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Physiological functions of seminal vesicle secretions in male fertility

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Summary. In many mammals, accessory gland secretions are ejaculated into the female reproductive tract, along with sperm, and the prostates and seminal vesicles are the main glands responsible for these secretions. Cauda epididymal sperm can efficiently fertilize eggs *in vitro*; however, we found that seminal vesicle secretions improved sperm fertilization rates *in vivo* by artificial insemination. Furthermore, using the seminal vesicle-removed mice, other studies have shown that seminal vesicle secretions contribute to embryogenesis and offspring health by regulating the environment in the female reproductive tract. These results indicate the significance of accessory gland secretions in fertilization and development *in vivo*. More than 700 proteins are present in the accessory glands, and genome editing accelerates the functional analysis of these proteins at the individual level. For example, some studies reported results from phenotypic analyses of genetically modified mice that were different from those of *in vitro* experiments. In this review, we discuss the current findings on the effects of accessory gland secretions on male fertility and the future prospects. Histol Histopathol

Key words: Fertilization, Genetically modified mice, Seminal vesicle, Sperm

Introduction

Sperm produced in the testes acquire fertilization abilities, such as motility and capacitation, during epididymal sperm transit, and are stored in the cauda epididymis (Robaire, 2015). Cauda epididymal sperm are ejaculated into the female reproductive tract along with secretions from accessory glands, mainly the prostates, seminal vesicles, and bulbourethral glands. In human, more than 70% of seminal plasma contains accessory gland secretions that originate from the seminal vesicles (Prins, 2015). We and other researchers surgically removed the seminal vesicles or the prostates in mice, rats and hamsters to examine the physiological functions of the accessory glands in male fertility. Although a significant decrease in male fertility is not found in many studies by removing the prostates, the removal of seminal vesicles causes subfertility (Table 1), indicating that the seminal vesicles mainly have a function in male fertility. Herein, we introduce the physiological function of seminal vesicles in male fertility at the molecular level based on recent studies and discuss future prospects.

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Physiological function of seminal vesicle-secreted proteins in male fertility

Copulatory plug formation

Rodents and some primates, such as chimpanzees, produce a copulatory plug in their vagina during mating. Camus and Gley (Camus and Gley, 1896; Engle, 1926; Dean, 2013) found that seminal vesicle secretions were coagulated by an enzyme from the anterior lobe of the prostate (also known as the coagulating glands) (Walker, 1910a,b; Engle, 1926; Dean, 2013) in an *in vitro* experiment. A later study revealed that coagulation was dependent on the concentration of proteins from the seminal vesicles and prostates (Gotterer et al., 1955). Furthermore, to examine the physiological function of seminal vesicles and prostates *in vivo*, we surgically removed the seminal vesicles or coagulating glands (a part of prostates). As shown in Figure 1A, the weight of the copulatory plug in the coagulating gland-removed males was reduced by half compared with that in control males (Noda and Ikawa, 2019; Noda et al., 2019). Furthermore, the seminal vesicle-removed males showed more severe subfertility than the coagulating gland-removed males and failed to generate copulatory plugs (Noda and Ikawa, 2019; Noda et al., 2019). Based on these studies, secretions from both coagulating glands and seminal vesicles are required for copulatory plug

formation. Therefore, to determine the influence of the lack of a copulatory plug on male fertility, the seminal vesicle-removed males were caged with the females. Immediately after mating, we observed semen leakage in the vagina of females mated with these males (Fig. 1B), leading to a decreased sperm count in the uterus and reduced fertilization rates (Noda and Ikawa, 2019; Noda et al., 2019). Furthermore, when females lacking the copulatory plug after mating were immediately re-caged with other males, they succeeded in sequential mating (Noda and Ikawa, 2019; Noda et al., 2019). Thus, we concluded that the copulatory plug has a dual function in preventing semen leakage and sequential mating.

