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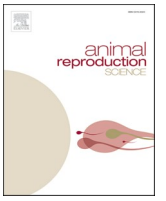

Extracellular vesicles in seminal plasma: A safe and relevant tool to improve fertility in livestock?

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ABSTRACT

Seminal plasma (SP) is not a pre-requisite for pregnancy. Yet, this heterogeneous, composite SP has proven relevant for fertility, as mediator for cell-to-cell communication between producing cells, spermatozoa and the female internal genital tract, regulating complex reproductive processes. Bearing hormones, proteins, cytokines as well as nuclei acids in nano-sized lipid bilayer seminal extracellular vesicles (sEVs), the SP concerts signaling to the female. Signals influence timing of ovulation, sperm transport and, particularly, enable the female immune system to balance her cryptic choice to engage into pregnancy or reject an eventual fertilization. This essay, focusing on livestock in general and pigs in particular, discusses the intrinsic roles of sEVs with regards to reproductive homeostasis, while binding and internalizing their cargo in spermatozoa and female tract epithelia to regulate their functional activity. Since prior studies had inconclusive results using bulk SP or single SP-contained free molecules, argumentation is hereby provided to increase the current incipient research on livestock sEVs, where fragile molecules relevant for fertility are shielded from degradation during handling. Seminal EVs isolated from SP can be used for andrological diagnosis and perhaps to select breeders with optimal fertility. Moreover, sEVs can be laboratory-uploaded with specific molecules or even engineered as lipid nanodroplets used as additives for extenders to improve fertility after artificial insemination (AI) or reproductive biotechnologies.

1. Introduction

Once we embrace theriogenology (greek *therio*: animal, *gen*: generation, *ology*: study of) as a working discipline, we study the ultimate purpose of any species: to reproduce. Capacity to procreate is not always optimal, often constrained by disorders and the iatrogenic pressure we impose on either females or males when using assisted reproductive techniques (ARTs) (Siqueira et al., 2019; Wang and Ibeagha-Awemu, 2021). If one selects andrology as branch within theriogenology, the interest focuses on what rules the fertility of a male, aiming to safely identify sub-fertile sires and exclude them from programmed breeding, i.e., artificial insemination (AI). Obviously, the first step usually is to clinically determine the capacity of the male to detect estrus and to mate followed by

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controlling the quality of the semen produced, upon which we expect to foresee potential fertility. This prognosis seems utopic still today (Rodriguez-Martinez, 2019), particularly when desiring to disclose levels of fertility for sires genotypically selected even prior to puberty (Taylor et al., 2018).

The development of AI has both eased breeding and advanced genetic selection. To favour gene dissemination from best sires, attempts were made to increase the number of AI-doses of liquid, cooled or deep-frozen semen produced per ejaculate, yet maintaining similar levels of fertility as after natural mating. Such plan, intended for all livestock, remains burdened by species- and individual-differences observed when lowering sperm numbers per AI-dose, likely by the modification of the fluids surrounding the spermatozoa (de Andrade et al., 2022). In most livestock species, the original seminal plasma (SP) is largely extended, whereas in others -as goats or humans- even eliminated (Chang, 1957; Pavaneli et al., 2019), questioning is role or its potential damaging effects for cryopreserved semen or when performing in vitro fertilization (IVF) (reviewed by de Andrade et al., 2022). For IVF, epididymal or ejaculated, washed spermatozoa, i.e., spermatozoa deprived from almost all SP are customarily used (Henkel, 2012). Eggs are fertilized, viable embryos are produced in vitro, and embryo transfer is successful, evidence that the exposure to SP is simply not essential for a pregnancy generated via ARTs (Sakkas et al., 2015; Leung et al., 2022; Rodriguez-Martinez et al., 2021).

