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Extracellular Vesicles in Male and Female Reproduction: A Comprehensive Review

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ABSTRACT

Extracellular vesicles (EVs) are dynamic, nanoscale membrane-bound structures actively secreted by cells that are recognized for their pivotal role in intercellular communication and as circulating biomarkers for diagnosing and prognosticating diseases. These vesicles effectively transfer proteins, lipids, and nucleic acids, modulating diverse physiological and pathological processes in both source and recipient cells. In the context of reproductive biology, EVs play multifaceted roles. They participate in numerous reproductive processes ranging from transcriptional and translational activity modulation to granulosa cell proliferation, cumulus expansion, gametogenesis, normal follicular growth, oocyte maturation, and fertilization rate regulation, ultimately influencing embryo development, blastocyst formation, implantation, and fertility outcomes. Additionally, EVs contribute to energy production via oxidative phosphorylation in the follicular fluid, facilitating ATP generation, which is crucial for proper oviductal cell function. They also engage in the thermogenesis pathway, which is instrumental in sperm thermotaxis for guided fertilization. Amniotic-derived EVs exhibit proinflammatory and procoagulant activities and have been successfully used to treat endometritis for enhanced pregnancy outcomes. Milk-derived EVs, especially long non-coding RNAs, participate in bone formation, immune modulation, and gene expression regulation. Cervical mucus-derived EVs possess sialidase activity, which aids spermatozoa in accessing the uterine cavity and fallopian tubes. Placental exosomes play a significant role in the management of infectious diseases during pregnancy, potentially enhancing the protective placental immune responses. Understanding the diverse functions of EVs in reproductive processes offers promising avenues for diagnosing, monitoring, and treating pregnancy-related diseases, and underscores their importance in physiological and pathological contexts.

HIGHLIGHTS

- Extracellular vesicles (EVs) are essential for intercellular communication and are involved in various aspects of reproductive biology.
- EVs influence transcription, granulosa cell proliferation, cumulus expansion, gametogenesis, etc.

Keywords: Cell communication, Biomarker, Extracellular vesicle, Reproduction, Therapeutics

Extracellular Vesicles (EVs) are minute, membrane-bound phospholipid bilayer structures that are released by diverse cells into the extracellular milieu. They consist of lipids, signaling proteins, small non-coding RNAs (sncRNAs), and regulatory RNAs, which facilitate intercellular information exchange (El-Andaloussi *et al*., 2013; Doyle and Wang, 2019; Wang *et al*., 2018). EVs originate either from the endosomal system or are released from the plasma membrane of parent cells and tissues. EVs include

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Exosomes, Microvesicles, and Apoptotic bodies based on their biogenesis, release mechanisms, size, contents, and functions (Akers *et al*., 2013; Frydrychowicz *et al*., 2015). Microvesicles, microparticles, or ectosomes form through outward budding and fission of the plasma membrane, whereas exosomes arise within the endosomal network and are liberated upon fusion of multivesicular bodies with the plasma membrane. Apoptotic bodies are released as cellular blebs during apoptosis (Doyle and Wang 2019; Giacomini *et al*. 2020; Margolis *et al*. 2019).

EVs interact with target cells through ligand-receptor interactions, thereby facilitating cell communication. This interaction involves ligand binding to membrane receptors, which triggers intracellular phosphorylation cascades. Uptake of EVs by target cells predominantly occurs via phagocytosis, and the extent of uptake may depend on the phagocytic capabilities of the recipient cell. Direct fusion of EVs with the plasma membrane may be constrained under acidic pH conditions. For instance, seminal fluid extracellular vesicles (SFEVs) from horses can bind to sperm at the site of semen deposition and adhere until they reach the oviduct, where they fuse and release their molecular components (Sahlen *et al*., 2010; Stewart *et al*., 2019). MicroRNAs (miRNAs) enclosed within EVs can be transferred to neighboring cells, subsequently modulating the gene expression and phenotype of recipient cells. Communication between EVs and target cells is facilitated through various mechanisms, including interactions between membrane proteins that activate intracellular signaling pathways within target cells, cleavage of membrane-bound exosomal proteins near target cell receptors, molecular transfer of EV contents through fusion with the target cell, and uptake of EVs by recipient cells through phagocytosis (Wang *et al*., 2018).

