Extracellular vesicles and assisted reproductive technology

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Abstract

Research in biogenesis, composition, uptake, and functionality of extracellular vehicles (EVs) has dramatically expanded in recent decades. These lipid membrane bound particles secreted from all cells contain regulatory molecules (proteins, peptides, RNAs, DNA fragments, and lipids) that are transferred to target cells upon binding to the plasma membrane. Roles of EVs were studied in cell to cell communication, mediation of cancer development and immune response, as biomarkers for disease and potential drug delivery systems. More recently, studies were conducted to investigate roles of EVs in reproduction, ranging from gamete maturation, fertilization, and embryonic development to implantation. As such, potential use of EVs for assisted reproductive technologies (ART) has garnered substantial interest. Objectives of this review are to present current understanding of EVs in reproduction, with particular regard to recent research in understanding influence of reproductive cycles on EV production and cargo composition, and to discuss therapeutic potential of EVs in reproductive technologies, including cryopreservation and areas of research that remain to be explored for efficient application of EVs in ART.

Keywords: Extracellular vesicles, gametes, cryopreservation, organs on a chip, synthetic vesicle

Introduction

Extracellular vehicles (EVs) are lipid membrane bound vesicles secreted by all cells.[1] EVs include exosomes (30 - 100 nm in size), released via fusion of a multivesicle body with a cell's plasma membrane; microvesicles (MV, also known as ectosomes, 100 - 1000 nm), shed via budding from plasma membrane;[2] and apoptotic bodies (up to 5000 nm), formed through contraction and membrane blebbing of dying cells[3] (Figure 1). EVs contain regulatory molecules, including proteins, peptides, RNA species, lipids, and DNA fragments which may be taken up by nearby cells via either fusion or endocytosis of vesicles, contributing to cell to cell communication.[4] Owing to size overlap between exosomes and MV, it is difficult to separate 1 population from another and the 2 groups are often studied together as extracellular vesicles, or "EVs" in healthy cells (i.e. excluding apoptotic bodies). Multiple methods have been evaluated for EV isolation (reviewed[5]), including ultracentrifugation (most commonly utilized method[6]), density gradient centrifugation,[7] size-exclusion chromatography,[8] ultrafiltration,[9] and sequential filtration.[10] Differences in isolation method become significant in that subpopulations of EVs (i.e. more exosomes versus MV, or EV with specific surface markers) may be preferentially purified, based on isolation technique. For example, biological activity of endometrial EVs isolated via a commercially available system were compared with those purified via ultracentrifugation and ultrafiltration.[11] Although ultracentrifugation had highest EV yield, EVs isolated via Total Exosome Isolation Kit were more readily taken up by trophoblast cells, indicating better preservation of biological activity. Further, regardless of purification method, copurified protein aggregates can confound the results of 2 most commonly utilized EV quantification methods, namely, colorimetric protein assays (e.g. BCA) and nanoparticle tracking analysis (NTA).[12]

Additionally, our ability to fully characterize EVs (both in terms of their composition and functionality) is challenged by the heterogenous nature of isolated EVs. Nevertheless, some characteristic features of EV subpopulations have been described. For example, owing to differing methods of release, proteins related to endosomal sorting complexes required for transport pathway are common to exosomes.[13] Exosomes are also enriched in tetraspanins (involved in cell adhesion and signaling[14]) and heat shock proteins.[15,16] MV contain higher concentrations of proteins associated with plasma membrane.[1] Although lipid characterization has been particularly understudied in EVs field as a whole,[17] lipid

composition and high cholesterol/phospholipid ratio of EVs results in notable membrane stability, which presumably allows for greater protection of cargo and EVs in vitro longevity.[18] In response to confusion associated with the heterogeneity of EVs and diversity of purification and quantification methods, in 2018, the International Society for Extracellular Vesicles published updated standards for minimal information for EV studies.[19] These serve as a guide for EV isolation, classification, and nomenclature, and are important to reduce variability in research results among laboratories which are due to inconsistent appellations, or attribution of functions specifically to MV/exosomes when isolation protocols produce heterogenous populations.

Improved understanding of EV sub-population compositions are also poised to hasten elucidation of the mechanisms of EV uptake into 'recipient' cells. It is currently understood that EVs are taken up by cells through a variety of pathways, including clathrin-mediated endocytosis, caveolin-mediated uptake, and lipid raft-mediated internalization (reviewed[20]). Whereas EV cargo transfer to recipient cells is documented,[21] understanding molecular mechanisms of delivery is an area necessitating further study. Still, EVs are well studied for their role in cell to cell communication, including in mediating cancer development (reviewed[5]) and immune response (reviewed[22]). Release of EVs by cancer cells and immune system stimulated interest in them as a source of biomarkers for early disease detection (reviewed[23]). As a result of their natural in vivo stability, cargo carrying capacity, and ability to be taken up by cells, there is also growing interest in utilizing EVs for in vivo drug and therapeutic protein delivery.[24,25]

Although the field of reproduction-specific EVs is still relatively new, several excellent reviews published in recent years highlighted growing interest and understanding of roles of EVs in successful gamete functions, fertilization, embryo development, and pregnancy.[26-30] Objectives are to outline current understanding of EVs in reproduction, including recent evaluations of reproductive stage on EV production and composition, and to discuss potential practical/therapeutic applications of EVs in assisted reproduction, with a primary focus on cryopreservation.

