Extracellular vesicles in seminal fluid and effects on male reproduction. An overview in farm animals and pets

Extracellular vesicles in seminal fluid and its impact on male reproduction. An overview in farm animals and pets

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ABSTRACT

Extracellular vesicles (EVs) are lipid bilayer nanovesicles released by most functional cells to body fluids, carrying bioactive molecules, mainly proteins, lipids, and nucleic acids deliverable to target cells. The EVs play an essential role in cell-to-cell communication by regulating different biological processes in the target cells. Male genital fluids, including seminal plasma, contain many extracellular vesicles (sEVs), which are less explored than those of other body fluids, particularly in farm animals and pets. The few existing studies demonstrated that epithelial cells of the testis, epididymis, ampulla of ductus deferens and many accessory sex glands release sEVs mainly following an apocrine mechanism. The released sEVs are morphologically heterogeneous and would bind to neighboring secretory cells, spermatozoa, and cells of the functional tissues of the female genital tract after mating or insemination. The sEVs encapsulate proteins and miRNAs useful for sperm function and male fertility. Therefore, sEVs could be strong candidates as reproductive biomarkers in breeding sires. However, it should also be noted that many of the current findings remain open to speculation and therefore pending experimental confirmation. Further studies are particularly needed to characterize both the membrane and contents of sEVs, as well as to examine the interaction between sEVs and target cells (spermatozoa and functional cells of the internal female genital tract). Priority for these studies is the development of methods that can be standardized and that are scalable, cost-effective and time-saving for the isolation of pure different subtypes of EVs present in the sEV pool.

Keywords: extracellular vesicles, epididymis, accessory sex glands, seminal plasma, pets, livestock species.

1. Introduction

The fertility potential of a sire is ruled not only by the delivered spermatozoa, but also by the accompanying seminal plasma (SP), the fluid built from the secretions of the male genital tract, mainly epididymis and accessory sex glands, that surrounds sperm during and after ejaculation. The SP is a complex fluid, rich in many active biomolecules that play key roles in regulating sperm function, fertilizing ability and signaling uterine immune tolerance to facilitate embryo and placental development (Rodriguez-Martinez et al., 2021a). Indeed, some SP-biomolecules directly affect key sperm functions such as motility or capacitation (López Rodríguez et al., 2013; Pereira et al., 2017). Other components of SP regulate the uterine immune system, promoting a tolerogenic and healthy environment (Waberski et al., 2018). Although some SP-biomolecules are free in the SP, many of them may be encapsulated into extracellular vesicles (EVs) where they remain safeguarded from the many natural inactivators existing in SP, such as proteases or nucleases. In this regard, SP, like other body fluids, contains a large number of EVs (e.g., billions in pig SP; Barranco et al., 2021), which are released by the functional secretory cells of the different organs of the male reproductive system.

Extracellular vesicles are lipid bilayer nanovesicles, 30 to 350 nm in diameter, released by the vast majority of functional cells to the body fluids, carrying bioactive molecules, mainly proteins, lipids and nucleic acids to be delivered to target cells (Jeppesen et al., 2019). The EVs play an essential role in cell-to-cell communication and regulate different biological processes in the target cells (Doyle and Wang, 2019). The presence of EVs in the fluids of the male genital tract was reported more than 50 years ago. In fact, these body fluids would be among the first where nanometer-sized vesicles surrounded by a membrane were identified. The first study reporting vesicle-like

membranous structures in semen was performed by Metz et al. (1968) in rabbits. Such membranous vesicles were later identified in the semen of human (Brody et al., 1983; Ronquist and Brody, 1985), and livestock: ovine (Breitbart et al., 1983; Breitbart and Rubinstein, 1982), bovine (Agrawal and Vanha-Perttula, 1988, 1987, 1986), equine (Arienti et al., 1998; Minelli et al., 1999, 1998) and porcine species (Ghaoui et al., 2004). These pioneering studies, based mainly on electron microscopy, were exploratory and provided elementary, yet relevant data, such as the size and shape of seminal EVs (sEVs). Despite this accumulation of early, exciting studies, the sEVs remaining poorly explored and their biogenesis, characterization and functional roles are far from being fully understood. In fact, sEVs are among the least explored among the EVs in the body. A global survey recently conducted by the International Society for Extracellular Vesicles (ISEV) highlighted that research on EVs has mainly focused on those circulating in blood, cerebrospinal fluid and urine; demoting those delivered in semen or colostrum to the miscellaneous group so called "other fluids", which together account for barely 1% of the total research carried out on EVs (Royo et al., 2020). Moreover, very few of these already limited investigations on sEVs have been conducted in livestock species, even though SP contains comparatively more EVs than cerebrospinal fluid or blood plasma, as demonstrated in the porcine species (Skalnikova et al., 2019).

Although sEV-research remains limited and is mostly conducted in humans or biomedical model species, there have been some very interesting research studies published in recent years in pets and livestock that provides both relevant findings for understanding sEV performance and a solid basis for future research. The objectives of this review are to showcase such research, highlighting the main findings, and also to offer a particular view of where future studies should be focused. Some findings from humans and animal models have helped to clarify critical methodological issues about

sEVs and to provide insights that could be extrapolated to sEVs from farm animals as well as pets, specifically dogs and cats.

2. Biogenesis and characterization of seminal extracellular vesicles

2.1.Biogenesis

Conventionally, EVs are clustered into two subsets, namely exosomes (<150 nm) and microvesicles (>100 nm) and this subdivision entails differences in their release mechanism. Exosomes are released from cytoplasmic multivesicular bodies that fuse with the plasma membrane, whereas microvesicles are budded directly from the plasma membrane (Hessvik and Llorente, 2018). While these releasing mechanisms are also present among the epithelial cells of male reproductive tissues, sEVs are primarily delivered by apocrine secretion in many cell types (Foot and Kumar, 2021). This mechanism involves the cytoplasmic protrusion of apical vesicles containing even smaller vesicles in addition to other molecular components. These apical blebs, so-called storage vesicles, detach from the secretory cells into the lumen, and decompose and release the smaller vesicles (Hermo and Jacks, 2002). These small vesicles, show different shapes and sizes and would be the EVs that freely appear in the fluids of the male genital tract (**Figure 1**). Some of these newly released vesicles would have a very short journey, at least in the epididymis, as they would mainly bind to neighboring epithelial cells to influence their functional activity to promote a favorable microenvironment for sperm maturation (Belleannée et al., 2013; Tamessar et al., 2021).