So, why does SP exist, at all? Why did animals with internal fertilization evolve having ejaculates built by emitted aliquots of epididymal spermatozoa embedded in a mixture of secretions from accessory sexual glands? Why does this composite SP, containing electrolytes, proteins, enzymes, hormones, and discrete, nano-sized membrane-bound extracellular vesicles (EVs) of epithelial (epididymal or sexual accessory gland) origin (Roca et al., 2021; Rodriguez-Martinez et al., 2021), accompany the emitted spermatozoa to the species-specific site of semen deposition? Evidence indicates so far that optimal fertility after natural mating depends on semen quality and the capacity and interest of the female to support a successful gestation (Firman et al., 2017; Schjenken and Robertson, 2020). Using AI, optimal fertility can also be obtained when a certain volume of SP from a fertile male, or even from specific SP-fractions, embeds spermatozoa maintaining their homeostasis (Rodriguez-Martinez et al., 2021a). This "SP-pheromone" (Robertson and Martin, 2022) signals to the female epithelial lining which reacts, in the species studied to date, by modulating timing of ovulation (Adams and Ratto, 2013), handling uterine inflammation (Rozeboom et al., 2000), tolerating the foreign spermatozoa in the oviduct (Alvarez-Rodriguez et al., 2019a, 2020a) and, beyond fertilization, enabling the female to decide whether to embark into a rather resource-demanding endeavor, as pregnancy to term is, or to resume cyclicity (Schjenken and Robertson, 2020).

There is also evidence, although we still ignore many underlying mechanisms, that the SP can, per se, i.e., if added separately from spermatozoa, modulate fertility (de Andrade et al., 2022). Embryo survival, implantation, placental development, farrowing and litter size improved when additional homologous/pools of SP were infused to the female genital tract of several species (reviewed by Rodriguez-Martinez et al., 2021).

Yet, some of these studies have not been duplicated (Parrilla et al., 2022), indicating that the use of a composite, complex fluid suspension as the SP requires further consideration. Indeed, identifying what it is in SP that induces these changes is mandatory. Logically, the task is major, and many studies have been already done or are undergoing, particularly focusing on the differences between the actions of SP under natural mating, and those registered when the SP is retrieved from collected semen and differentially handled in vitro, preserved, and used later for analyses and/or as additive (de Andrade et al., 2022). We strongly suggest, to diminish reiteration of data being presented and discussed here, that readers consult recent extensive comparative reviews we and our long-lasting co-authors have already published, where we attempted to dissect the composition of the SP, considering various species for their specific reproductive anatomy, mating behavior, and semen deposition sites during mating or AI (Rodriguez-Martinez et al., 2021). We further considered the role of SP's various components, from its proteome to the action of sEVs, both in vivo and when eventually used for semen preservation or in vitro development (Roca et al., 2021).

In this invited assay we intend to critically review our humble current knowledge on the SP and its eventual value for fertility when ARTs, primarily AI, are used. We insist that most free SP-molecules, from hormones to peptides, act on spermatozoa or the female genital lining rapidly in vivo, as they face enzymatical degradation by proteases and nucleases in the SP. Extra-corporeal handling of semen or of separated SP accelerates this degradation of specific biomolecules free in the SP, including peptides, proteins and nucleic acids reported elsewhere as directly related to sperm function and fertility (Roca et al., 2020, 2021). The perspective that such molecules would escape degradation if contained in the apparently phagocytosis-free sEVs obviously makes these vesicles very attractive for further research. One could argue that sEVs are a small component within the SP. Yet, considering these lipid vesicles can preserve most relevant biomolecules for handling under artificial breeding, their importance to enhance andrological diagnostics and further improve fertility after ARTs deserves to be examined in detail.

Our aim is to review: (i) the roles of sEVs in cell-to-cell communication with spermatozoa and the female internal genital tract; and (ii) which molecules they carry can act as pheromone signals to the female, be biomarkers, or whether they can be isolated to be used as additives for fertility improvement. The area is incipient regarding research in livestock and more ought to be done, as identified in a very recent systematic meta-review of the literature in this area (Parra et al., 2022). Having spent our careers mostly working with pig reproduction, we would beg the reader for forgiving our insistence in particularly addressing this species where further research in this area of sEVs is, although initiated, yet to be developed.

