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

3

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25 **ABSTRACT**

26

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

47

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

## 50 **1. Introduction**

51

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

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

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

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

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

102

## 103 **2. Biogenesis and characterization of seminal extracellular vesicles**

104

### 105 *2.1. Biogenesis*

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

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

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

146

## 147 2.2. Characterization

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

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

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

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

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

200 biogenesis, metabolism, and membrane adhesion and remodeling functions, the latter  
201 including several reproductive-specific proteins directly related to sperm fertilizing  
202 ability. The study by Rowlison et al. (2020), focused on epididymal EVs from domestic  
203 cats, comparing the proteome of EVs isolated from different epididymal segments and  
204 showing that the expression of several EV-proteins changes between segments. Some of  
205 these proteins are related to the epididymal sequential maturation of spermatozoa,  
206 specifically with their acquisition of motility and their ability to bind to the zona pellucida  
207 (ZP). Similar results were previously obtained by Girouard et al. (2011) on EVs isolated  
208 from the caput and cauda of the bull epididymis. In addition, there are other studies based  
209 on one- or two-dimensional gel electrophoresis (2-DE) and first reported by Gatti et al.  
210 (2005) in EVs collected from ovine epididymal cauda. They compared the sodium  
211 dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) banding pattern of these  
212 epididymal cauda EVs with that of epididymal cauda fluids (raw fluid and the supernatant  
213 fluid from ultra-centrifugation, i.e., EVs-free), SP, cytoplasmic droplets, and mature  
214 spermatozoa, and reported that the protein bands of epididymal cauda EVs were singular  
215 and different from that of other samples. The most highly expressed proteins in  
216 epididymal cauda EVs were grouped as membrane-bound proteins, metabolic enzymes  
217 and cytoskeleton-associated proteins. Frenette et al. (2006) compared the protein profile  
218 of EVs collected from caput and cauda bull epididymis and from ejaculated semen. The  
219 protein 2-DE profile varied among the sources of EV-origin, with those from caput  
220 epididymis showing many unique spots, which matched specific proteins such as heat  
221 shock protein HSP90B1 and HSPA5, with both relevant for oocyte fertilization (Dun et  
222 al., 2012). In contrast, other proteins related to sperm functionality, such as P25b, a  
223 protein involved in the binding of sperm to the ZP (Caballero et al., 2010), were only  
224 present in EVs isolated from epididymal cauda and ejaculates. It is worth mentioning that

225 sEVs carry immunoregulatory proteins such as transforming growth factor  $\beta$  isoforms 1-  
226 3 (Barranco et al., 2019). Piehl et al. (2013) analyzed the protein composition of porcine  
227 sEVs, identifying a total of 28 distinct proteins by MALDI-TOF (Matrix Assisted Laser  
228 Desorption/Ionization Mass Spectrometry mass spectrometry). The identified proteins  
229 were grouped as structural proteins (mainly actin), enzymes, intracellular ion channels  
230 and spermadhesins, the most abundant proteins in porcine SP (Rodriguez-Martinez et al.,  
231 2021a). Ronquist et al. (2013) compared the SDS-PAGE banding patterns of sEVs from  
232 four species, namely human, canine, bovine and equine. Most of the protein bands were  
233 within the molecular weight in the range of 10 to 150 kDa, as in pig sEVs (Piehl et al.,  
234 2013), and with similar banding pattern among these species. However, there were  
235 differentially expressed protein bands as in the case of canine EVs, where bands were  
236 expressed with less intensity.

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

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

265

### 266 **3. Interaction between seminal EVs and spermatozoa**

267

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

275 entirely clear how sEVs deliver their contents to sperm. Two alternative delivery  
276 mechanisms are currently contemplated; using either direct membrane fusion or the  
277 formation of transient fusion pores (Björkgren and Sipilä, 2019). The first mechanism  
278 would involve tetraspanins, such as CD9, and integrins to promote competent fusion sites  
279 after glycosylphosphatidylinositol-anchored mediated docking (Al-Dossary et al., 2015).  
280 The second mechanism would involve the mechanoenzyme dynamin 1 in the formation  
281 of transient fusion pores (Zhou et al., 2019). Milk fat globule factor 8 (MFG8) protein,  
282 identified in ovine sEVs (Leahy et al., 2020), could also be relevant for efficient  
283 trafficking of biomolecules between sEVs and sperm (Trigg et al., 2021). It is also worth  
284 mentioning that the sEVs, in addition to delivering their contents to the sperm, could also  
285 remove "non-useful" proteins from the sperm membranes. Leahy et al. (2020) reached  
286 this conclusion after analyzing the protein contents of ovine sEVs and spermatozoa. Then,  
287 the interaction between sEVs and sperm would be transient, and the sEVs would bind,  
288 fuse, interchange and detach.