Traditionally, the EVs present in the fluids of the male genital tract are mainly released by the epididymis and the prostate gland. In fact, epididymosomes and prostasomes are the terms commonly used to refer to EVs released in the male genital

tract (Saez et al., 2003; Sullivan and Saez, 2013), with prostasomes being an inaccurate term to refer to all EVs present in SP. In this review, we will use the umbrella name of seminal extracellular vesicles (sEVs) to refer to all EVs released by the male genital tract, regardless of the specific site of release. Conceptually, functional cells of any tissue of the male genital tract should be able to release EVs, as occurs in the rest of the body (Hessvik and Llorente, 2018). In addition to the epididymis and the prostate gland, epithelial cells of vesicular glands (they are also anatomically referred to as seminal vesicles) and the ampulla of the ductus deferens in the bull release EVs (Agrawal and Vanha-Perttula, 1987; Renneberg et al., 1995). Moreover, the mechanism of apocrine secretion for releasing EVs has also been demonstrated in the ductus deferens of mice (Manin et al., 1995). Indirect evidence supports that sustentacular cells in the testis would also be able to release EVs. Mancuso et al. (2018) demonstrated that porcine Sertoli cells cultured *in-vitro* release EVs with microRNAs (miRNAs) and protein contents that vary according to hormonal levels of FSH and testosterone, suggesting Sertoli cells provide the seminiferous epithelium and beyond with signals mediated by EVs, which could even include other sustentacular cells, such as the rete testis. Currently, there are no reports of bulbourethral glands releasing EVs; these glands in the pig deliver all their secretion via an apocrine, goblet-cell like mechanism (Badia et al., 2006). In summary, most internal organs of the male genital system would deliver EVs, contributing to the heterogeneous pool of EVs present in SP. Unfortunately, as the present time, we still lack specific markers capable of differentiating EVs according to their releasing tissue source.

2.2. *Characterization*

In terms of morphological characterization and in the absence of specific studies performed in pets and livestock species, the cryo-electron microscopy study performed

by Höög and Lötvall (2015) on human sEVs is uniquely illustrative. They identified morphologically distinct subtypes of sEVs: spherical or oval in shape and with electron dense or translucent contents. Extracellular vesicles with morphology similar to the above subtypes can also be identified in the SP of the pig (Barranco et al., 2019; Skalnikova et al., 2019) and chicken (Cordeiro et al., 2021). The transmission electron microscopy images of **Figure 2** show porcine sEVs exhibiting some of these morphological subtypes. These studies confirm the diversity of EVs in the SP-pool and Höög and Lötvall (2015) postulated that each subtype of sEVs would have a specific cellular origin.

At present, there are limited reports characterizing the membrane of sEVs and very few performed in pets and livestock. The only one of note would be that of Piehl et al. (2006) that characterized the membrane of EVs and sperm isolated from the sperm-rich fraction (SRF) of porcine ejaculates and identified the high concentration of cholesterol and sphingomyelin, alike the sperm membrane basic constitution. One of the most interesting tools to characterize EVs is the use of specific markers, as they allow differentiation of EVs from other co-isolated nanoparticles and can also identify specific EV subtypes. The EVs are also enriched in tetraspanins, a transmembrane protein family (Jankovičová et al., 2020), in addition to other proteins. Accordingly, the International Society for Extracellular Vesicles (ISEV) recommends analyzing some of these transmembrane proteins, such as CD9, CD63, CD81, to characterize the isolated EVs (Théry et al., 2018). Using these markers, Barranco et al. (2019) identified different subtypes of EVs in porcine SP, which could indicate differences in the releasing tissue, contents and also target cells of sEVs, as tetraspanins play a determining role in the selective anchoring of EVs to cell target membranes (Gurung et al., 2021). In porcine semen, Alvarez-Rodriguez et al. (2019) also cytometrically found sEVs expressing CD44, a cell surface protein active in cell-to-cell interaction and adhesion. Interestingly,

the percentage of CD44-positive sEVs was found to vary according to objectively collectable ejaculate fractions (10 first mL of SRF, rest of SRF and post-SRF), being proportionally higher in the first 10 mL of SRF. The authors suggested that these CD44-positive sEVs would come from the epididymis, since the SP of the first 10 mL of SRF comes mostly from the epididymal cauda (Rodriguez-Martinez et al., 2021a). The same authors also intended to characterize chicken sEVs showing that there were few and that these did not express either CD9 or CD44 proteins (Alvarez-Rodriguez et al., 2020), but contrasted with the more recent findings of Cordeiro et al. (2021). In sum, these studies clearly show that the SP contains a heterogeneous mixture of EVs, which would have different origin, contents and probably also target cells. For example, Sahlén et al. (2010) reported in men that specific markers such as CD10, CD13 and CD26 are present in sEVs released by the prostate, but not in those secreted by the vesicular glands.

