regulating sperm selection by the ovine cervix

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Abstract

In species where semen is deposited in the vagina, the cervix has the unique function of facilitating progress of spermatozoa towards the site of fertilisation while also preventing the ascending influx of pathogens from the vagina. For the majority of species, advances in assisted reproduction techniques facilitate the bypassing of the cervix and therefore its effect on the transit of processed spermatozoa has been largely overlooked. The exception is in sheep, as it is currently not possible to traverse the ovine cervix with an inseminating catheter due to its complex anatomy, and semen must be deposited at the external cervical os. This results in unacceptably low pregnancy rates when frozen-thawed or liquid stored (>24 h) semen is inseminated. The objective of this review is to discuss the biological mechanisms which regulate cervical sperm selection. We assess the effects of endogenous and exogenous hormones on cervical mucus composition and discuss how increased mucus production and flow during oestrus stimulates sperm rheotaxis along the crypts and folds of the cervix. Emerging results shedding light on the sperm-cervical mucus interaction as well as the dialogue between spermatozoa and the innate immune system are outlined. Finally, ewe breed differences in cervical function and the impact of semen processing on the success of fertilisation, as well as the most fruitful avenues of further investigation in this area are proposed.

Introduction

In vaginal depositors, the cervix has the unique function of preventing the influx of pathogens from the vagina to the uterus while serving to regulate sperm passage. Of the millions to billions (species-dependent) of spermatozoa deposited in the female reproductive tract (FRT), less than 100 spermatozoa arrive at the site of fertilisation and the cervix plays a critical role in the selection of the successful few (see review by Sakkas et al. 2015). The ability to bypass the cervix during assisted reproduction in most species, including humans, has meant that much of the recent research has focused on the interaction of sperm with the utero-tubal junction and oviduct (see review by Holt & Fazeli 2016). Despite intensive research efforts, the sheep is the only large domestic animal species in which it remains currently impossible to traverse the cervix during artificial insemination (AI) and therefore the furthest semen can usually be deposited is at the external cervical os (opening). While pregnancy rates following cervical AI with liquid semen inseminated on the day of collection are generally acceptable (~60%) (O’Hara et al. 2010), pregnancy rates fall to below 30% when frozen-thawed (F/T) spermatozoa are inseminated (Salamon & Maxwell 1995). The reasons for this dramatic decline in pregnancy rates with F/T semen remains elusive. However, with the deposition of F/T spermatozoa laparoscopically into each uterine horn, pregnancy rates recover to approximately 70%, identifying the cervix as the principal site of sperm transport inhibition (Salamon & Maxwell 1995). Laparoscopic AI is labour intensive, requires specialist veterinary expertise and is not considered welfare friendly. Therefore, for the extensive uptake of sheep AI, which provides widespread access to elite genetics, an improved approach for routine cervical deposition of semen must be devised. However, the understanding of the factors regulating sperm selection in the cervix remain elusive, and this precludes the development of sheep AI. This review takes a systematic approach to the problem by reviewing the salient scientific literature on the anatomy and micro-anatomy of the cervix. Focusing on sheep, we describe the sperm interactions within the cervix, not only with its secretions which change according to hormonal influences, but also with the immune system. Interesting observations can be made...
through the study of ewe breed differences and lessons can be learned from studies on the impact of semen processing and seminal plasma (SP) on the number and function of spermatozoa crossing the cervix. A summary of these factors are presented in Fig. 1. We conclude by proposing likely fruitful avenues of future investigation.

**Anatomy of the cervix**

**Gross anatomy limits cervical penetration**

The cervix is a long fibrous structure composed primarily of connective tissue (fibrillar collagen and high-molecular-weight proteoglycan complexes), an outer serosal layer and inner epithelial layer. The ectocervix protrudes into the vagina, the opening of which is referred to as the external cervical os, while the endocervix acts as the passageway to the uterus. The ovine cervix is approximately 4–7 cm in length but this varies between individual ewes as well as with parity, age, breed and physiological state (Kershaw et al. 2005). The inner lumen is dominated by the presence of 4–7 angular folds, which point caudally but are not concentrically aligned and thus obscure the central lumen (Fig. 1). It is these angular folds that pose the challenge to the use of an AI catheter in sheep. The number of folds and their level of interdigitation is directly correlated to the depth of penetration of an inseminating pipette. The lumen is at its narrowest (2–3 mm) nearest the vagina and the second and third fold is out of alignment with the first so that the inseminating pipette is misdirected away from the central lumen, typically resulting in less than 1 cm penetration into the cervical canal in the majority of ewes. While under the influence of oestrogen, there is some softening of the cervix during oestrus, through the rearrangement of collagen bundles within the cervical extracellular matrix (Kershaw et al. 2007), but this is not enough to allow passage of an insemination pipette. When deeper penetration is achieved, there is a 7–12% per cm increase in fertility as depth of insemination is increased (Salamon & Maxwell 2000). There have been reports of successful cervical penetration through redesigning the insemination catheters (Wulster-Radcliffe et al. 2004) or clamping the external cervical os and physically retracting it into the vagina allowing for deeper cervical penetration which has led to marginally improved fertility (Halbert et al. 1990). However, cervical penetration rates were influenced by inseminator skill and experience, ewe parity, interval from previous lambing and season of the year and due to inconsistent...