The molecular mechanisms underlying copulatory plug formation were also investigated. Specifically, Williams-Ashman et al. showed that transglutaminase caused the crossbridge between glutamine and lysin in seminal vesicle-secreted proteins via γ-glutamyl-ε-lysine dipeptide bonds, leading to the enzymatic coagulation of the semen (Notides and Williams-Ashman, 1967; Williams-Ashman et al., 1972; Williams-Ashman, 1984). Later studies identified the prostate-specific transglutaminase TGM4 in human and mouse ejaculates (Pilch and Mann, 2006; Dean et al., 2011). Seven major secreted proteins in mouse seminal vesicles, known as seminal vesicle secretory protein (SVS1-7), were identified by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie brilliant blue (Lundwall et al., 1997; Luo et al., 2001). Out of these seminal vesicle secreted proteins, SVS1-3 conserve the substrate for transglutaminase (Lin et al., 2002; Tseng et al., 2008). Lin et al. identified tandem repeats of QXK(S/T) in SVS3 as the transglutaminase-catalyzed sites, and found the same peptide sequence in SVS2 (Lin et al., 2002). Although SVS1 does not conserve this peptide sequence, Q232 and Q254 in SVS1 have been identified as the two major catalytic sites for TGM4 (Tseng et al., 2009). Thus, it is thought that the interaction between SVS1-3 in seminal vesicles and TGM4 in coagulating glands is required for copulatory plug formation.

To verify this hypothesis, the physiological functions of proteins present in the seminal vesicles and prostates were analyzed in genetically modified mice. In fact, the single-knockout (KO) mice lacking *Tgm4* or *Svs2*, and mice lacking the genomic region encoding *Svs2-6* could not form copulatory plugs, resulting in subfertility (Dean, 2013; Kawano et al., 2014; Shindo et al., 2019). In

Table1. Effects of the surgical removal of accessory glands on male fertility.

Species	Excised	Pregnancy rate (%)	Litter size	References
Hamster	Control	76.7±5.5	8.7±0.5	Chow et al., 1986
	AG	61.1±6.6	9.0±0.6	
	VP	56.1±6.1	7.4±0.6	
	DP	64.3±7.4	8.3±0.7	
	CG	69.8±7.0	7.8±0.7	
	SV	69.1±7.1	8.9±0.7	
	AG, VP, DP, CG and SV	33.3±6.4	8.6±0.9	
Mouse	Control	73	9.4±0.3	Pang et al., 1979
	VP and DP	38	9.8±0.9	
	CG	73	9.2±2.8	
	SV	7	4	
	Control	95.2±1.9	8.3±0.3	Peitz and Olds-Clarke, 1986
	SV	77.8±5.1	8.0±0.4	
	Control	N.D	13.6±0.5	Kawano et al., 2014
	CG	N.D	9.6±2.0	
	SV	N.D	0	
	Control	1.4±0.3[#]	8.8±2.0	Noda et al., 2019
	CG	1.5±0.0[#]	9.1±2.4	
	SV	0.6±0.5[#]	6.1±3.7	
Rat	Control	61.4±1.9	9.9±0.4	Gunn and Gould, 1958
	DP	58.4±7.8	9.1±0.4	
	Control	100[†]	5.5±0.5†	Queen et al., 1981
	VP	100[†]	5.3±0.4†	
	DP	0[†]	0†	
	CG	25[†]	5.2±1.6†	
	SV	0[†]	0†	
	Control	N.D	14.8±0.6	Carballada and Esponda, 1992
	CG	33.3	13.0±4.6	
	SV	N.D	0	

[†], Some data were used from Table 1 of the previous paper (Queen et al., 1981); [#], No. of litters/female/month; AG, ampullary gland; CG, coagulating gland; DP, dorsal prostate; N.D., not determined; SV, seminal vesicle; VP, ventral prostate.

addition, we recently found that the seminal vesicles of *Aoc1l3* (also known as *Svs1*) KO mice became transparent. However, the copulatory plug weight and male fertility of *Aoc1l3* KO mice were comparable to those of control mice (Fig. 2) (Noda et al., 2023). Transparency of organs caused by the loss-of-function of targeted genes has also been observed in another study (Ohmuraya et al., 2005), but the cause remains unclear. Furthermore, we examined the physiological function of *Pate4* (prostate and testis expressed 4, also known as *Svs7* and *Caltrin*) which mainly exists in seminal vesicles, using genetically modified mice. Previous studies showed that PATE4 is involved in Ca^{2+}-dependent events related to sperm fertilization abilities, such as sperm capacitation, sperm motility, acrosome reaction, and sperm–egg interaction by *in vitro* experiments (Coronel et al., 1992; Dematteis et al., 2008; Luo et al., 2001). However, we found that PATE4 was dispensable for sperm fertilization via artificial insemination using *Pate4* KO SVSs (Fig. 1C) (Noda et al., 2019). However, *Pate4* KO males could not produce copulatory plugs in the vagina, although major seminal vesicle-secreted proteins, including SVS2, were present in the seminal vesicles of *Pate4* KO mice (Fig. 1A). Disruption of copulatory plug formation in *Pate4*