1.1. What makes the SP relevant for fertility?

In most (if not all) species with internal fertilization, spermatozoa bathe in a surrounding fluid, from the testis to the ejaculate. The interval from spermiation to ejaculation is variably long depending on species and the intended use of the males, and the fluid changes in composition, particularly during sperm maturation along the epididymis (Rodriguez-Martinez et al., 2022) as well as when the SP is built, is also variable, depending on the species and their sexual accessory glands (Rodriguez-Martinez et al., 2021). In principle, the SP

contains biomolecules secreted by cells of the male reproductive system freely contained in an electrolyte-defined fluid. As well, epithelia of the male reproductive tract deliver, mainly via blebbing apocrine secretion, nanovesicles generically defined as sEVs (Roca et al., 2021; Foot and Kumar, 2021) whose biogenesis and characteristics resemble, but not entirely, those of other body fluids (Salomon et al., 2022). Experimental evidence is registered that the SP components act in concerted action on spermatozoa and the mated female, a concept that must be considered, although most evidence has -for obvious methodological reasons- been gathered by analysis of artificially retrieved SP for different molecules at a time, from the determination of organic to inorganic components (Barranco et al., 2015a,b), hormonal determinations (Padilla et al., 2021), to proteomics (Perez-Patiño et al., 2018), transcriptomics and genomics (Rodriguez-Martinez et al., 2021) or metabolomics (Mateo-Otero et al., 2021). Detailed information is available to assert the SP as a "pheromone" where specific components, as nerve growth factor-ß (NGF-ß), are present and able to stimulate ovulation (Robertson and Martin, 2022). As well, SP-proteins are considered to maintain stability of the sperm plasma membrane issuing a preservation of sperm potential fertilizing capacity during sperm transport and storage in the female genital tract, yet being interactive with other factors, as temperature, pH, specific capacitating ions, and sperm receptors relevant for interaction with the lining epithelium and the oocyte vestments, and ultimately for fertilization (Rodriguez-Martinez et al., 2021). Enzymes are present to keep Reactive Oxygen Species (ROS) concentrations at physiological limits (Parrilla et al., 2020). Other SP-proteins trigger the invasion of polymorphonuclear leukocytes (PMNs) to counteract eventual pathogens and to eliminate defective and surplus spermatozoa and foreign proteins via phagocytosis and enzymatic challenge (Rodriguez-Martinez et al., 2010). Lastly, a relevant number of SP-proteins is significantly associated with male fertility data (Perez-Patiño et al., 2018; Roca et al., 2020). Adding to this somewhat complicated view of actions of the free SP-components on spermatozoa, the SP further influences the female pig immune system (Alvarez-Rodriguez et al., 2019a, 2020a) via fragile and short-lived cytokines (Barranco et al., 2019b), attempting to issue a decisive cryptic female choice, either to reject or to tolerate the presence of foreign cells, the spermatozoa, and of the hemi-allogeneic products of fertilization, the embryo, the fetus and the extraembryonic placenta (Firman et al., 2017; Martinez et al., 2020). Such state of maternal immune tolerance is established promptly after semen deposition and it is to last to term, a most peculiar reproductive phenomenon in eutherian mammals (Schjenken and Robertson, 2020). Such female decision is based on a multitude of factors, considering the substantial resource investment the female embarks in when getting pregnant, including innate immunity (Piccinni et al., 2021). Apparently, the female immune system is able to consider not only the gamete set up, but also components of the SP as essential, components that can elicit dramatic changes in the expression of genes related to sperm function and the immune status of the female organs to warrant fertilization and conceptus development (Alvarez-Rodriguez et al., 2019a, 2020a; Schjenken and Robertson, 2020; Martinez et al., 2020). Needless to say, it seems evident that the SP particularly signals the female immune system, the ultimate ruler for fertility. By now we have simply described the action of free-SP components; however, what about the particulate component, the sEVs?