**Figure 1** Graphical illustration of (A) the gross anatomy of the ovine cervical canal (B), a transverse section showing secondary grooves and (C) the micro-anatomy demonstrating the channels within which spermatozoa progress. Also detailed are some of the key physiological processes regulating sperm transport through the cervix. Not to scale.
fertility results, as well as welfare concerns, is not widely used. Other studies have focused on understanding the mechanisms of remodelling of the extracellular matrix leading to cervical dilation. A plethora of studies have used pharmacological approaches toward cervical dilation through the use of prostaglandin E₂, FSH and LH, oxytocin, 17 β-oestradiol and hyaluronan. While cervical softening has been achieved, and led in some cases to deeper penetration of the cervix, none have resulted in acceptable levels of fertility following cervical AI with F/T semen.

Cervical micro-anatomy and its role in sperm transport

In cows, Mullins and Saacke (1989) characterised the anatomy of the bovine cervix. They described folds which run the length of the cervix and apparently provide ‘privileged pathways’ for spermatozoa to migrate the full length of the endocervical canal to the uterus. These folds have also been described in women (Kessel 1979) but not in other species studied to date. Assessment of the folds following mating using transmission electron microscopy demonstrated that beating cilia were orientated in the direction of the vagina, while spermatozoa were orientated towards the uterus, indicating that spermatozoa swim against waves created by the ciliary beating (Mullins & Saacke 1989). In Fig. 2, representative scanning electron microscopy images of the luminal epithelium of the sheep cervix are presented. These demonstrate the crypts and folds of the ovine cervix are similar to that reported in other species, although it is not clear if these run continuously all the way from the cervical os to the endocervix. The presence of ciliated epithelial cells in the ovine cervix (Fig. 2F) was unexpected and to the best of our knowledge has not been previously reported. It is not known if spermatozoa interact with these cilia in a similar manner to the ciliated epithelial cells in the oviduct. The presence of folds along the FRT suggests that they may have evolved to accommodate sperm transit as evidenced by the presence of spermatozoa deep within the cervical channels in cervices from ewes recovered shortly after insemination with F/T semen (Richardson et al. 2019). The flow of mucus within these crypts and folds is thought to differ in composition and be slower than the stronger flow in the lumen of the cervix, which is critical for the protection of the upper FRT against the infiltration of pathogens (Cone 2009). Indeed when ewes were inseminated with immotile spermatozoa, these spermatozoa were found in the lumen and not in the crypts of the cervix (Mattner & Braden 1969).

A number of studies have focused on the biophysical benefits of fluid flow on human, mouse and bull sperm transit using in vitro microfluidics approaches (see review by Suarez & Wu 2017). Motile spermatozoa orientate and swim against a flow (positive rheotaxis), which has been proposed as a major determinant of sperm guidance over long distances in the mammalian FRT (Miki & Clapham 2013). Conversely, mechanisms such as chemotaxis and thermotaxis are likely to only be effective once spermatozoa are in close proximity within the oviducts. Given the fibrous nature of the cervix, smooth muscle contractions are likely to play less of a role than in the uterus and oviducts and thus rheotaxis may be a major determinant of cervical sperm transport.

It has been suggested that rheotaxis is the result of the flagella beating forming a conical surface behind the sperm head, directing the sperm head upstream (Miki & Clapham 2013). However, others have proposed that sperm orientation against a flow is governed by near-surface hydrodynamic interactions (wall tracking behaviour), via the interaction of a front-back asymmetric microswimmer with a solid boundary. Both human and bull spermatozoa not only swim against the fluid flow, but tend to swim upstream in spiral-shaped trajectories along the walls of the microchannel. This wall-tracking behaviour is known as thigmotaxis, although it is yet to be established if this is only a feature of in vitro systems or indeed regulates sperm rheotaxis in vivo. Tung et al. (2014) studied the migration of bull spermatozoa against fluid flow in a microfluidic device that recreated the
biophysical environment of mammalian spermatozoa with microgrooves (20 µm in width) embedded on a microchannel surface. They reported that microgrooves allow spermatozoa to swim faster and more efficiently in the presence of the flow which suggests that the grooves present along the FRT have evolved, in part at least, to accommodate spermatozoa transport. Interestingly, research investigating the pathogenesis of Tritrichomonas foetus, a puller swimmer pathogen, was swept back against a flow (Tung et al. 2015) suggesting that motility alone does not lead to positive rheotaxis but the faster outward flow of mucus in the cervical lumen during oestrus has the ability to prevent pathogens from crossing the cervix. Taken together, the evidence suggests that cervical sperm migration is stimulated by rheotaxis and occurs deep within the crypts and folds of the cervix in an environment which is structurally distinct to that of the lumen. The outward flow of mucus in the lumen aids in the removal of pathogens, white blood cells as well as defective spermatozoa and provides protection to the upper FRT suggesting a coevolution of females and males to support fertilisation while suppressing infection.

