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Author's contribution to the original publications:

Study 1

Designed and performed the experiments; bovine oviductal epithelial cells' isolation and culture. Spermatozoa processing, co-culture of bovine oviductal epithelial cells with spermatozoa, isolation of RNA, quality and quantity measurement of RNA. Primer designing and qPCR. Analyzed the data and wrote the original manuscript.

Study 2

Designed and performed the experiments; bovine oviductal epithelial cells' isolation and culture. Spermatozoa processing, co-culture of bovine oviductal epithelial cells with spermatozoa, isolation of extracellular vesicles from oviductal conditioned media, RNA isolation from extracellular vesicles, small RNA library preparation. Wrote the original manuscript.

Study 3

Designed and performed the experiments; isolation and characterization of extracellular vesicles from bovine follicular fluids. Co-culture of spermatozoa with extracellular vesicles, analysis of spermatozoa viability, capacitation and acrosome reaction. Analyzed the data and contributed to writing the original manuscript.

ABBREVIATIONS

AI	Artificial Insemination
ASRM	American Society of Reproductive Medicine
ARTs	Assisted reproductive technologies
BOECs	Bovine oviductal epithelial cells
CPM	Counts per million
CTC	Chlortetracycline
DC	Direct contact
DE	Differential gene expression
DEG	Differentially expressed gene
DPBS	Dulbecco phosphate-buffered saline
DLS	Dynamic light scattering
EV	Extracellular vesicles
ESCRT	Endosomal Sorting Complexes Required for Transport
FBS	Fetal bovine serum
FDR	False discovery rate
FF	Follicular fluid
GSEA	Gene set enrichment analysis
GO	Gene ontology
ILVs	Intraluminal Vesicles
ISEV	International Society for Extracellular Vesicles
IVF	*In vitro* fertilization
KEGG	Kyoto encyclopedia of genes and genomes
miRNA	MicroRNA
MVBs	Multivesicular bodies
NC	Non-contact
NTA	Nanoparticle tracking analyser
OS	Oxidative stress
oEVs	Oviductal Extracellular vesicles
PCA	Principal component analysis
RIF	Recurrent implantation failure
RNAseq	RNA sequencing
ROS	Reactive oxygen species
SDF	Spermatozoa DNA fragmentation
SEC	Size-exclusion chromatography
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
tRPS	Tunable resistive pulse sensing
UTJ	Utero-tubal junction
WHO	World Health Organization

1. INTRODUCTION

The intricate molecular mechanisms involved in the pre-fertilization as well as post-fertilization events can provide a strong foundation for developing reproductive technologies to treat infertility. The technological advancements in assisted-reproductive technologies (ART) in the past decade have led to the innovation of diverse tools (Schroeder et al., 2022). The ART-based tools primarily leverage the cellular and molecular mechanisms related to fertilization. Although ARTs has led to successful pregnancies, it is still linked with higher risks for both the mother and the fetus compared to natural conception. (Qin et al., 2015, 2016). Reports have suggested an increase in the incidence of congenital abnormalities found in singletons born after ARTs (Liu et al., 2022; J. Zhao et al., 2020). In cases of natural conception, the interactions that take place before fertilization i.e. the periconception period, are of paramount importance in determining the fate of pregnancy including early embryo development. The majority of molecular interactions between male and female gametes during the period of periconception take place in the oviduct of the maternal reproductive tract. The oviduct actively communicates with male gametes making them competent to penetrate the oocyte by inducing capacitation and the acrosome reaction. In addition, the oviduct also aids spermatozoa transportation, survival, and also establishes proper physiological conditions that are conducive for fertilization.

Prior to fertilization, these interactions occurring between male gametes (spermatozoa) and oviductal cells both at the cellular and molecular levels play an important role in spermatozoa selection (Holt & Fazeli, 2016). Spermatozoa communicate with the epithelial cells of the oviduct primarily by binding to their surface receptors which affects their gene expression (Alvarez-Rodriguez et al., 2019; Fazeli et al., 2004). Furthermore, a previous study revealed that female mice mated with male mice displayed oviductal-transcriptomic upregulation of adrenomedullin as well as prostaglandin endoperoxidase synthase 2 genes compared to the female mice mated with mutant male mice with arrested spermatogenesis. These results suggest the presence of viable spermatozoa are necessary for inducing gene expression changes in the oviduct (Fazeli et al., 2004). The relevance of these changes remained relatively underexplored. In addition, very little has been known regarding the molecular mediators between spermatozoa and the oviductal epithelial cells that led to the alteration in oviductal gene expression. The impact of oviductal proteins that might be engaged in the contact between the spermatozoa and the oviduct has been the focus of countless research studies. In both bovine and porcine species, representatives of the annexin proteins found in the oviductal epithelium have been recognized as vital receptors for spermatozoa surface proteins (Ignotz et al., 2007; Teijeiro et al., 2009). Altogether, these studies suggested that sperm-oviductal interactions are mediated by diverse secreted proteins as well as

receptors facilitating the cross-talk between male gametes and the female reproductive tract.

Apart from the secreted proteins of oviductal epithelial cells and their interaction with male gametes, another class of messengers seem to mediate these interactions. These interactions seem to be carried out by exosomes and microvesicles that are collectively called extracellular vesicles (EVs). EVs are a heterogenous group of lipid bilayer enclosed nanoparticles that act as mediators of intercellular communication (Machtinger et al., 2016a; Pitt et al., 2016; Simon et al., 2018). EVs transmit their messages to the recipient cells by releasing their diverse molecular cargo containing DNA, RNA, proteins, lipids and other metabolites utilizing endocytosis as well as cellular fusion processes (Raposo & Stoorvogel, 2013). Exosomes are vesicles that have a diameter of about 30 to 100 nm and are generated by a process of endocytosis where as microvesicles bud off from the plasma membrane (Valadi et al., 2007). Apart from the EVs from oviductal epithelium playing an important role in supporting spermatozoa motility, and viability as well as transport (Almiñana et al., 2018) the EVs derived from oviduct (oEVs), also interact with the female gamete (oocyte). Microvesicles derived from canine oviducts have proven to play an essential role in oocyte maturation (Lange-Consiglio et al., 2017). Moreover, during the process of oocyte release from the ovary some of the follicular fluid (FF) also reaches the oviduct (Brüssow et al., 1999). The FF is a rich source of EVs, that have been reported to influence the oviductal transcriptome (Hasan et al., 2020). At the same time, progesterone present in FF is also known to induce capacitation and the acrosome reaction in spermatozoa (Bravo & Valdivia, 2018a) but the impact of FF derived EVs on capacitation and the acrosome reaction of spermatozoa remains elusive.