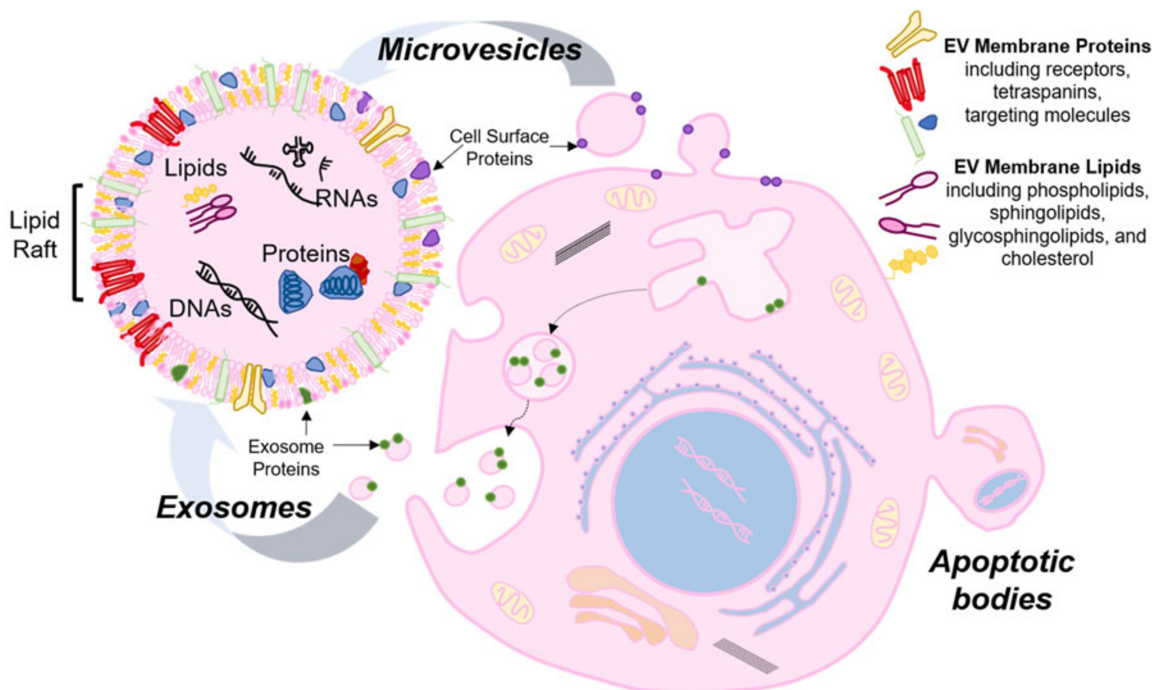

Figure 1. Schematic of extracellular vesicle (EV) biogenesis and composition, with exosomes secreted via fusion of multivesicle bodies from early endosome fusing with cell's plasma membrane, microvesicles secreted via budding, and apoptotic bodies released via blebbing. Collectively termed EVs, these membrane-bound vesicles carry lipid, RNA, DNA, and protein cargo from their cells of origin.

Extracellular vesicles in reproduction

Overview of male reproductive tract extracellular vesicles

To date, studies on male reproductive tract EVs have focused on vesicles derived from epididymis (epididymosomes), and prostate gland (prostasomes). Epididymosomes are released into lumen from large blebs that form and detach from parent epithelial cells.[31] Epididymosomes fuse with sperm during their transit through epididymis and deliver small RNA,[32,33] and protein cargo,[34,35] and alter lipid composition of sperm's plasma membrane,[36] essential for sperm maturation process (reviewed[37]). Specifically, epididymosomes reduce cholesterol/phospholipid ratio of bovine caput epididymal sperm,[36] that results in increased membrane fluidity necessary for the sperm's future ability to capacitate. Gene ontology analyses of bovine epididymosomes classified proteins into 6 functional groups, including adhesion molecules, chaperones, enzymes, structural proteins, signal transducers, and transporters.[37] Many of these proteins are involved in sperm egg interactions, motility, plasma membrane modification, and elimination of defective gametes.[38] Transfer of small RNAs (e.g. miRNA and tsRNA both small, noncoding RNA that primarily function in RNA silencing and posttranscriptional regulation[39]) from epididymosomes to sperm is a mechanism for transgenerational epigenetic inheritance.[32,40] Coincubation of immature sperm with epididymosomes in domestic cats sustained in vitro motility.[41] Delivered miRNA from epididymal EVs is linked to pregnancy success in mice, as intracytoplasmic sperm injection with sperm from caput epididymis displayed significant implantation failure following embryo transfer; a phenotype rescued by injection of cauda-specific miRNA into embryos.[42] It is apparent that these vesicles have a profound influence on not only male gametes, but also pregnancy success and, potentially, offspring/subsequent generation traits via epigenetic inheritance.

Prostasomes (reviewed[43]) are challenging to characterize, as they are mixed in with EVs from other sources in seminal fluids. Proteomic analyses of human prostasomes identified proteins in broad categories (enzymes, transport or structural integrity, GTP proteins, chaperones, signal transduction, and unannotated).[44] Supplementation of sperm with prostasomes improved sperm motility, attributed to delivery of calcium signaling[45] and ATP-generating[46] machinery to the gamete. Human prostasome membranes are high in cholesterol, sphingomyelin and glycosphingolipids.[47,48] Fusion with prostasomes enriched sperm's plasma membranes in key lipids, including cholesterol.[49] This, in turn, is apparently responsible for the prevention of premature acrosomal exocytosis in sperm exposed to prostasomes in cattle[50] and humans.[51,52] Prostasomes also protect sperm against female immune system, including decreasing natural killer cell activity in vitro,[53] indicating prostasomes function both to maintain sperm function and support survival.

Overview of female reproductive tract extracellular vesicles

Extracellular vesicles derived from vaginal (vaginosomes[54]), uterine (uterosomes, reviewed [55,56]), oviductal (oviductosomes or oEVs, reviewed[27]) and follicular (ffEVs, reviewed[30,57,58]), fluids, and secretions from in vitro cultured embryos (reviewed[59]) are characterized in a variety of species. Supplementation of EVs from these sources benefit gamete function and/or embryo development. For example, coincubation of vaginal EVs with murine sperm increased rates of progesterone-induced acrosome exocytosis compared to unsupplemented controls.[54] This effect was attributed to transfer of tyrosine phosphorylated proteins and plasma membrane Ca2+ ATPase ([PMCA], which removes calcium from cells) from EVs to sperm, essentially priming cells for capacitation while preventing premature acrosome reaction. Endometrial mesenchymal stem cell EVs improved blastomere count and hatching rates in murine embryos.[60] Coincubation with EVs also stimulated embryos to release vascular endothelial growth factor and platelet derived growth factor, which in turn may modulate endometrial receptivity to implantation.[60]

Our laboratory recently demonstrated that domestic cat oviductal extracellular vesicles (oEVs) bound to acrosomal region and midpiece of sperm and prevented premature acrosomal exocytosis (in vitro), while improving sperm motility and fertilizing capacity[61] (Figure 2). We postulated that enrichment in proteins identified in oEVs was related to energy metabolism, calcium transport and

membrane function, may have regulated this effect. In cattle, oEVs modulate sperm calcium concentrations[62] and they also modulated sperm capacitation in cattle and mice.[62,63] Porcine oEVs reduced rates of polyspermy in vitro,[64] and murine oEV supplementation during in vitro fertilization and embryo culture improved live birth rates after embryo transfer.[65] Hence, recently, oEVs have been utilized to improve in vitro embryo production technologies.[27]

Follicular fluid EVs, apparently produced by granulosa and cumulus cells, are taken up into cumulus cell layer of domestic cat oocytes (Figure 2) and, in cattle, transferred to an oocyte via transzonal projections.[66] Bovine ffEVs stimulated granulosa cell proliferation[67] and cumulus cell expansion,[68] and improved blastocyst production.[68] Bovine embryos produced in vitro in the presence of ffEVs exhibited varying levels of global DNA methylation compared to nonffEV counterparts.[69] Taken together, it is clear that supplementation of EVs to in vitro embryo production systems promises to improve not only production rates, but also embryo quality and potentially live birth success.

