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# Sperm interaction with the uterine innate immune system: toll-like receptor 2 (TLR2) is a main sensor in cattle

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## ABSTRACT

During the passage through the female reproductive tract, sperm interact with various compartments and their immune systems. The immune system that protects the female against pathogens also could destroy sperm or prevent them from reaching the site of fertilisation. In particular, the uterine innate immune response is crucial from the perspectives of both the sperm and the uterus. Following insemination, sperm immediately start to trigger inflammation in the uterus by entering uterine glands and activating an innate immune response. In cattle, the activation occurs mainly via TLR2 signalling, if not the only one, between sperm and the uterine epithelium lining the glands. This acute immune response is manifested as the upregulation of mRNA expression of *IL8, TNFA, IL1B*, and *PGES*. As a consequence, many sperm are trapped by polymorphonuclear neutrophils, the first and major component of innate immunity. The sperm-induced uterine innate immune responses apparently serve to clear the uterus of excess sperm and, importantly, prepare the endometrium for implantation. Pathophysiological conditions in the uterus seriously disrupt this phenomenon, and thus could directly decrease fertility.

**Keywords:** cattle, endometrium, innate immunity, mucus, sperm, toll-like receptor 2, uterine gland, uterus.

## Introduction

In mammals, during copulation or artificial insemination (AI), a massive number of sperm are introduced into the female reproductive tract (FRT) to boost the probability of fertilisation. In the course of passage through the FRT, sperm interact with various compartments and their immune systems. The immune system that protects the female against pathogens could also destroy sperm or prevent them from reaching the site of fertilisation. The immunological interactions of sperm with the cervix, the utero-tubal junction, and the oviduct have been intensively studied but remain poorly understood (Suarez 2016; Wigby *et al.* 2019). Here we discuss the activation and the importance of the sperm-induced uterine innate immunity from the perspectives of the sperm and the uterus.

The uterus, particularly its endometrium, has crucial roles in support of embryological and fetal development, as well as parturition (Spencer *et al.* 2005). Further, the uterus regulates and supports sperm passage as well as clearing excess sperm from the FRT (Katila 2012). During natural coitus, uterine inseminators such as pigs and horses deposit semen directly into the uterine body. However, in vaginal inseminators, such as humans and cattle, semen is deposited in the anterior part of the vagina and sperm must pass through the uterine cervix to access the uterine lumen. During AI of cattle, sperm are deposited into the uterus, bypassing the cervix, such that the endometrium is the initial region where sperm come into contact with the FRT. Regardless of the site of insemination, sperm interact with the endometrium before migrating into the oviduct and these interactions regulate sperm passage and survival. Moreover, when semen is naturally or artificially deposited into the uterus, seminal plasma components directly affect the uterine responses to insemination. More often than not, the uterus is exposed

to pathogens at insemination and during parturition (Philpott 1993; Sheldon *et al.* 2020). Hence, the uterine environment is equipped with a well-developed and strictly controlled immune system that can respond to various antigens to which it is exposed (Robertson 2000).

The immune system in the uterus and other compartments of the FRT have evolved to meet the unique requirements of balancing immune reactions against pathogens, allogenic sperm, and an immunologically distinct fetus (Ochiel *et al.* 2008). The immune system in the FRT consists of both innate and adaptive immune components. As in the vagina and cervix, sperm in the uterus are also affected by female innate immune defenses (Suarez and Pacey 2006; Elweza *et al.* 2018). Therefore, sperm immune interactions with the uterus need attention. Notably, the processes of activation of uterine immune responses by sperm are not well understood. In this review, the activation of uterine innate immunity by sperm is discussed giving special emphasis on our recent investigations in *Bos taurus* cattle. Moreover, we discuss how interactions between sperm and the uterine innate immune system can be important from the standpoint of both sperm and the uterus. The effects of seminal plasma on the female immune responses will not be considered in this review, but are reported in Robertson (2005), Robertson (2007), Rodríguez-Martínez *et al.* (2011) and McGraw *et al.* (2015).

## Overall features of sperm–uterine interaction and immune communication

The FRT has various adaptations to impede the invasion and proliferation of pathogens. This includes physical, chemical and immunological impediments. These impediments probably evolved for use against pathogens, but they also have the potential to harm sperm or prevent them from reaching the site of fertilisation (Wigby *et al.* 2019). Out of the various impediments, innate immunity is essential in protecting females from infectious diseases. Innate immunity is the non-specific defense system to protect against infection during the critical initial period of exposure to a new pathogen. Adaptive immunity, the other major defense system against the infection, remembers previous encounters with specific pathogens and destroys them when they attack again. Adaptive immune responses are slow to develop on first exposure to a new pathogen, as specific clones of B and T cells have to become activated and expand; therefore, it can take some time before the responses are effective. Innate immunity is mediated by a variety of signalling molecules such as prostaglandins and cytokines and phagocytic cells such as polymorphonuclear neutrophils (PMNs) and macrophages that recognise conserved features of invaders and become quickly activated to help destroy them. PMNs are the first cells recruited in large numbers to the site of the new infection and are considered one of the major components of the innate immune system (Alberts *et al.* 2002).

