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Extracellular vesicles in seminal fluid and effects 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, containing bioactive molecules, mainly proteins, lipids, and nucleic acids having actions at target cells. The EVs have essential functions in cell-to-cell communication by regulating different biological processes in target cells. Fluids from the male reproductive tract, including seminal plasma, contain many extracellular vesicles (sEVs), which have been evaluated to a lesser extent than those of other body fluids, particularly in farm animals and pets. Results from the few studies that have been conducted indicated epithelial cells of the testis, epididymis, ampulla of ductus deferens and many accessory sex glands release sEVs mainly via apocrine mechanisms. The sEVs are morphologically heterogeneous and bind to functional cells of the male reproductive tract, spermatozoa, and cells of the functional tissues of the female reproductive tract after mating or insemination. The sEVs encapsulate proteins and miRNAs that modulate sperm functions and male fertility. The sEVs, therefore, could be important as reproductive biomarkers in breeding sires. Many of the current findings regarding sEV functions, however, need experimental confirmation. Further studies are particularly needed to characterize both membranes and contents of sEVs, as well as the interaction between sEVs and target cells (spermatozoa and functional cells of the internal female reproductive tract). A priority for conducting these studies is development of methods that can be standardized and that are scalable, cost-effective and time-saving for isolation of different subtypes of EVs present in the entire population of sEVs.

Keywords: Extracellular vesicles; Epididymis; Accessory sex glands; Seminal plasma; Pets; Livestock species.

1. Introduction

The fertility potential of a sire is determined not only by the spermatozoa produced, but also by the accompanying seminal plasma (SP), a fluid produced by the secretions of the male reproductive tract, mainly from the epididymis and accessory sex glands, and which is the medium that surrounds sperm during and after ejaculation. The SP is a complex fluid, containing many active biomolecules that have important functions in regulating sperm, fertilizing capacity and signaling uterine immune tolerance to facilitate embryo and placental development (Rodriguez-Martinez et al., 2021a). Indeed, some SP-biomolecules directly affect important 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 environment for optimal embryo development (Waberski et al., 2018). Although some SP-biomolecules are free in the SP, many others may be encapsulated in extracellular vesicles (EVs) where they would remain protected from the many natural inactivators 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 into the body fluids, containing bioactive molecules, mainly proteins, lipids and nucleic acids that are transported to target cells (Jeppesen et al., 2019). The EVs have an essential function 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 reproductive 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 from which there was reporting of 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: sheep (Breitbart et al., 1983; Breitbart and Rubinstein, 1982), cattle (Agrawal and Vanha-Perttula, 1988, 1987, 1986), horses (Arienti et al., 1998; Minelli et al., 1999, 1998) and pigs (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). Even though there has been this conducting of these early important studies, the sEVs remain poorly evaluated and the biogenesis, characterization and functions of sEVs 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; with there being few studies with semen or colostrum, which are included in the miscellaneous group of so called "other fluids", which together account for about 1% of the total research conducted on EVs (Royo et al., 2020). Furthermore, very few of these already limited investigations on sEVs have been conducted in livestock species, even though the SP contains comparatively more EVs than cerebrospinal fluid or blood plasma, as reported for pigs (Skalnikova et al., 2019).

Although sEV-research remains limited and has been conducted mostly in humans or biomedical model species, there have been some very interesting studies from which results have been published in recent years in pets and livestock that provides both relevant findings for understanding sEV functions and a basis for future research. The objectives of this review are to highlight such research, by providing a review of the most