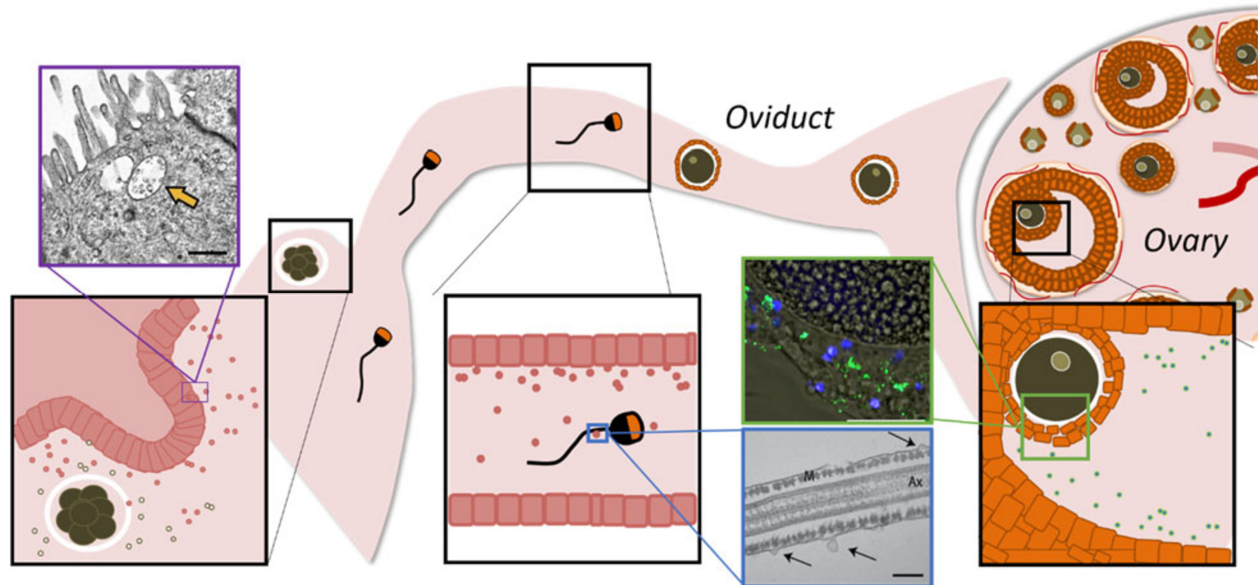

Figure 2. Schematic of female reproductive tract EVs and interaction with gametes/embryos, including pre-release uterosomes in a multivesicular body in ovine endometrial luminal epithelium (top left, TEM image from Burns et al 2016), oviductosomes or oEV from domestic cats (lower middle, TEM image fusing with sperm tail from Ferraz et al 2019), and feline follicular fluid (top middle, fluorescent image with EV lipids labeled in green (DiO) and EV proteins in blue (Ghost Dye) taken up by cumulus cells, manuscript under review)

Extracellular vesicles and reproductive cycles

Despite strong evidence of beneficial actions of EVs on reproduction, our understanding of how EV biogenesis and composition are modulated by reproductive cycle is limited. Nevertheless, there is evidence that changes in secreted EVs are under hormonal control.[66,70-73] For example, ovariectomized sheep treated with progesterone for 14 days had higher EV count (based on NTA) and mode diameter in their uterine fluid compared to untreated cohorts, indicating biogenesis and secretion of endometrial EVs were modulated by progesterone.[71] In an in vitro study with primary cell cultures of human endometrial epithelial cell lines, there were no differences in EV amounts (based on protein concentrations) from cells cultured only with estrogen (i.e. proliferative phase-mimetic) versus estrogen and progesterone (i.e. secretory phase mimetic).[72] However, there were 380 differentially expressed proteins between EVs of 2 treatment groups, many of which were involved in cytoskeletal organization, cell migration, cell adhesion, and extracellular matrix organization. These studies indicated that uterine EV production and their protein compositions are under hormonal control.

Recently, counts, transcriptomes, and proteomes of oEVs recovered during various stages of reproductive cycles have been characterized in several mammalian species. In murine oEV, very few differences in miRNA content were observed among reproductive stages (metestrus/diestrus versus proestrus/estrus).[73] In domestic cats, we observed an increase in concentration, but not size distribution, in oEVs recovered during midluteal phase compared to early luteal and late follicular phases (manuscript under review). Conversely, protein and mRNA/small RNA content of bovine oEV isolated during postovulatory and early luteal phases were enriched in proteins related to protein translation and transport, whereas those obtained during late luteal phase contained proteins related to vesicles, cytoskeleton, metalloexopeptidase activity, and innate immune system.[70] Furthermore, miRNA with high abundance in recently ovulated and late luteal phase included those involved in fatty acid biosynthesis and metabolism, cell cycle, oocyte meiosis, and multiple signaling pathways.[70] These results were further evidence for potential targeted messaging to gametes and/or embryos to promote fertilization and development by reproductive tract.

Also in a bovine model, miRNA from ffEV of 3 - 6 mm diameter follicles at various stages of the estrous cycle were compared.[66] Follicular fluid EVs from early luteal ovaries (with evidence of recent ovulation, low follicular fluid progesterone concentration) were enriched in miRNA involved in multiple signaling pathways, including FoxO, Hippo, MAPK, and TGF_β compared to ffEVs from midluteal phase ovaries (tan corpus luteum, high progesterone).[66] When early luteal phase ffEVs were supplemented to culture media during 24 hour in vitro oocyte maturation, expression of genes associated with pathways related to oocyte development, ovulation, and Notch signaling were upregulated in cumulus cells, indicating that reproductive stage specific cargo of ffEVs may have key roles in regulating gamete function. More tangentially, there is also evidence linking metabolic status as result of lactation to miRNA composition of bovine follicular fluid EVs.[74] Namely, cows in negative energy balance had ffEV with downregulation of miRNA associated with metabolic processes and oocyte growth compared to metabolically normal lactating heifers, which has implications for folliculogenesis during lactational anestrus. Although in the earlier bovine ffEV study, supplementing ffEVs during IVM upregulated gene expression in the cumulus cell, no differences in oocyte maturation rates were observed between ffEVs gametes versus controls.[66] It is possible that 24 hour time frame of IVM was not long enough to facilitate transfer of EV cargo into the oocyte and influence meiotic maturation outcome. In another bovine study, ffEVs were detected in cumulus cells via confocal microscopy 16 hours after incubation.[60] We have similarly observed uptake of ffEV lipids and proteins only in cumulus cells of feline oocytes after 18 hours coincubation (manuscript under review). However, in ova from domestic dogs, oEV contents were observed in the oocyte after 72 hours incubation.[75] It is clear, therefore, that an improved understanding of the kinetics of EV uptake into oocytes is necessary to further our understanding of their functional effects and any subsequent reproductive stage-specific differences.

Potential for dramatic differences in EV composition by reproductive stage/status are not limited to female reproductive tract. Differences in protein composition of seminal plasma during breeding versus nonbreeding seasons have been characterized in sheep[76,77] and goats.[78,79] Notably, there is some evidence that certain protein components of breeding season (autumn/winter) seminal plasma improved motility of frozen-thawed ram sperm; however, this effect was not observed in nonbreeding season or unsupplemented sperm.[80] While focus of these studies was on seminal plasma, rather than isolated EVs, it is possible that some proteins identified as interacting with sperm membranes are derived from EVs. This assertion is supported by the finding that there were no quantitative differences in abundance of most metabolic enzymes isolated from ram seminal plasma extracellular vesicles versus whole seminal plasma.[81]

Extracellular vesicles and assisted reproductive technologies of cryopreservation

Potential of EV for assisted reproductive technologies, especially in vitro embryo production, has been elegantly reviewed recently.[28] Here, we focus on new research into potential beneficial effects of EVs in cryopreservation. Recent reports of EV supplementation to gamete and/or embryo cryopreservation yielded varying results. Significantly improved survival and pregnancy success were

observed with cryopreserved and thawed in vitro-derived bovine embryos cultured in amniotic MV (from days 5 - 7 following IVF), compared to unsupplemented controls.[82] Zygotes coincubated with oEVs from either the isthmus or ampulla of the oviduct were compared to unsupplemented or fetal calf serum supplemented controls.[83] Blastocysts developing from isthmic oEV-supplemented cultures displayed improved post-thaw survival rate (determined via reexpansion of blastocoel cavity) compared to control and FBS supplemented groups. Conversely, bovine embryos cultured with varying concentrations (based on protein content) of postovulatory oEVs had improved day 7 blastocyst rates, but supplementation had no effect on cryotolerance of blastocysts, as evaluated by hatching rates.[84] Further, postthaw survival of bovine blastocysts sequentially cultured in oEVs and uterus-derived EVs was not significantly improved compared to controls.[85] As each study utilized different EV sources (amniotic, oviductal and uterine fluid alone or in combination), isolation methods, and concentrations, as well as coculture timings and metrics of success, the full influence of reproductive EVs on bovine embryo cryopreservation is not yet clear.