In addition to protecting females from infectious diseases, the female innate immunity along with other features of the FRT controls the migration of sperm and also selects against sperm with abnormal morphology and motility. As with the other compartments of the FRT, the uterus contributes toward the success of fertilisation and development by providing a passage for sperm and supporting the removal of abnormal and excess sperm; the uterine innate immune system has a major role in this process (Suarez and Pacey 2006; Katila 2012; Elweza *et al.* 2018).

Soon after mating or AI, a substantial portion of sperm is lost from the vagina through fluid backflow (Dobrowolski and Hafez 1970; Suga and Higaki 1971; Hawk 1987). Out of the rest of the sperm in the uterus, only thousands or tens of thousands of behaviourally and morphologically sound sperm reach the oviduct (Suarez *et al.* 1997). As a matter of course, the remaining sperm in the uterine cavity must be cleared away to prepare the uterus for implantation (Troedsson *et al.* 2001). Of note, uterine innate immunity, particularly phagocytosis of sperm by PMNs, performs an important role in this clearing process (Rozeboom *et al.* 1998).

The rate of sperm migration from the site of deposition to the site of fertilisation is influenced by distinct features of the tract (Suarez and Pacey 2006). Information on the rate of sperm passage through the uterus is limited due to practical constraints. In cattle, after natural mating, sperm need approximately 6–8 h to reach the oviduct in sufficient numbers to fertilise the ovum (Wilmut and Hunter 1984). It takes 1–2 h in swine (Hunter 1981) and 4 h in horses (Scott 2000) to accomplish this event. In cattle, sperm were detected in the oviducts within 1 h after natural mating or AI (Hawk 1987). The rapidly transported sperm might contribute to fertilisation in cattle; however, further investigations are necessary to confirm whether rapidly transported bull sperm to the oviduct contribute to the fertilisation population in the oviduct.

Recently, we have conducted an *in vivo* study in multiparous beef cows to investigate sperm passage and distribution in the uterus and vagina after AI to the uterine body. Lavages of the uterine body, horn (ipsilateral to ovulatory follicle) and vaginal smears were examined at various time points. The outcomes indicated that sperm rapidly transported simultaneously either forward into the uterine horn or backward into the vagina within 1 h after AI. Most of the sperm were eliminated from the uterus and vagina 6 h after AI. Moreover, the uterine lavages and vaginal smears were completely free from sperm at 10 h after AI (Marey *et al.* 2019). In addition, it was reported that the majority of the sperm are eliminated by 6 h after AI and by 12–24 h only a few sperm were left in the tract particularly in the vagina (Mitchell *et al.* 1985; Hawk 1987). In horses, sperm were present in high but decreasing numbers in the uterus from 0.5–4 h and had completely disappeared at 48 h after AI (Katila 1995). In swine, a higher number of

sperm in the uterus at 5–6 h and moderate numbers at 20–25 h were detected after AI (Kaeoket *et al.* 2003). These results indicate that majority of sperm either migrate to the oviduct or are eliminated rapidly from the uterus into the vagina. Clearly, there are differences among mammalian species in sperm migration through the uterus.

It is likely that the uterus co-evolved with sperm to assist and regulate the sperm passage by adapting distinct features such as the uterine smooth muscle contractions and the thick, clear, viscoelastic mucus that layers the surface of the endometrial epithelium. Moreover, as is the case in the vagina and cervix, PMNs, which are the major component of the innate immune system also have an impact on sperm passage through the uterus (Suarez and Pacey 2006).

### Uterine contractions

Smooth muscle contractions in the walls of the female tract can facilitate the transport of sperm toward the site of fertilisation in the ampulla of the oviduct. In the uterus myometrial contractions can aid passage of sperm through the uterus. In cows and ewes, strong contractile activity occurs during estrus, whereas contractions are weak and localised during the luteal phase (Hawk 1987). An ultrasonographic study in humans revealed that cranially directed waves of uterine smooth muscle contractions which increase in intensity during the late follicular phase have a major role in the progression of sperm through the uterus (Kunz *et al.* 1996). In mares, myometrial contractions start immediately after natural mating. During AI, myometrial contraction may be induced by mechanical stimulation of the vagina and cervix. The contractions are mediated by neurogenic oxytocin release (Madill *et al.* 2000).