important findings, and also provide the perspective of where future studies should be focused. The review also shows some findings from humans and animal models that clarify methodological issues about sEVs and 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 release mechanisms. Exosomes are released from cytoplasmic multivesicular bodies that fuse with the plasma membrane, whereas microvesicles are released by budding 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 released following apocrine secretion mechanisms (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 disintegrate, releasing the smaller vesicles (Hermo and Jacks, 2002). These small vesicles are of different shapes and sizes and would be the EVs that are present in the fluids of the male reproductive tract (Figure 1). Some of these newly released vesicles would have a very short journey, at least in the epididymis, as they bind to proximate epithelial cells, where the contents of sEVs 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 reproductive 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 reproductive 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, there will be use of the "umbrella" term for seminal extracellular vesicles (sEVs) to refer to all EVs released by the male reproductive tract, regardless of the specific site of release. Conceptually, functional cells of any tissue of the male reproductive tract should have the capacity to release EVs, as occurs in other parts of the body (Hessvik and Llorente, 2018). In addition to the epididymis and the prostate gland, epithelial cells of vesicular glands (anatomically referred to as seminal vesicles) and the ampulla of the ductus deferens in bulls release EVs (Agrawal and Vanha-Perttula, 1987; Renneberg et al., 1995). Furthermore, apocrine secretion of EVs in the ductus deferens has been reported in mice (Manin et al., 1995). Indirect evidence indicates sustentacular cells in the testis have the capacity to release EVs. Mancuso et al. (2018) reported that Sertoli cells of pigs cultured *in-vitro* release EVs with microRNAs (miRNAs) and protein contents that vary with FSH and testosterone concentrations in the surrounding milieu, suggesting Sertoli cells have signaling pathways to the seminiferous epithelium and to other tissues that are 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; the secretions form these glands occur via an apocrine, goblet-cell like mechanism (Badia et al., 2006). In summary, most internal organs of the male reproductive system produce EVs, contributing to the heterogeneous population of EVs present in SP. Unfortunately, as the present time, there remains to not be sufficient knowledge about the specific markers associated with differentiating EVs and how this is affected by 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. There was identification of morphologically distinct subtypes of sEVs: spherical or oval in shape and with electron dense or translucent contents. Extracellular vesicles morphologically similar to these 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 depicted in Figure 2 are indicative of sEVs containing some of these morphological subtypes. These studies confirm there is a diversity of EVs in the SP-population and Höög and Lötvall (2015) postulated that each subtype of sEVs were from a specific cellular origin.

At present, there are limited reports in which there are data reported for characterizing the membrane of sEVs and very few of these studies were performed in pets and livestock. The only noteworthy study is that of Piehl et al. (2006) where there was reporting on the characterization of the membrane of EVs and sperm isolated from the sperm-rich fraction (SRF) of boar ejaculates and identification that there was a large concentration of cholesterol and sphingomyelin similar to that of the sperm membrane contents for these compounds. One of the most interesting techniques used to characterize EVs is the specific markers because these markers allow for procedures to be utilized to ascertain the differentiation of EVs from other co-isolated nanoparticles and can also be utilized to 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 SP of boars, which could provide insights into the differences in cells producing the EVs and in the contents and also target cells of sEVs because tetraspanins have functions in the selective binding of EVs to target cell membranes (Gurung et al., 2021). In boar semen, Alvarez-Rodriguez et al. (2019) utilized cytometric procedures to determine which sEVs contained CD44, a cell surface protein that is important in cell-to-cell interaction and adhesion. Interestingly, the percentage of CD44-positive sEVs varied according to objectively collectable ejaculate fractions (10 first mL of SRF, rest of SRF and post-SRF), being proportionally greater in the first 10 mL of SRF. There were suggestions, based on the findings, that these CD44-positive sEVs are produced by the epididymis, because the SP of the first 10 mL of SRF comes mostly from the epididymal cauda (Rodriguez-Martinez et al., 2021a). There has also been attempts to characterize chicken sEVs with results indicating there were few sEVs and that these did not contain either CD9 or CD44 proteins (Alvarez-Rodriguez et al., 2020), but these earlier findings are inconsistent with the more recent findings of Cordeiro et al. (2021). In summary, the results from studies clearly indicate the SP contains a heterogeneous mixture of EVs, which would be produced by different cells, have different contents and probably also have different target cells. For example, Sahlén et al. (2010) reported that in men there were specific markers such as CD10, CD13 and CD26 that were 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 these compounds from natural inactivators in body fluids (e.g., proteases and nucleases in SP). The complex contents of sEVs is customized by the