Extracellular vesicles encapsulate a diversity of active biomolecules, mainly lipids, a wide range of proteins, including cytokines and regulatory enzymes, and nucleic acids, including DNA and both small non-coding and regulatory RNAs (Keerthikumar et al., 2016), and protect them from natural inactivators in body fluids (e.g., proteases and nucleases in SP). This complex contents is tailor-made by the releasing cells for delivery to target cells. Consequently, there may be substantial differences in the contents of EVs among body fluids. For instance, a study in cows comparing EVs revealed differences in protein contents if isolated from milk or blood plasma (Koh et al., 2017). Looking at sEV contents, the few existing studies in pets and livestock have focused mainly on proteomic and transcriptomic profiling. In proteomics, two large-scale studies have been recently performed, namely, Leahy et al. (2020) in ovine sEVs and Rowlison et al. (2020) in feline sEVs. They identified a total of 520 and 3,008 proteins, respectively. The study by Leahy et al. (2020) revealed that ovine sEVs are enriched in proteins related to vesicle

biogenesis, metabolism, and membrane adhesion and remodeling functions, the latter including several reproductive-specific proteins directly related to sperm fertilizing ability. The study by Rowlison et al. (2020), focused on epididymal EVs from domestic cats, comparing the proteome of EVs isolated from different epididymal segments and showing that the expression of several EV-proteins changes between segments. Some of these proteins are related to the epididymal sequential maturation of spermatozoa, specifically with their acquisition of motility and their ability to bind to the zona pellucida (ZP). Similar results were previously obtained by Girouard et al. (2011) on EVs isolated from the caput and cauda of the bull epididymis. In addition, there are other studies based on one- or two-dimensional gel electrophoresis (2-DE) and first reported by Gatti et al. (2005) in EVs collected from ovine epididymal cauda. They compared the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) banding pattern of these epididymal cauda EVs with that of epididymal cauda fluids (raw fluid and the supernatant fluid from ultra-centrifugation, i.e., EVs-free), SP, cytoplasmic droplets, and mature spermatozoa, and reported that the protein bands of epididymal cauda EVs were singular and different from that of other samples. The most highly expressed proteins in epididymal cauda EVs were grouped as membrane-bound proteins, metabolic enzymes and cytoskeleton-associated proteins. Frenette et al. (2006) compared the protein profile of EVs collected from caput and cauda bull epididymis and from ejaculated semen. The protein 2-DE profile varied among the sources of EV-origin, with those from caput epididymis showing many unique spots, which matched specific proteins such as heat shock protein HSP90B1 and HSPA5, with both relevant for oocyte fertilization (Dun et al., 2012). In contrast, other proteins related to sperm functionality, such as P25b, a protein involved in the binding of sperm to the ZP (Caballero et al., 2010), were only present in EVs isolated from epididymal cauda and ejaculates. It is worth mentioning that

sEVs carry immunoregulatory proteins such as transforming growth factor β isoforms 1-3 (Barranco et al., 2019). Piehl et al. (2013) analyzed the protein composition of porcine sEVs, identifying a total of 28 distinct proteins by MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry mass spectrometry). The identified proteins were grouped as structural proteins (mainly actin), enzymes, intracellular ion channels and spermadhesins, the most abundant proteins in porcine SP (Rodriguez-Martinez et al., 2021a). Ronquist et al. (2013) compared the SDS-PAGE banding patterns of sEVs from four species, namely human, canine, bovine and equine. Most of the protein bands were within the molecular weight in the range of 10 to 150 kDa, as in pig sEVs (Piehl et al., 2013), and with similar banding pattern among these species. However, there were differentially expressed protein bands as in the case of canine EVs, where bands were expressed with less intensity.

Using transcriptomics, four recent studies focused on disclosing the miRNA cargo of bovine, porcine and equine sEVs. In bovine, Alves et al. (2021) explored the load of miRNAs of sEVs, identifying 380 miRNAs. They listed all miRNAs but did not provide possible relationships to reproductive functions as it was not the goal of the study. In porcine sEVs, Xu et al. (2020) identified 325 mRNAs, predicting reproductive roles for some of them. Specifically, roles in spermatogenesis (ssc-miR-148a-3p; ssc-miR-10a-5p) and fertility (miR-10b, miR-191, miR-30d, and let-7a), with one of them (ssc-miR-200b) particularly related to the number of piglets born per litter. They also noted that pig sEVs are rich in PIWI-interacting RNAs (piRNAs, they found 19,749), although they did not link them to any reproductive function because of the lack of consultative databases. Also in pigs, Zhang et al. (2020) explored the miRNA cargo of EVs isolated from urine, blood plasma, SP and bile, and found that all EVs expressed well-defined miRNAs related to immune functions. Also recently, Twenter et al. (2020) explored the miRNAs cargo of

equine sEVs from caput, corpus and cauda epididymis, showing some of the identified miRNAs putative roles in sperm motility and viability and also in oocyte maturation and embryo development. They also reported epididymal EVs are carriers of miRNAs from epididymal epithelial cells to maturing spermatozoa in transit through the duct. In addition to this delivery of miRNAs to maturing sperm, sEVs also deliver their contents to mature sperm, including miRNAs, after ejaculation as long as the sperm remain surrounded by SP (Trigg et al., 2019). Together these proteomics and transcriptomic studies clearly demonstrate that sEVs encapsulate biomolecules useful for sperm functionality and show that the biomolecule loaded in the sEVs varies between releasing tissues and between species. Besides these two variables, there are other factors influencing the contents of sEVs. The contents would be testosterone-dependent, and the sEVs released under low testosterone levels would be less effective for sperm function (Ma et al., 2018). Similarly, environmental factors, such as excessive air temperature, would also influence the load of sEVs, at least on the load of miRNAs, as shown in heat-stressed bulls (Alves et al., 2021).