Cervical mucus

Mucus production and its mechanical properties

Cervical mucus is a complex non-Newtonian viscoelastic bodily fluid comprised of secretions from the oviducts, endometrium and cervix. It is primarily composed of water (95–99%) but also includes cellular material, ions, plasma proteins, bactericidal proteins, enzymes and mucins (Curlin & Bursac 2013). In vaginal depositors such as sheep and humans, it regulates sperm migration to the upper FRT while, at the same time, acts as a protective barrier against infection. It has also been reported to limit the progression of immotile and membrane-damaged spermatozoa as well as DNA-fragmented spermatozoa (Bianchi et al. 2004) towards the oviduct, suggesting that the sperm surface morphology may reflect their DNA status.

Sperm transport from the vagina to the oviducts is dependent on the properties of cervical mucus in which spermatozoa must travel, including mucus quantity, viscosity and hydration – all of which are regulated by ovarian steroids. Cervical mucus production is continuous and its production is under the influence of oestrogen, whereas progesterone has a modifying effect. As a result, its composition varies across the reproductive cycle. In humans, the cervix produces approximately 20–60 mg of mucus per day during the luteal phase and increases 10–20 fold, up to 700 mg per day, in the peri-ovulatory period (Moghissi & Syner 1976). Similar increases in mucus production during oestrus have been demonstrated in sheep (Maddison et al. 2016). These increases are likely due to increased para-cellular permeability of the ectocervical cells as has been shown in in vitro culture systems using human ectocervical cells supplemented with oestrogen (Gorodeski 2000). In both sheep and humans, the levels of hydration peaks in the follicular phase and is inversely related to the protein content, with a less proteinaceous, viscoelastic mucus produced in the follicular phase (Maddison et al. 2016). This is essential for progression of spermatozoa with normal mobility and morphology. This natural variation in cervical mucus production and composition seems to facilitate sperm transport during the follicular phase (oestrogen dominant) while during the luteal phase (progesterone dominant), it acts as an antimicrobial barrier as well as priming the FRT for an impending pregnancy.

For sperm transport, the viscoelastic properties of mucus appear to be more influential than the viscosity alone (Tung et al. 2017). Mucus viscoelasticity is largely regulated by mucins, which are large polymeric glycosylated proteins that are widely expressed within the oviduct, endometrium, cervix and vagina and make up approximately 45% of all proteins in cervical mucus. These large complex glycoproteins consist of a central core protein domain, rich in the amino acids serine, threonine and proline, which provide a high number of attachment points for branching oligosaccharide side chains and are terminated with either sialic acid (NeuAc) or fucose (Fuc). Unlike the majority of glycoproteins, the oligosaccharides of mucins are predominantly O-linked. It is this complex arrangement of side chains that give mucin its filamentous properties and a bottle-brush-like appearance. The carbohydrate side chains can be neutral, sulphated or sialyted (Andersch-Bjorkman et al. 2007), with the latter two being partly responsible for conferring a net-negative charge to mucus. Mucins can be characterised into three classes (i) the secreted mucins, (ii) membrane-associated mucins and (iii) small soluble mucins. Secreted mucins can be further classified into either gel forming or non-gel forming.

The mucin core proteins are produced in the rough endoplasmic reticulum of the endocervix epithelial cell where a small amount of N-glycosylation is required for normal processing following which they are then shuttled to the Golgi apparatus in which O-glycosylation takes place. The highly condensed mucin is then transported to the mucin granule of goblet cells, in which high intergranular levels of calcium and hydrogen ions shield negatively charged sites on mucins from electrostatic repulsion (Verdugo 2012). This allows the highly condensed polyanionic macromolecular mucins to be packaged into mucin storage vesicles within goblet cells (Muchekehu & Quinton 2010). Mucin secretion can be modulated by pathogens, hormones and neurotransmitters, while, after leaving the vesicles, bicarbonate (HCO₃⁻) appears to have the greatest modulating effect. Extracellular HCO₃⁻ removes
the cationic shields from mucins via sequestering of \( \text{Ca}^{2+} \) and buffering of \( \text{H}^+ \). This allows for rapid expansion of the mucin via electrostatic repulsion into an extracellular network of ‘tangled strings’ as is evident in Fig. 2. In the process, the volume increases by as much as 1000-fold in less than a couple of seconds upon exposure to high concentrations of \( \text{HCO}_3^- \) (Muchekehu & Quinton 2010). The structure of mucins are modified during the cycle from globular-ovulatory to fibrous-pre-ovulatory mucus which appears to be regulated by pH changes and, combined with a reduction in viscosity, allows sperm penetration in the peri-ovulatory period (Baumber et al. 2002).