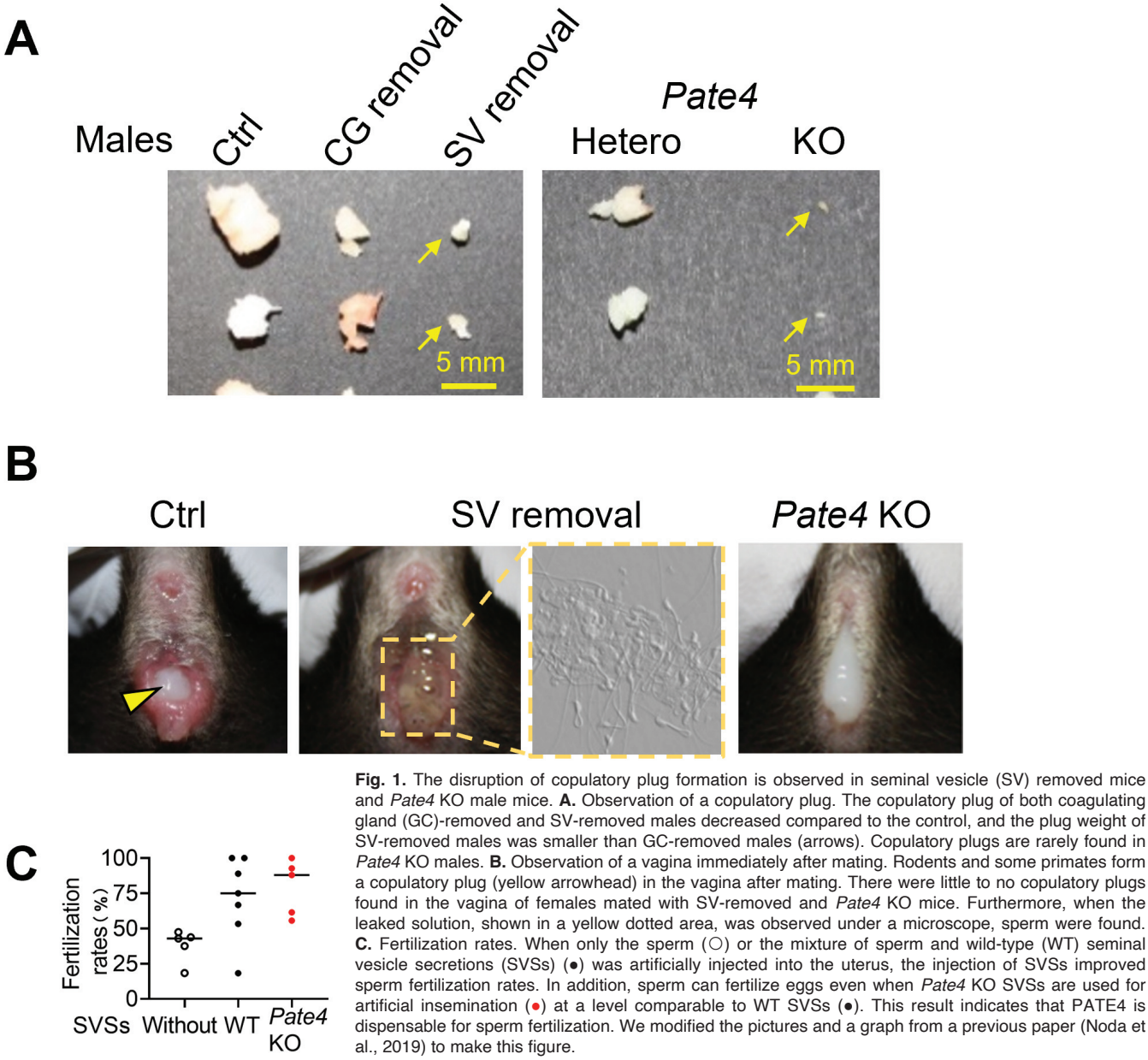

Fig. 1. The disruption of copulatory plug formation is observed in seminal vesicle (SV) removed mice and *Pate4* KO male mice. **A.** Observation of a copulatory plug. The copulatory plug of both coagulating gland (GC)-removed and SV-removed males decreased compared to the control, and the plug weight of SV-removed males was smaller than GC-removed males (arrows). Copulatory plugs are rarely found in *Pate4* KO males. **B.** Observation of a vagina immediately after mating. Rodents and some primates form a copulatory plug (yellow arrowhead) in the vagina after mating. There were little to no copulatory plugs found in the vagina of females mated with SV-removed and *Pate4* KO mice. Furthermore, when the leaked solution, shown in a yellow dotted area, was observed under a microscope, sperm were found. **C.** Fertilization rates. When only the sperm (○) or the mixture of sperm and wild-type (WT) seminal vesicle secretions (SVSs) (●) was artificially injected into the uterus, the injection of SVSs improved sperm fertilization rates. In addition, sperm can fertilize eggs even when *Pate4* KO SVSs are used for artificial insemination (●) at a level comparable to WT SVSs (●). This result indicates that PATE4 is dispensable for sperm fertilization. We modified the pictures and a graph from a previous paper (Noda et al., 2019) to make this figure.

KO males led to semen leakage and male subfertility (Fig. 1B) (Noda et al., 2019). Furthermore, we could not find the reported peptide sequence [QXK(S/T)] as a substrate for TGM4 in the PATE4 amino acid sequences. Based on these phenotypic analyses, we speculated that the molecular mechanisms that generate the copulatory plug are more complex than in TGM4-dependent copulatory plug formation.

Sperm fertilization

Cauda epididymal sperm and accessory gland secretions are ejaculated into the female reproductive tract. It takes approximately four hours for mouse sperm to migrate to oocytes in the oviductal ampulla (Muro et al., 2016). In 1951, Chang and Austin, using ejaculated sperm and cauda epididymal sperm of rabbits and rats, revealed that sperm acquire the ability to fertilize oocytes by staying in the female reproductive tract (Austin, 