3. Interaction between seminal EVs and spermatozoa

Once released from the secretory functional cells to the ductal lumen, sEVs interact with spermatozoa. The interaction involves three sequential events, namely, binding, fusion and cargo trafficking. Seminal EVs bind to specific sperm membrane receptors such as Rab family proteins and soluble N-ethylmaleimide-Sensitive Factor attachment protein receptor (SNARE), both identified in sperm and sEVs (Girouard et al., 2011). Components of membrane lipid raft microdomains would be involved in the fusion between sEVs and spermatozoa (Candenas and Chianese, 2020). It is still not

entirely clear how sEVs deliver their contents to sperm. Two alternative delivery mechanisms are currently contemplated; using either direct membrane fusion or the formation of transient fusion pores (Björkgren and Sipilä, 2019). The first mechanism would involve tetraspanins, such as CD9, and integrins to promote competent fusion sites after glycosylphosphatidylinositol-anchored mediated docking (Al-Dossary et al., 2015). The second mechanism would involve the mechanoenzyme dynamin 1 in the formation of transient fusion pores (Zhou et al., 2019). Milk fat globule factor 8 (MFGE8) protein, identified in ovine sEVs (Leahy et al., 2020), could also be relevant for efficient trafficking of biomolecules between sEVs and sperm (Trigg et al., 2021). It is also worth mentioning that the sEVs, in addition to delivering their contents to the sperm, could also remove "non-useful" proteins from the sperm membranes. Leahy et al. (2020) reached this conclusion after analyzing the protein contents of ovine sEVs and spermatozoa. Then, the interaction between sEVs and sperm would be transient, and the sEVs would bind, fuse, interchange and detach.

Some sEVs bind to sperm immediately after their release, during the journey of sperm through the male genital duct system. Others are free in the SP and are projected out together with spermatozoa at ejaculation. Some of these free sEVs bind to sperm after ejaculation (Du et al., 2016) and others do so once in the female genital tract after mating or AI (Aalberts et al., 2013). Interestingly, Aalberts et al. (2013), in an experiment conducted with equine sEVs, proposed that the three sequential interaction events, namely binding, fusion and cargo-release or -exchange, would not occur immediately one after the other for sEVs that bind to sperm in the female genital tract. They postulated that the binding would occur in the uterus and the fusion in the oviduct shortly before fertilization, under the strongly progesterone-dominated environment that follows ovulation. The pH of the environment would be a modulating factor of sEV-sperm

interaction although it is open to controversies. In humans, Murdica et al. (2019a) indicated that sEV-sperm binding would occur at neutral pH and fusion at acidic pH, which occurs in the vagina, the site of semen delivery during intercourse in humans. This would be feasible in species with vaginal deposition of semen but not in those with deposition in the cervix uteri and uterine body deposition, as it occurs in most farm animals. In equine, Aalberts et al. (2013) demonstrated that the binding of sEVs to viable sperm was optimal at pH of 7.5-8.0. Of note, to remember is that the spermatozoa entering the cervix in human are those present in the prostate-dominated, non-coagulating first part of the ejaculate, while those sperm in the vagina are entrapped in a coagulum formed by semenogelins, and not necessarily involved in fertilization (Rodriguez-Martinez et al., 2011).

The interaction between sEVs and sperm would be selective. Bovine (Schwarz et al., 2013) and ovine (Gatti et al., 2005) EVs from the caput epididymis have more fusogenic affinity for spermatozoa than those from the cauda segment. Moreover, among epididymal cauda EVs, CD-9-positive EVs would be the ones to transfer their contents to spermatozoa (Caballero et al., 2013) and for such delivery, the cooperation of the dipeptidyl peptidase-4 protein, also known as CD-26, would be required. Interestingly, the epididymal EV-population lacking CD9 shows greater affinity for non-viable sperm, transferring epididymal sperm-binding protein 1 to them (D'Amours et al., 2012). Binding between spermatozoa and sEVs depends not only on sEVs, but also on spermatozoa. The *in vivo* sEV-to-sperm binding in the epididymal lumen is segment-dependent, greater in the caput and less in the cauda, as demonstrated in ovine (Gatti et al., 2005). However, such binding is also greater between caput EVs and cauda spermatozoa when they are cultured *in vitro* (Frenette et al., 2010). These findings would indicate that epididymal sperm would be more or less "attractive" to sEVs depending on

their level of maturation. The sEVs would also be selective in choosing the binding site on sperm. Sperm have three structurally well-defined compartments, namely the head, the mid-piece and the tail, each of them with well-defined functions. Vesicles from the epididymis would have a greater targeting affinity for the post-acrosomal region of the head (Zhou et al., 2019), whereas those derived from the accessory sex glands would exhibit affinity for all head membrane domains (acrosome ridge, acrosome, and post-acrosome) (Aalberts et al., 2013; Du et al., 2016). In this regard, our research group has evidence that sEVs bind to sperm in the three main sperm compartments (**Figure 3**). The different binding site would be linked to its functional impact and those bound to the sperm head would influence capacitation, acrosomal reaction and oocyte binding capacity, whereas those bound on the mid-piece and main piece of the tail would have a greater impact on mitochondrial activity, energy metabolism and motility.

4. Involvement of seminal EVs in sperm maturation and functionality

Sperm maturation occurs during their journey through the epididymis and is orchestrated by the sequential interaction of maturing sperm with changing intraluminal fluids. This interaction leads to structural and compositional changes that enable sperm to acquire the ability to move forward and fertilize the oocytes (Björkgren and Sipilä, 2019). Key players in this interaction are the sEVs released in the epididymis, the so-called epididymosomes, that deliver bioactive molecules to maturating sperm for the acquisition of forward motility and the ability to fertilize the oocyte (Sullivan, 2015). Research conducted in bovine showed that epididymosomes influence sperm maturation in two ways (Belleannée et al., 2013). The first, more direct, is by fusing with the membrane of maturing sperm and delivering their contents to them. The second, indirect,

by interacting with neighboring epithelial epididymal cells to modulate their secretions to provide a better epididymal environment for sperm maturation. The epididymis environment and the involvement of epididymosomes in sperm maturation is discussed in more detail in another review in this special issue (Rodriguez-Martinez et al., 2021b).