Extracellular vesicles (EVs) are versatile carriers of molecular cargo, including RNA, proteins, and drugs, with notable therapeutic potential. Mesenchymal stem cell-derived EVs, such as those derived from the placenta, exhibit the unique ability to modulate immune responses and enhance tissue repair. Seminal fluid EVs, including epididymosomes and prostasomes, influence sperm development, function, and interaction with the female reproductive tract. These vesicles carry an array of proteins, including CLU, MIF, CRISP, ADAM, SORD, B4GALT, HSP70, and PDIA, contributing to sperm maturation and protection from oxidative stress. Uterine and oviductal EVs facilitate embryo-maternal communication, aiding processes such as implantation, angiogenesis, and immune regulation. The communication network extends to follicular fluid EVs, which affects follicular development, oocyte maturation, and gene expression. Differentially expressed genes in follicular fluid affect oxidative phosphorylation, thermogenesis, and sperm thermotaxis, potentially guiding the sperm to the fertilization site. Overall, EVs play pivotal roles in reproductive physiology and hold promise as therapeutic agents for various medical applications (Yáñez-Mó *et al*., 2015).

Role of Extracellular vesicles in male reproduction

Extracellular vesicles present in seminal plasma include epididymosomes and prostasomes that support sperm development and function as well as influence the physiology of female reproductive tract cells (Aalberts *et al*., 2014; Baixaul *et al*., 2014; Girouard *et al*., 2011; Pons-Rejraji *et al*., 2011; Tamessar *et al*., 2020). Epithelial cells of the epididymis produce a population of EVs that bud directly from the plasma membrane (Caballero *et al*., 2013; D'Amours *et al*., 2012). These vesicles, called epididymosomes, fuse with sperm cells to transfer proteins that contribute to their maturation of sperm cells (Martin-DeLeon, 2015; Saez *et al*., 2003; Zhou *et al*., 2018). Epididymosomes are 50–250 nm in size, are produced by epithelial cells lining the epididymis, and help in sperm maturation, delivery of antioxidants, and elimination of defective spermatozoa (Amann *et al*., 1993; Belleannee *et al*., 2013; D'Amours *et al*.,2012; Sullivan *et al*., 2013; Trigg *et al*., 2019; Zhou *et al*., 2018). Proteins such as clusterin (CLU), macrophage migration inhibitory factor (MIF), members of the cysteine-rich secretory protein (CRISP) family, disintegrin metalloprotease (ADAM), sorbitol dehydrogenase (SORD), beta-1,4-galactosyltransferase (B4GALT), heat shock protein 70 (HSP70), and protein disulfide-isomerase A (PDIA) have been identified in epididymosomes (Sullivan *et al*., 2013 ;Trigg *et al*., 2019). Collectively, these molecules are postulated to promote the acquisition of sperm motility, oxidation-reduction, metabolism, capacitation, acrosome reaction, and ultimately, fertilization. Epididymosomes exhibit an overall enrichment of sphingomyelin and cholesterol, as well as distinct lipid profiles, which not only enforce the stabilization of the sperm plasma membrane compatible with their long-term storage, but also help

prepare sperm for capacitation upon entering the female reproductive tract and regulating sperm membrane fluidity (Belleannee *et al*., 2013; Girouard *et al*., 2011; Martin-DeLeon, 2015; Sullivan *et al*., 2013). Epididymosomes contain an abundance of antioxidant enzymes, including the reactive oxygen species (ROS) scavenger glutathione peroxidase (GPX) family and other antioxidants, such as serum albumin, gamma-glutamyltransferase, superoxide dismutase, ferroxidase, haptoglobin, and apolipoprotein, which protect mature sperm from oxidative stress due to the presence of high PUFA in the plasma membrane (Aitken, 2017). The ubiquitin-proteasome system in epididymosomes targets proteins for selective enzymatic degradation, thereby aiding in the elimination of defective sperms (Sutovsky *et al*., 2001). Epididymal sperm binding protein 1 (ELSPBP1), present in epididymosomes, is selectively transferred to epididymal sperm that are dead at ejaculation, suggesting that epididymosomes contribute to the identification of poor-quality sperm for elimination (Caballero *et al*., 2013; Légaré *et al*., 2017; Sullivan *et al*., 2013; Trigg *et al*., 2019).