1.2. Seminal extracellular vesicles and their role in the SP

The SP is far from being a simple fluid containing diverse molecules, a matter the reader is already aware of; it contains a large amount of nano-sized cell-derived proteolipid membrane-bound sEVs, delivered along the process of formation of the SP from the tract

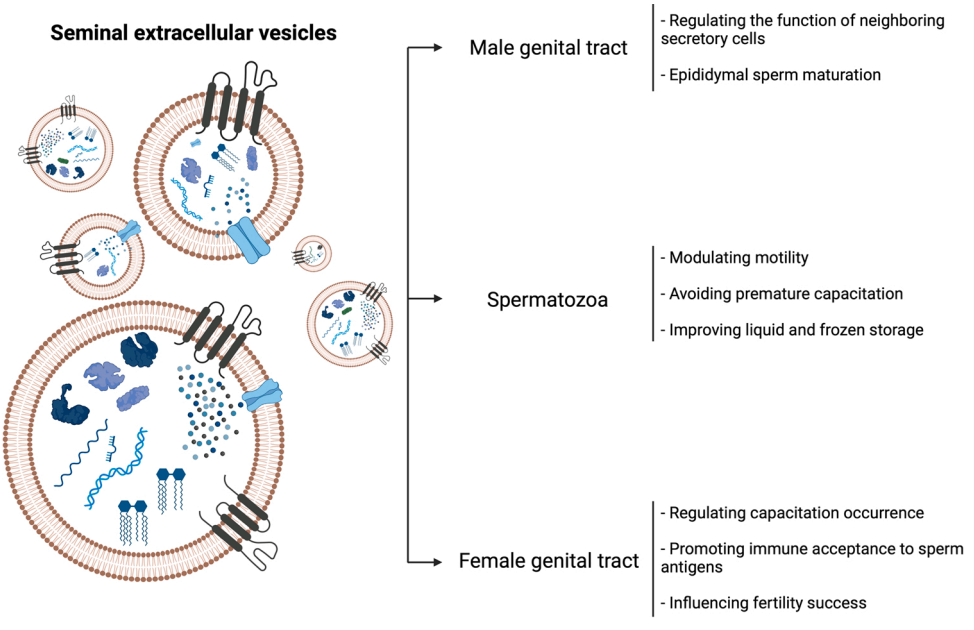

Fig. 1. Assumed functions of seminal extracellular vesicles. The drawings were created in BioRender.com.

and sexual gland epithelial lining, an evolutionarily conserved process (Cordeiro et al., 2021; Ghanam et al., 2022; Aleksejeva et al., 2022). The sEVs are apocrine-shed either as exosomes (processed multivesicular bodies of 30–100 nm diameter released by exocytosis) or as microvesicles (100–1000 nm size out-budded plasma membrane vesicles). The sEVs encapsulate a rather enriched complex load of often fragile biological components, i.e., lipids, signaling proteins, small non-coding and regulatory RNAs (ncRNAs) (Jeppesen et al., 2019; Xu et al., 2020). The sEVs are able to interact (i.e., add, fuse, and internalize) with specific cell targets, becoming information carriers to spermatozoa (Murdica et al., 2019; Foot and Kumar, 2021; Roca et al., 2021) or epithelial cells, particularly those of the female genital tract (Aleksejeva et al., 2022). Per definition, the sEVs delivered by Sertoli cells, efferent duct and particularly the epididymis (their so-called epididymosomes) interact intensely with spermatozoa during their maturational transit, uploading lipids, proteins and RNAs relevant for acquiring essential functional capacities as motility and fertilization (see review by Rodriguez-Martinez et al., 2022 and references therein). At ejaculation, the population of sEVs becomes more heterogenous (Barranco et al., 2019a), with new EVs being shed from the deferent ducts and specific sexual accessory glands. Being difficult to determine the specific source for each type of sEVs when lacking specific identificatory markers, it is perhaps mandatory to reiterate our claim to avoid source-related names and use a basic nomenclature: sEVs (Parra et al., 2022). Overall, as shown in Fig. 1, the concerted mixture of sEVs exert specific actions on capacitation and fertilizing capacity (reviewed by de Andrade et al., 2022) or even the immune responsiveness of the female against paternally derived antigens (Tamessar et al., 2021). It has been established that ncRNA in semen differentially contributes to reproduction, their relative expression even explaining levels of fertility when explored within spermatozoa (Alvarez-Rodriguez et al., 2020b; Martinez et al., 2022). Small ncRNAs are also present in sEVs, including miRNAs (9%, almost 300 variants) as the ssc-miR-10b (Xu et al., 2020) with a clear relation to fertility (Alvarez-Rodriguez et al., 2020b), and related to the cytokine-related modulation of immune responses (Barranco et al., 2020).