Most studies relating sEVs and sperm functional parameters have been conducted in humans and mostly in men showing severe alterations of seminal parameters, such as oligozoospermia, azoospermia, asthenozoospermia and teratozoospermia (Candenas and Chianese, 2020). Highlighted should be the study by Murdica et al. (2019b), demonstrating the influence of sEVs on the regulation of sperm motility and time of capacitation after incubating ejaculated sperm with sEVs isolated from the SP of astheno- or normozoospermic men. Specifically, they found that sEVs from normozoospermic men but not from asthenozoospermic men, enhanced sperm motility and triggered capacitation. This differential performance of sEVs would be related to differences in the expression of proteins and miRNAs involved in reproductive processes between sEVs from individuals with normal and altered semen parameters (Barceló et al., 2018; Murdica et al., 2019a). Similar studies have not been conducted in livestock species, perhaps because breeding sires are selected not only for their genetic traits, but also for yielding ejaculates with satisfactory sperm quantity and quality, while those with poor semen quality are culled.

The few studies in pets and farm animals relating sEVs and sperm functionality also reported that sEVs would influence motility and capacitation, in addition to the acrosomal reaction (Figure 4). In pigs, Piehl et al. (2013) and Du et al. (2016) conducted similar studies by incubating/extending ejaculated sperm with sEVs and evaluating effects on motility and capacitation. Regarding sperm motility, while Piehl et al. (2013) found no differences between treated sperm incubated with sEVs and control sperm

incubated with extender without EVs. Du et al. (2016) noted that EVs enhanced sperm motility. Beyond the disagreement regarding sperm motility, both studies agree that sEVs stabilize sperm membranes and prevent premature capacitation and consequent acrosome exocytosis. However, in an earlier study in pigs, Siciliano et al. (2008) found that the acrosome rupture was triggered in sperm incubated with sEVs. In an experimental study conducted in equine semen, Aalberts et al. (2013) reported that incubation of ejaculated sperm with sEVs did not influence the timing of capacitation. In pets, Mogielnicka-Brzozowska et al. (2015) reported that the total and progressive motility of canine sperm improved after incubation with sEVs. The mechanism of action of sEVs in influencing sperm motility would be related to their ability to regulate sperm intracellular Ca^{2+} (Palmerini et al., 1999; Park et al., 2011). Recently, Zhang et al. (2021) proposed that sEVs would play this role by activating a cation channel of sperm (CatSper), which regulates motility during capacitation-related events (Vicente-Carrillo et al., 2017). Other EV-mechanisms could also be involved. For instance, sEVs synthesize ATP through glycolysis and this ATP would modulate sperm mitochondrial metabolism and, consequently, sperm motility (Guo et al., 2019). Further, sEVs would control the delivery of zinc ions to spermatozoa, an essential ion to stabilize sperm membranes and thus promote motility (Mogielnicka-Brzozowska et al., 2015). The mechanism of action of sEVs on regulating the timing of sperm capacitation is still unclear. In humans, Bechoua et al. (2011) suggested that sEVs modulate protein tyrosine phosphorylation, a pivotal event in sperm capacitation. However, Aalberts et al. (2013) conducted an experiment incubating equine ejaculated spermatozoa with sEVs showing that sEVs would have limited influence on tyrosine phosphorylation.

The above studies in pets and livestock showed some contradictory results regarding the influence of sEVs on sperm functionality, as also occurs in those performed

in humans (Foot and Kumar, 2021). Several explanations can be issued for these inconsistencies, the most plausible being differences in methodologies employed between studies to isolate sEVs and the intrinsic diversity in the contents and membrane composition of isolated sEVs. Not all isolation methods used in the studies mentioned above guarantee the purity of isolated sEVs, and some of the isolated sEVs may be contaminated with proteins and miRNAs free in the SP (Royo et al., 2020). Another differentiating factor would be the inherent diversity of isolated sEVs. Several subtypes of EVs are present in the SP of farm animals (Alvarez-Rodriguez et al., 2019; Barranco et al., 2019) and each of these subtypes would have a different cellular origin and, therefore, also a different contents (Greening and Simpson, 2018). This diversity of EVs transported through semen can selectively interact with target cells, whether spermatozoa or cells of the male or female genital tract, providing a highly complex and yet, little understood mode of cellular communication.

Successful long-term semen preservation in mammals still remains a challenge. Current sperm freeze-thaw methods, even the most successful, remain suboptimal, as they induce structural as well as biochemical and functional changes in sperm, impairing their functional performance after thawing, including fertilization capacity (Khan et al., 2021; Kumar et al., 2019; Yeste, 2016). To date, to our knowledge, there is only one study that has explored the potential of EVs to mitigate the detrimental impact of freeze-thawing on spermatozoa. The study of Rowlison et al. (2021) conducted in domestic cats showed that frozen-thawed sperm improved motility after thawing when incubated with epididymal EVs. However, a number of studies investigated the usefulness of EVs secreted outside the male genital tract in improving sperm cryopreservation (reviewed by Saadeldin et al., 2020). *In vitro* experiments conducted by Alcantara-Neto et al. (2020) demonstrated the effectiveness of porcine oviductal EVs for improving the survival of thawed pig sperm.

Similar results were achieved by De Almeida Monteiro Melo Ferraz et al. (2020) in frozen-thawed spermatozoa from red wolves and cheetahs incubated with dog and cat oviductal EVs, respectively. Mesenchymal cell derived EVs have also been shown to be effective. Qamar et al. (2019) improved the motility and integrity of plasma and acrosomal membranes of frozen-thawed canine sperm by adding mesenchymal cell-derived EVs to the freezing medium. Similar results were also reported by Mokarizadeh et al. (2013) in mouse sperm. These studies did not demonstrate causal mechanisms for this improvement, but Qamar et al. (2019) attributed the positive effect on the ability of EVs to repair sperm membranes and reduce oxidative stress associated with cryopreservation. In that study, they demonstrated expression changes in genes related to membrane repair, modulation of mitochondrial reactive oxygen species and chromatin integrity. Mokarizadeh et al. (2013) also reported an increased expression of specific EVs biomolecules in the membranes of thawed spermatozoa, namely CD29, CD44, ICAM-I and VCAM-I. However, not all EVs would have positive effects on sperm functionality. Extracellular vesicles from human embryonic kidney-derived cells, a scalable cell line used for mass EV-production, did not influence the functionality of pig sperm after 5 h of co-culture (Vilanova-Perez et al., 2020).