1951; Chang, 1951). This physiological change of sperm observed in the female reproductive tract is known as capacitation. In the later study, Chang showed that sperm fertilization ability was lost by treatment with seminal plasma, even if sperm capacitation occurred in the uterus, and that the fertilization ability of seminal plasma-treated sperm was recovered after staying in the female reproductive tract (Chang, 1957). As previously mentioned, most seminal plasma is delivered from the seminal vesicles. Thus, to examine the influence of the inhibition of sperm fertilization ability by seminal plasma on fertilization rates, we artificially injected only cauda epididymal sperm or a mixture of cauda epididymal sperm and seminal vesicle secretions into a uterus. The fertilization rate of cauda epididymal sperm with seminal vesicle secretions was higher than that of cauda epididymal sperm alone (Fig. 1C) (Noda et al., 2019). Yanagimachi et al. showed that capacitated sperm have difficulty passing through the utero-tubal junction (UTJ) when capacitated sperm are artificially injected into the uterus, leading to reduced fertilization (Shalgi et al., 1992). Thus, these results indicate that the temporary suppression of sperm fertilization ability by the seminal plasma contributes to sperm migration into the oviductal ampulla and improves fertilization rates *in vivo*.

To identify proteins from seminal plasma that regulate sperm fertilization ability, the influence of some parameters, such as cholesterol efflux from the sperm membrane, sperm motility and acrosome reaction, was investigated by mixing cauda epididymal sperm with seminal vesicle-secreted proteins *in vitro*. Thus, some candidates required for sperm fertilization ability were identified (Table 2). In later studies, the physiological functions of the candidates were analyzed in genetically modified mice. Specifically, the number of pups delivered from the mating of CEA cell adhesion molecule 10 (*Ceacam10*) KO females and males were decreased, but the reason for this result remains unclear (Finkenzeller et al., 2003). The disruption of *Serpine2*, serine (or cysteine) peptidase inhibitor, clade E, member 2, results in altered seminal vesicle fluid compositions,