Waves of myometrial contractions can also be directed caudally and therefore act to clear sperm from the uterus (Hawk 1987; Suarez and Pacey 2006; Katila 2012). In rats, after mating a higher degree of both cranially and caudally propagating contractions of the uterine horns were reported (Crane and Martin 1991). Further, in estrous domestic cats, both ascending and descending uterine contractions were observed (Chatdarong *et al.* 2002). In cattle, when live and heat-killed sperm were inseminated into the uterus, the majority of the heat-killed sperm were quickly discharged into the vagina, whereas only a few live sperm experienced the same fate (Suga and Higaki 1971). Thus, it is apparent that the cranially directed myometrial contractions of the uterus aid the migration of viable sperm through the uterus towards the oviduct.

### The mucus of the uterine cavity

The presence of mucus in the uterus can also have an important role in assisting sperm migration through the uterine cavity as in the other segments of the tract. Uterine mucus can originate from the cervix but it is also secreted by uterine epithelium. In humans, the uterine cavity is filled with mucus in the late follicular phase; this is due to the assistance of uterine myometrial contractions that draw mucus in from the cervix (Fukuda and Fukuda 1994). The uterine cavity contains only a scarce amount of fluid space during midcycle (Casslén 1986) and cervical mucus is enough to fill the space. In cattle, uterine lavages around estrus recovered thick mucus (Alghamdi *et al.* 2009). Further, we have reported that in bovine endometrial explants, a thick, clear, viscoelastic mucus is present on the surface of the endometrial epithelium (Akthar *et al.* 2020*b*). Mucins are large highly glycosylated proteins that are the major component of mucus throughout the body and are responsible for the highly viscoelastic nature of mucus (Lagow *et al.* 1999; Andersch-Björkman *et al.* 2007). Expression of mucin genes has been detected in the endometrium of cows (Kasimanickam *et al.* 2014; Wagener *et al.* 2017) and several other mammalian species (Lagow *et al.* 1999). A few studies in mammals reported that some mucin genes are expressed differentially in glandular and luminal epithelial cells (Lagow *et al.* 1999). Altogether, cervical mucus and local secretion by the endometrium may account for the overall mucus in the uterus.

Bull sperm have been demonstrated to orient themselves along the long axis of threads of bovine cervical mucus (Tampion and Gibbons 1962). In mammals, sperm with normal motility and morphology readily swim through the mucus that fills the cervix of estrous females (Katz *et al.* 1997; Anilkumar *et al.* 2001). We have reported that sperm appeared to glide over the mucus coating of the endometrial surface (Akthar *et al.* 2020*b*). It seems that sperm swim through the cervical mucus whereas they glide over the top of the mucus in the uterus. Altogether, it is apparent that mucus in the uterus could also assist sperm migration.

Cervical mucus promote sperm selection by providing a greater barrier to abnormal sperm that cannot swim properly or that have a poor hydrodynamic profile compared to morphologically normal, vigorously motile sperm (Katz *et al.* 1990). Further, analysis of backflow sperm revealed that cervical mucus itself acted to filter out sperm with poor motility and morphology (Kölle 2015). However, there is no direct evidence of whether the mucus in the uterus acts as a barrier for abnormal sperm.

### PMNs in the uterus

In mammals, insemination results in an influx of PMNs into the uterine cavity (Austin 1957; Mattner 1968; Alghamdi *et al.* 2009). These PMNs assist and regulate the removal of sperm from the uterine cavity (Katila 1995; Kaeoket *et al.* 2003; England *et al.* 2013). The elimination of sperm by PMNs is mediated by two processes: phagocytosis of sperm and the formation of DNA-based neutrophil extracellular traps (Alghamdi *et al.* 2009; Hong *et al.* 2017). The influx of PMNs after semen deposition is essential not only to eliminate excess sperm which remained in the uterus too long but also to remove pathogens that may be introduced during mating (Mattner 1968; Alghamdi *et al.* 2009).

In cattle, during the pro-estrus and estrus periods, PMNs gather in large numbers in the endometrial stroma (Mattner 1968). In swine, PMNs assemble around estrus throughout the uterine endometrium along the basal lamina of the surface epithelium (Bischof *et al.* 1994). Insemination causes a rapid influx of these PMNs to pass through the basal lamina into the uterine lumen (Kaeoket *et al.* 2003). In cows, PMNs were recovered from uterine lavages in similar numbers at 2, 3 and 4 h after AI (Alghamdi *et al.* 2009). Our recent findings in cows demonstrated that after AI, PMNs start to appear in the uterus at 3 h, peak at 6 h, and completely disappear by 10 h (Marey *et al.* 2019). In mares, PMNs influx starts within 30 min, peaks at 4–6 h, and remains elevated for up to 24 h after AI (Katila 1995). This rapid and relatively short duration of PMNs infiltration into the uterus ensures effective removal of excess, dead and damaged sperm (Austin 1957; Bedford 1965; Mattner 1968). However, normal sperm may also be attacked, particularly in vaginal inseminators, because their sperm have lost much of the immune protection produced by the seminal plasma constituent (Suarez and Oliphant 1982). Nevertheless, rapid transport of active sperm through the uterus into the oviduct before a substantial number of PMNs arrive may be required to enhance fertilisation (Suarez and Pacey 2006; Marey *et al.* 2019). However, in cattle, there is no direct evidence of whether the sperm that are rapidly transported into the oviduct contribute to the fertilisation.