Therefore, taken together, these studies imply that there exists a two-way dialogue between the spermatozoa-oviduct and EVs and can be playing a vital role in this interaction. The spermatozoa influence the oviductal transcriptome and in turn, the oviductal components affect the vital functional parameters of the spermatozoa. However, any imbalances in this two-way communication process could significantly affect the fertilization process and because of this, such molecular interactions could serve as potential targets for the diagnosis and treatment of infertility. While ARTs like IVF and AI have been helpful in treating infertility, failure in fertilization or implantation may occur due to dysregulated spermatozoa-oviduct interactions. (Bashiri et al., 2018; Tian et al., 2022). Therefore, in this thesis, we investigated whether spermatozoa can communicate with the oviductal epithelial cells remotely and alter the gene expression of the oviduct without directly interacting with them. In addition, we also aimed to study the changes in the cargo and production of EVs derived from the oviduct in response to spermatozoa. Furthermore, we also showed the dose/concentration-dependent effects of FF derived EVs on the vital functions of spermatozoa which includes viability, capacitation and the acrosome reaction. It has been established that spermatozoa capacitation and the acrosome reaction is enhanced by progesterone that is already present in the FF (Calogero

et al., 2000). Therefore, we also evaluated whether FF-EVs and progesterone have synergistic effects in impacting the viability, capacitation, and the acrosome reaction of spermatozoa. Furthermore, unless stated otherwise in the experiments documented in this thesis, the term “EVs” pertains to microvesicles and exosomes.

2. REVIEW OF LITERATURE

2.1 Periconception and Infertility

Infertility is a significant issue in contemporary society and it is believed to affect 8% – 12% of reproductive-aged couples worldwide (Vander Borght & Wyns, 2018). Infertility is defined by the American Society of Reproductive Medicine (ASRM) as the inability to conceive despite more than one year of efforts at natural insemination (Habbema et al., 2004). Infertility in women could arise from a plethora of factors which are broadly seen as issues in the pre-conception or the post-conception processes. The various organs, fluids, tissues, and glands in the female reproductive system play essential roles in creating a favourable environment for the fusion of male and female gametes, leading to a successful pregnancy. The oviduct/fallopian tubes play a key role in maintaining the periconception environment and facilitate the transport of gametes, maturation of male gametes, fertilization, and early embryonic development (Croxatto, 2002; S. Li & Winuthayanon, 2017a).

Despite these notable events taking place in the oviduct, the information regarding the periconception milieu in the oviduct is very limited. The scientific community has historically overlooked the importance of the oviduct due to the success of ARTs. However, there is now a growing recognition of its significance in developing new tools to treat various forms of infertility (Fazeli & Holt, 2016; Ménézo et al., 2015). The oviductal environment plays a critical function in supplying the embryo with enough metabolic support and shielding it from oxidative stress (Avilés et al., 2010; Maillo et al., 2016). The oviductal fluid is a complex mixture containing numerous proteins with various effects on the fertilization process and the development of preimplantation embryos. Fertilization may occur *in vitro*, and it is evident that the presence of these unique proteins is not necessary for this procedure; nonetheless, they may aid in preparing the oviduct for the arrival and development of an early embryo (Coy & Yanagimachi, 2015). Moreover, a study has reported distinct modifications in the proteomic profile of oviductal secretions induced by the presence of both male and female gametes. These alterations appear to create a conducive environment for the gametes and prime the oviductal milieu for the arrival of the embryo (Georgiou et al., 2005). The success of ARTs led researchers to believe that the pre-conception microenvironment, the major part of which is the oviductal fluid, might be substituted by a simple, buffered, balanced salt solution with a sufficient energy supply. Despite the significant advances in ARTs since the birth of the first *in vitro* fertilization (IVF) baby in 1978, achieving pregnancy through ART is still not guaranteed, and many couples require multiple attempts (Garrido et al., 2011). During natural conception, among millions of spermatozoa that are deposited in the vagina, approximately 1,000 make it to the fallopian tubes, where one spermatozoon might potentially fertilize an oocyte. Due to *in vivo* selection of fertilization-competent spermatozoa that can interact with the oocyte, the quantity of spermatozoa

accessing the fallopian tube is drastically reduced (Holt & Fazeli, 2010). In ARTs, spermatozoa selection is typically based on morphological characteristics and motility. However, these attributes do not necessarily reflect the fertilization potential of spermatozoa, leading to cases of unexplained infertility (Leung et al., 2022; Lipitz et al., 1993).

Approximately 30% of couples diagnosed with infertility globally have idiopathic or unexplained infertility, which means that there is no clear cause for their inability to conceive. (Sadeghi, 2015). Infertility is becoming increasingly prevalent across generations, and has garnered significant attention due to its impact on a large number of couples in their reproductive years. Despite the rapid development of ARTs, there are still significant limitations, such as an unacceptable rate of success, unexplored mechanisms, and restricted therapies. Comprehending the intricate cellular and molecular mechanisms that take place before, during, and after conception is essential for enhancing the efficacy of current ARTs. Within these mechanisms, intercellular communication facilitated by EVs is gaining increasing attention among developmental biologists as a potential target for improving ARTs to diagnose and treat infertility causes (Zhou et al., 2021). Effective intercellular communication is critical for the normal function of multicellular organisms, including reproductive physiology. Researchers have conducted numerous scientific and clinical studies in recent years to explore the possible relationship between EVs and reproductive health or disorders (Zhou et al., 2021). EVs, as a collective entity, play an essential role in regulating various physiological and pathological processes through intercellular communication and cargo exchange. Therefore, they represent a promising avenue for better understanding and treating infertility.

2.2 EVs: Nanocontainers filled with messages

EVs are a heterogenous group of double lipid layered, nano-sized semi-spherical particles released from diverse prokaryotic and eukaryotic cells (Gill et al., 2019; Pol et al., 2012). In addition, the presence of EVs has also been reported in various biological fluids e.g. urine, blood, saliva, milk, semen, and FF (Carnino et al., 2019; Monguió-Tortajada et al., 2019). Initially, the EVs were not considered of any biological importance but this viewpoint considerably changed once they were recognized as nano shuttles facilitating cell-cell communication (Simons & Raposo, 2009). EVs transport various molecules, including miRNAs, mRNAs, lipids, proteins, and DNA, and deliver them to the target cell, where they alter physiological responses (Waqas et al., 2022). Several reports have suggested that EVs are involved in malignancies, immunological responses, pregnancy, and other pathophysiological conditions (Dietz et al., 2021; Kim et al., 2020). Emerging studies have revealed that EVs derived from several categories of cells are intricately involved in the reproductive signalling events and are playing an active role in the successful establishment

of pregnancy by regulation of gamete maturation, fertilization as well as in the implantation of the embryo (Machtinger et al., 2016b).