**Cervical mucus proteome**

Characterisation of gene expression changes in the endocervix as well as the quantification of proteins in its secretions over the duration of the oestrous cycle are key to understanding the relationship between mucus proteome, mechanical properties of mucus and sperm interaction with it. While over 800 proteins have been identified in cervical mucus (Soleilhavoup et al. 2016), its viscosity during oestrus is mainly due to the elevated amounts of secreted mucus as well as their level of glycosylation. Biochemical changes in cervical mucins, such as dramatic changes in O-glycosylation at ovulation, may also contribute to the hydration of cervical mucins, as well as promote sperm penetration and survival in the FRT due to altered sialic acid content (Ma et al. 2016). Five mucins have been identified in the FRT (Andersch-Bjorkman et al. 2007) of which three are gel forming (MUC 5B, MUC 5AC, MUC 6) and two are transmembrane proteins (MUC 1 and MUC 16). MUC 5B is the main gel-forming mucin responsible for the viscoelastic properties of cervical mucus (Portal et al. 2017). It has been shown to be more abundant in cervical mucus collected during the follicular than the luteal phase in both sheep (Soleilhavoup et al. 2016, Maddison et al. 2017) and humans (Gipson et al. 2001) when progesterone is at its lowest. The degradation of mucins is partly controlled by the enzymatic action of sialidase (NEU1) which cleaves terminal sialic acid residues from the carbohydrate end chains, leading to a decrease in the viscoelastic properties of mucus. Indeed recently, NEU1 was found to be present in sheep cervical mucus during oestrus and absent during the mid-luteal phase (Maddison et al. 2017).

Several transcripts associated with mucin biosynthesis and intracellular transport as well as their post secretory modifications have been shown to be upregulated in bovine cervical epithelium during oestrus (Pluta et al. 2012). Histological staining revealed that sialylated mucins dominate at the bases of bovine cervical folds, within the so-called privileged pathways, whereas sulphated mucins were more abundant at their apices. Interestingly, mucus consists mainly of sialomucins with lesser amounts of sulphomucins present. This balance is under endocrine control as when ewes were ovariectomised, mucus production declined and consisted mainly of sulphomucins but following supplementation with oestradiol benzoate, mucus production was restored as were the levels of sialomucins mucus (Adams & Tang 1986). The core mucin glycans also appears to be altered during the transition from follicular to luteal phases, whereas terminal glycans change mainly in the peri-ovulatory period and are associated with changes in glycosidase activity (Pluta et al. 2011).

Despite anatomical barriers, 55% of proteins have been shown to be common in luminal fluid from the cervix, uterus and oviducts of sheep (Soleilhavoup et al. 2016). In the same study, the cervix and the oviduct had an increased number of proteins during oestrus, while the luteal phase was characterised by a higher abundance of proteins in uterine fluid associated with the preparation for supporting embryonic development. This pattern of protein abundance in the fluids along the cycle is most likely a result of the regulation of secretions by the endocrine and immune systems (Lee et al. 2015). The next phase of this work must be to identify which proteins are important for sperm transit across the cervix and to characterise their interaction with spermatozoa. Currently the only example of this is in the macaque, where the glycoprotein beta-defensin 126 (DEFB126) binds to the sperm surface in the corpus and cauda epididymis (Yudin et al. 2003) forming part of the complex sperm glyocalyx. The addition of sialic acid moieties to defensin peptides confers a negative charge to spermatozoa, thereby repelling the negatively charged mucus and facilitating migration of non-capacitated sperm through electronegative mucus. It plays a major role in immune recognition and its release during capacitation is required for spermatozoa to interact effectively with the zona pellucida (Tollner et al. 2004). Spermatozoa from men with DEFB126 mutations have lower lectin-binding which is associated with fewer O-linked oligosaccharides, altered ability to penetrate synthetic mucus and reduced fertility (Tollner et al. 2011). Beta-defensins are conserved across mammalian species and are expressed within both male and female reproductive tissues (Equine; Narciandi et al. 2011, Johnson et al. 2015, Ovine, Hall et al. 2017). In cattle, DEFB126 is preferentially expressed in the caudal epididymis (Narciandi et al. 2016) with similar binding patterns on the sperm surface to macaque (Fernandez-Fuertes et al. 2016) and increases sperm motility and mucus penetration (Fernandez-Fuertes et al. 2016) as well as sperm binding to oviductal epithelial explants in vitro (Lyons et al. 2018). Further detailed characterisation of the functional role of proteins within the wider beta-defensin cluster, and others, both on spermatozoa and within mucus are needed to better understand sperm-cervical mucus interaction.

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Sperm transport across the cervix

Characterising the problem

Establishment of a population of functional spermatozoa in the oviduct occurs over a period of 4–9 h with sperm numbers peaking in the oviducts approximately 24 h post insemination. Significant numbers of spermatozoa have been found in the ovine cervix within 1 h of insemination indicating that sperm entry into the cervix is relatively quick and there is a relationship between the numbers of spermatozoa in the cervix 1–2 h post insemination and the numbers in the oviducts at 24 h and resulting fertility (Croker et al. 1975). Irrespective of whether fresh or frozen-thawed semen is inseminated, the majority of spermatozoa are lost to the exterior through the vagina. Phagocytosis of spermatozoa also plays a major role in the elimination of sperm from the FRT due to the infiltration of leukocytes into the uterine lumen, and cervix (Pini et al. 2017) which, through the interaction of L-selectin, bind sialic acid on the sperm surface (Yu et al. 2018). The production of reactive oxygen species by phagocytes has also been demonstrated to decrease sperm motility, which is likely to adversely affect sperm progression (Shi et al. 2012).