Collectively, it is evident that the influx of PMNs, a major component of the innate immune response, modulate sperm passage through the uterus. Notably, in the process of eliminating excess, dead, and abnormal sperm, uterine innate immunity performs a major role.

## Sperm trigger uterine innate immune responses

The process of sperm triggering innate immune responses in the uterus is summarised in Fig. 1. We used an array of approaches to elucidate the inflammatory responses of the bovine endometrium to bull sperm, from a simple *in vitro* co-culture of bovine endometrial epithelial cells (BEECs) with frozen-thawed sperm (Elweza *et al.* 2018; Ezz *et al.* 2019), to an endometrial explant incubation model with fresh (Akthar *et al.* 2020*b*) and frozen-thawed (Elesh *et al.* 2021) sperm, to the *in vivo* experiments that were described above (Marey *et al.* 2019). Initially, with our *in vitro* culture model, only live bull sperm stimulated an inflammatory cascade in BEECs, characterised by increased mRNA expression of the proinflammatory cytokines interleukin 8 (*IL8*), tumor necrosis factor-alpha (*TNFA*), interleukin 1 B (*IL1B*), and nuclear factor-kappa B2 (*NFKB2*), as well as complement factor 3 (*C3*) and prostaglandin E synthase

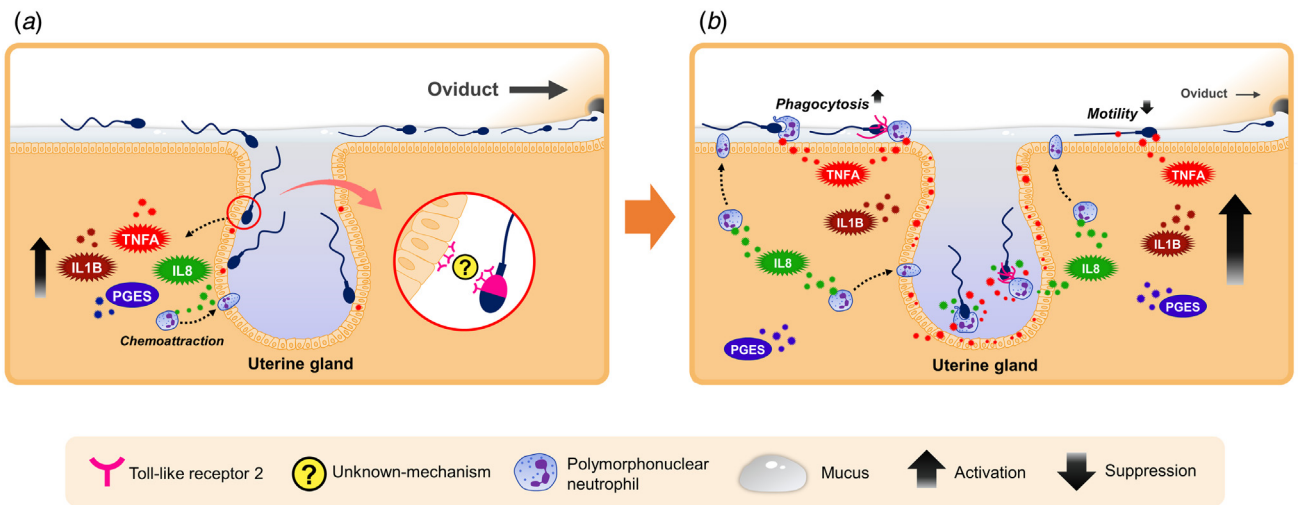


**Fig. 1.** Schematic of the interaction between bovine sperm and preovulatory endometrium to induce the first step of cascade for innate immune responses. (*a*) The sperm appeared to glide over the mucus layer on the surface epithelium and immediately enter the uterine glands to activate innate immune responses. Sperm–uterine gland interaction triggers the responses by inducing proinflammatory cytokines TNFA, IL1B and IL8, a strong chemoattractant for polymorphonuclear neutrophils (PMNs), as well as PGES expression in the endometrium, and enhances the influx of PMNs into uterine glands. TLR2 on both sperm and the uterine epithelium is involved in the innate immune responses of the uterus to sperm. Meanwhile, active sperm rapidly migrate towards the oviduct before a significant number of PMNs arrive. (*b*) Afterward, the immune response is quickly amplified. The attachment of sperm to PMNs upregulates the innate immune response by causing the PMNs to produce massive proinflammatory cytokines. The response spreads to the surface epithelium, and additional PMNs pass through the surface epithelium into the uterine cavity. As a consequence, sperm are attacked and destroyed by PMNs. The proinflammatory cytokines, particularly TNFA enhance sperm phagocytosis and may decrease sperm motility. Perhaps, a few active sperm overcome the sperm-induced innate immune response and migrate towards the oviduct.