secretory cells for delivery to specific target cells. Consequently, there may be substantial differences in the contents of EVs among body fluids. For example, results from a study in cattle in which there was comparison of EVs, indicated there were differences in protein contents if EVs were isolated from milk or blood plasma (Koh et al., 2017). There are a few studies in which there has been evaluation of sEV contents in pets and livestock and they have focused mainly on proteomic and transcriptomic profiling. In the proteomics area, there have been two large-scale studies that have recently been performed, namely, the study of Leahy et al. (2020) of sheep sEVs and Rowlison et al. (2020) with sEVs of cats. There was identification of a total of 520 and 3,008 proteins, respectively. The results of the study by Leahy et al. (2020) indicated sEVs of sheep 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 capacity. Results from the study by Rowlison et al. (2020) that focused on epididymal EVs of domestic cats, comparing the proteome of EVs isolated from different epididymal segments, indicated there were several EV-proteins were differentially abundant between segments of the epididymis. Some of these proteins are related to the epididymal sequential maturation of spermatozoa, specifically with acquisition of motility and capacity to bind to the zona pellucida (ZP). Similar results were previously reported 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) with the first reported by Gatti et al. (2005) in EVs collected from cauda epididymis of sheep. In this study, there was comparison of 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 collected using ultra-centrifugation procedures, i.e., EVs-free), SP,

cytoplasmic droplets, and mature spermatozoa, and results indicate there were protein bands of epididymal cauda EVs that were singular and different from that of other samples. The most abundant 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 having many unique spots, which were consistent in gel migration to that of specific proteins such as heat shock protein HSP90B1 and HSPA5, with both of these proteins being relevant for oocyte fertilization (Dun et al., 2012). In contrast, other proteins related to sperm functions, 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 noteworthy that sEVs contain immunoregulatory proteins, such as transforming growth factor β isoforms 1-3 (Barranco et al., 2019). Piehl et al. (2013) analyzed the protein composition of boar sEVs, with there being identification of a total of 28 distinct proteins using MALDI-TOF procedures (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry mass spectrometry). The proteins were grouped as structural proteins (mainly actin), enzymes, intracellular ion channels and spermadhesins, the most abundant proteins in boar 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, such as with the boar sEVs (Piehl et al., 2013), and with similar banding pattern among the four species. Protein bands, however, stained differentially among species, with bands from dog sEVs less stained.

Using transcriptomics, four recent studies were conducted that focused on evaluating the miRNA contents of bull, boar and stallion sEVs. In bulls, Alves et al. (2021) evaluated the contents of miRNAs of sEVs, identifying 380 miRNAs. There was a listing of all miRNAs provided but there was not information provided regarding the possible associations with reproductive functions because that was not the goal for conducting the study. In boar sEVs, Xu et al. (2020) identified 325 miRNAs, predicting reproductive functions for some of these miRNAs. Specifically, there were functions of spermatogenesis (ssc-miR-148a-3p; ssc-miR-10a-5p) and fertility (miR-10b, miR-191, miR-30d, and let-7a), with one of these (ssc-miR-200b) particularly related to the number of piglets born per litter. It was also noted that boar sEVs are rich in PIWI-interacting RNAs (piRNAs = 19,749 in number), although there was no evaluation of associations of these piRNAs with any reproductive function because of the lack of consultative databases. Also in boars, Zhang et al. (2020) evaluated the miRNA contents of EVs isolated from urine, blood plasma, SP and bile, and reported that all isolated EVs contained well-defined miRNAs related to immune functions. Also recently, Twenter et al. (2020) evaluated the miRNA contents of stallion sEVs from the caput, corpus and cauda epididymis, and reported that some of the miRNAs that were identified had putative functions in sperm motility and viability and also in oocyte maturation and embryo development. It was also reported that epididymal EVs have miRNA contents from epididymal epithelial cells that are transferred to maturing spermatozoa as these are circulating through the epididymis. In addition to this transfer of miRNAs to maturing sperm, sEVs are also involved in the transfer of contents to mature sperm, including miRNAs, after ejaculation as long as the sperm remain surrounded by SP (Trigg et al., 2019). Together the results from these proteomic and transcriptomic studies clearly indicate sEVs encapsulate biomolecules that modulate sperm functions and also indicate