To the best of our knowledge, there is only one scientific report linking sEVs to male *in vivo* fertility. Cordeiro et al. (2021) isolated sEVs from rooster ejaculates with clear differences in sperm viability and motility and showed that ejaculates from more fertile males had smaller sEVs than those from less fertile males. They also found compositional differences between sEVs, showing higher HSP90AA1 expression in those isolated from more fertile males. In addition to influencing the functional performance of sperm and thus male *in vivo* fertility, sEVs would also contribute to the fertility success of males through their interaction with the epithelial cells of the female genital tract after

mating or insemination delivering (Figure 4). Seminal EVs have the ability to be bound and internalized by the endometrial cells (Paktinat et al., 2019). Bai et al. (2018) demonstrated, in an *in vitro* experiment, that pig sEVs were able to up-regulate the expression of genes related to immune and inflammatory responses in endometrial epithelial cells. Accordingly, sEVs would play and essential role in regulating the immune response of the female genital tract, facilitating the survival and functionality of sperm and subsequent embryo and placental development. It should be noted that sEVs, like those present in other body fluids, contain a large number of miRNAs with well-documented immune-related functions (Zhang et al., 2020).

5. Conclusions and targets for future research

This review reveals that sEVs remain underexplored compared to those found in other body fluids, such as those circulating in blood or cerebrospinal fluids, even though there are comparatively more EVs in SP than in any other body fluid. This lack of knowledge is particularly striking for those present in the SP of pets (dog and cat) and farm animals. Summarizing the few existing research studies, it seems clear the epithelia of the male genital tract releases EVs, including testes, epididymis, vas deferens ampulla and some accessory sex glands, and they would do so mainly following an apocrine mechanism. The released sEVs would bind to and regulate neighboring secretory cells, using paracrine pathway, spermatozoa and cells of the functional tissues of the female genital tract, following mating or insemination. In sperm, sEVs bind, fuse with the plasma membrane and deliver their contents that, according to the current knowledge, would influence epididymal maturation, motility and capacitation. Moreover, sEVs would also remove non-functional proteins from spermatozoa. Once inside the female genital tract,

the sEVs would be bound and internalized by the epithelial cells modulating the immune response against spermatozoa and embryos. The limited data accumulated so far provide valuable information on sEVs, but many of these findings remain open to speculation and therefore need to be confirmed in future studies. Consequently, the research of sEVs in pets and livestock remains a challenge and different research approaches should be considered.

Further characterization studies of both the membrane and contents of SVs are essential, but to do so, will first require methods that can be standardized scalable, inexpensive, and time-saving for isolation of pure sEVs. Currently, different isolation methods are being used, generating some inconsistent and sometimes even contradictory results, making their comparison difficult and limiting their clinical usefulness (Mercadal et al., 2020). In addition, methods should be able to separately isolate the different subtypes of EVs present in SP, as each subtype may have a different contents in active biomolecules and thus different effects on target cells. These studies would allow characterization of the different subtypes of EVs present in SP and allow labeling of the distinctive molecules of each sEV-subtype for easy and rapid identification and selection. Once the sEV subtypes are identified, it will be possible to better understand the involvement of sEVs in sperm functionality and male fertility, which currently remains unclear and controversial.

Finding biomarkers of male fertility remains a challenge today, both for domestic animals as well as for humans. Seminal plasma biomolecules influence sperm functionality, embryo development, and implantation (Bromfield, 2018; Druart et al., 2019; Pérez-Patiño et al., 2018; Szczykutowicz et al., 2019). Consequently, some SP-biomolecules have been posted as candidates for biomarkers of sperm functionality and male fertility (Rodriguez-Martinez et al., 2021a). We now know that some of these

seminal biomolecules are encapsulated in sEVs, where they remain active by being protected from the natural inactivators present in SP (e.g., proteases and nucleases). Moreover, we also know that sEVs bind and interchange molecules with spermatozoa and epithelial cells of the endometrium. Overall, these findings strongly point out to sEVs as serious candidates for use as biomarkers of sperm functionality and male fertility. Today, the search for biomarkers in sEVs is negligible, unlike those circulating/present in other body fluids as in blood plasma or urine, which have been widely explored for their use as biomarkers for diverse pathologies, include cancer (Simeone et al., 2020; Street et al., 2017; Yekula et al., 2020). Only three papers listed in PubMed in May 2020 address this issue and they have been conducted in humans (Barceló et al., 2018; Larriba and Bassas, 2021; Vickram et al., 2020). Consequently, finding out whether sEVs are useful biomarkers of fertility is an exciting challenge. However, before tackling this task, it is imperative to fully characterize all subtypes of vesicles circulating in male genital tract fluids (Pucci and Rooman, 2017). Unfortunately, this is a research task that has not yet been completed in pet and livestock species, making it a pending challenge.

The complete characterization of the sEV subtypes will facilitate that they can be used as therapeutic tools (Peng et al., 2020; Sil et al., 2020). Today we know that sEVs from normozoospermic ejaculates improve sperm motility while those of asthenozoospermic ejaculates reduces it (Murdica et al., 2019b). These findings raise the possibility of using sEVs to improve sperm quality in individuals showing idiopathic poor sperm quality. It has also been shown that sEVs can improve sperm freezability (Qamar et al., 2019). In some farm animals there are clear differences between sires in sperm freezing capacity, impairing the use of poor sperm freezers as semen cryobankers (Roca et al., 2006). Here, sEVs could be used to improve sperm cryotolerance in bad sperm freezers by supplementing the freezing medium with sEVs from good sperm freezers. In

this case, EVs can be artificially enriched with specific molecules. Specific subtypes of sEVs could be loaded with molecules of interest using proven procedures, such as electroporation (for miRNAs), sonication (for proteins), or passive diffusion of hydrophobic molecules (for soluble chemicals) (Lim and Kim, 2019). Thus, "engineered" sEVs would be used to improve the *in vivo* bioavailability of molecules of interest to both sperm and uterine cells and thus improve their functionality. Full characterization of sEVs subtypes will also facilitate further studies for designing and producing synthetic EVs, structurally similar to those of SP, which would load with specific biomolecules for particular applications. For instance, as additives to semen extenders for improving both sperm preservability and/or *in vivo* fertility of seminal AI-doses. These synthetic EVs added to seminal AI-doses can also be used for delivering drugs to improve the tolerogenic female local immunity.