Cervical immunological response to spermatozoa

Multiple studies have now reliably established that SP is immunogenic in the FRT across multiple species including humans, mice and cattle. These studies have identified a common inflammatory profile of innate immune transcripts including recruitment of immune cells and activation of cytokines and chemokines (Sharkey et al. 2012). Rather than be detrimental to fertility, this physiological inflammation is associated with improved reproductive outcomes, and the orchestration of the immune response is now thought to be a key factor in establishing local adaptations that promote the tolerance of the allogenic foetus during pregnancy (reviewed by Schjenken & Robertson 2014).

In the assessment of the immune response in the FRT, the focus traditionally has been on the uterus, the site where semen is deposited during natural mating in some species or when AI is used, and the detail of the cervix has been overlooked. However, given that the anatomy of the ovine cervix precludes the successful use of cervical AI, the immune response to semen in this region is of critical importance in the ewe. What analysis has been performed to date suggests that the response to SP is site specific and varies between cervical and vaginal epithelial cells (Sharkey et al. 2007). Few studies, in any species, have examined the impact of the cervical immune system on fertility, and therefore, it is timely that a reappraisal of the role of the cervix is now beginning (Martyn et al. 2014). Even within the cervix, distinct regions have been identified, where the endocervix, which together with the uterus and oviduct comprise the upper FRT, is composed of a single layer of columnar epithelial cells. The lower FRT consisting of the vagina and ectocervix are made up of keratinised, stratified squamous epithelium. Multiple studies have shown that it is the ectocervix that represents the primary site of responsiveness in terms of immune activation (Sharkey et al. 2007), which is logical, given its critical defence role to preventing ascending infection in the FRT. However, studies establishing the responsiveness and regional variation, particularly after the deposition of spermatozoa, are limited in the ewe.

Due to the aforementioned focus on SP, as the usual transport medium for spermatozoa during natural mating, it is still unclear if spermatozoa alone are directly immunogenic. One interesting study found that immune cells were recruited to the uterus of female mice mated with vasectomised males but the response was absent after mating with males from which seminal glands were removed (Johansson et al. 2004). This clearly indicates that, at least in mice, the predominant driver of the immune response in the FRT is SP and not spermatozoa. In contrast, however, the presence of antisperm antibodies (ASAs), both in circulation and in tissues of the FRT across multiple species, suggest otherwise. The identification of ASA in cervical mucus has also been reported, and interestingly, intrauterine insemination has been found to be an effective method to achieve pregnancy in humans. The fact that ASAs are generated against spermatozoa in the first place confirms that spermatozoa can be immunogenic, and it is possible that antigenic peptides are exposed on the sperm surface from some males and are reported to account for a significant proportion of unexplained infertility cases in humans (Cui et al. 2015). It is thought that impaired sperm–mucus interactions could contribute to the generation of ASA, and result in what is known as immunological infertility. Antisperm IgA on spermatozoa or in cervical mucus can severely inhibit sperm penetration of cervical mucus and migration through it (Kremer & Jager 1992).

The processing of ram semen prior to AI dilutes the immunomodulatory peptides usually resident in SP and may contribute to higher, and breed-specific inflammatory responses in cervical tissue of the ewe, which may ultimately preclude passage of spermatozoa through, and survival in the cervix. The sperm glycocalyx is known to be composed of highly glycosylated peptides (Tecle & Gagneux 2015), one important family of which are β-defensins. Small cationic peptides secreted in the epididymis to expansively coat spermatozoa – these multifunctional effector proteins – not only contribute to the charge-mediated passage of spermatozoa through mucus (discussed earlier) but also prevent immunoreognition and protect spermatozoa through prevention of the binding of ASA (Yudin et al. 2005).

Ewe breed differences in the immune response have been well established in response to infectious conditions of the cervix and vagina, but reports on their reproductive outcomes are limited (Cui et al. 2015). This is likely, in part, due to the relative inability of the cervix to defend against ascending infections. A full understanding of sperm–mucus–immune cell interactions and the implications for fertility and local adaptations to act as a barrier against the dissemination of pathogens is critical.
agents, and there is no reason to suspect that similar differences in local FRT responsiveness to spermatozoa and/or SP also do not exist. Considerable variation in the inflammatory potential of SP samples from different humans has been reported (Sharkey et al. 2007), and this may explain why spermatozoa from the same ram can transmigrate the cervix of some ewe breeds better than others. It is possible therefore that inherent basal or induced immune response differences between breeds contribute to the success or otherwise of sperm passage, but this theory remains to be investigated in sheep.