the biomolecules contained in the sEVs varies with the tissues secreting and between species producing the sEVs. Besides these two variables, there are other factors influencing the contents of sEVs. The contents of sEVs would be testosterone-dependent, and the sEVs released when testosterone concentrations are basal would be less effective in modulation of sperm functions (Ma et al., 2018). Similarly, environmental factors, such as excessive air temperature, would also have effects on the contents of sEVs, at least on the content of miRNAs, as reported for 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 processes, namely, binding, fusion and content 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 contents to sperm. Two alternative delivery mechanisms are currently contemplated; 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 binding (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 sEVs of sheep (Leahy et al., 2020), could also be relevant for efficient trafficking of biomolecules between sEVs and sperm (Trigg et al., 2021). It is also

noteworthy that the sEVs, in addition to transferring contents to the sperm, could also be involved in processes that lead to removal of "non-useful" proteins from the sperm membranes. Leahy et al. (2020) reached this conclusion after analyzing the protein contents of sheep sEVs and spermatozoa. The interaction between sEVs and sperm would be transient, and the sEVs would bind, fuse, interchange contents and detach.

Some sEVs bind to sperm immediately after release, during the period of transport of sperm through the male reproductive duct system. Others are free in the SP and are transported together with spermatozoa at the time of ejaculation. Some of these free sEVs bind to sperm after ejaculation (Du et al., 2016) and others do so after transport into the female reproductive tract after mating or AI (Aalberts et al., 2013). Interestingly, Aalberts et al. (2013), in an experiment conducted with stallion sEVs, proposed that the three sequential interaction processes, namely binding, fusion and content-release or -exchange, would not occur in a timely sequence after the sEVs bind to sperm in the female reproductive tract. There was postulation that the binding would occur in the uterus and the fusion in the oviduct, shortly before fertilization, under influence of progesterone released at ovulation. The pH of the environment would be a modulating factor of sEV-sperm interaction although the matter is still controversial. In humans, Murdica et al. (2019a) stated that sEV-sperm binding would occur at a neutral pH and fusion at an acidic pH, which occurs in the vagina, the site of semen deposition during intercourse in humans. This would be feasible in species with vaginal deposition of semen but not in those where there is semen deposition in the cervix or uterine body, as occurs in most farm animals. In horses, Aalberts et al. (2013) reported that the binding of sEVs to viable sperm was optimal at pH of 7.5 to 8.0. Noteworthy to remember is that the spermatozoa entering the cervix in humans are those present in the prostate-dominated SP, the non-coagulating first portion 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. Bull (Schwarz et al., 2013) and ram (Gatti et al., 2005) EVs from the caput epididymis have more fusogenic affinity for spermatozoa than those from the cauda segment. Furthermore, among epididymal cauda EVs, CD-9-positive EVs would be those for which there was transfer of contents to spermatozoa (Caballero et al., 2013) and for such transfer to occur there would be actions of dipeptidyl peptidase-4 protein, also known as CD-26, that would be required. Interestingly, the epididymal EV-population lacking CD9 has a greater affinity for non-viable sperm, transferring epididymal sperm-binding protein 1 to these cells (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, being greater in the caput and less in the cauda, as occurs in sheep (Gatti et al., 2005). Such binding, however, is also greater between caput EVs and cauda spermatozoa when these are cultured *in vitro* (Frenette et al., 2010). These findings indicate epididymal sperm are more or less likely to bind with sEVs depending on stage of maturation of these spermatozoa. There would also be selectivity with sEV binding to sperm because of specificity of binding sites. Sperm have three structurally well-defined compartments, namely the head, mid-piece and tail, each of these 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 have an affinity for all head membrane domains (acrosome ridge, acrosome, and post-acrosome) (Aalberts et al., 2013; Du et al., 2016). In this regard, sEVs bind to sperm in the three main sperm compartments (Figure 3). The different binding site would be linked to the functions and those sEVs 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 effect on mitochondrial activity, energy metabolism and motility.