(*PGES*). Live bull sperm also suppressed mRNA expression of the anti-inflammatory cytokine, transforming growth factor-beta 1 (*TGFB1*). In addition, medium conditioned by sperm co-incubated with BEECs stimulated phagocytosis of sperm by PMNs *in vitro*. Moreover, incubation of PMNs with fresh media supplemented with a combination of IL1B, TNFA and PGE2 increased sperm phagocytosis by PMNs (Elweza *et al.* 2018). It was reported that treatment of PMNs with IL1B, TNFA, and IL8 enhanced neutrophil extracellular trap formation (Keshari *et al.* 2012), and incubation of bovine PMNs with TNFA and IL1A enhanced their phagocytic activity (Kabbur *et al.* 1995). Therefore, it is apparent that proinflammatory cytokines, particularly TNFA enhance sperm phagocytosis by PMNs. Further, using our *in vitro* culture model, we reported that the number of sperm that remained attached to BEECs decreased gradually over time of co-culture (Elweza *et al.* 2018). It is likely that proinflammatory cytokines are cytotoxic to sperm, and thus weaken or kill them, thereby accelerating sperm detachment. In support of this, TNFA was reported to decrease human sperm motility and induce sperm chromatin and DNA damage in a concentration and time-dependent manner (Perdichizzi *et al.* 2007). Altogether, the outcomes revealed that active bull sperm attach to BEECs and trigger an acute uterine local innate immune response, with the initiation of a proinflammatory response that increases sperm phagocytosis by PMNs.

## Exploring the uterine innate immune responses to sperm using explants

Information on sperm interactions with the uterine innate immune system obtained by incubating sperm with cultured monolayers of endometrial epithelium is limited, due to the partially de-differentiated state of the epithelial cells and the absence of a connective tissue stroma to provide uterine PMNs. Results from co-incubation of sperm with the cultured monolayers indicated that sperm bind to uterine epithelium and induce inflammatory responses (Elweza *et al.* 2018). In order to more fully investigate the stimulation of inflammatory responses, we developed an *ex vivo* model that closely resembles the endometrium *in vivo*. Briefly, after obtaining the reproductive tracts of cows, the uterine horns were incised longitudinally. Using an 8 mm biopsy punch, disks of endometrial tissues with 2 mm intact endometrium were dissected from the glandular endometrial regions. These endometrial explants contained surface and glandular epithelium, as well as underlying stroma. The surface epithelium expressed microvilli and some were ciliated (Fig. 2). In addition, a clear, tenacious mucus coated the surface epithelium. It could not be removed by gentle rinsing or suction (Akthar *et al.* 2020*b*). The responses of the explants to sperm are discussed in the next two subsections.

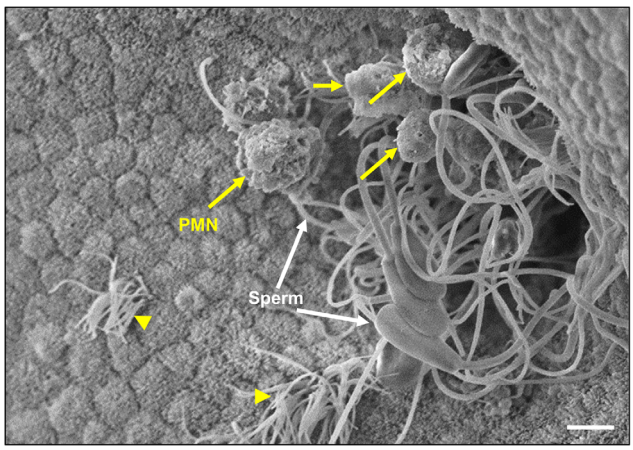


**Fig. 2.** A scanning electron micrograph of bull sperm (white arrows) associated with a uterine gland of an explant of preovulatory endometrium. Near the opening of the gland, polymorphonuclear neutrophils (PMNs) (yellow arrows) appear to bind to sperm. Ciliated surface epithelial cells are indicated by yellow arrowheads; the remainder of the surface epithelial cells show microvilli on their apical surfaces. Bar = 5 μm. Adapted from Akthar *et al.* (2020*b*).