In our laboratory, ffEV supplementation during cryopreservation and thawing of domestic cat oocytes did not improve survival based on morphology, but did enhance ability of frozen and thawed oocytes to resume meiosis in vitro (manuscript under revision). Still, proteomic analyses of cat ffEVs identified proteins with known cryomodulating effects, including heat shock proteins (HSP), cytoskeletal elements, and oxidoreductases. For example, treatment of oocytes with cytoskeleton stabilizing agents improved cryopreservation success in mice[86] and cows.[87] Significant loss of chaperone proteins like HSPs and oxidoreductases have been observed following sperm cryopreservation in sheep[88] and carp,[89] respectively. Transfer of these and other potentially cryoprotective proteins may have supported postthaw recovery of gametes; however, much more work is necessary in this area as well.

For sperm, the addition of bovine oEVs postthaw maintains viability while supporting capacitation during incubation, compared to sperm incubated under capacitating conditions in oEV absence.[62] Cryopreservation significantly alters sperm membrane's lipid compositions (reviewed[90]), including reducing sterols, phosphatidyl choline, and unsaturated fatty acids in caprine sperm membranes.[91] As previously mentioned, EVs contain high concentrations of lipids and proteins associated with lipid-rafts, which likely supports their membrane fusion and stability in vitro,[92] but also could support recovery of cryodamaged sperm membranes upon fusion. Lipid component is part of rationale behind addition of egg yolk to sperm cryopreservation media.[93] More recently, a liposome-based medium (OptiXcell) was evaluated for cryopreservation of ram,[94] buffalo,[95] bull,[96,97] deer,[98] and rhino[99] sperm, with promising results overall, even when compared to egg yolk, the current 'gold-standard'. Similar to EVs, liposomes are phospholipid membrane vesicles, though they lack protein cargo. It is feasible that combining our understanding proteins present in EV membranes, particularly those likely to target gametes and promote uptake, we may be able to engineer vesicles with specific lipid and/or protein compositions to support cryosurvival while maintaining high affinity for gametes.

Future extracellular vesicles production for therapeutics

Applications of EVs to assisted reproduction is faced with several challenges, including the aforementioned need to improve our ability to isolate, purify and characterize EVs. However, even once we have a better understanding of EVs and optimal (and standardized) methods to purify populations of interest, EVs with consistent characteristics with low variability must be producible in large quantities. This is not feasible with in vivo sources. Beyond inherent variability, flushing both oviducts (Figure 3a) of a single individual with the aforementioned Total Exosome Isolation Kit, we isolate on average 3.1×10^{10} and 4.5×10^{10} EV/ml (in 1 ml) from domestic cats and dogs, respectively. In contrast, for our experimental purposes, we typically utilize $\sim 3 \times 10^{7}$ EVs/ml. Though this is sufficient in a laboratory setting, high throughput production of EVs is necessary for clinical applications.

In vitro extracellular vesicles production

To improve our ability to consistently increase EV production, much work on EVs utilize in vitro produced vesicles from cell lines. Impact of isolation and culture conditions on EV production has been reviewed,[6] yet much is still unknown, in large part because our understanding of biogenesis of these

vesicles is not fully elucidated. Different cell types secreted different amounts of EVs in vitro,[6] but whether this is intrinsic to cells or perhaps in response to pre-established culture methods is not certain. Presence or absence of serum in culture medium for neuroblastoma cells has been demonstrated to alter both quantity of produced EVs as well as their protein expression levels.[100]Alterations in lipid composition are also apparent from cell lines from same organ, as lipid profiling of prostate-derived cell line EVs identified differences in molecular lipids between cell lines with different tumorgenicity.[101]

Although culture conditions, once optimized, should allow for production of EVs with definable characteristics, high throughput production remains challenging.[102] Owing to broad interest in EVs in regenerative medicine and cancer research, this area has already begun to be explored. EV production from prostate cancer cells is significantly improved when cells are cultured in a bioreactor compared to a conventional system,[103] with EV morphology and select surface markers not different between these 2 conditions. However, metabolic analyses indicated differences in EV composition between conventional versus bioreactor derived EVs, particularly with regard to lipid contents. A 3D printer fabricated bioreactor has also been shown to enhance EV production for human endothelial cells.[104] Still, as trauma and cell stress have also been demonstrated to increase EV secretion[105] and mRNA/protein compositions,[106] respectively. Mechanical forces on cells are known to promote exocytosis,[107] therefore it is also possible that shear forces on cells grown in bioreactor systems may be stimulating mechanical, stress-driven EV production. Under these conditions, EV composition is likely altered and, potentially, less efficacious. More studies are needed to assess: 1) if dynamic culture systems consistently result in improved EV production across cell types, compared to conventional culture; and 2) how quality/composition/functionality of these EVs compare with in vivo-derived counterparts and/or those produced by conventional culture systems.

In a sense, platforms for improved in vitro production of reproductive EV are already in progress. Organs on a chip technology, which has been applied to recreate in vitro microenvironment in more naturalistic conditions by mimicking tissue architecture, cell heterogeneity, and dynamic exposure to nutrients, growth factors and hormones, represent microfluidic bioreactors. Automated and controlled flow with specifically timed hormone supplementations can mimic normal pulsatile hormone pattern of reproductive cycle, and flow-through containing EVs readily collected (Figure 3b).