It appears logical to state that the SP is a composite secretion that promotes cell-to-cell communication between reproductive and non-reproductive cells, following evidence from experimental studies in vivo (Alvarez-Rodriguez et al., 2019a; 2020a) and in vitro (Reshi et al., 2020). Of interest, however, is to insist in the fact that sEVs are, at least in vitro (Bai et al., 2018), able to elicit specific changes in the lining epithelium. Why would this be so interesting? Well, simply because active biomolecules are better protected from degradation and loss-of-function within the EVs than free in the SP. This becomes essential when we discuss the extra-corporeal use of SP and, as mentioned above, if we intend to use specific components as identificatory biomarkers or even to increase fertility following AI or other ARTs.

1.3. Can we increase fertility after AI using specific components of the SP?

Over the past 20–25 years, strategies to maintain fertility after AI to similar levels as those registered after natural mating have not only focused on sperm basic quality variables (particularly motility) but also on the inclusion of SP. This is not surprising, even if the rationale was mostly empirical; the fertility of a male after mating must depend on both semen components. Yet the components can be separated for IVF or ICSI, but apparently not for ejaculated spermatozoa intended for AI. The latter can be SP-cleansed, but their membrane stability is substantially affected (owing to the depletion of specific lipids from the plasma membrane; de Andrade et al., 2022) and the spermatozoa must be used shortly thereafter. Exclusion of SP is claimed as beneficial for some routines of preservation (as in pigs) but this assertion is, in principle, wrong. Ejaculated boar spermatozoa that ought to be frozen are firstly exposed for hours to SP (holding time, Eriksson et al., 2001) and then spermatozoa reconcentrated (and most SP removed) ahead of a secondary extension for cryopreservation. Noteworthy, boar sperm viability is best restored after thawing when SP is again added (de Andrade et al., 2022), especially if the SP belongs to the sperm-rich fraction (Torres et al., 2016). Addition of SP from good male sperm freezers has improved sperm cryosurvival of bad freezers in pigs (Hernández et al., 2007), and stallions (Heise et al., 2010). The moment of SP-addition has varied, appearing most efficient when added post-thaw (de Andrade et al., 2022; Rodriguez-Martinez et al., 2021 and references therein). Additional AIs with only homologous/pools of SP could increase relevant reproductive outcomes as rates of early embryo survival (Parrilla et al., 2020; Schjenken and Robertson, 2020; Martinez et al., 2021), implantation (Waberski et al., 2018; Schjenken and Robertson, 2020), placental development (Martinez et al., 2020), farrowing rates, litter size and even offspring development and health (Morgan et al., 2020). These beneficial effects of SP can be achieved by including a minimal proportion of SP left in AI-doses, usually of the order of 10% (Rozeboom et al., 2000; Recuero et al., 2019; Ortiz et al., 2019).

What is most relevantly present in those proportions of SP left or added? Is there any molecule that could be extracted, purified and used instead of bulk SP? Proteins, as enzymes or even cytokines, have been proposed, and would be most practical to use. Yet, the results of trials aiming relationships with fertility in livestock between a certain protein (Rodriguez-Martinez et al., 2010, Perez-Patiño et al., 2018; Druart et al., 2019, Kang et al., 2019, Parrilla et al., 2020), or cytokines (Barranco et al., 2019b; Roca et al., 2020), had been variable, not to say contradictory. Surprising? Not really, considering inter- or intra-male variations in composition and relative amounts of these molecules and even the effects of handling (Padilla et al., 2020; Parrilla et al., 2020). Inter-male variation has led to ambiguous statistical relations, either positive or negative (Novak et al., 2010; De Lazari et al., 2020; Kang et al., 2019; Pavaneli et al., 2019; de Andrade et al., 2022). An additional complication is that the intactness of such molecules is short-lived, jeopardizing their possibly reliable use under current handling. A recent example is built by studies aiming to disclose if intrauterine pre-AI infusion of transforming growth factor-ß (TGF-ß, as TGF-$ß_1$), which is conspicuously present in pig SP (Barranco et al., 2015a, 2018), could replace native SP and affect pre-implantation embryo development. Both SP and TGF-$ß_1$ influenced endometrial cytokines, but only SP impacted pre-implantation pig embryo development, meaning the effects of SP must include other molecules, which would work in a coordinated way (Martinez et al., 2021; Parrilla et al., 2022).