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

300 interaction although it is open to controversies. In humans, Murdica et al. (2019a)  
301 indicated that sEV-sperm binding would occur at neutral pH and fusion at acidic pH,  
302 which occurs in the vagina, the site of semen delivery during intercourse in humans. This  
303 would be feasible in species with vaginal deposition of semen but not in those with  
304 deposition in the cervix uteri and uterine body deposition, as it occurs in most farm  
305 animals. In equine, Aalberts et al. (2013) demonstrated that the binding of sEVs to viable  
306 sperm was optimal at pH of 7.5-8.0. Of note, to remember is that the spermatozoa entering  
307 the cervix in human are those present in the prostate-dominated, non-coagulating first  
308 part of the ejaculate, while those sperm in the vagina are entrapped in a coagulum formed  
309 by semenogelins, and not necessarily involved in fertilization (Rodriguez-Martinez et al.,  
310 2011).

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

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

337

#### 338 **4. Involvement of seminal EVs in sperm maturation and functionality**

339

340 Sperm maturation occurs during their journey through the epididymis and is  
341 orchestrated by the sequential interaction of maturing sperm with changing intraluminal  
342 fluids. This interaction leads to structural and compositional changes that enable sperm  
343 to acquire the ability to move forward and fertilize the oocytes (Björkgren and Sipilä,  
344 2019). Key players in this interaction are the sEVs released in the epididymis, the so-  
345 called epididymosomes, that deliver bioactive molecules to maturing sperm for the  
346 acquisition of forward motility and the ability to fertilize the oocyte (Sullivan, 2015).  
347 Research conducted in bovine showed that epididymosomes influence sperm maturation  
348 in two ways (Belleannée et al., 2013). The first, more direct, is by fusing with the  
349 membrane of maturing sperm and delivering their contents to them. The second, indirect,

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

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

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

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

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

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

413         Successful long-term semen preservation in mammals still remains a challenge.  
414 Current sperm freeze-thaw methods, even the most successful, remain suboptimal, as they  
415 induce structural as well as biochemical and functional changes in sperm, impairing their  
416 functional performance after thawing, including fertilization capacity (Khan et al., 2021;  
417 Kumar et al., 2019; Yeste, 2016). To date, to our knowledge, there is only one study that  
418 has explored the potential of EVs to mitigate the detrimental impact of freeze-thawing on  
419 spermatozoa. The study of Rowlison et al. (2021) conducted in domestic cats showed that  
420 frozen-thawed sperm improved motility after thawing when incubated with epididymal  
421 EVs. However, a number of studies investigated the usefulness of EVs secreted outside  
422 the male genital tract in improving sperm cryopreservation (reviewed by Saadeldin et al.,  
423 2020). *In vitro* experiments conducted by Alcantara-Neto et al. (2020) demonstrated the  
424 effectiveness of porcine oviductal EVs for improving the survival of thawed pig sperm.

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

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

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

459

## 460 **5. Conclusions and targets for future research**

461

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

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

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

494 Finding biomarkers of male fertility remains a challenge today, both for domestic  
495 animals as well as for humans. Seminal plasma biomolecules influence sperm  
496 functionality, embryo development, and implantation (Bromfield, 2018; Druart et al.,  
497 2019; Pérez-Patiño et al., 2018; Szczykutowicz et al., 2019). Consequently, some SP-  
498 biomolecules have been posted as candidates for biomarkers of sperm functionality and  
499 male fertility (Rodriguez-Martinez et al., 2021a). We now know that some of these

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

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

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

537

### 538 **Ethical Statement**

539 The experiments with animals and specimens in the aforementioned studies developed by  
540 the authors of this review were performed according to the European Directive  
541 2010/63/EU, 22/09/2010 for animal experiments and approved by the Bioethics  
542 Committee of Murcia University (research code: 639/2012).