4. Involvement of seminal EVs in sperm maturation and functionality

Sperm maturation occurs during the transport through the epididymis and is modulated 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 capacity for forward motility and for the fertilization of oocytes (Björkgren and Sipilä, 2019). Important factors in this interaction are the sEVs released in the epididymis, the so-called epididymosomes, that are involved in the transfer of bioactive molecules to maturating sperm for the acquisition of forward motility and the capacity to fertilize the oocyte (Sullivan, 2015). Results from research conducted in bulls indicates epididymosomes have dual effects on sperm maturation (Belleannée et al., 2013). The first, more direct, is by fusing with the membrane of maturing sperm and transfer of contents into sperm. The second, indirect action, is by interacting with epithelial epididymal cells through paracrine actions to modulate secretions to provide an optimal epididymal environmental milieu for sperm maturation. The epididymal environmental milieu and the involvement of epididymosomes in sperm maturation are discussed in more detail in another review in this Special Issue of *Animal Reproduction Science* (Rodriguez-Martinez et al., 2021b).

Most studies relating sEVs and sperm functional parameters have been conducted in humans and mostly in men where there are marked alterations of values for seminal variables, such as oligozoospermia, azoospermia, asthenozoospermia and teratozoospermia (Candenas and Chianese, 2020). Highlighted is the study by Murdica et

al. (2019b), where results indicated there were effects 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, sEVs from normozoospermic men but not from asthenozoospermic men, enhanced sperm motility and induced capacitation. This differential function of sEVs would be related to differences in the abundance of proteins and miRNAs involved in reproductive processes that are contained in 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 producing ejaculates with satisfactory sperm quantity and quality, while those with lesser semen quality are culled.

The results from the few studies in pets and farm animals relating sEVs and sperm functions indicated sEVs would have effects on sperm 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) reported there were 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 this inconsistency in findings regarding sperm motility, results from both studies were consistent in that sEVs stabilized sperm membranes and prevented premature capacitation and consequent acrosome exocytosis. In an earlier study with pigs, Siciliano et al. (2008) reported the acrosome rupture was induced in sperm incubated with sEVs. In a study conducted with stallion semen, Aalberts et al. (2013) reported that incubation of ejaculated sperm with sEVs did not have effects on the timing of capacitation. In pets, Mogielnicka-Brzozowska et al. (2015) reported that

the total and progressive motility of dog sperm improved after incubation with sEVs. The mechanism of action of sEVs in affecting sperm motility would be related to the regulation of the sperm intracellular Ca^{2+} concentrations (Palmerini et al., 1999; Park et al., 2011). Recently, Zhang et al. (2021) proposed that sEVs would have functions in activating a cation channel of sperm (CatSper), which regulates motility during capacitation-related processes (Vicente-Carrillo et al., 2017). Other EV-mechanisms could also be involved. For example, sEVs synthesize ATP through glycolysis and this ATP would modulate sperm mitochondrial metabolism and, consequently, sperm motility (Guo et al., 2019). Furthermore, sEVs would control the transfer of zinc ions into 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 process in sperm capacitation. Aalberts et al. (2013), however, conducted an experiment incubating ejaculated spermatozoa of stallions with sEVs with results indicating sEVs would have limited effects on tyrosine phosphorylation. The results for these studies in pets and livestock were inconsistent regarding the effects of sEVs on sperm functions, and results were also inconsistent in studies performed in humans (Foot and Kumar, 2021). There are several explanations for these inconsistencies, the most plausible being differences in methodologies utilized between studies to isolate sEVs and the intrinsic diversity in the contents and membrane composition of isolated sEVs. The isolation methods used in the studies did not result in a similar 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 factor contributing to inconsistent results 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 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 reproductive tract, providing for a very complex and yet, little understood mode of cellular communication.