A bovine oviduct-on-a-chip, mimicking in vivo conditions in this organ, demonstrated epigenetic patterns of chip derived embryos to be more similar to fresh controls than those produced via conventional IVF.[108] An artificial uterus has similarly been produced via microfluidic chip, consisting of

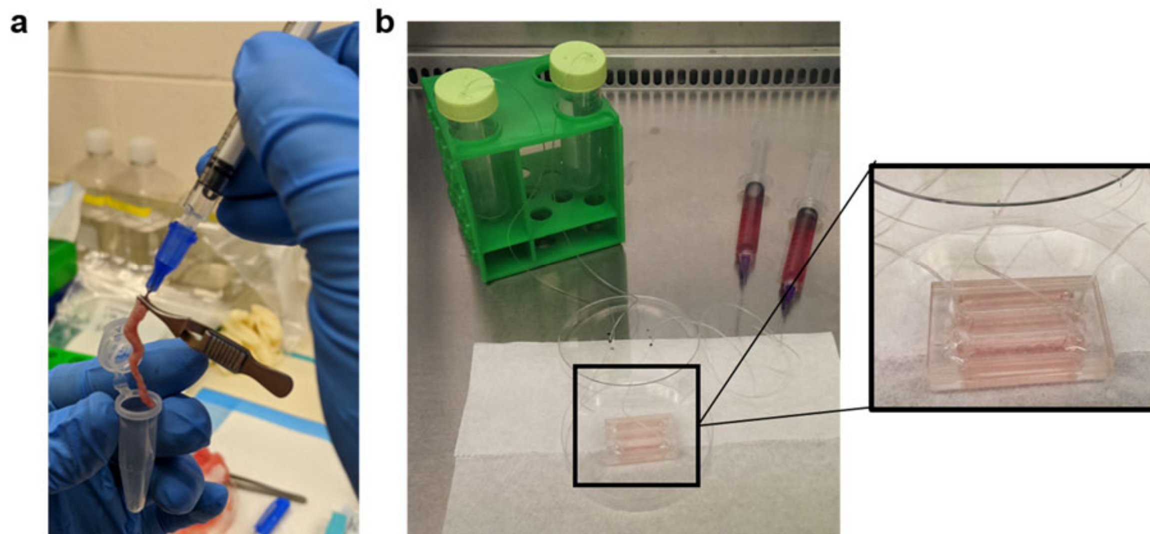

Figure 3. In vivo and vitro alternatives for collection of EVs, with: a) flush of fluids containing EV from domestic cat oviducts, versus b) dynamic fluidic culture system where tissues/cells of interest can be maintained in channels (inset) with culture medium delivered via syringe pump and flow through containing EV collected in tubes.

two channels separated with a porous membrane, with cell culture (endometrial and embryos) on apical side and perfusion on basolateral.[109] Higher rates of blastocyst development were obtained in chip compared to a conventional Petri dish. No microfluidic systems for vaginal cells have been reported, though a human vaginal epithelial cell model with 3D organoid culture has been described.[110] A combination ovary, fallopian tube, uterus, cervix, and liver reproductive system on a chip with human and murine tissues/cells has been produced (termed EVATAR), that mimics hormonal profiles of human menstrual cycle in vitro.[111] We have previously described an ovary-on-a-chip for culture of domestic dog and cat ovarian tissues,[112] and substantial strides have been made in development of in vitro spermatogenesis with testis on chip in recent years.[113-115] Conversely, microfluidic or bioreactor systems for accessory sex glands and/or epididymis are lacking. Still, 3D prostate organoid cultures (human and mouse sources) have been described[116] for application primarily to prostate cancer research. Moreover, coculture of human cauda epididymal epithelial cells (spheroids) with caput epididymal sperm improved sperm motility and ZP-binding compared to non-cocultured controls.[117] Although EV production has not been specifically compared with regards to culture system in any of described dynamic and/or 3D culture systems, we are optimistic that tools being developed to produce improved in vitro models of reproductive organs have potential to serve as controllable sources of reproductive EVs for ART as well.

Synthetic or manufactured extracellular vesicles

Several methods to manufacture EVs have also been reported outside of those secreted 'naturally' in cell culture. These have been of particular interest for their potential as nanoscale drug delivery systems, taking advantage of their inherent cargo transfer (reviewed[118-121]) and gamete/embryo (reviewed[29]) abilities. Most nanovesicles or "exosome mimetics" have been produced via disruption of cell membranes. For example, filter-based extrusion of natural killer cells were shown to maintain antitumor effects of parental/source cells, in both in vitro and in vivo mouse models.[122] EV mimetics have been produced by treating cells with cytochalasin B, by which dissociated cell membranes roughly size of MV can be obtained.[123,124] These 'nanovesicles' have similar morphology to natural EVs (based on TEM) and reportedly have similar content and functionality as parental cells.[124]

Membrane extrusion has also been utilized to engineer 'hybrid' EVs. For example, EVs secreted from 3T3 fibroblast and A549 tumor cell line were fused with synthetic lipids.[125] This method allowed for a significant increase in number of controlled size vesicles compared to EVs purified from cell line secretions. Further, engineered EVs were demonstrated to be effective at drug delivery, as small interfering RNA (siRNA) loaded by electroporation into EV induced gene silencing in target cells comparable to commercially available kits. Fusion of EVs secreted from murine macrophages with engineered liposomes increased yield while maintaining targeted binding functionality of EVs.[126,127] These fusion EVs also had demonstrable success as drug delivery vehicles when tested in vitro. Getting further away from in vivo derived EVs and cell sources, synthetic vesicles have been produced (reviewed[92]), including liposomes (previously discussed in cryopreservation section), composed of natural lipids and fully synthetic 'polymersomes', composed of amphiphilic block copolymers.[128] Though cargo of naturally secreted EVs is not replicated in these manufactured vesicles, above studies demonstrated their ability to be loaded with specific cargos of interest. As a result, engineered EVs have potential for drug delivery or other clinical and ART applications.

Conclusion

Extracellular vesicle research represents a rapidly evolving field with diverse potential clinical applications. Although work on reproductive EVs is relatively new, epididymosomes, prostasomes, vaginasomes, uterosomes and oviductosomes have been characterized in numerous studies. This interest is owed in no small part to important effects observed on gamete maturation and function observed thus far. Substantially more work is needed to fully understand biogenesis, uptake, and downstream effects of varying EV, particularly within context of reproductive tract. However, if new methods for in vitro production can consistently generate high quality and quantity vesicles, there is broad clinical potential of EVs in assisted reproduction.

Conflict of interest

There are no conflicts of interest to declare.