The cervix possesses a potent ability to produce antimicrobial defence molecules and increased lysozyme activity after intercourse has been shown. Increased β-defensin (HBD2 and HBD3) expression has also been documented with inflammation of the cervix (Meng et al. 2013), supporting an important role for peptides in mucus in defence of the FRT. This specific gene family is copy number variable across the genomes of multiple species, including in cattle (Bickhart et al. 2012). A lower DEFB4 copy number was associated with susceptibility to cervical cancer in humans (Abe et al. 2013) and variation in the transcript content between breeds will undoubtedly contribute to the functional differences in fertility in sheep. Some of these transcripts are also expressed in the FRT leading to speculation that their roles may firstly contribute to regulation of immune system in the FRT as well as affecting sperm survival and transport. The advent of complete and accurately annotated genome sequences in sheep means that this is a fertile area for future research.

**Exogenous hormones impair cervical sperm transport**

One of the most pertinent examples of impaired cervical sperm transport is in ewes grazing oestrogenic pastures. These ewes have altered cervical morphology, significantly increased mucus production and severely reduced numbers of spermatozoa in the oviducts 24 h post mating, leading to impaired fertility (Lightfoot et al. 1967). In women, it is known that exogenous hormones impact mucus characteristics such as viscosity and protein content and that these changes negatively impact sperm penetration through mucus (Lewis et al. 2010). Impaired sperm transport due to cervical mucus thickening is the major contraceptive action of the levonorgestrel-releasing intrauterine system as well as an important secondary mechanism of the combined oral contraceptive pill. The use of exogenous hormones in the synchronisation of oestrus in sheep is essential for AI in most countries, but this has been long associated with reduced fertility. Several studies have reported reduced fertility rates and reduced numbers of spermatozoa in the FRT of the progestagen-treated ewe compared to naturally cycling animals. The precise cause of impaired sperm transport in hormonally treated ewes is unknown, although it is presumably due to an altered endocrinological balance within the animal, but other factors such as neutrophil recruitment in response to the physical presence of a synchronisation device may also play a role (Mitchell et al. 2005). Observed phenomena in progestagen-treated ewes include increased production of cervical mucus (Maddison et al. 2016), altered cervical mucus proteome (Maddison et al. 2017), increased sperm breakage and loss (Gillan et al. 1999) as well as reduced functionality and viability of spermatozoa in vitro, when suspended in mucus from progestagen-treated ewes (Manes et al. 2016). In addition, synchronised ewes have higher enzymatic activity and protein content of the endometrium (Murdoch & White 1968), downregulation of Interleukin-8 in the cervical epithelium (Mitchell et al. 2002), earlier infiltration of leucocytes (Quinlivan 1967), delayed secretion from the oviducts (Murdoch & White 1968) as well as increased degeneration of the glandular epithelium of the FRT (Hawk & Conley 1971). Similar issues of impaired sperm transport have been shown following superovulation in cattle where most fertilised oocytes have no accessory spermatozoa (Hawk et al. 1988).

**Ewe breed differences in cervical function**

The exception to the poor fertility achieved following cervical AI with frozen-thawed semen is in Norway, where lambing rates following insemination into the external cervical os or indeed the vagina (so-called ‘shot-in-the-dark’) with frozen-thawed semen to a natural oestrus have been reported to be greater than 70% (Paulenz et al. 2007). This success clearly demonstrates that cervical penetration is not essential for successful sheep AI; Donovan et al. (2004) evaluated the procedures used in Norway under Irish conditions and reported higher pregnancy rates using fresh compared to frozen-thawed semen but found no significant difference in pregnancy rate following a natural or synchronised oestrus. While there is variation between individual rams (O’Meara et al. 2005), as well as between ejaculates of the same ram, there is no difference between rams of Irish and Norwegian origin; however, there is a significant effect of ewe breed on pregnancy rate (Donovan et al. 2004) with values of 8, 28, 44 and 77% reported for Suffolk, Texel, Belclare and Finnish Landrace, respectively. Parallel, albeit higher, ewe breed differences in pregnancy rates have also been reported following cervical insemination with liquid-stored semen (O’Hara et al. 2010) and following natural mating (Hanrahan 2003). Further studies examining breed differences, gross anatomy of the cervix as well as in-timing of ovulation and endocrinological profiles failed to explain the ewe breed differences in fertility (Donovan et al. 2004, Fair et al. 2007). Using a combination of fertilisation rates and accessory sperm number, significantly more spermatozoa were shown to have reached the site of fertilisation in Belclare than in Suffolk ewes following
cervical insemination with frozen-thawed semen, and this difference was eliminated following laparoscopic insemination (Fair et al. 2005). This illustrates that it is the inability of spermatozoa to traverse the cervix of low (Suffolk) compared to high (Belclare) fertility breeds and is in agreement with other studies which have reported that the migration of spermatozoa through the cervix appears to be the critical limiting factor.