2.2.1 Biogenesis of EVs

EVs can be classified into three subtypes based on their biogenesis and size: exosomes, microvesicles, and apoptotic bodies, as depicted in Fig 1. Microvesicles, the size of which ranges from 50 nm – 1000 nm are also called ectosomes and are shed by the outward budding of the cells surface. The requisite for the formation of microvesicles is the relocation of phospholipid phosphatidylserine from the inner leaflet of the plasma membrane towards the outer leaflet. Once a sufficient amount of phospholipid phosphatidylserine is translocated by the action of aminophospholipid translocases, the release of the microvesicle is mediated by the contraction of the actin-myosin system (Petrovčíková et al., 2018). The size of exosomes overlaps with microvesicles which range from 40 nm – 100 nm, however, the mechanism of formation of exosomes includes the endosomal sorting complex required for the transport (ESCRT) pathway. The syndecan-synthenin pathway is adopted by ESCRT for the formation of multivesicular bodies (MVBs) which contain intraluminal vesicles (ILVs) (Petrovčíková et al., 2018). Once the MVBs mature, the inward invagination of the endosomal membrane takes place which eventually leads to the fusion of MVBs with the plasma membrane leading to the release of exosomes into the extracellular space (Teng & Fussenegger, 2021). Cholesterol plays a key role in determining the fate of the contents present in ILVs which are packed within MVBs. The MVBs which have higher amounts of cholesterol are destined for secretion as exosomes whereas the MVBs with lower cholesterol content fuse with the lysosome wherein the contents of the ILVs are degraded (Möbius et al., 2002). Apoptotic bodies are larger in size among the subtypes of EVs ranging from 500 nm – 4000 nm. Apoptotic bodies are formed by membrane blebbing as a result of programmed cell death. The cells undergoing apoptosis have nuclear chromatin condensation, followed by blebbing and eventually enclosure of the disintegrated products into vesicles which are called apoptotic bodies (Akers et al., 2013).

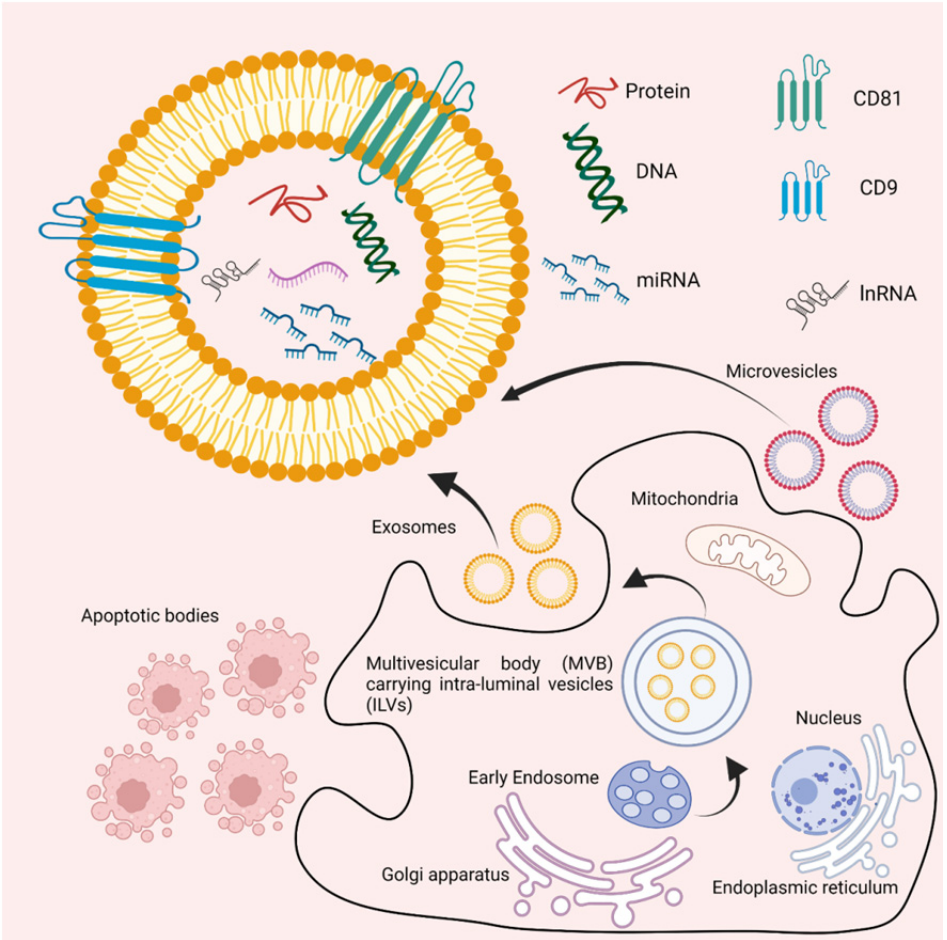

Figure 1: Illustration depicting the biogenesis of EVs: EVs are formed through two main pathways: the endosomal pathway and the plasma membrane budding pathway. After internalization of extracellular material through endocytosis, some early endosomes mature into multivesicular bodies (MVBs). MVBs can either fuse with lysosomes for cargo degradation or undergo fusion with the plasma membrane, releasing intraluminal vesicles (ILVs) as exosomes. In the plasma membrane budding pathway, EVs are generated directly from the plasma membrane through outward budding and subsequent pinching off. This process gives rise to microvesicles, which are released directly into the extracellular environment. Both exosomes and microvesicles are enriched with bioactive molecules, including proteins, lipids, and nucleic acids, which can be selectively packaged and transferred to recipient cells, thereby mediating intercellular communication.

2.2.2 EV Cargo: Composition of the messengers

Substantial investigations have been conducted to elucidate the composition of EVs. As a consequence, many databases including datasets from various EV research have been assembled which are publicly available (Mathivanan & Simpson, 2009; Simpson et al., 2012). EVs are capable of harbouring a diverse array of biomolecules which include a wide variety of proteins, lipids, and nucleic acids and this cargo may trigger a functional response in the recipient cell.