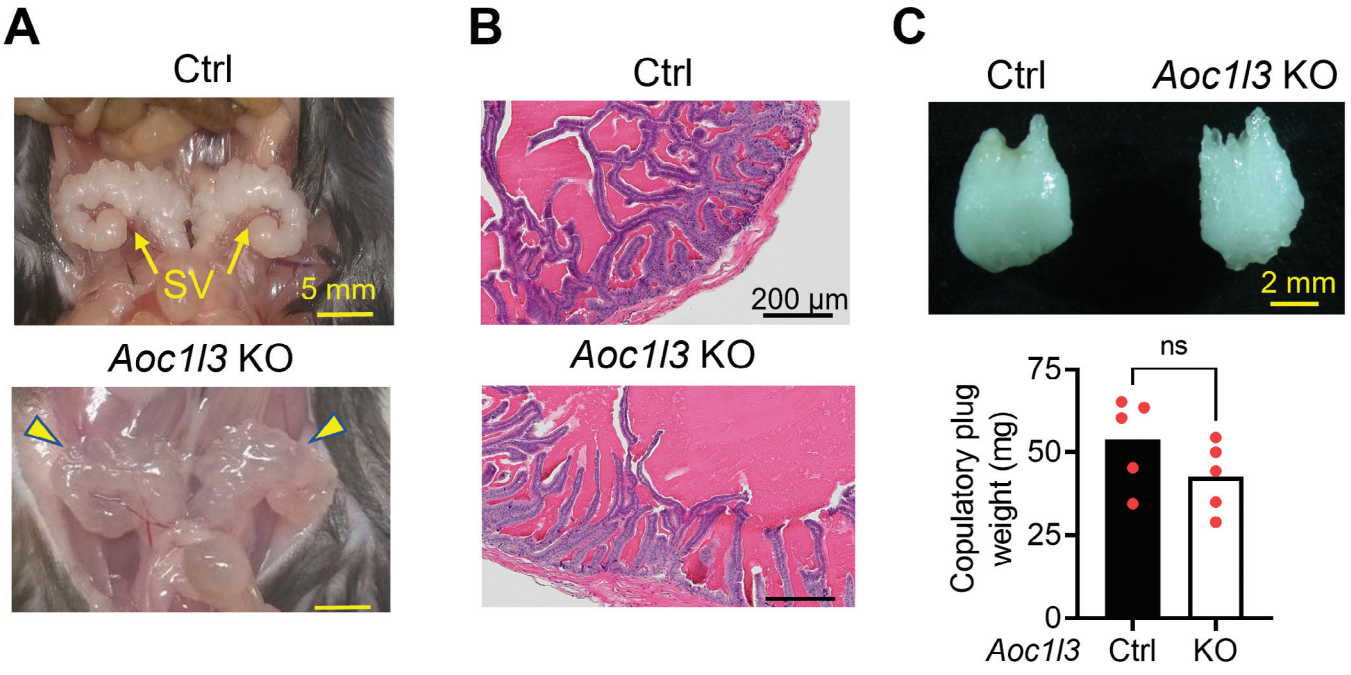

Fig. 2. *Aoc1l3* KO male mice can form a copulatory plug. **A.** Gross morphology of SVs. *Aoc1l3* KO SVs became transparent (yellow arrowheads). **B.** Histological analysis of SVs using hematoxylin and eosin staining. No obvious structural differences between the control and *Aoc1l3* KO SVs were observed. **C.** Observation of copulatory plugs. *Aoc1l3* KO male mice generated copulatory plugs, with no significant difference in weight observed between control and KO plugs. ns: not significant. We modified the pictures and a graph from a previous paper (Noda et al., 2023).

leading to a lack of copulatory plugs and reduced fertility (Murer et al., 2001). *Spink1*, serine peptidase inhibitor, Kazal type 1 (also known as *Spink3*), KO mice died 14.5 days after birth because of autophagic cell death and impaired regeneration of acinar cells (Ohmuraya et al., 2005). Disruption of SVS2 in seminal vesicles increases the number of dead sperm in the uterus, indicating that SVS2 binding to sperm protects sperm from uterine immunity (Kawano et al., 2014). SVS4 forms a cluster with SVS2-6 in a specific region of mouse chromosome 2, and the SVS family is thought to originate from gene duplication of the ancestral gene of whey acidic protein (WAP)-four-disulfide core (WFDC)-type protease inhibitors (Clauss et al., 2005; Karn et al., 2008), suggesting that the physiological function of SVS4 is similar to that of SVS2. In fact, the phenotype of male mice lacking mouse *Svs2-6* is similar to that of *Svs2* single KO males (Shindo et al., 2019). As described above, we found that PATE4 is required for copulatory plug formation but not sperm fertilization (Fig. 1) (Noda et al., 2019). Thus, the influence of seminal plasma proteins on sperm fertilization ability remains unclear at the individual level.