Epithelial cells of the prostate also secrete EVs. These vesicles, sometimes termed prostasomes, are thought to interact with sperm cells in the female reproductive tract to facilitate their reaching the oocyte maturation, potentiate sperm motility capacitation, and initiate the acrosomal reaction (Carlini *et al*., 1997; Pons-Rejraji *et al*., 2011). Prostasomes protect sperm cells from the female immune system on their way to the ovum, inhibit phagocytosis by monocytes and neutrophil granulocytes and interfere with lymphocyte proliferation. Additionally, NK cell activity is inhibited by prostasomes (Ronquist, 2012; Sahlen *et al*., 2010; Saez *et al*., 2003).

Seminal fluid EVs (SFEVs) contain large amounts of protein, nucleic acid, and lipids, and these vesicles also interact with sperm and exert effects on female reproductive tract cells (Stewart *et al*., 2019). Specific factors that may contribute include key elements of calcium signaling pathways, such as CD38, cholesterol, cyclic adenosine diphosphoribose-synthesizing enzymes, progesterone receptors, and ryanodine receptors, which are transferred from the SFEVs to the sperm. A myriad of proteins carried by SFEVs perform various immunological functions. In bull, SFEVs inhibit lymphocyte proliferation, neutrophil phagocytic capacity, and superoxide production. Markers like CD59 are detected in both bull and horse, while CD46 is detected in horse and sheep SFEVs (CD46 and CD59; protection of sperm from complement attack) (Stewart *et al*., 2019). The female reproductive tract is susceptible to opportunistic bacterial infections. SFEVs may counter this through the delivery of antimicrobial molecules, including chromogranin B, secretolytin, synaptophysin, and CAP-18, all of which can curtail bacterial growth (Carlsson *et al*., 2000).

Role of extracellular vesicles in female reproduction

EVs regulate various reproductive physiological functions, including ovarian follicle development, oocyte maturation and fertilization, early embryo development, and endometrial–conceptus crosstalk (Li *et al*., 2017).

EVs present in the uterine fluid may directly transfer information such as miRNAs or proteins (CD52) and leukemia inhibitor factor (LIF), contributing to the endometrial-embryo crosstalk essential for the embryo implantation process. Plasma membrane calcium-transporting ATPase 4 (PMCA4) protein, which is transported through EVs within the uterine fluid during estrus, is likely to be key in the maintenance of calcium homeostasis and sperm viability during storage in the oviduct, capacitation, and the acrosome reaction (Al-dossary *et al*., 2013). Oviductosomes (EVs in the oviductal fluid) contain a membrane protein called Plasma Membrane Ca^{2+} - ATPase 4 (PMCA4), which plays a role in sperm capacitation and fertilization (Avilés *et al*., 2010). Additionally, this study demonstrated the in vitro uptake of exosomal PMCA4 by sperm cells, suggesting an important role of these vesicles during fertilization (Al-dossary *et al*., 2013). Similarly, specific oviductal secretions affect oocyte and sperm function because oviductins, osteopontin, glycodelins, and lactoferrin may play a role in gamete interactions (Machtinger *et al*., 2015).

Proteomic analysis revealed that EVs secreted proteins, such as oviductal glycoprotein (OVGP), heat shock protein A8 (HSPA8), and myosin 9 (MYH9) by bovine oviduct epithelial cells (BOECs), are involved in fertilization, early pregnancy development, and zona pellucida maturation (Lopera-Vásquez *et al*., 2016). Embryos treated with EVs from BOEC culture media induced an increased number of total cells and better survival rate after vitrification compared to embryos cultured without

EVs (Lopera-Vásquez *et al*., 2016). Uterine EVs are involved in embryo–maternal communication through the modulation of biological processes, including the differentiation of trophoblast binucleate cells, apoptosis (EVs induce apoptosis of immune cells), and cellular proliferation prerequisite for uterine receptivity to conceptus implantation (Fazeli, 2011).

Successful pregnancy relies on intricate molecular communication between the embryo and the female reproductive tract, commencing in the oviduct and extending until placental formation. Cytokines and growth factors, such as interleukin-1β (IL-1β), heparin-binding epidermal growth factor (HB-EGF), integrins, and leukemia inhibitory factor (LIF), collaboratively participate in the dialogue between the embryo and maternal tissues (Fazeli, 2011; Nakamura *et al*., 2019). During pregnancy, exosomes released from the fetal membrane have the potential to convey signals originating from the fetus to the maternal uterus and cervix. Extracellular vesicles (EVs) isolated from both trophoblast (TE) cells and maternal endothelial cells, indicative of the endometrial vasculature, contain abundant proteins and microRNAs (miRNAs), such as miR-126-5P, miR-296-5P, miR-16, and miR-17-5P, which may play a crucial role in angiogenesis. Moreover, the upregulation of miR-150 in umbilical cord blood-derived exosomes enhances the proliferation, migration, and tube formation of umbilical vein endothelial cells (Fazeli, 2011; Hu *et al*., 2012).