2.2.2.1 Proteins

A comprehensive study of the protein cargo of EVs has been conducted, analyzing the contents of varied-sized vesicles formed by distinct cell types (Conde-Vancells et al., 2008; Demory Beckler et al., 2013; Turiák et al., 2011). The majority of proteins identified in EVs are those involved in biogenesis processes, including endosomal pathway proteins. Components of the ESCRTs, such as ALIX and TSG101, are enriched in vesicles. Furthermore, EVs include a variety of tetraspanins, such as CD63, CD81, and CD9. Signal transduction proteins such as MHC I and MHC II, and transmembrane proteins LAMP1 have also been detected in EVs (Abels & Breakefield, 2016).

2.2.2.2 Lipids

According to a research article, the lipid composition of EVs has been observed to have similarities with the cells they originate from. Several lipids including sphingomyelin, cholesterol, ganglioside GM3, phosphatidylserine, and ceramide are found in high abundance in EVs. EVs derived from MVBs have a greater amount of phosphatidylserine facing the extracellular environment compared to the plasma membrane of the cell, which may facilitate their uptake by recipient cells (Fitzner et al., 2011; Llorente et al., 2013).

2.2.2.3 Nucleic acids

The transfer of DNA by EVs has been observed, with evidence indicating that EV-DNA can increase mRNA and protein expression in recipient cells (Cai et al., 2013). However, EVs are mostly enriched in diverse species of RNA such as mRNA, miRNA, rRNA, t-RNA, piwi-interacting RNA, and Y-RNA (Crescitelli et al., 2013; C. C. Li et al., 2013; Nolte-'t Hoen et al., 2012). Among all the diverse forms of RNA, miRNA is one of the most studied and enriched forms present in the EVs (Munir et al., 2020; Smith et al., 2020). miRNAs are non-coding RNAs that are relatively short (often containing less than 22 nucleotides) and play an important part in the post-transcriptional regulation of gene expression (Chekulaeva & Filipowicz, 2009; He & Hannon, 2004). Recent studies have focused on EV derived full-length tRNAs and tRNA fragments (tRFs), as a prospective source of biomarkers and new mediators in eukaryotic cell-to-cell communication (Sharma et al., 2016; Tosar & Cayota, 2020; Wilusz, 2015).

2.2.3 Characterization of EVs: Tools to decode the messages

Over the past decade, there has been a notable surge in research into EVs and the discovery of new functional applications for these vesicles. However, it has been challenging to obtain pure EV elutes which are devoid of non-vesicular components for functional research. As a result, it was necessary to assemble a set of specifications for the analysis of these vesicles and reporting of studies based on EVs. The International Society for EVs (ISEV) provides researchers with a minimal set of biochemical, biophysical, and functional standards that researchers should follow to verify that specific nanoparticles are EVs (Lötvall et al., 2014; Théry et al., 2018a). These guidelines aim to ensure transparency, consistency, and reproducibility in the reporting and characterization of EVs in research articles. The key components of these guidelines include:

- **Sample Collection and Processing:** ISEV suggests thoroughly describing methods used for sample collection, including appropriate anticoagulants and handling procedures. In addition, details regarding techniques employed for EV isolation and the removal of cell debris must be provided. The pre-analytical variables that could potentially influence EVs and their characterization must also be documented.
- **EV Isolation and Characterization:** Clear explanation of EV isolation methods used and also description of any additional purification steps performed, such as immunocapture or immunoaffinity-based methods. EVs must be characterized based on size, morphology and EV markers such as tetraspanins (CD9, CD63, CD81).
- **Nucleic Acid Analysis:** The methods used for isolating nucleic acids (RNA or DNA) from EVs must be reported including explanations for RNA quality control methods such as RNA integrity number (RIN) analysis or Agilent Bioanalyzer profiles. The techniques used for RNA quantification, such as qPCR or RNA sequencing must be described. In addition ISEV also recommends to address any potential contamination of EV samples with non-vesicular RNA and strategies to control it.
- **Functional Assays and Functional Validation:** The detailed descriptions of functional assays conducted on EVs, including uptake studies, recipient cell response assays, or functional cargo delivery assays. Furthermore, according to ISEV guidelines, there must be inclusion of appropriate controls, both positive and negative, to validate the functionality of the EV preparations.
- **Terminology and Nomenclature:** ISEV also suggests to adhere to accepted nomenclature and terminology, making a clear distinction between EV subtypes (exosomes, microvesicles, apoptotic bodies) and non-vesicular particles. Usage of the generic term “extracellular vesicles” accurately to refer to the vesicular particles under investigation.

2.2.3.1 Characterization based on size and concentration of EVs

Precise measurement of EV size is crucial for their accurate characterization, as it plays a key role in determining their biological function. Therefore, technologies that can accurately measure particle size in the nanometer range are essential. Several commonly used instruments that can measure the size distribution of EVs are listed below:

Nanoparticle tracking analysis (NTA):
In order to measure the size and concentration of EVs, a light scattering technology NTA is one of the techniques that can be employed (C. Gardiner et al., 2013). The Brownian motion of these particles in a solution is the underlying basis for quantifying EVs. The laser inside the NTA instrument illuminates the particles due to which the particles scatter and their movement is captured by a camera. Afterwards using the Stokes-Einstein equation particle size is calculated based on the distance travelled by it (Dissanayake, Midekessa, et al., 2021). NTA is a well-suited tool as compared to DLS for measuring the size of particles in a sample containing heterogeneous populations of nanoparticles (Chan et al., 2017; Wright, 2012).

Dynamic Light scattering (DLS):
DLS is another technique for nanometer-scale particle size measurements. DLS also measures the size of nanoparticles in suspension based on the Brownian motion of dispersed particles and is considered ideal to measure monodispersed nanoparticles (Chan et al., 2017).

Tunable Resistive Pulse Sensing (tRPS):
tRPS is a highly precise technology that enables researchers to measure the size, charge, and concentration of EVs with great accuracy. This technology employs a pore-based sensor that evaluates each particle individually as it passes through the filter. However, there is a risk of filter clogging due to larger-sized particles, which can impair the instrument's functionality (Administrator, 2013; Maas et al., 2017; Phan et al., 2021).

2.2.3.2 Characterization based on the morphology of EVs

Sophisticated and optimized microscopy is crucial in identifying the structural characteristics of EVs. Numerous imaging technologies have now been developed to assist researchers in evaluating the morphology of EVs. Some of them are described below:

Transmission electron microscopy (TEM): TEM is the most used electron microscopy technique adopted for imaging EVs. In order to get the samples ready for TEM imaging, the EVs are embedded onto a carbon-coated grid following which they are fixed, and then dehydrated (Hasan et al., 2020). The electron gun generates a stream of electrons, which are then directed at the

specimen. As the electrons pass through the sample, the electrons scatter at different angles and based on the scattering of electrons and the resulting contrast generates image on the screen (Malenica et al., 2021).