Environment in the female reproductive tract

The female reproductive tract is a potentially hostile environment containing cells and molecules that protect the female from infection (Wigby et al., 2019). Low pH, reactive oxygen species (ROS), antimicrobial peptides, scavenging macrophages, and antibodies generated by the immune systems are present in the female reproductive tract. This environment is harmful to ejaculated sperm, and the chemical and cellular defenses potentially impair sperm viability and fertilization ability. The seminal plasma contributes to the survival of ejaculated sperm in this environment, enabling them to reach and fertilize the egg in the ampulla. In addition, prostaglandins in seminal plasma cause uterine contractions to increase, which support sperm transport (Prins, 2015). Uterine contractions also contribute to sperm migration at the UTJ (Ryu et al., 2024). We recently revealed that the interaction between UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-like 5 (GALNTL5) (also known as pp-GalNAc-T19), a sperm surface protein, and N-acetylgalactosamine (GalNAc) on the UTJ is required for sperm-UTJ binding and migration into the oviduct (Noda et al., 2025). The acid environment in the vagina (approximately pH 4.0) becomes neutral after exposure to seminal plasma, creating slightly alkaline environmental conditions (pH 7.2-7.8) (Fox et al., 1973; Prins, 2015), suggesting that seminal plasma is required for sperm survival in the vagina. Artificial injection of a mixture of cauda epididymal sperm and seminal vesicle secretions improves fertilization, but the injection of cauda epididymal sperm alone into the uterus leads to damage

Table 2. Comparisons of functions of seminal vesicle-secreted proteins obtained by *in vitro* and *in vivo* experiments.

Proteins	*in vitro* experiments	*in vivo* experiments
CEACAM10	CEACAM10 increased sperm motility (Li et al., 2005)	The litter size obtained by mating with *Ceacam10* KO females and *Ceacam10* KO males decreased (Finkenzeller et al., 2003)
LYZ3	LYZ3 inhibited sperm capacitation (Ou et al., 2021)	Based on the International Mouse Phenotyping Consortium (IMPC) database (https://www.mousephenotype.org/data/genes/MGI:1924647), *Lyz3* was dispensable for male fertility
PATE4	PATE4 was required for Ca^{2+}-dependent events relating to sperm fertilization abilities, such as sperm motility and capacitation (Coronel et al., 1992; Luo et al., 2001; Dematteis et al., 2008)	*Pate4* KO males disrupted the copulatory plug, leading to semen leakage and subfertility (Noda et al., 2019)
QSOX1	QSOX1 increased sperm progressive motility, but suppressed sperm capacitation (Balu et al., 2022)	*Qsox1* straight-KO mice showed cardiovascular disease (Caillard et al., 2018)
SERPINE2	SERPINE2 inhibited sperm capacitation (Li et al., 2018)	Proteolysis was activated in the seminal vesicle of *Serpine2* KO mice, resulting in impaired copulatory plug formation and male infertility (Murer et al., 2001)
SPINK1 (also known as SPINK3)	SPINK1 inhibited sperm capacitation and hyperactivation (Ou et al., 2012; Zalazar et al., 2020; Ramachandran et al., 2021)	*Spink1* KO mice showed postnatal lethality due to the activated proteases in the pancreas (Ohmuraya et al., 2005)
SVS2	SVS2 suppressed sperm capacitation (Kawano and Yoshida, 2007). Also, SVS2 has TGM4 cross-linking sites, indicating that SVS2 is considered a constituent protein of a copulatory plug (Seitz and Aumuller, 1990; Tseng et al., 2012)	*Svs2* KO males showed a disrupted copulatory plug. Furthermore, the lack of SVS2 in seminal vesicle secretions decreased sperm survival rates in the uterus. Hence, *Svs2* KO males became almost infertile (Kawano et al., 2014)
SVS4	SVS4 suppressed sperm capacitation (Araki et al., 2016). As SVS4 has substrates for TGM4, SVS4 may be required for copulatory plug formation (Porta et al., 1990)	The phenotype of mice lacking the genome region encoding *Svs2-6* was comparable to that of *Svs2* single-KO mice (Shindo et al., 2019)

of the sperm membrane, causing sperm death (Kawano et al., 2014). This result indicates that the binding of seminal vesicle secretions to sperm protects from the uterine immune system. In fact, Kawano et al. showed that SVS2, a seminal vesicle–secreted protein, is a critical factor for sperm protection (Kawano et al., 2014).