Ethical Statement

The experiments with animals and specimens in the aforementioned studies developed by the authors of this review were performed according to the European Directive 2010/63/EU, 22/09/2010 for animal experiments and approved by the Bioethics Committee of Murcia University (research code: 639/2012).

Funding

The research of the authors was funded by MICINN (Spain) and FEDER EU-funds (Grant PID2020-113493RB-I00), Madrid, Spain, the Research Council FORMAS, Stockholm (Grants 2017-00946 and 2019-00288) and European Union´s Horizon 2020 research and innovation programme (Grant H2020-MSCA-IF-2019-891382).

Acknowledgments

The authors are grateful to Topics Norsvin España (Madrid, Spain) for providing semen samples and reproductive tissues from male pigs. The authors are indebted to Antonio García Lorca for his disinterested work on the drawings showed in the figures.

Author contributions

Conceptualization, J.R. and I.B.; writing—original draft preparation, J.R.; writing—review and editing, J.R.; H.R.-M., L.P. and I.B.; funding acquisition, J.R. and H.R.-M.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest. The funders had no influence on the contents of the manuscript.

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Figure legends

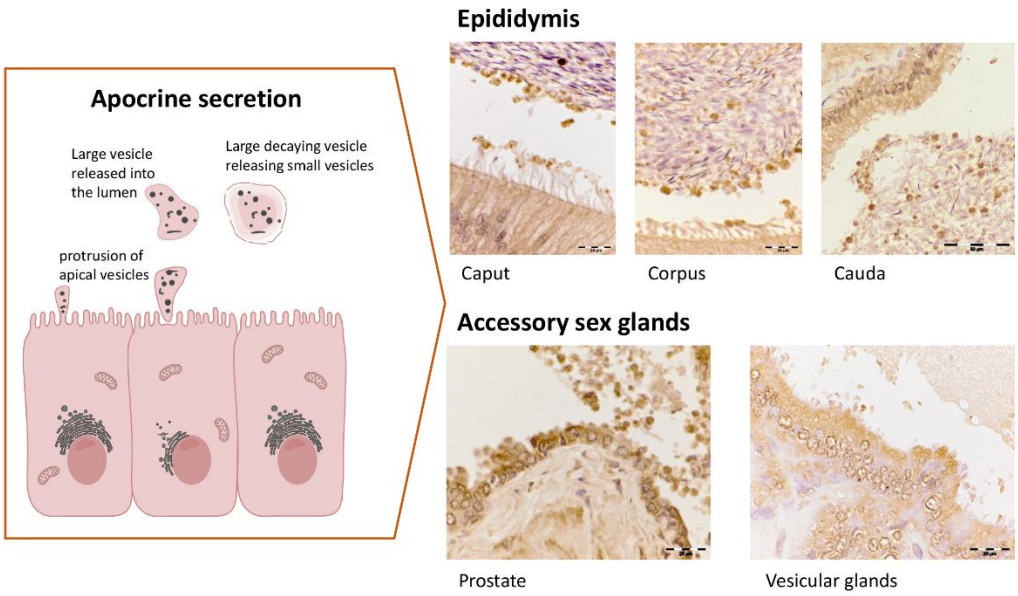

Figure 1. Schematic drawing illustrating the mechanism of apocrine secretion, including formation of apical vesicles and the fate of large released and decaying vesicles in the lumen of the genital tract of the male pig (segments of the epididymis and accessory sex glands) to finally deliver extracellular vesicles. The drawing was created in BioRender.com.

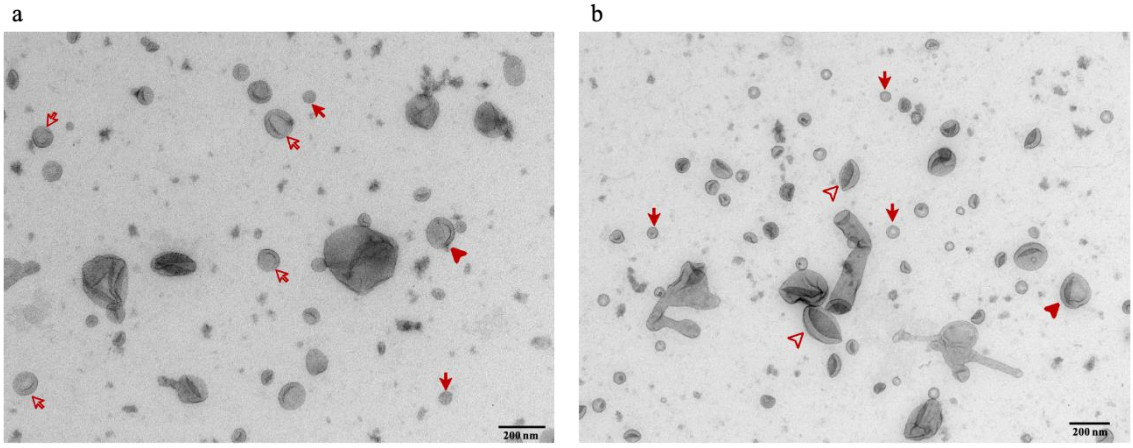

Figure 2a-b. Transmission electron micrographs showing extracellular vesicles from porcine seminal plasma, and their diversity in size and shape. Extracellular vesicles were isolated by ultrafiltration (0.22µm plus Amicon®-100K) with size exclusion liquid chromatography (Barranco et al., 2021). The arrows identify some morphological subtypes of seminal extracellular vesicles according to the classification made by Höög and Lötvall (2015) in human semen: (1) single spherical vesicle (unfilled arrow), double spherical vesicle (filled arrow), oval vesicle (unfilled arrowhead) and double vesicle (filled arrowhead). Images, belonging to the authors, were generated at the Central Experimental Research Service (SCSIE) of the University of Valencia.