Scanning electron microscopy (SEM): SEM is another imaging technique used for visualizing EVs, which utilizes a high-energy electron beam to generate magnified images of the surface of EVs. Prior to imaging, EVs are chemically or cryogenically fixed and dehydrated to prepare them for imaging. The scanning of the EVs with the electron beam generates emitted electrons, which are used to obtain images of the EVs. This technique provides topographical details of the surface of EVs (Malenica et al., 2021).

Cryo-electron microscopy (cryo-EM): Cryo-EM involves fixing samples by using a technique called cryo-immobilization, where water is vitrified enabling samples to remain in their native state. The specimens are imaged at low temperatures which ensures that the EVs retain their characteristic spherical form (Koifman et al., 2017).

2.2.3.3 Characterization based on the presence of EV markers

When the contents or function of EVs are described, it is necessary to establish the enrichment of EV markers and the depletion of contaminants. The following methods described are adopted for the biochemical characterization of EVs:

Western Blotting: The standard western blot method is used to identify protein markers associated with EVs to validate the enrichment of EVs. The presence of markers such as CD9 and CD63 are important to confirm the isolated particles are EVs (Lo et al., 2020).

EV array: This technique generates a multiplexed phenotyping for EVs and captures EVs using antibodies. This method of detection is sensitive and requires low sample volumes, however, this technique is not widely available (Jørgensen et al., 2013).

Mass Spectrometry: The proteome analysis of EVs using mass spectrometry (MS) has attracted a lot of interest as a way to find new protein biomarkers. Developments in high-performance mass spectrometry and comprehensive proteome databases have resulted in successful analysis of EV based proteins (Jalaludin et al., 2023).

The overlapping similarities between exosomes and microvesicles are the major hindrances in isolating the two sub-population of EVs. However, many isolation protocols have been proposed by various studies which are listed below in Table 1.

Table 1. Description of methodologies used for purification of EVs

Method	Principle	Advantages	Disadvantages	Purity	Yield	References
Size Exclusion Chromatography (SEC)	SEC separates molecules depending on their size. When molecules of various sizes are incorporated or omitted from the matrix's pores, separation occurs.	Simple, cost-effective, and preserving the biological function and structure of EVs	Cannot be used if the EVs and contaminants are of the same size as contaminants co-elute with EVs	High	High	(Mohammadi et al., 2021)
Ultracentrifugation	Separation based on the density of the particles	Appropriate for large sample volumes, inexpensive	Exosomes damaged by the laborious, time-consuming, and low-yield procedure	Medium	Low	(Lin et al., 2020)
Density gradient ultracentrifugation	Under centrifugal force, molecules settle until they reach a medium with the same density as them	High purity and no EV damage	Labour-intensive planning and complex procedure.	High	Low	(Kamerkar et al., 2017)
Filtration	Size based separation while passing through filters that have a defined pore size	Simple, no special equipment or reagents are required	Blockage of filtration membrane and loss of exosomes	High	Medium	(Ding et al., 2021)
Immunoaffinity	Interaction of antibodies with particular membrane proteins of EVs	EV subtype separation with a high degree of specificity	Expensive	High	Medium	(Coumans et al., 2017)
Polymer based precipitation	The precipitation of EVs is carried out using polymers	Appropriate for large-volume sample	Contamination and aggregation	Low	High	(Coumans et al., 2017)

2.3 Impact of EVs on Fertilization

The process of fertilization involves a series of synchronized molecular events, the most crucial of which is the amalgamation of a spermatozoon and the oocyte, leading to the formation of a diploid zygote (Georgadaki et al., 2016a). Fertilization requires mammalian spermatozoa to travel a considerable distance in the female reproductive tract to reach the site of fertilization in the oviduct (Suarez & Pacey, 2006). Although only one spermatozoon is required to fertilize the egg, millions of spermatozoa are deposited into the vagina after natural mating. The acidic vaginal pH is not conducive to spermatozoa survival, and they must travel through the cervix, which is lined with mucus-filled grooves, in order to reach the uterus and fallopian tubes. The mucus that is present inside the canal serves as a significant barrier for spermatozoa, especially those with impaired motility and affected morphology. In addition, the hostile immune system also targets spermatozoa and only some of them make their way into the uterus. Spermatozoa move through the uterus in the direction of the oviduct with the assistance of contractions of the smooth muscles of the uterine lining (Siu et al., 2021). The viscosity, pH, and immunological reactions all operate as fine selection barriers to prevent spermatozoa from passing through the lower female reproductive tract. The process of a fine selection of spermatozoa takes place in the oviduct where normal sperm morphology and motility are insufficient for passage across the utero-tubal junction (UTJ). There is evidence suggesting that in order to pass through the UTJ, spermatozoa are required to possess surface proteins, particularly a disintegrin and metalloprotease 3 (ADAM3) (Yamaguchi et al., 2006),(Yamaguchi et al., 2009). Apart from ADAM3, another barrier may be the viscous mucus present in the grooves of UTJs which might hinder the transportation of spermatozoa (Georgadaki et al., 2016b; Tienthai, 2015). A small number of spermatozoa travel via the utero-tubal joints to reach the oviducts where they are kept viable or preserved in a reservoir by mediating contact with epithelial cells of the oviduct (Suarez & Pacey, 2006). The oviduct is a safe place for spermatozoa to reside and as soon as they reach the oviduct, careful regulation of the maternal immune system is required to allow survival of male gametes and the developing fetus (Pérez-Cerezales et al., 2018). In order to ascend towards the oocyte, spermatozoa needs to attain competence to fertilize the oocyte successfully. The process of fertilization is reliant on the functional maturation of spermatozoa, which occurs in the oviduct. These maturation processes includes the necessary steps of capacitation, followed by the acrosome reaction, and without these steps, fertilization cannot occur. During the process of capacitation, cholesterol and many other sterols are stripped away from the plasma membrane of the spermatozoon head (Ikawa et al., 2010). The removal of cholesterol increases the permeability of the membrane due to which the concentration of intracellular calcium ions increase eventually modulating protein tyrosine phosphorylation (Naz & Rajesh, 2004; Shadan et al., 2004). Capacitation results in hyperactivated motility and initiates

the acrosome reaction of the spermatozoa (Aitken & Nixon, 2013). All the events beginning with the arduous trek of spermatozoa to fertilization and eventually to implantation require a fine tuning of the intercellular communication. EVs have been reported as one of the essential mediators of the cross-talk that exists during pre and post fertilization. Some of the roles played by EVs in reproduction related events are described in Table 2 below:

Table 2. Description of some of the studies related to the effects of EVs on pre and post fertilization events

Process	Findings	Species
Maturation of spermatozoa	EVs derived from epididymal fluid (epididymosomes) include proteins related to spermatozoa maturation, including macrophage migration inhibitory factor (MIF) and aldose reductase (AKRB1) (Frenette et al., 2003).	Bovine
Inhibition of premature acrosome reaction and premature capacitation	The spermatozoon membrane after fusing with EVs (prostasomes) present in the seminal fluid, becomes enriched with cholesterol, sphingomyelin, and saturated glycophospholipids as a result of which the membrane fluidity of spermatozoa reduces, which inhibits an early acrosome reaction (Pons-Rejraji et al., 2011).	Human
Capacitation, acrosome reaction, and fertilization	Prior to fertilization, CD9-labeled EVs from the plasma membranes of oocytes transport proteins to fertilizing spermatozoa in the perivitelline space (PVS). Transfer of these EVs is essential for remodelling of the spermatozoon membrane and fusion with the oocyte (Barraud-Lange et al., 2007).	Mice
Prevention of polyspermy	Following fertilization, Juno is shed from the oolemma and redistributed in EVs. These EVs can attach and neutralize acrosome reacted spermatozoa to avoid polyspermy (Bianchi & Wright, 2014).	Mice
Communication between embryos	Improvement of the cloned embryo *in vitro* when co-cultured with porcine embryos. Labelled EVs from porcine embryos were taken up by the cloned embryos (Saadeldin et al., 2014).	Porcine
Maturation of oocyte	EVs derived from FF play a role in the cumulus expansion of the oocyte (Hung et al., 2015).	Bovine

The final maturation of gametes, fertilization, and early embryo development take place in the oviduct which makes it a key organ to determine the fate of pregnancy. In recent decades, there have been significant advancements in procedures such as cryopreservation and IVF yet, in terms of efficiency these techniques are far from optimal as compared to *in vivo* conception. Multiple studies using human and animal models suggest that technologies ARTs, such as intracytoplasmic sperm injection or *in vitro* fertilization, which circumvent these gamete/embryo-oviductal interactions, are linked to genomic imprinting abnormalities (Duranthon & Chavatte-Palmer, 2018; Lazaraviciute et al., 2014). Hence a detailed understanding of oviduct biology and its secretory components including EVs will help to enhance various ART procedures, which will have significant effects on human health.

2.4 Oviduct: The site of fertilization and source of EVs

The oviduct is a particular region in the female reproductive tract also known as the fallopian tube (in humans), which is connected to the uterus and is situated near the ovary as shown in Fig. 2. The oviduct is a site where fertilization occurs in a vast number of animals. The oviduct was termed as *tuba uteri* by Gabriele Fallopius who was the first person to give the anatomical description of the oviduct (Leese, 1988). The oviduct/fallopian tube is the site of fertilization, pre-implantation embryo development, and the place where spermatozoa attains functional maturation (Killian, 2004; S. Li & Winuthayanon, 2017b). The oviduct is a fibromuscular structure with many layers including the mucosa, connective serosa and muscular layer (Avilés et al., 2015). Anatomically, the oviduct comprises the following parts:

a) The infundibulum which is funnel-shaped and has a fimbriated end lies in close proximity to the ovary. It is responsible for transporting oocytes from the ovary to the ampullar region of the oviduct (Talbot et al., 1999).
b) The ampulla is the middle portion with the expanded lumen. This part of the oviduct hosts fertilization and cleavage of the early diploid zygote (Coy et al., 2012; S. Li & Winuthayanon, 2017b).
c) The isthmus is the narrow part of the oviduct, acting as a reservoir for storing spermatozoa. The binding of spermatozoa with epithelial cells of the oviduct occurs in this region. This region of the oviduct governs spermatozoa function, including viability and capacitation (Miller, 2015).
d) The UTJ connects the endometrial cavity of the uterus to the fallopian tube. The presence of epithelial crypts makes it act as a selective gate that allows individual spermatozoa with proper morphology to move towards the isthmus (Hunter, 1995).

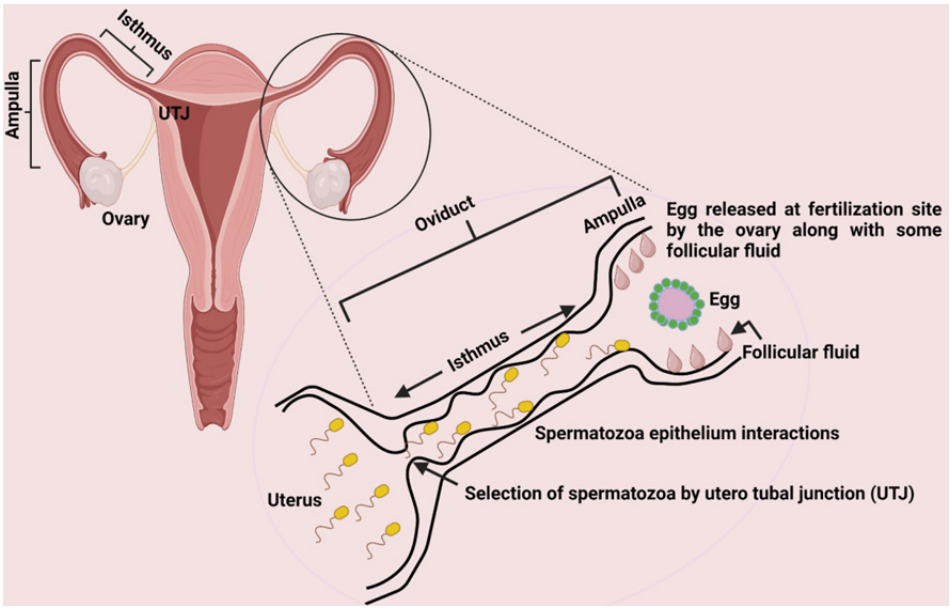

Figure 2: A schematic depicting the various parts of the oviduct along with their functions

The simple columnar epithelial cells that line the lumen of the oviduct may be divided into two categories – ciliated cells and nonciliated or secretory cells. The components released by secretory cells are driven onto the surface of the oviduct epithelium by ciliary action, facilitating the transit of gametes and zygotes. The number of secretory cells steadily rises towards the isthmus, with ciliated cells predominating in the fimbria area (Abe, 1994; Özen et al., n.d.). The oviductal fluid includes components released by secretory epithelial cells and plays a pivotal role in influencing functions of spermatozoa, fertilization, and early embryo development (Killian, 2004; Saint-Dizier et al., 2020). The constituents present in the oviductal fluid are listed in Table 3.