The influence of seminal fluid on physiological functions after fertilization has also been investigated. For example, paternal alloantigens, such as the major histocompatibility complex and other conceptus antigens, persist during pregnancy, and regulatory T (Treg) cells mediate immune tolerance (Aluvihare et al., 2004; Firmal et al., 2020; Jiang and Vacchio, 1998; Tafuri et al., 1995). $CD4^+CD25^+$ Treg cells expressing *Foxp3* increase in the para-aortic lymph nodes draining the uterus; however, Treg cells are diminished in females that mated with seminal vesicle-removed males (Robertson et al., 2009). Transforming growth factor β (TGFβ), which is required for inducing Treg cells, is contained in the seminal plasma, including seminal vesicle secretions (Robertson et al., 2002; Ghiringhelli et al., 2005; Wang et al., 2023). Intravaginal injection of TGFβ was shown to prevent spontaneous abortion in CBA/J females that mated with DBA/2 males (Clark et al., 2008). Thus, TGFβ from seminal plasma is thought to contribute to immune tolerance during pregnancy. Furthermore, the removal of seminal fluid causes a change in gene expression profiles related to the Toll-like receptor 4 (TLR4) signaling cascade in the endometrium (Schjenken et al., 2015), and *Tlr4* KO females increased gestation length and perinatal mortality (Wahid et al., 2015). Thus, TLR4 signaling cascades activated by seminal fluid may be key pathways for immune tolerance during pregnancy (Bromfield et al., 2014; Firmal et al., 2020). Furthermore, impaired embryogenesis and placental hypertrophy have been observed in females mated with seminal vesicle-removed males; the offspring of these females showed altered growth and weight due to changes in their metabolic parameters (Bromfield et al., 2014). These studies indicate that seminal plasma, containing seminal vesicle secretions, modifies the environment of the female reproductive tract, contributing to normal embryogenesis and offspring health. However, other studies have failed to find overt defects in sperm viability, pregnancy rates, and litter size when a sufficient number of mouse sperm from the cauda epididymis were artificially injected into a uterus (Leckie et al., 1973; Watson et al., 1977; Noda et al., 2019). Some studies showed that females were impregnated by insemination of cauda epididymal sperm from bulls and boars (Igboeli and Foote, 1968; Holtz and Smidt, 1976). Because of the differences in some conditions, such as mouse strains and successful mating stimulation by artificial insemination, there may be gaps in results obtained among studies; thus, more careful analysis may be required to examine the effect of seminal plasma on the environment of the female reproductive tract.

Conclusion

While cauda epididymal sperm can fertilize oocytes *in vitro*, we revealed that seminal vesicle secretions injected into the female reproductive tract during ejaculation support fertilization ability of sperm *in vivo*. Other studies have found that the seminal fluid alters the environment of the female reproductive tract after mating, leading to normal embryogenesis and healthy offspring. Thus, since seminal plasma, including seminal vesicle secretions, improves fertilization and development *in vivo*, further studies are required to reveal the molecular mechanisms underlying these functions.

In this review, we have predominantly cited studies that used mice and rats as experimental organs, but the significance of seminal plasma in fertilization and development has been reported in bulls and human (Bromfield, 2016; Nederlof et al., 2017). Globally, one in six people is affected by infertility (Cox et al., 2022; WHO, 2023), and approximately half of these cases are due to male factors (Agarwal et al., 2021). Spermatogenic dysfunction, obstructive azoospermia, and sexual dysfunction have been shown to be some of the causes of male infertility (Jose-Miller et al., 2007; Agarwal et al., 2021), but approximately half of the causes of male infertility are poorly understood (Jose-Miller et al., 2007; Punab et al., 2017). Furthermore, cases of bull subfertility have been reported even in individuals with sperm displaying normal morphology and motility (Barbat et al., 2010; Dochi et al., 2010), and variations at the molecular level were found in their sperm (Harayama et al., 2017). However, the major causes of subfertility in bulls remain unclear. In these situations, functional analysis of proteins secreted from accessory glands, such as the seminal vesicles, is valuable for the investigation of causes of male infertility, and for the development of new molecular markers to predict male reproductive performance. More than 700 proteins have been identified in the accessory gland using proteomics (Dean et al., 2009), but the physiological functions of only limited number of proteins are known. As genome editing has led to the generation of genetically modified mice with cost- and labor-effective approaches, the utilization of genome editing will accelerate the elucidation of the functions of proteins in seminal plasma in sperm fertilization ability and the regulation of the environment in female reproductive tract.

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A conflict of interest statement. The authors declare that there are no conflicts of interest.

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