543

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554

555 **Author contributions**

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

558

559 **Declaration of Competing Interest**

560 The authors declare that they have no conflicts of interest. The funders had no influence  
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562

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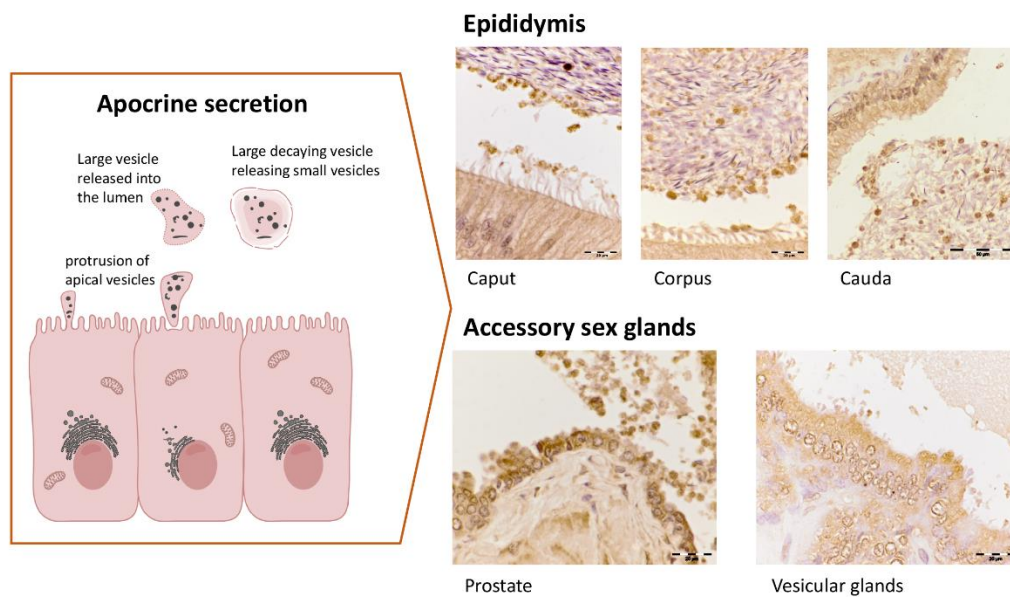
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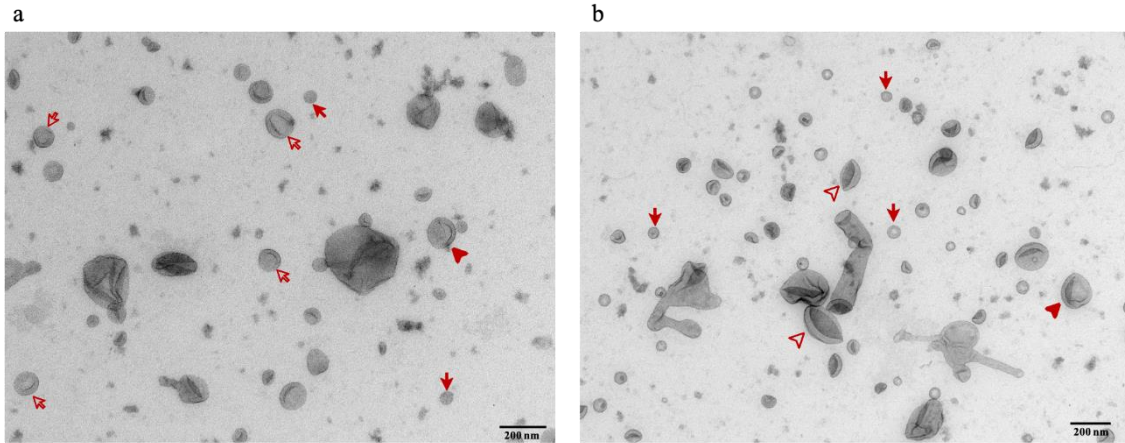
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 972