Table 3. List of some of the important components in oviductal fluid

Constituents present in oviductal fluid	Examples
Growth Factors	• Embryotrophic factor-3 (Xu et al., 2004) • Fibroblast growth factor (Pinto-Bravo et al., 2021) • Granulocyte-macrophage colony stimulating factor (de Moraes & Hansen, 1997) • Hepatocyte growth factor (Srivastava et al., 1999) • Insulin growth factor 1 (Stevenson & Wathes, 1996)
Cytokines	• Interleukin-1 (Giudice & Saleh, 1995) • Interleukin-6 (Jiwakanon et al., 2010) • Interleukin-8 (Palter et al., 2001) • Interleukin-10 (Jiwakanon et al., 2010) • Interleukin-12 (Strandell et al., 2004) • Transforming growth factor α (Giudice & Saleh, 1995) • Transforming growth factor β (Strandell et al., 2004)
Hormones	• Adrenomedullin (H. W. R. Li et al., 2010) • Angiotensin (Wijayagunawardane et al., 2009) • Gonadotropin releasing hormone (Sengupta & Sridaran, 2008) • Leptin (Archanco et al., 2007) • Nitric oxide synthase (Kobayashi et al., 2016) • Oxytocin (Schaeffer et al., 1984) • Progesterone (Lamy et al., 2016)
Proteases	• Matrix Metalloproteases (Zampini et al., 2014) • Protease nexin-1(A. Kouba et al., 2000) • Plasminogen activator (A. Kouba et al., 2000)
Antioxidant protection	• Catalase (Lapointe & Sirard, 1998) • Cu-Zn Superoxide dismutase (Guerin, 2001) • Mn-Superoxide dismutase (Guerin, 2001) • Glutathione (C. S. Gardiner et al., 1998)
Defence agents	• Complement component 3 (Lee et al., 2009) • Defensin-5 (Quayle et al., 1998) • Haptoglobin (Lavery et al., 2004) • Immunoglobulins (Kimijima et al., 1990) • Lactoferrin (Zumoffen et al., 2013)
Glycosidases and Glycosyltransferases	• Amylase (Bruns et al., 1982) • β-D-galactosidase (Rios et al., 2002) • α-D-mannosidase (Sukeno et al., 1972) • β-N-Acetyl glucosaminidase (Solomon, 1979)
Enzymes	• Acid phosphatase (Aire & Steinbach, 1976) • Choline acetyltransferase (Steffl et al., 2006) • Cyclooxygenase (Popli et al., 2015)
Chaperones	• Heat shock proteins (Lloyd et al., 2009) • GRP 58 (Bauersachs et al., 2004) • GP 96 (Bauersachs et al., 2004)

Constituents present in oviductal fluid	Examples
Proteins	• Albumin (Hendler et al., 1957) • Annexin (Teijeiro et al., 2016) • AWN (sperm adhesin) (C. Y. Song et al., 2010) • LAMP-1 (Töpfer-Petersen et al., 2008) • LAMP-2 (Töpfer-Petersen et al., 2008) • Oviduct specific glycoprotein (Y. Zhao et al., 2022)
Glycosaminoglycans and proteoglycans	• Hyaluronan (Fouladi-Nashta et al., 2017) • Syndecan (Tienthai, 2011) • Betaglycan (Kubota et al., 2009)

In addition to above mentioned components, a study in pigs also revealed that there is a minimal contribution of FF in the oviductal fluid during the ovulation period (Brüssow et al., 1999). Furthermore, a study revealed that the negligible amount of FF entering the oviduct might have some stimulatory effects on spermatozoa (Hansen et al., 1991a). Al-Dossary et al. reported for the first time the existence of EVs in the oviductal fluid (oEVs) of the mouse (Al-Dossary et al., 2013). Several studies since then have detected EVs in the oviducts of several animals, including cattle, pigs, dogs, birds, mice, and humans (Alcântara-Neto et al., 2020; Bathala et al., 2018; Lange-Consiglio et al., 2017; Waqas et al., 2017). Some of the findings from the aforementioned studies are listed as follows in Table 4.

Table 4. Studies from the literature regarding oviductal EVs and their function

Species	Findings
Bovine	• oEVs generated *in vitro* enhance embryo quality and cryosurvival (Sidrat et al., 2022). • oEVs improve the development and quality of the embryo (Lopera-Vasquez et al., 2017).
Canine	• miRNAs derived from oEVs are associated with oocyte maturation and enhancing canine IVM (Lange-Consiglio et al., 2017).
Red Wolves and Cheetahs	• oEVs cargo plays an essential role in maintaining the vital functions of spermatozoa (de Almeida Monteiro Melo Ferraz et al., 2020)
Porcine	• oEVs regulate polyspermy during IVF (Alcântara-Neto et al., 2020).
Murine	• oEVs improve the efficiency of embryo transfer (Qu et al., 2019).

2.5 Bidirectional interactions of Spermatozoa and Oviduct

The fate of various aforementioned events e.g. sperm capacitation, fertilization, and early embryo development are determined by the interactions between the oviduct and spermatozoa, which constitutes an essential part of the periconception milieu. There are multiple facets surrounding the interactions that take place between spermatozoa and the oviduct. One repercussion of spermatozoa–oviduct interaction is the alteration of the gene expression and proteome of the oviductal epithelial cells in response to the arrival of spermatozoa. A growing body of literature has reported that the arrival of spermatozoa in the oviduct regulates gene expression in oviductal epithelial cells, leading to the up and down-regulation of numerous genes. A study in mice revealed that the delivery of spermatozoa into the reproductive tract by natural mating resulted in the alteration of gene expression levels in 214 genes and the same pattern was not induced by spermatozoa from infertile mice (T145H mice) (Fazeli et al., 2004; López-Úbeda et al., 2015). In addition, a study has demonstrated that the oviduct can differentiate between X and Y chromosome bearing spermatozoa as the gene expression of oviduct alters differentially in response to the X and Y chromosome bearing spermatozoa. These results suggest that oviduct acts as a sensor differentiating between X and Y chromosome bearing spermatozoa (Almiñana et al., 2014). It is estimated that only around 1000–5000 spermatozoa reach the oviduct in porcine and bind to the oviductal cells out of 30 billion spermatozoa deposited into the female reproductive tract after insemination (Rodriguez-Martinez et al., 2020). This oviductal selection mechanism seems to be based on the DNA integrity of the spermatozoon or some other characteristics of the spermatozoon (Holt & Fazeli, 2010). The relevance of spermatozoa-induced gene expression in the oviduct remains poorly understood concerning its impact on the periconception environment and the development of the early embryo. A diagram illustration depicting the gene expression changes induced by spermatozoa is depicted in Fig 3.