Acknowledgement

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References

1. Doyle LM, Wang MZ: Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. Cells 2019;8:727-751.
2. Abels ER, Breakefield XO: Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. Cell Mol Neurobiol 2016;36:301-312.
3. Wickman G, Julian L, Olson M: How apoptotic cells aid in the removal of their own cold dead bodies. Cell Death Differ 2012;19:735-742.
4. Zaborowski MP, Balaj L, Breakefield XO, et al: Extracellular vesicles: Composition, biological relevance, and methods of study. Bioscience 2015;65:783-797.
5. Willms E, Cabañas C, Mäger I, et al: Extracellular vesicle heterogeneity: subpopulations, isolation techniques, and diverse functions in cancer progression. Front Immunol 2018;9:738.
6. Gudbergsson JM, Johnsen KB, Skov MN, et al: Systematic review of factors influencing extracellular vesicle yield from cell cultures. Cytotechnology 2016;68:579-592.
7. Zhang Z, Wang C, Li T, et al: Comparison of ultracentrifugation and density gradient separation methods for isolating Tca8113 human tongue cancer cell line-derived exosomes. Oncol Lett 2014;8:1701-1706.
8. Davis CN, Phillips H, Tomes JJ, et al: The importance of extracellular vesicle purification for downstream analysis: A comparison of differential centrifugation and size exclusion chromatography for helminth pathogens. PLOS Neglect Trop D 2019;13:e0007191.
9. Nordin JZ, Lee Y, Vader P, et al: Ultrafiltration with size-exclusion liquid chromatography for high yield isolation of extracellular vesicles preserving intact biophysical and functional properties. Nanomed-Nanotechnol 2015;11:879-883.
10. Heinemann ML, Ilmer M, Silva LP, et al: Benchtop isolation and characterization of functional exosomes by sequential filtration. J Chromatogr A 2014;1371:125-135.
11. Niu Z, Pang RT, Liu W, et al: Polymer-based precipitation preserves biological activities of extracellular vesicles from an endometrial cell line. PloS One 2017;12:e0186534.
12. György B, Módos K, Pállinger É, et al: Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters. Blood 2011;117:e39-e48.
13. Colombo M, Moita C, Van Niel G, et al: Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci 2013;126:5553-5565.
14. Termini CM, Gillette JM: Tetraspanins function as regulators of cellular signaling. Front Cell Dev Biol 2017;5:34-34.
15. Gastpar R, Gehrmann M, Bausero MA, et al: Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. Cancer Res 2005;65:5238-5247.
16. Théry C, Regnault A, Garin J, et al: Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73. J Cell Biol 1999;147:599-610.
17. Pocsfalvi G, Stanly C, Vilasi A, et al: Mass spectrometry of extracellular vesicles. Mass Spectrom Rev 2016;35:3-21.
18. Pfrieger FW, Vitale N: Cholesterol and the journey of extracellular vesicles. J Lipid Res 2018;59:2255-2261.
19. Théry C, Witwer KW, Aikawa E, et al: Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7:1535750.
20. Mulcahy LA, Pink RC, Carter DRF: Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles 2014;3:24641.
21. Lai CP, Kim EY, Badr CE, et al: Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. Nat Commun 2015;6:7029.
22. Robbins PD, Morelli AE: Regulation of immune responses by extracellular vesicles. Nat Rev Immunol 2014;14:195-208.
23. Lane R, Korbie D, Hill M, et al: Extracellular vesicles as circulating cancer biomarkers: opportunities and challenges. Clin Transl Med 2018;7:14-25.
24. Usman WM, Pham TC, Kwok YY, et al: Efficient RNA drug delivery using red blood cell extracellular vesicles. Nat Commun 2018;9:2359.
25. Yang Y, Hong Y, Cho E, et al: Extracellular vesicles as a platform for membrane-associated therapeutic protein delivery. J Extracell Vesicles 2018;7:1440131.
26. Machtinger R, Laurent LC, Baccarelli AA: Extracellular vesicles: roles in gamete maturation, fertilization and embryo implantation. Hum Reprod Update 2016;22:182-193.

27. Almiñana C, Bauersachs S: Extracellular vesicles in the oviduct: Progress, challenges and implications for the reproductive success. Bioengineering 2019;6:32-57.
28. Andrade GM, Bridi A, Gimenes LU, et al: Extracellular vesicles and its advances in female reproduction. Anim Reprod 2019;16:31-38.
29. Barkalina N, Jones C, Wood MJA, et al: Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature. Hum Reprod Update 2015;21:627-639.
30. De Ávila ACFCM, Da Silveira JC: Role of extracellular vesicles during oocyte maturation and early embryo development. Reprod Fertil Dev 2020;32:56-64.
31. Hermo L, Jacks D: Nature's ingenuity: bypassing the classical secretory route via apocrine secretion. Mol Reprod Dev 2002;63:394-410.
32. Reilly JN, Mclaughlin EA, Stanger SJ, et al: Characterisation of mouse epididymosomes reveals a complex profile of microRNAs and a potential mechanism for modification of the sperm epigenome. Sci Rep 2016;6:1-15.
33. Trigg NA, Eamens AL, Nixon B: The contribution of epididymosomes to the sperm small RNA profile. Reproduction 2019;157:R209-R223.
34. Sullivan R, Frenette G, Girouard J: Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. Asian J Androl 2007;9:483-491.
35. Nixon B, De Iuliis GN, Hart HM, et al: Proteomic profiling of mouse epididymosomes reveals their contributions to post-testicular sperm maturation. Mol Cell Proteomics 2019;18:S91-S108.
36. Schwarz A, Wennemuth G, Post H, et al: Vesicular transfer of membrane components to bovine epididymal spermatozoa. Cell Tissue Res 2013;353:549-561.
37. Sullivan R: Epididymosomes: role of extracellular microvesicles in sperm maturation. Front Biosci (Schol Ed) 2016;8:106-114.
38. Girouard J, Frenette G, Sullivan R: Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. Int J Androl 2011;34:e475-e486.
39. Cai Y, Yu X, Hu S, et al: A brief review on the mechanisms of miRNA regulation. Genom Proteom Bioinf 2009;7: 147-154.
40. Rompala GR, Mounier A, Wolfe CM, et al: Heavy chronic intermittent ethanol exposure alters small noncoding RNAs in mouse sperm and epididymosomes. Front Genet 2018;9:32.
41. Rowlison T, Ottinger MA, Comizzoli P: Key factors enhancing sperm fertilizing ability are transferred from the epididymis to the spermatozoa via epididymosomes in the domestic cat model. J Assist Reprod Genet 2018; 35:221-228.
42. Conine C, Sun F, Song L, et al: Small RNAs gained during epididymal transit of sperm are essential for embryonic development in mice. Dev Cell 2018;46:470-480.e473.
43. Aalberts M, Stout TA, Stoorvogel W: Prostasomes: extracellular vesicles from the prostate. Reproduction 2014;147:R1-R14.
44. Utleg AG, Yi EC, Xie T, et al: Proteomic analysis of human prostasomes. Prostate 2003;56:150-161.
45. Park K-H, Kim B-J, Kang J, et al: Ca2+ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. Sci Singal 2011;4 ra31. doi: 10.1126/scisignal.2001595.
46. Ronquist KG, Ek B, Morrell J, et al: Prostasomes from four different species are able to produce extracellular adenosine triphosphate (ATP). BBA Gen Subjects 2013;1830:4604-4610.
47. Brouwers JF, Aalberts M, Jansen JW, et al: Distinct lipid compositions of two types of human prostasomes. Proteomics 2013;13:1660-1666.
48. Arvidson G, Ronquist G, Wikander G, et al: Human prostasome membranes exhibit very high cholesterol/phospholipid ratios yielding high molecular ordering. BBA Biomembranes 1989;984:167-173.
49. Burden HP, Holmes CH, Persad R, et al: Prostasomes—their effects on human male reproduction and fertility. Hum Reprod Update 2005;12:283-292.
50. Frenette G, Sullivan R: Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface. Mol Reprod Dev 2001;59:115-121.
51. Pons-Rejraji H, Artonne C, Sion B, et al: Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. Int J Androl 2011;34:568-580.
52. Carlini E, Palmerini CA, Cosmi EV, et al: Fusion of sperm with prostasomes: effects on membrane fluidity. Arch Biochem Biophys 1997;343:6-12.
53. Tarazona R, Delgado E, Guarnizo M, et al: Human prostasomes express CD48 and interfere with NK cell function. Immunobiology 2011;216:41-46.
54. Fereshteh Z, Bathala P, Galileo DS, et al: Detection of extracellular vesicles in the mouse vaginal fluid: Their delivery of sperm proteins that stimulate capacitation and modulate fertility. J Cell Physiol 2019;234:12745-12756.
55. O'neil EV, Burns GW, Spencer TE: Extracellular vesicles: Novel regulators of conceptus-uterine interactions? Theriogenology 2020; in press.
56. Kurian NK, Modi D: Extracellular vesicle mediated embryo-endometrial cross talk during implantation and in pregnancy. J Assist Reprod Genet 2019;36:189-198.
57. Tesfaye D, Hailay T, Salilew-Wondim D, et al: Extracellular vesicle mediated molecular signaling in ovarian follicle: Implication for oocyte developmental competence. Theriogenology 2020; in press.