## The uterine gland is a niche for sperm in the uterus

In our recent investigation using the sperm-endometrial explant co-incubation model, epifluorescence video microscopy was used to observe the real-time interactions of fluorescently labelled sperm with endometrial explants. Sperm appeared to glide over the mucus coating of the endometrial surface. In the preovulatory phase endometrial explants, when sperm encountered uterine glands, they entered the glands and remained in them during the 30 min observation period. Sperm were not observed to attach to the surface epithelium. Scanning electron microscopy (SEM) images of explants that were fixed after incubation with sperm showed sperm associated with the uterine glands (Fig. 2). When sperm were incubated with luteal phase endometrial explants, it seemed that fewer sperm were gliding over the surface epithelium of luteal endometrium; however, sperm did not enter into the glands they encountered. These observations indicated that the uterine gland is a niche where sperm remain and interact with the uterus (Akthar *et al.* 2020*b*; Elesh *et al.* 2021). In cattle, this phenomenon was also reported *in vivo*, where glandular retention of sperm has been observed in histological sections made 24 h after AI (Koyama *et al.* 1986).

Studies of sperm–uterine interactions in several other mammalian species provide empirical support that sperm are held in uterine glands. In dogs, sperm remained clustered in uterine glands for as long as 7–8 days after copulation (Doak *et al.* 1967). Further, in dogs, glandular retention of sperm has been observed in histological sections and SEM images made 24 h after AI (Rijsselaere *et al.* 2004). In rabbits, 2–48 h after natural mating, in histological assessments, more

sperm were present in uterine glands than in the uterine lumen (Koyama *et al.* 1986). In swine, histological sections and SEM images revealed that most of the glandular openings were filled with a large number of sperm following natural mating (Koyama *et al.* 1986). The commonality of the evidence across the mammalian species affirms that the uterine gland is a niche where sperm are retained in the uterus.

### Sperm interact with uterine glands to trigger uterine innate immune responses

Using the model of co-incubating sperm with endometrial explants, there was evidence that motile sperm interact with the glandular epithelium to induce local uterine innate immune responses. After co-incubation with explants, fresh active bull sperm upregulated mRNA expression of proinflammatory cytokines *TNFA* and *IL1B*, as well as *PGES* to activate the immune system. Further, sperm upregulated the mRNA expression of *IL8*, a strong chemoattractant for PMNs. Markedly, heat-inactivated (immotile) sperm were not present within glands nor did they upregulate the mRNA expression of inflammatory markers. Of note, fresh bull sperm induced TNFA protein expression mostly in glandular epithelium. These observations confirmed that uterine glands act as a sensor for sperm to switch on the uterine innate immune responses to clear the uterus in preparation for embryo implantation. It is also possible that the immune response prepares for implantation in ways other than simply clearing the uterus of excess sperm.

After sperm entered uterine glands, PMNs were present in the glands among the clusters of sperm and some sperm appeared to bind to the PMNs during the observation period of 30 min (Fig. 2). However, PMNs were not detected in the uterine glands in the absence of sperm in controls in which sperm were not added to explants. These results indicate that, in the case of cattle, the initial route of PMNs into the uterine cavity is through the walls of the glands. The origin of PMNs would be the endometrial stroma, which was present in explants. After PMNs enter the glands, attachment of sperm to PMNs could upregulate the innate immune response by causing the PMNs to produce immune-regulatory cytokines, which in turn, could increase additional migration of PMNs through the surface epithelium. PMNs have been observed phagocytising sperm within uterine glands in mammals such as little brown bats, guinea pigs, horseshoe bats, rabbits, and pigs (Austin 1960; Koyama *et al.* 1986; Racey *et al.* 1987). The PMNs in the bovine uterine glands could phagocytise sperm within the glands as well. The existence of PMNs along with sperm in uterine glands indicated an apparent uterine innate immune activation in response to sperm. Since PMNs are an important component of the innate immune system, this phenomenon is consistent with the hypothesis that sperm–gland interaction triggers uterine innate immunity.

Single/many complex molecular mechanisms could involve in sperm interaction with the uterine gland to trigger the uterine innate immunity, possibly a similar or distinct ligand/receptor mechanism to other compartments of the FRT.

### Toll-like receptor 2 plays a major role in sperm–uterine immune interactions in cattle

Endometrial epithelium express specific pattern recognition receptors (PRRs) that can recognise pathogens through pathogen-associated molecular patterns (PAMPs), and can detect tissue injuries through damage-associated molecular patterns (DAMPs; Janeway and Medzhitov 2002). Among PRRs, Toll-like receptors (TLRs) efficiently recognise pathogens or their PAMPs, and mount an early immune response, resulting in the expression of inflammatory mediators that affect immune cells to the site of infection, ensuring the elimination of invading pathogens or cell fragments (Schaefer *et al.* 2004; Gabler *et al.* 2010).