An investigation into the rheology of the mucus between the breeds found that Suffolk ewes tended to have higher elastic and complex moduli than that from Belclare ewes leading to greater mucus penetration, as assessed in vitro (Richardson et al. 2011). Glycosylation of cervical mucins also varied between breeds, with low fertility breeds (Suffolk) containing a significantly higher sialic acid content in the cervical channels than high fertility breeds (Belclare), while in vitro, the addition of sialic acid to spermatozoa increased mucus penetration (Richardson et al. 2019). The immunoprotection of spermatozoa against immune recognition in the female uterus has been shown to be mediated by sialic acid (Alkhodair et al. 2018), and therefore, differential glycosylation levels may mediate higher immunoreactivity and lower fertility in vivo. The larger variation in ewe breed cervical function is certainly a useful biological model to better understand sperm transport across the cervix in both animals and women.

**Processing of semen reduces cervical penetration**

Over the last 50 years, semen preservation methods for both frozen-thawed and liquid storage of semen have improved so as to yield acceptable levels of sperm quality for insemination. While mass motility of fresh semen has been shown to be correlated with field fertility (David et al. 2015, 2018), there is a poor correlation between post-thaw in vitro parameters and in vivo fertility in sheep (O’Meara et al. 2005). It is clear that processing of semen such as freezing, thawing or liquid storage reduces the longevity of spermatozoa in the FRT. Where possible, intrauterine insemination aids in overcoming this such as in humans (Kop et al. 2015) as well as cattle (Murphy et al. 2017). In sheep, when semen is collected, diluted and stored for more than 24h, fertility declines rapidly when cervically inseminated (O’Hara et al. 2010). A meta-analysis by Maxwell and Salamon (1993) reported a reduction in fertility of 10–35% per day of storage following cervical AI. This decline occurred irrespective of the sperm number, semen diluent, storage temperature or conditions employed. Using fibered confocal microscopy, Druart et al. (2009) observed in excess of three times less spermatozoa in the body of the uterus 4h following cervical insemination of ram semen stored for 24h compared to fresh semen despite identical motility and velocity between sperm populations at insemination. When frozen-thawed semen is cervically inseminated, fertility rates can fall to 10–30%; however, when laparoscopically inseminated fertility rates of >60% are the norm (Salamon & Maxwell 2000). Modifications, such as double cervical inseminations of frozen-thawed semen a number of hours apart, have resulted in increased fertility, but this is due to increased sperm number rather than widening the time spermatozoa are present in the FRT (Salamon 1977). Similarly, increasing the number of frozen-thawed spermatozoa cervically inseminated increased the fertility but was still lower than the fertility achieved following laparoscopic insemination of frozen-thawed or cervical insemination of fresh semen (Maxwell & Hewitt 1986). Collectively, this demonstrates that the fertilising potential of frozen-thawed spermatozoa is maintained (albeit with sublethal damage; Pini et al. 2018a), but similar to that of liquid-stored semen, it is the inability of a sufficient number of spermatozoa to pass the cervical barrier into the uterus that results in low fertility following cervical AI.

**Seminal plasma supports cervical sperm transport**

Seminal plasma (SP) is a complex assortment of inorganic ions, organic salts, citric acid, sugars, prostaglandins, hormones, proteins and bioactive agents secreted mainly from the accessory glands but with contributions from the testes and epididymis (Mann 1964). Recently, studies have demonstrated that bioactive signalling agents in SP interact with the FRT, across a range of species, irrespective of the site of semen deposition (see review by Robertson & Sharkey 2016). As outlined earlier, SP has been shown to evoke gene expression and cellular changes in the innate immune system, aid in the protection from pregnancy disorders, improve embryo implantation following in vitro fertilisation (IVF) and even offspring health. In addition to these benefits, its role in nourishing spermatozoa and supporting their transit in the FRT is well established. Despite this, caudal epididymal spermatozoa of a wide range of species which have not been coated with SP are fertile when used in intracytoplasmic sperm injection (Human; Silber et al. 1995) and IVF (Cattle; Holden et al. 2017) and has yielded similar fertility to ejaculated spermatozoa, when deposited into the uterus in sheep (Rickard et al. 2014). It should be noted that in all of these cases, spermatozoa did not have to cross the cervical barrier and when vaginal insemination of epididymal spermatozoa was performed in dogs, it led to poor pregnancy rates (Thomassen & Farstad 2009). In sheep, a number of studies have shown addition of SP to liquid stored (Lopez-Perez & Perez-Clariget 2012) and cryopreserved (Maxwell et al. 1999) ram spermatozoa prior to cervical AI led to improved pregnancy rates, while others found no effect (O’Meara et al. 2007) or an inconsistent effect (Leahy et al. 2010). These conflicting results may be due to individual ram effects from which the SP was collected (Rickard et al. 2016). Rickard et al. (2014) exposed caudal
epididymal ram spermatozoa to SP prior to cervical and intrauterine insemination. Using a combination of probe-based Confocal Laser Endomicroscopy and in vivo fertility data, they reported more spermatozoa at the utero-tubal junction and higher pregnancy rates when epididymal spermatozoa were pre-exposed to SP prior to being deposited at the external cervical os. Exposure of epididymal spermatozoa to SP had no effect when laparoscopically deposited into the uterus, clearly demonstrating that the beneficial effect of SP is localised to spermatozoa traversing the tortuous ovine cervix. Seminal plasma appears to have a protective effect on liquid semen stored for 24h especially when SP is from rams of predetermined high preservation ability (Soleilhavoup et al. 2014). When SP from rams deemed to be good or ‘poor freezers’ was added back to spermatozoa, the SP from the ‘good freezers’ improved post-thaw sperm motility (Rickard et al. 2015, 2016), although similar effects were not observed with bull spermatozoa (Holden et al. 2017). When exposed for a short period of time, SP seems to act as a protective medium during in vitro processing of ram spermatozoa and may ameliorate the processing induced stressors of cryopreservation (Leahy & de Graaf 2012), while this does not seem to be the case in other species (reviewed by Bromfield (2016)). It is unknown if the protective effects of ram SP during processing and cervical transit is by exposing spermatozoa to new proteins, altering the abundance of existing membrane-bound proteins, protection of spermatozoa in the FRT or indeed through other non-proteomic factors.