EVs play a significant role in the maternal recognition of pregnancy, with the trophectoderm of the elongating conceptus secreting interferon tau (IFNT). Uterine EVs contain IFNT, which in turn regulates the expression of genes related to elongation and implantation in the endometrium while inhibiting luteolytic mechanisms. In addition, intrauterine EVs stimulate the production of IFNT protein by conceptus trophoblast cells. EVs carry a diverse range of lipid cargos, including prostaglandins, which are potentially responsible for conceptus elongation and implantation, working in conjunction with progesterone (P4) and IFNT. Various prostaglandins synthesized and secreted by both the conceptus and endometrium exert autocrine and paracrine effects on conceptus development, endometrial function, and responses to P4 and IFNT during early pregnancy.

Mammalian preimplantation embryos develop naturally in the female genital tract (i.e., the oviduct and uterus) and interact with these dynamic and adaptable environments, which are vital for their development and survival (Paria and Dey, 1990). Studies in bovine, porcine, murine, and human models have demonstrated that zygotes and embryos at various stages, including cleavage, morula, and blastocyst stages, release EVs into the extracellular medium. In the absence of a female genital tract, such as during in vitro culture, embryos reside in a semi-defined culture medium that is devoid of endocrine or paracrine factors. This communication gap with the maternal genital tract can be bridged by co-culturing embryos with somatic cells, such as cumulus and oviduct cells, or by using medium conditioned by somatic cells. However, even in the absence of direct contact with genital tract cells, preimplantation embryos can enhance their development in vitro by producing autocrine factors, enabling communication among embryos when cultured in groups. These autocrine factors present in the culture medium, possibly encapsulated within exosomes, may serve as biomarkers predictive of high-quality blastocysts, offering a noninvasive approach for improving in vitro fertilization (IVF) outcomes. EVs in spent embryo culture media may be valuable predictive biomarkers for selecting high-quality IVF blastocysts. Expanding our understanding of EV content and function holds great potential for their use as non-invasive biomarkers in embryo culture and as therapeutic tools in addressing infertility and early pregnancy loss (Glazer *et al*., 2017).

In bovine studies, the addition of EVs collected from uterine flushing around the time of implantation led to downregulation of transcripts associated with the immune system in endometrial epithelial cells (Robbins and Morelli, 2014). MiRNA sequencing of EVs collected at different stages of pregnancy revealed dynamic miRNA profiles, with miR-98 being a potential regulator of the maternal immune system. Pregnancy and embryonic mortality in animals can be diagnosed by detecting individual miRNAs in circulating EVs. Analysis of miRNA profiles from EVs in the maternal blood of cattle on pregnancy day 21 revealed a lower abundance of 27 miRNAs in somatic cell nuclear transfer-derived embryonic loss groups than in successful pregnancy groups. This suggests that the delivery of specific cargo via EVs into the uterine cavity

conditions the uterine environment for improved embryo development and endometrial receptivity, enhancing pregnancy success.

Cell-free RNAs have the potential to regulate gene expression between cells, making them valuable diagnostic markers for describing tissue environments (Giacomini *et al*. 2020). Some evidence suggests that miRNAs encapsulated in follicular fluid (FF) EVs may target mRNA associated with critical signaling pathways, such as the wingless (WNT), transforming growth factor-beta (TGF-β), mitogen-activated protein kinase (MAPK), neurotrophin, epidermal growth factor receptor (ErbB), and ubiquitin-mediated pathways. These pathways play pivotal roles in follicular development, granulosa cell proliferation, cumulus expansion, steroidogenesis, meiosis, and embryo mitosis in mammals. In studies involving equine in vitro models, fluorescently labeled EVs isolated from FF were taken up by ovarian granulosa cells, suggesting that RNA exchange is a significant means of communication in normal ovarian physiology (DaSilveira *et al*., 2012; Hung *et al*., 2017; Sohel *et al*., 2013).