Toll-like receptors are transmembrane proteins that form an important component of the innate immune system which involves pathogen recognition (Mogensen 2009). In cattle, the endometrium expresses TLRs 1–10; in particular, endometrial epithelial cells express TLRs 1–7 and 9, and stromal cells express TLRs 1–4, 6, 7, 9 and 10 (Davies *et al.* 2008). Among TLRs, TLR2 is involved in the recognition of a wide variety of pathogens and their products, such as lipoproteins, and peptidoglycans (PGNs) of Gram-positive bacteria (Mogensen 2009). Further, TLR2 mediates physiological inflammation at the interaction of sperm with cumulus-oocyte complexes during fertilisation (Shimada *et al.* 2008).

Recently, using a series of *in vitro* experiments, we demonstrated the association of TLR2 with sperm–uterine immune interactions in cattle. Sperm have been identified as inducers of *TLR2* mRNA expression and subsequent protein production, particularly in glandular and surface epithelium (Akthar *et al.* 2020*b*; Elesh *et al.* 2021). In one study, we investigated the functional role of TLR2/4 in sperm-BEECs interaction. Here, BEECs monolayers were incubated with either TLR1/2 antagonist or TLR4 antibody before sperm were added; these treatments prevented the stimulatory effect of sperm on transcription of pro-inflammatory genes by BEECs. Furthermore, sperm increased the phosphorylation levels of TLR2/4 downstream targets such as p38MAPK and JNK in BEECs. Treatment of BEECs with TLR1/2 antagonist before sperm addition inhibited JNK phosphorylation, whereas TLR4 antibody inhibited the phosphorylation of both p38MAPK and JNK. These findings indicated that BEECs respond to sperm via TLR2/4 signal transduction (Ezz *et al.* 2019). Further, in the explant incubation model, SEM images and fluorescence video microscopy revealed that the addition of TLR1/2 antagonist before adding sperm subsequently reduced the sperm numbers in the uterine glands and inhibited the increase of the major proinflammatory marker *TNFA* mRNA expression seen in the presence

of sperm, which indicated the involvement of endometrial TLR2 in sperm–uterine immune interactions (Akthar *et al.* 2020*b*). Moreover, a recent study in mice identified TLR4 as a mediator of the uterine immune response to sperm (Schjenken *et al.* 2021). Thus, it is evident that sperm uterine interactions induce an acute inflammatory response involving the endometrial toll-like receptors (TLRs) signalling pathway mainly via TLR2 at least in cattle. Further, sperm induced the cluster of differentiation 44 (*CD44*) mRNA expression in BEECs and protein expression in glandular and surface epithelium (Elesh *et al.* 2021). This suggests that CD44, which is known to regulate the TLR2 responses (Qadri *et al.* 2018), is at least partly involved in sperm–uterine immune interaction. The link between CD44 and TLR2 in sperm attachment and the subsequent immune response is unknown.

Recently, we reported that TLR2 is localised in the posterior segment of the bull sperm head (Akthar *et al.* 2020*a*). Further, the involvement of sperm TLR2 in sperm–uterine immune interaction was investigated by the means of the sperm-endometrial explant co-incubation model. At first, sperm were pre-treated with TLR1/2 antagonist to block the TLR2 of sperm. After washing, TLR1/2 antagonist pre-treated sperm were co-incubated with endometrial explants. Here, we determined that TLR1/2 antagonist reduced retention of sperm in glands; further, the TLR1/2 antagonist pre-treated sperm did not increase mRNA expression of *TNFA, IL1B*, and *PGES* in the endometrium, whereas non-treated sperm induced these genes (Akthar *et al.* 2020*a*). These findings validate that TLR2 on bull sperm and endometrium is involved in the innate immune response to sperm in glands. However, the detailed molecular mechanism of TLR2 involvement in the uterine gland response to sperm is not yet known. Collectively, it is evident that, in cattle, TLR2 acts as a main sensor, if not the only one, that activates the glandular innate immune response to sperm.

Further, we have determined that TLR2 in the oviduct epithelium is also involved in the innate immune response to sperm. We have developed an explant model of the oviductal ampulla, which is the site of fertilisation, to examine the interactions of sperm with oviduct epithelium. Primary mucosal folds were cut from the ampulla of the oviduct for use as explants. Because sperm must be capacitated in order to fertilise, we pre-treated bull sperm with heparin to induce capacitation (Parrish *et al.* 1988). To investigate the role of TLR2 in sperm–oviduct interaction, TLR1/2 antagonist was added to explants before the addition of sperm, after which sperm attachment to explants and explant mRNA were quantified. Here the addition of TLR1/2 antagonist to the heparin treated or non-treated sperm-explant co-incubations reduced sperm attachment to the epithelium. Further, addition of TLR1/2 antagonist to the heparin treated sperm-explant co-incubation, inhibited TLR2 protein expression and sperm-induced anti-inflammatory cytokine *TGFB1* mRNA expression. These results indicate that the attachment of sperm to the ampullary epithelium launches a generally tolerogenic immune response, and this is mediated by TLR2 (Morillo *et al.* 2020).