58. Di Pietro C: Exosome-mediated communication in the ovarian follicle. J Assist Reprod Genet 2016;33:303-311.
59. Giacomini E, Alleva E, Fornelli G, et al: Embryonic extracellular vesicles as informers to the immune cells at the maternal–fetal interface. Clin Exp Immunol 2019;198:15-23.
60. Blázquez R, Sánchez-Margallo FM, Álvarez V, et al: Murine embryos exposed to human endometrial MSCs-derived extracellular vesicles exhibit higher VEGF/PDGF AA release, increased blastomere count and hatching rates. PloS One 2018; 13: e0196080.
61. De Amm Ferraz M, Carothers A, Dahal R, et al: Oviductal extracellular vesicles interact with the spermatozoon's head and mid-piece and improves its motility and fertilizing ability in the domestic cat. Sci Rep 2019;9:1-12.
62. Franchi A, Moreno-Irusta A, Domínguez EM, et al: Extracellular vesicles from oviductal isthmus and ampulla stimulate the induced acrosome reaction and signaling events associated with capacitation in bovine spermatozoa. J Cell Biochem 2020;121:2877-2888.
63. Bathala P, Fereshteh Z, Li K, et al: Oviductal extracellular vesicles (oviductosomes, OVS) are conserved in humans: murine OVS play a pivotal role in sperm capacitation and fertility. Mol Hum Reprod 2018;24:143-157.
64. Alcântara-Neto A, Fernandez-Rufete M, Corbin E, et al: Oviduct fluid extracellular vesicles regulate polyspermy during porcine in vitro fertilisation. Reprod Fertil Dev 2019;32:409-418.
65. Qu P, Zhao Y, Wang R, et al: Extracellular vesicles derived from donor oviduct fluid improved birth rates after embryo transfer in mice. Reprod Fertil Dev 2019;31:324-332.
66. De Ávila ACFCM, Bridi A, Andrade GM, et al: Estrous cycle impacts microRNA content in extracellular vesicles that modulate bovine cumulus cell transcripts during in vitro maturation†. Biol Reprod 2019;102:362-375.
67. Hung W-T, Navakanitworakul R, Khan T, et al: Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. Biol Reprod 2017;97:644-655.
68. Hung W-T, Hong X, Christenson LK, et al: Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biol Reprod 2015;93:117,111-119.
69. Da Silveira JC, Andrade GM, Del Collado M, et al: Supplementation with small-extracellular vesicles from ovarian follicular fluid during in vitro production modulates bovine embryo development. PLoS One 2017; 12: e0179451.
70. Almiñana C, Tsikis G, Labas V, et al: Deciphering the oviductal extracellular vesicles content across the estrous cycle: implications for the gametes-oviduct interactions and the environment of the potential embryo. BMC Genomics 2018;19:622-649.
71. Burns GW, Brooks KE, O'neil EV, et al: Progesterone effects on extracellular vesicles in the sheep uterus. Biol Reprod 2018;98:612-622.
72. Greening DW, Nguyen HPT, Elgass K, et al: Human endometrial exosomes contain hormone-specific cargo modulating trophoblast adhesive capacity: Insights into endometrial-embryo interactions. Biol Reprod 2016 Feb;94(2):38. doi: 10.1095/biolreprod.115.134890.
73. Fereshteh Z, Schmidt SA, Al-Dossary AA, et al: Murine oviductosomes (OVS) microRNA profiling during the estrous cycle: Delivery of OVS-borne microRNAs to sperm where miR-34c-5p localizes at the centrosome. Sci Rep 2018;8:16094.
74. Hailay T, Hoelker M, Poirier M, et al: Extracellular vesicle-coupled miRNA profiles in follicular fluid of cows with divergent post-calving metabolic status. Sci Rep 2019;9:1-14.
75. Lange-Consiglio A, Perrini C, Albini G, et al: Oviductal microvesicles and their effect on in vitro maturation of canine oocytes. Reproduction 2017;154:167-180.
76. Gündoğan M: Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi rams. Turk J Vet Anim Sci 2006;30:95-100.
77. Cardozo J, Fernández-Juan M, Forcada F, et al: Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. Theriogenology 2006;66:841-850.
78. La Falci VSN, Tortorella H, Rodrigues JL, et al: Seasonal variation of goat seminal plasma proteins. Theriogenology 2002;57:1035-1048.
79. Aguiar GV, Van Tilburg MF, Catunda AGV, et al: Sperm parameters and biochemical components of goat seminal plasma in the rainy and dry seasons in the Brazilian Northeast: the season's influence on the cooling of semen. Arq Bras Med Vet Zootec 2013;65:6-12.
80. Domínguez M, Falcinelli A, Hozbor F, et al: Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. Theriogenology 2008;69:564-573.
81. Leahy T, Rickard JP, Bernecic NC, et al: Ram seminal plasma and its functional proteomic assessment 2019;157:R243-R256.
82. Lange-Consiglio A, Lazzari B, Pizzi F, et al: Amniotic microvesicles impact hatching and pregnancy percentages of in vitro bovine embryos and blastocyst microRNA expression versus in vi vo controls. Sci Rep 2020;10:1-10.
83. Lopera-Vasquez R, Hamdi M, Maillo V, et al: Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro. Reproduction 2017;153:461-470.
84. Le Bourhis D, Janati Idrissi S, Mermillod P, et al: Effect of oviductal fluid extracellular vesicle supplementation during *in vitro* culture on development and quality of bovine embryos. Reprod Fertil Dev 2020;32:158-158.
85. Leal C, Cañon-Beltrán K, Cajas Y, et al: Extracellular vesicles from oviduct and uterus in sequential culture improve the quality of bovine embryos produced *in vitro*. Reprod Fertil Dev 2020;32:164-164.