The continuous progression in methods of protein identification using high resolution strategies have provided extensive information about the human SP proteome, which can be used as a tool to identify biomarkers of reproductive function. A comparative study of the protein composition of goat buck, boar, ram, bull, stallion, alpaca and camel SP revealed considerable divergence of SP proteomes between the species (Druart et al. 2013), while ram sperm proteins appear to be highly conserved across species with 95% proteins reported in other species (Pini et al. 2016). As with all ‘Oomics’ studies, the major challenge now is to characterise the biological functions of these proteins. There have been more than 700 proteins identified in ram SP (of which 40 were identified as being of sperm origin) including a high abundance of Binder of Sperm family proteins (BSP1, BSP5, SPADH1, SPADH2), the spermidhesin family (bodhesins2), lactoferrin and newly identified proteins like UPF0762 (C6orf58 transcript; Soleilhavoup et al. 2014). Under in vitro-capacitating conditions, BSP1 has recently been shown to stabilise the ram sperm membrane, reduce protein tyrosine phosphorylation while increasing cholesterol efflux and induced spontaneous acrosome reactions, while BSP5 had minimal effects on capacitated ram spermatozoa (Pini et al. 2018b).

The process of cryopreservation as well as the use of egg yolk extenders have recently been shown to alter the proteome of ram spermatozoa (Pini et al. 2018c). SP proteins have been reported to protect ram sperm from cold shock damage prior to cryopreservation (Pini et al. 2018d). Some studies have suggested that spermidhesins are responsible for these protective properties (Barrios et al. 2005), while others found no association of this protein family with liquid preservation of semen (Soleilhavoup et al. 2014). Zinc alpha 2-glycoprotein (ZAG) in the SP increased ram sperm motility initially but was negatively associated with sperm motility after 24h of semen preservation in liquid state, indicating a biphasic effect (Soleilhavoup et al. 2014). When the proteome of epididymal and ejaculated ram spermatozoa were compared, surprisingly, only two membrane-bound proteins were detected solely in ejaculated sperm lysates: liver-enriched transcript 1 (LEG1/C6orf58) and epidermal growth factor-like repeats and discoidin I-like domains 3 (EDIL3; Pini et al. 2016). This illustrates that despite its relatively complex composition, SP exposure leads to a very limited number of proteins binding tightly to the ram sperm plasma membrane. Further investigation of the effect of these three proteins on the function of ram spermatozoa are warranted.

Conclusions and future avenues of investigation

The problem of impaired cervical transit in sheep is well characterised and represents an ideal model of fertilisation failure due to impaired sperm transport in humans. Given the complexity of the problem, it is clear that elucidating the reasons for this requires an inter-and multi-dimensional approach. Advances in ‘Oomics’ technologies have provided biologists with the toolbox to complete in-depth characterisation of changes in the cervix in the lead up to ovulation as well as the variation in SP composition and the effects of processing on the sperm membrane. It is likely that a combination of these changes which alter the delicate balance of sperm interaction with the cervix, and its secretions, is responsible for the impaired cervical sperm transit. Interpreting how these changes relate to cervical sperm transport and ultimately fertility is a difficult task, but one which must be undertaken. In the absence of achieving cervical dilation, the use of experimental models which have the highest levels of variation in cervical sperm transit are critical to advancing our knowledge and solving this enigma. The most promising of these is the interrogation of differences in cervical function in ewe breeds known to have divergent fertility as well as the factors within SP which increase epididymal sperm penetration of the cervix. Taking this approach will enable us to identify the molecular markers that mediate the dynamic sperm signalling responses which are crucial for cervical sperm transport similar to those already identified as being required by spermatozoa.
to cross the utero-tubal juncture and interact with the oviduct. This intriguing area of reproductive biology will not only lead to the development of protocols for effective cervical AI of sheep but also increase our basic understanding of sperm interaction with the cervix, applicable to all vaginal depositories.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

S. Fair drafted the manuscript with the assistance of K. Meade, S. de Graaf and X. Druart. K Reynaud provided the images for Fig. 2. All authors contributed to the content of the manuscript and proof read it.

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