Successful long-term semen preservation in mammals still remains a challenge. Current sperm freezing-thawing methods, even the most efficacious, remain suboptimal, because by imposing these procedures there is inducing of structural as well as biochemical and functional changes in sperm, impairing functions of sperm after thawing, including fertilization capacity (Khan et al., 2021; Kumar et al., 2019; Yeste, 2016). To the best of our knowledge, there is only one study where there has been exploration of the potential of EVs to mitigate the detrimental effects of freezing-thawing processes on spermatozoa. The results of the study of Rowlison et al. (2021) conducted in domestic cats indicate that frozen-thawed sperm improved motility after thawing when incubated with epididymal EVs. In a number of studies, there was investigation of the usefulness of EVs secreted outside the male reproductive tract in improving sperm cryopreservation (reviewed by Saadeldin et al., 2020). Results from *in vitro* experiments conducted by Alcantara-Neto et al. (2020) indicted the effectiveness of pig oviductal EVs for improving the survival of thawed boar sperm. Similar results were reported by De Almeida Monteiro Melo Ferraz et al. (2020) when frozen-thawed spermatozoa from red wolves and cheetahs were incubated with dog and cat oviductal EVs, respectively. Mesenchymal cell derived EVs also have been effective in these regards. Qamar et al. (2019) reported that there was greater motility and integrity of plasma and acrosomal membranes of frozen-thawed dog sperm by adding mesenchymal cell-derived EVs to the freezing medium. Similar results were also reported by Mokarizadeh et al. (2013) for mouse sperm. The results from

studies did not indicate there were causal mechanisms for these outcomes, but Qamar et al. (2019) attributed the positive effect to the capacity of EV contents to repair sperm membranes and reduce oxidative stress associated with cryopreservation. In that study, there were changes in the expression of some genes associated with membrane repair, modulation of mitochondrial reactive oxygen species and chromatin integrity. Mokarizadeh et al. (2013) also reported that there was an increased abundance of specific EV biomolecules in the membranes of thawed spermatozoa, namely CD29, CD44, ICAM-I and VCAM-I. Not all EVs, however, would have positive effects on sperm functions. Extracellular vesicles from human embryonic kidney-derived cells, a scalable cell line used for mass EV-production, did not have effects on the functions 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 marked differences in sperm viability and motility, and ejaculates from more fertile males having smaller sEVs than those from less fertile males. It was also reported that there were compositional differences between sEVs, with there being a larger abundance of HSP90AA1 in the sEVs isolated from more fertile males. In addition to having effects on the functions of sperm and thus male *in vivo* fertility, sEVs would also contribute to the fertility of males through interactions with the epithelial cells of the female reproductive tract after mating or insemination (Figure 4). Seminal EVs have the capacity to bind and be internalized by the endometrial cells (Paktinat et al., 2019). Bai et al. (2018) reported, in an *in vitro* experiment, that pig sEVs had the capacity to increase the production of proteins related to immune and inflammatory responses in endometrial epithelial cells. Accordingly, sEVs would have essential functions in regulating the immune response of the female reproductive tract, facilitating the survival and functions of sperm and

subsequent embryo and placental development. It is noteworthy 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

Results from studies addressed in this review indicate 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 fluids. This lack of knowledge is particularly striking for sEVs present in the SP of pets (dog and cat) and farm animals. Summarizing the results from the few research studies that have been conducted, it seems clear the epithelia of the male reproductive system releases EVs, including the testis, epididymis, vas deferens ampulla and some accessory sex glands, and that these tissues do so mainly through apocrine mechanisms. The released sEVs would bind to and regulate secretory cells in close proximity, with the paracrine pathway being the mode of action, spermatozoa and cells of the functional tissues of the female reproductive tract, following mating or insemination. In sperm, sEVs bind, fuse with the plasma membrane and transfer contents that, based on the current knowledge, would affect epididymal maturation, motility and capacitation. Furthermore, sEVs would also have functions in the removal of non-functional proteins from spermatozoa. Once inside the female reproductive tract, the sEVs would be bound and internalized by the epithelial cells modulating the immune response against spermatozoa and embryos. The limited results that have been reported provide valuable information on sEVs, but many of these findings remain open to speculation and, therefore, findings need to be evaluated 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 sEVs 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, contributing to some inconsistent and sometimes contradictory results, making comparisons difficult and limiting clinical usefulness (Mercadal et al., 2020). In addition, isolation methods should be able to isolate separately the different subtypes of EVs present in SP, because each subtype may have different contents of active biomolecules and thus have different effects on target cells. These studies would allow for characterization of the different subtypes of EVs present in SP and allow for 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 functions and male fertility, which currently remains unclear and controversial.