86. Park SE, Chung HM, Cha KY, et al: Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol™, a cytoskeleton stabilizer. Fertil Steril 2001;75:1177-1184.
87. Morató R, Izquierdo D, Albarracín JL, et al: Effects of pre-treating in vitro-matured bovine oocytes with the cytoskeleton stabilizing agent taxol prior to vitrification. Mol Reprod Dev 2008;75:191-201.
88. Pini T, Rickard JP, Leahy T, et al: Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. Proteomics 2018;181:73-82.
89. Li P, Hulak M, Koubek P, et al: Ice-age endurance: the effects of cryopreservation on proteins of sperm of common carp, Cyprinus carpio L. Theriogenology 2010;74:413-423.
90. Mandal R, Badyakar D, Chakrabarty J: Role of membrane lipid fatty acids in sperm cryopreservation. Advances in Andrology 2014;2014:1-9.
91. Chakrabarty J, Banerjee D, Pal D, et al: Shedding off specific lipid constituents from sperm cell membrane during cryopreservation. Cryobiology 2007;54:27-35.
92. Fernandez-Trillo F, Grover LM, Stephenson-Brown A, et al: Vesicles in nature and the laboratory: Elucidation of their biological properties and synthesis of increasingly complex synthetic vesicles. Agnew Chem 2017;56:3142-3160.
93. Manjunath P: New insights into the understanding of the mechanism of sperm protection by extender components. Anim Reprod 2012;9:809-815.
94. Luna-Orozco JR, González-Ramos MA, Calderón-Leyva G, et al: Comparison of different diluents based on liposomes and egg yolk for ram semen cooling and cryopreservation. Iran J Vet Res 2019; 20: 126-130.
95. Ansari MS, Rakha BA, Akhter S, et al: OPTIXcell improves the postthaw quality and fertility of buffalo bull sperm. Theriogenology 2016;85:528-532.
96. Singh A, Bhakat M, Mohanty TK, et al: Effect of Tris-egg yolk, soya milk, and liposome-based extenders on Sahiwal (*Bos indicus*) sperm quality during pre- and post-cryopreservation stages. Cryoletters 2019;40:94-102.
97. Röpke T, Oldenhof H, Leiding C, et al: Liposomes for cryopreservation of bovine sperm. Theriogenology 2011;76:1465-1472.
98. Stewart JL, Shipley CF, Katich AS, et al: Cryopreservation of white-tailed deer (Odocoileus virginianus) semen using soybean-, liposome-, and egg yolk-based extenders. Anim Reprod Sci 2016;171:7-16.
99. Wojtusik J, Stoops MA, Roth TL: Animal protein-free OptiXcell and shortened equilibration periods can replace egg yolk-based extender and slow cooling for rhinoceros semen cryopreservation. Cryobiology 2019;89:21-25.
100. Li J, Lee Y, Johansson HJ, et al: Serum-free culture alters the quantity and protein composition of neuroblastoma-derived extracellular vesicles. J Extracell Vesicles 2015;4:26883.
101. Brzozowski JS, Jankowski H, Bond DR, et al: Lipidomic profiling of extracellular vesicles derived from prostate and prostate cancer cell lines. Lipids in health and disease 2018;17:211.
102. Davies OG, Rafiq QA: Considerations for the bioprocessing, manufacture and translation of extracellular vesicles for therapeutic and diagnostic applications. Cell Gene Ther Insights 2017;3:683-694.
103. Palviainen M, Saari H, Kärkkäinen O, et al: Metabolic signature of extracellular vesicles depends on the cell culture conditions. Journal of extracellular vesicles 2019;8:1596669.
104. Patel DB, Santoro M, Born LJ, et al: Towards rationally designed biomanufacturing of therapeutic extracellular vesicles: impact of the bioproduction microenvironment. Biotechnol Adv 2018;36:2051-2059.
105. Kuravi SJ, Yates CM, Foster M, et al: Changes in the pattern of plasma extracellular vesicles after severe trauma. PloS One 2017;12:e0183640.
106. De Jong OG, Verhaar MC, Chen Y, et al: Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. J Extracell Vesicles 2012;1:18396.
107. Apodaca G: Modulation of membrane traffic by mechanical stimuli. Am J Physiol-Renal 2002;282:F179-F190.
108. Ferraz MA, Henning HH, Costa PF, et al: Improved bovine embryo production in an oviduct-on-a-chip system: prevention of poly-spermic fertilization and parthenogenic activation. Lab Chip 201717:905-916.
109. Li W-X, Liang G-T, Yan W, et al: Artificial uterus on a microfluidic chip. Chinese J Anal Chem 2013 41:467-472.
110. Hjelm BE, Berta AN, Nickerson CA, et al: Development and characterization of a three-dimensional organotypic human vaginal epithelial cell model. Biol Reprod 2010;82:617-627.
111. Xiao S, Coppeta JR, Rogers HB, et al: A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. Nat Commun 2017;8:14584-14584.
112. Nagashima JB, El Assal R, Songsasen N, et al: Evaluation of an ovary-on-a-chip in large mammalian models: Species specificity and influence of follicle isolation status. J Tissue Eng Regen M 2018;12:e1926-e1935.
113. Komeya M, Hayashi K, Nakamura H, et al: Pumpless microfluidic system driven by hydrostatic pressure induces and maintains mouse spermatogenesis in vitro. Sci Rep 2017;7:1-8.
114. Komeya M, Kimura H, Nakamura H, et al: Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. Sci Rep 2016;6:1-10.
115. Yamanaka H, Komeya M, Nakamura H, et al: A monolayer microfluidic device supporting mouse spermatogenesis with improved visibility. Biochem Biophys Res Commun 2018;500:885-891.
116. Drost J, Karthaus WR, Gao D, et al: Organoid culture systems for prostate epithelial and cancer tissue. Nat Protoc 2016;11:347-358.
117. Moore HD, Curry MR, Penfold LM, et al: The culture of human epididymal epithelium and in vitro maturation of epididymal spermatozoa. Fertil Steril 1992;58:776-783.

118. Vader P, Mol EA, Pasterkamp G, et al: Extracellular vesicles for drug delivery. Adv Drug Delivery Rev 2016;106:148-156.
119. Batrakova EV, Kim MS: Using exosomes, naturally-equipped nanocarriers, for drug delivery. J Control Release 2015;219:396-405.
120. Ohno S-I, Drummen GP, Kuroda M: Focus on extracellular vesicles: development of extracellular vesicle-based therapeutic systems. Int J Mol Sci 2016;17:172-191.
121. Agrahari V, Agrahari V, Burnouf P-A, et al: Extracellular microvesicles as new industrial therapeutic frontiers. Trends Biotechnol 2019;37:707-729.
122. Zhu L, Gangadaran P, Kalimuthu S, et al: Novel alternatives to extracellular vesicle-based immunotherapy–exosome mimetics derived from natural killer cells. Artif Cells Nanomed Biotechnol 2018;46:S166-S179.
123. Gomzikova MO, Zhuravleva MN, Miftakhova RR, et al: Cytochalasin B-induced membrane vesicles convey angiogenic activity of parental cells. Oncotarget 2017;8:70496.
124. Gomzikova MO, Zhuravleva MN, Vorobev VV, et al: Angiogenic activity of cytochalasin B-induced membrane vesicles of human mesenchymal stem cells. Cells 2019;9:95-111.
125. Jhan Y-Y, Prasca-Chamorro D, Zuniga GP, et al: Engineered extracellular vesicles with synthetic lipids via membrane fusion to establish efficient gene delivery. Int J Pharm 2020;573:118802.
126. Sato YT, Umezaki K, Sawada S, et al: Engineering hybrid exosomes by membrane fusion with liposomes. Sci Rep 2016;6:21933.
127. Rayamajhi S, Nguyen TDT, Marasini R, et al: Macrophage-derived exosome-mimetic hybrid vesicles for tumor targeted drug delivery. Acta Biomater 2019;94:482-494.
128. Rideau E, Dimova R, Schwille P, et al: Liposomes and polymersomes: a comparative review towards cell mimicking. Chem Soc Rev 2018;47:8572-8610.