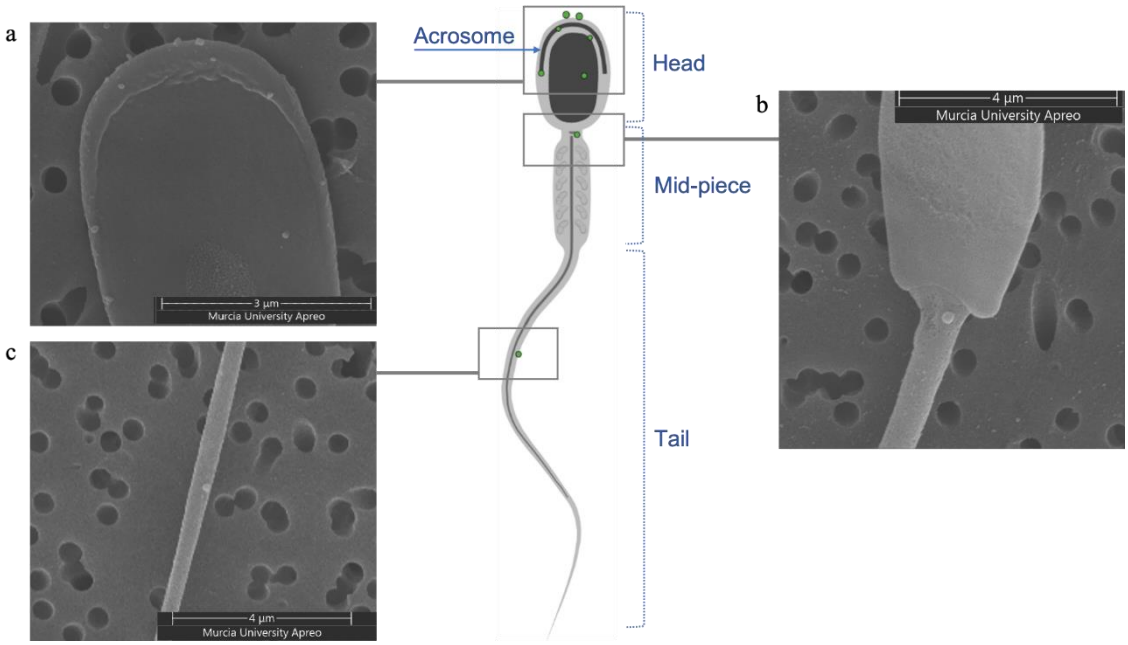

Figure 3a-c. Transmission electron micrographs showing extracellular vesicles bound to different porcine sperm membrane domains in the head (a), neck (b) and tail (c). Images, belonging to the authors, were generated at the Scientific and Technical Research Area of the University of Murcia. The drawing of spermatozoon was created in BioRender.com.

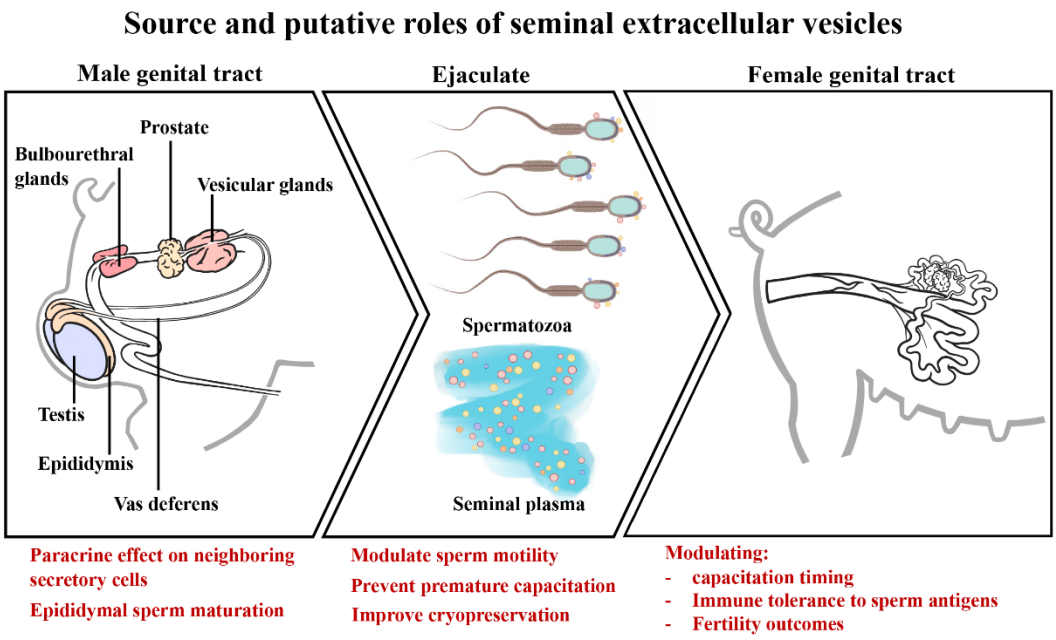

Figure 4. Scheme illustrating the seminal extracellular vesicle-releasing organs in the male reproductive tract and the putative functions of released seminal extracellular vesicles on both the spermatozoa, the male and the female reproductive tracts. The putative functions of sEVs are those reported in scientific studies in pet and livestock species. Drawings were created in BioRender.com.