Follicular cells have the capacity to secrete EVs found in FF, which facilitate information transfer between cells. Investigations in equine and bovine models have demonstrated that FF EVs are internalized by granulosa cells in vitro, affecting the expression of genes associated with follicle development (DaSilveira *et al*., 2012; Sohel *et al*., 2013). The majority of miRNAs in FF are found in the exosome fraction (Sohel *et al*., 2013). Some miRNAs in follicular EVs may also regulate oocyte growth, as changes in their expression have been observed in follicles at different stages of oocyte maturation (Sohel *et al*., 2013; Sohel *et al*., 2013). Follicular fluid exosomes have been implicated in the regulation of the TGF-β signaling pathway, a pivotal pathway in follicular development within granulosa cells (Sohel *et al*., 2013). The expression of miRNA-375 in granulosa cells and oocytes facilitates follicular growth, proliferation, and the modulation of apoptosis in cumulus cells. Conversely, overexpression of miR-375 inhibits the proliferation of cumulus cells, increases apoptosis, suppresses estradiol production, and hinders follicular development in porcine granulosa cells (Sohel *et al*., 2013). Polycystic ovarian syndrome (PCOS) is a complex syndrome characterized by reproductive, metabolic, and psychological features including hyperandrogenism, obesity, insulin resistance, polycystic ovarian morphology (PCOM), and anovulation. Differential expression of cellular and extracellular miRNAs has been observed between ovarian follicles in healthy individuals and those with PCOS, indicating molecular signaling disturbances at the preovulatory stage. Several miRNAs have been identified to be differentially expressed in granulosa cells and/or FF between fertile individuals and those with PCOS (Sohel *et al*., 2013).

Furthermore, differentially expressed genes in follicular fluid drive oxidative phosphorylation, a vital process for ATP production that is crucial for the proper function of oviductal cells (Sohel *et al*., 2013). This energy production can also trigger the thermogenesis pathway, a key component of sperm thermotaxis, where sperm adjust their swimming direction in response to temperature gradients, guiding them toward the fertilization site.

Exosomes derived from amniotic fluid exert their influence through inflammatory and procoagulant activities and have been used to treat endometritis in mares to facilitate successful pregnancies (Hell *et al*., 2017; Lange-Consiglio *et al*., 2020). Milk-derived exosomes participate in diverse processes, including bone formation, immune modulation, and regulation of gene expression, particularly long noncoding RNAs (Wang, 2017). Cervical mucus-derived exosomes contribute to fertility by virtue of their sialidase activity, which modifies the highly glycosylated mucus to ease spermatozoa access to the uterine cavity and fallopian tubes (Flori *et al*., 2007). Placental exosomes play a significant role in handling infectious diseases during pregnancy, as exosomes from macrophage-derived sources can amplify the release of pro-inflammatory cytokines, such as IL-6, IL-8, and IL-10, potentially bolstering protective placental immune responses (Nakamura *et al*., 2019). Placental EVs inhibit maternal immune rejection of the fetus through the display of ligands, such as Fas ligand and tumor necrosis factor–related apoptosis-inducing ligand, which inhibit T-cell signaling and induce lymphocyte apoptosis (Fazeli, 2011; Holder *et al*., 2016; Robbins and Morelli, 2014).

Thus, exosomes play multifaceted role in various aspects of reproductive biology and extend their influence. Exosomes are intricately involved in a multitude of reproductive processes, including modulation of transcription and translational activity, granulosa cell proliferation, cumulus

expansion, gametogenesis, normal follicular growth, oocyte maturation, fertilization rate, embryo development, blastocyst formation, and implantation, ultimately shaping pregnancy outcomes and fertility (Yanez-Mo *et al*., 2015).

CONCLUSION

In conclusion, extracellular vesicles (EVs) represent a diverse class of nanoscale membrane vesicles that are actively released by cells. They serve as potent mediators of intercellular communication and have great potential as circulating biomarkers for the diagnosis and prognosis of various diseases. EVs have the unique ability to transfer proteins, lipids, and nucleic acids, exerting an influence on both recipient and parent cells, thereby affecting a wide range of physiological and pathological processes. Recent studies have elucidated the presence and potential functions of EVs in reproductive organs and fluids, including the oviduct, uterus, and embryonic secretions. Deciphering this newly recognized mode of communication promises to advance our understanding of how maternal tissues and embryos regulate early embryo development and implantation. Extracellular vesicles play a crucial role in mediating a wide array of physiological and pathological processes via their involvement in intercellular communication and substance exchange. This opens up promising avenues for gaining insights into the functions of EVs during pregnancy and applying this knowledge to the diagnosis, monitoring, and treatment of pregnancy-related diseases. Further research is essential to unlock the full potential of EVs in reproductive biology and medicine.

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