Altogether, these findings indicate that TLR2 is involved in the innate immune responses of the uterus and oviduct to sperm.

## Pathophysiological conditions modulate sperm–uterine immune interactions

The sperm-induced uterine immune responses can be disrupted by various factors, particularly pathological conditions, that may negatively affect fertility. The uterine mucosa exerts its innate immune responses via efficient recognition and reaction to pathogens and sperm mainly via TLRs (Marey *et al.* 2020). Recently we illustrated the effects of pathogens on the response of endometrial epithelium to sperm. Since the bovine endometrium recognises PGN, lipopolysaccharide (LPS), and sperm via the TLR2/4 pathways, we investigated the effects of these PGN and LPS on the uterine innate immune response to sperm. We determined that PGN blocked sperm-induced inflammatory reactions, even at low concentrations, but no effect of LPS on sperm-induced response was observed in BEECs. We also determined that TLR2 signalling acts as a common pathway for PGN and sperm. At low concentrations, PGN blocked sperm-induced inflammation and inhibited sperm phagocytosis by PMNs. In the sperm-endometrial explant co-incubation model, PGN increased the retention of sperm within the uterine glands and relatively so in the surface epithelium. The addition of CD44 antibody into the PGN-sperm-explant co-incubation blocked sperm retention in glands and surface epithelium, indicating that the CD44 adhesion molecule is involved in this PGN-triggered sperm attachment to the endometrium. Thus, the presence of PGN residues disrupts sperm-induced uterine innate immune responses and prevents the physiological inflammation induced by the sperm in the endometrium via the TLR2 signalling pathway, possibly leading to impairment of uterine clearing and subsequent embryo implantation (Elesh *et al.* 2021).

Moreover, pathophysiological conditions also modulate the sperm–oviduct immune interactions. We have reported that Zearalenone, a non-steroidal estrogenic Fusarium mycotoxin disrupts the typical immune interactions between bovine sperm and oviductal epithelial cells at the level of cytokine expression and PGE2 production (Yousef *et al.* 2017). Taken together, it is evident that the pathophysiological conditions disrupt the immune interactions between sperm and the female reproductive tract, which leads to poor reproductive outcomes.

## Significance of sperm induced uterine innate immunity

The uterine innate immunity that is induced by the interaction between sperm and endometrial epithelium primarily helps

to clear the uterine environment from excess, abnormal and dead sperm as the ideal place for implantation. The rapid removal of sperm by the uterine innate immune response also prevents the development of acquired immune responses against sperm that could reduce the fertility of the female (Zralý *et al.* 2003; Hansen 2011). Moreover, sperm could modulate the uterine immune responses to facilitate embryo implantation and female immune adaptation for pregnancy, as reported in mice (Schjenken *et al.* 2021).

Following insemination, sperm immediately start to trigger physiological inflammation by switching on the uterine innate immune response. These sperm interact with the uterine gland to activate the innate immune response of the uterus and, as a consequence, many sperm are attacked and destroyed by PMNs. The complete picture is, therefore, the sperm that overcome all the impediments of the female reproductive tract, including sperm-induced uterine immunity, finally succeeds.

## Conclusions

Bovine sperm interact with uterine glands to induce the innate immune responses that are required to remove excess, dead, and abnormal sperm to facilitate embryo implantation. In cattle, the sperm–uterine interaction occurs via the TLR2 activation of both sperm and the uterine epithelium. Further, pathophysiological conditions in the uterus disrupt the immune interactions between the sperm and the uterus which leads to disruption of uterine clearing, which could decrease fertility. The bovine sperm-endometrial explant model, observed with epifluorescence microscopy and live video imaging, allows us to obtain new insights into sperm–uterine immune interactions under near *in vivo* conditions. However, this review has identified some gaps in the current information related to the uterine innate immune response to sperm. For instance, although, the evidence suggests that sperm trigger the uterine innate immunity involving the TLR2 signalling pathway, the detailed molecular pathways behind this phenomenon are still unclear. Further, it needs to be learned whether any particular kind of sperm enter the uterine glands to activate the innate immune response. Although we have learned much about the uterine innate immune response to sperm, there is much more to be learned about how this response supports the roles of the uterus in the reproductive process.

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