1.4. Are sEVs a better alternative to bulk- or specific free SP-molecules?

As stated earlier, sEVs contain most relevant molecules of SP, particularly those affecting spermatozoa, their survival and fertilizing capacity, including miRNAs of significant importance for early embryo development (Rodriguez-Martinez et al., 2021; Roca et al., 2021). That these molecules are protected from degradation while included in the EVs and that they can be delivered to target cells, as evidenced when sEVs were incubated with cells under simple in vitro conditions (Leahy et al., 2020), announce their broad functional capabilities. Isolation and preservation of sEVs derived from recognized and well-documented highly fertile males, could be infused into the female (Gervasi et al., 2020) to gain differential effects on fertility. That EVs can escape phagocytosis (Li et al., 2021) adds a most relevant asset for their use as selective bio-carriers. Whether the detected presence of CD44, the specific receptor for hyaluronan, in pig sEVs (Alvarez-Rodriguez et al., 2019b) would have a role in this "masking" of EVs by being covered by hyaluronan along their transit post-ejaculation (Rodriguez-Martinez et al., 2016), is a plausible hypothesis to be tested.

In most species of livestock the amounts of sEVs are huge compared to other body fluids; however, their use in practical terms for AI or other biotechnologies is constrained by shortcomings, including the reported sEVs-heterogeneity, so that their purification, modification, selective load enrichment and preservation for breeding use (Li et al., 2021) calls for alternatives.

At hand it appears approaches of exosome mimetics (Asai and Oku, 2014), e.g., the production of artificial EVs [so-called "hybrid nanovesicles" (Li et al., 2021; Amiri et al., 2022)] vesicles that are nano-technologically fabricated using top-down, bottom-up, or bio-hybrid strategies (Li et al., 2021). The first named strategy is basically the transfer of cells responsible for the EVs production of interest, i.e., the epididymal principal cells collected as offal from an abbatoir that are disrupted in the laboratory to gain the EVs. Such approach has the advantage of retrieving a particular single type of EV (epididymosomes), waiving heterogeneity, but it is superfluous in livestock, since the same result can be achieved by cannulation of the ductus deferens to singly collect the epididymal fluid from a recognized high-fertile sire separated from further breeding. Another approach could be the generation of fully artificial "synthetic Evs" where key components (i.e., specific proteins or miRNAs) are incorporated into lipid nanoparticles (liposomes) through supra-molecular chemistry. This bottom-up strategy is a potentially cheaper and repeatable option for the delivery of crucial components of sEVs during AI, which could also waive inter-male variation (Asai and Oku, 2014, 2021). The efficiency of fully synthetic vesicles is, however, still lower than when using EVs delivery systems (Murphy et al., 2021). The third approach, the so-called biohybrid strategy, can generate tailored-EVs by fusing native sEVs with liposomes previously uploaded with specific molecules of interest, as proteins or nucleic acids. In this case, the native sEVs are not affected in terms of their intrinsic properties, i.e., their coating, their receptors, etc., properties that help the sEVs to maintain intactness and resistance to phagocytosis. This approach combines the bottom-up approach with uploading of relevant biomolecules that would simply enrich native sEVs for immediate use without unsafe manipulation of the natural sEVs, thus facilitating transfer of relevant molecules to target cells without jeopardizing native sEVs funtionality.