973 **Figure legends**



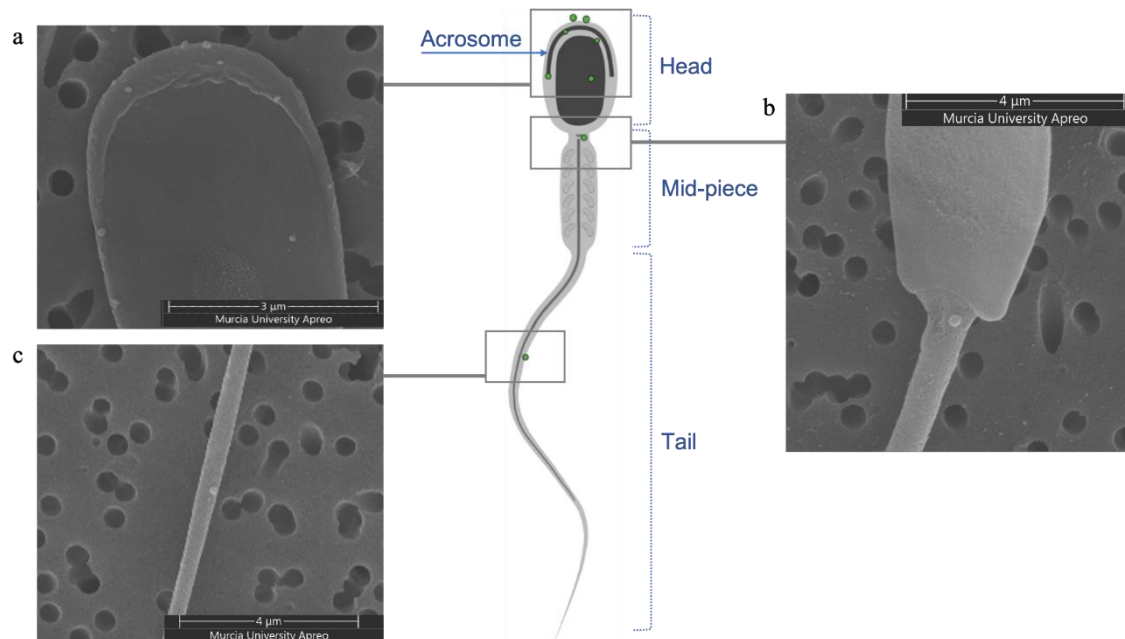
974

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



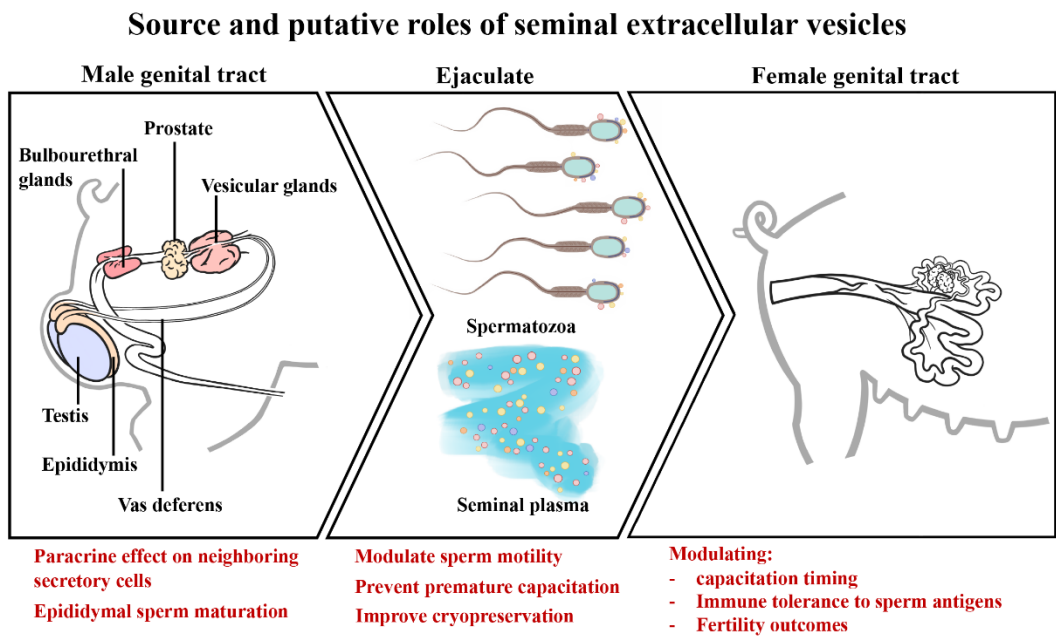
980

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



990

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



996

997 **Figure 4.** Scheme illustrating the seminal extracellular vesicle-releasing organs in the  
 998 male reproductive tract and the putative functions of released seminal extracellular  
 999 vesicles on both the spermatozoa, the male and the female reproductive tracts. The  
 1000 putative functions of sEVs are those reported in scientific studies in pet and livestock  
 1001 species. Drawings were created in BioRender.com.

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