Finding biomarkers of male fertility remains a challenge, both in domestic animals as well as humans. Seminal plasma biomolecules have effects on sperm functions, 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 postulated as candidates for biomarkers of sperm functions and male fertility (Rodriguez-Martinez et al., 2021a). It is known that some of these seminal biomolecules are encapsulated in sEVs, where these remain active by being protected from the natural inactivators present in SP (e.g., proteases and nucleases). Furthermore, sEVs bind and interchange molecules with spermatozoa and epithelial cells of the endometrium. Overall, these findings certainly indicate the sEVs are important candidates for use as biomarkers of sperm functions and male fertility. The emphasis on search for biomarkers in seminal EVs has been negligible, unlike those circulating/present in other body fluids such as in

blood plasma or urine, which have been widely explored for use as biomarkers for diverse pathologies, including 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 these were conducted in humans (Barceló et al., 2018; Larriba and Bassas, 2021; Vickram et al., 2020). Consequently, determining whether sEVs are useful biomarkers of fertility is an exciting challenge. Before addressing this task, it is imperative to fully characterize all subtypes of vesicles circulating in the male reproductive 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 these can be used as therapeutic agents (Peng et al., 2020; Sil et al., 2020). The sEVs from normozoospermic ejaculates improve sperm motility while those of asthenozoospermic ejaculates reduce sperm motility (Murdica et al., 2019b). These findings raise the possibility of using sEVs to improve sperm quality in individuals with idiopathic poor sperm quality. The sEVs can improve sperm freezability (Qamar et al., 2019). In some farm animals there are marked differences between sires in sperm freezing capacity, reducing the inclusion of males with less-than-optimal sperm freezing capacity in semen cryobanks (Roca et al., 2006). The sEVs could be used to improve sperm cryotolerance of males with sperm that have less-than-optimal freezing capacity by supplementing the freezing medium with sEVs from males with relatively greater sperm freezing capacity. In this case, EVs can be artificially enriched with specific molecules. Specific subtypes of sEVs could be loaded with beneficial molecules of interest using proven loading 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 functions of these cells. Complete characterization of sEV subtypes will also facilitate further studies for designing and producing synthetic EVs, structurally similar to those of SP, which would contain specific biomolecules for particular applications. For example, as additives to semen extenders for improving both sperm preservability and/or *in vivo* fertility of AI-doses. These synthetic EVs added to 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).

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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

Fig. 1. Schematic depictions of the mechanism of apocrine secretion, including formation of apical vesicles and the processing of large released and decaying vesicles in the lumen of the reproductive tract of the male pig (segments of the epididymis and accessory sex glands) to subsequent release of extracellular vesicles. The drawing was created in BioRender.com.

Fig. 2a-b. Transmission electron micrographs showing extracellular vesicles from boar seminal plasma, and the variation in size and shape. Extracellular vesicles were isolated using ultrafiltration (0.22 μm plus Amicon®-100K) and size exclusion liquid chromatography procedures (Barranco et al., 2021). The arrows identify some morphological subtypes of seminal extracellular vesicles according to the classifications described 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 produced by the authors were generated at the Central Experimental Research Service (SCSIE) of the University of Valencia.

Fig. 3a-c. Transmission electron micrographs showing extracellular vesicles bound to different boar sperm membrane domains in the head (a), neck (b) and tail (c). Images produced by 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.

Fig. 4. Depiction illustrating the seminal extracellular vesicle-releasing tissues in the male reproductive tract and the putative functions of released seminal extracellular vesicles on both the spermatozoa, male and 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.