In either case, suitable molecules can be uploaded, as exemplified in Fig. 2, by passive diffusion to these "semi-artificial" sEVs using available techniques (Amiri et al., 2022), i.e., electroporation for miRNAs, sonication for proteins or incubation for lipid-permeable chemicals (Lim and Kim, 2019), promoting their usefulness as nano-carrier additives in AI and other ARTs with the final aim of improving fertility. However, although major and steady advances in translational nanomedicine are being done using artificial nanovesicles as biomimetic nanocarriers, EVs appear as far to be more efficient than synthetic systems (Murphy et al., 2021) for

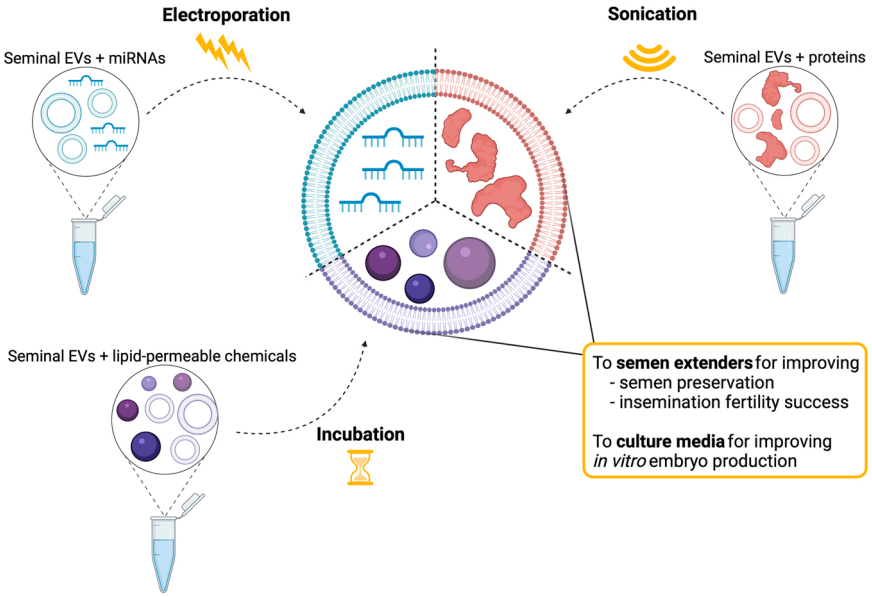

Fig. 2. Methods available for the incorporation of some biomolecules (lipid-permeable chemicals, proteins or small RNAs) into native seminal extracellular vesicles (sEVs) and possible applications of these retrofitted SEVs for artificial insemination or in vitro embryo production. The drawings were created in BioRender.com.

molecular therapy, e.g., for ischemic diseases (Aday et al., 2021), cancer (Lim and Kim, 2019), or reproductive diseases (Esfandyari et al., 2021). Therefore, the use of sEVs as strategy to increase fertility in livestock is still positive wishing. Unfortunately, too few studies have been done on livestock studying the strict effect of sEVs on fertility (Parra et al., 2022). Further steps are needed to properly characterize the contents, the most relevant subtype of sEVs to be considered for engineering, and most importantly, to make experiments linking isolated sEVs to fertility. Such studies are, luckily, initiated….

2. Concluding remarks

This invited essay has critically summarized current knowledge of the roles the complex SP apparently play in ruling fertility and prolificacy. It has focused on discussing the strengths, limitations, and future perspectives for a particular component, i.e., the sEVs. It particularly addressed their ability to harbor SP-molecules relevant to sperm survival and function, as well as fertility and embryo development, and to maintain these protected against enzymatic degradation or phagocytosis/immune rejection. Since the bilayer vesicles can be retrieved from SP and in vitro engineered, their use can be standardized for ARTs. However, we still need basic studies of sEVs on livestock species, concerning effective isolation, characterization, and testing for their eventual relation to fertility. The way forward is still long, albeit initiated and considered feasible.

2.1. Ethical statement

Animal husbandry and experimental handling whenever cited for individual own studies were performed according to the European Directive 2010/63/EU, 22/09/2010 for animal experiments and current Swedish legislation (SJVFS 2017:40. Date: 06/2016) and approved by the Bioethics Committee of Murcia University (research code: 639/2012) and the "Regional Committee for Ethical Approval of Animal Experiments" (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permits Dnr 75–12, ID1400 and 03416–2020).

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Author contribution statement

HRM and JR have conceptualized, written and acquired funding for own studies included in this essay paper.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design, writing or in the decision to publish the manuscript.

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