Moreover, Ellington et al. demonstrated that when cultured oviductal epithelial cells are co-incubated with spermatozoa, the bovine epithelial cells respond by the synthesis of new proteins (Ellington et al., 1993). Another *in vivo* study in pigs revealed that the interaction of spermatozoa with the oviduct resulted in a more than two-fold increase in the synthesis of 19 proteins (Georgiou et al., 2007). Studies have also revealed that one protein in particular (oviduct-specific glycoprotein; OSG) is crucial and increases the rate of fertilization (A. J. Kouba et al., 2000) and also regulates polyspermy (Coy et al., 2008).

Another aspect of spermatozoa-oviduct interaction includes the physiological processing of spermatozoa within the female reproductive tract. This process induces the spermatozoa to undergo capacitation and the acrosome reaction, both of which are essential in terms of fertilization. In addition to EVs from the male reproductive tract such as prostasomes, EVs of the female reproductive system also play an active role in spermatozoa activation, motility, survival, and

capacitation, as well as the acrosome reaction. EVs released from various regions of the female genital tract may serve as signaling mediators that potentially play a critical role in regulating spermatozoa functions (Bathala et al., 2018; Borges et al., 2013; de Lamirande et al., 1997). Several studies reported that EVs isolated from the oviductal fluids of the female reproductive tract had a profound effect on sperm capacitation and the acrosome reaction. A study conducted in cattle revealed that oviduct derived EVs induced phosphorylation of tyrosine based receptors in spermatozoa which is a key event associated with capacitation (Franchi et al., 2020). Reports also suggest that oviductosomes transfer calcium efflux pumps namely Ca^{2+}ATPase-4 (PMCA4), to the plasma membrane of the spermatozoon that then play a vital part in maintaining the capacity of spermatozoa to fertilise the egg (Al-Dossary et al., 2013, 2015). Immunofluorescence-based fusion studies have demonstrated that oviductosomes have the ability to interact and fuse with spermatozoa through the interaction between the v-integrin subunit and CD9. These markers are typically present on the head and mid portion of the spermatozoa (Al-Dossary et al., 2015). Such exchange of protein cargos between spermatozoa and EVs of the oviduct regulates both spermatozoa capacitation as well as a timely acrosome reaction during the periconception period. It is imperative to determine the mechanism of transfer of oviductal EVs to the plasma membrane of spermatozoa, as these EVs are expected to have a significant impact on conception and infertility (Al-Dossary et al., 2015; Barbaux et al., 2020; Bathala et al., 2018).

The collective notion garnered from these studies is that the interactions that take place between spermatozoa and the oviduct are bidirectional. However, the mediators of the spermatozoa-oviduct dialogue and the potential communication pathways by which spermatozoa influence the transcriptome and proteome of the oviduct are still insufficiently explored. A comprehensive investigation of the role of EVs in various stages of reproduction, from periconception to fertilization and implantation, under different pathological conditions, could provide valuable tools for promoting reproductive health (Machtinger et al., 2016a). Hence it is important to focus on elucidating the mechanisms of cargo transfer between EVs and gametes, as well as the differential composition of male and female reproductive tract EVs, to gain insights into the causes and management of infertility problems in young couples struggling to conceive.

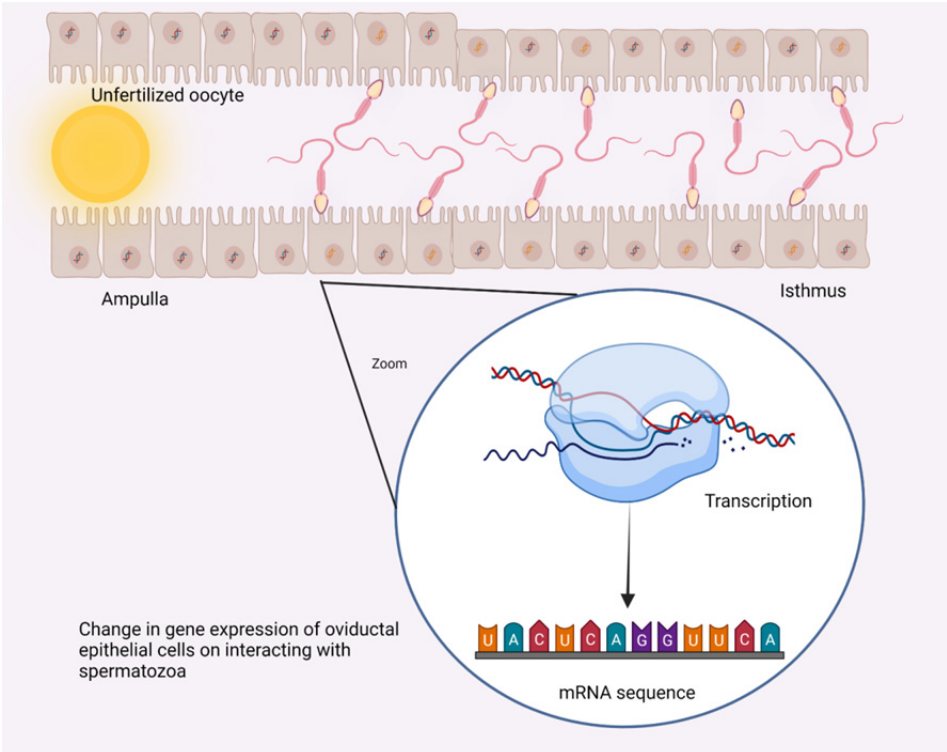

Figure 3: Schematic showing where spermatozoa are interacting with the oviductal cells: As spermatozoa make contact with the oviductal epithelial cells, intricate signaling pathways are activated, orchestrating the modulation of transcriptional activity and consequently influencing the transcriptomic landscape.

2.6 Triad of Oviduct, Spermatozoa and FF in establishing periconception milieu

Another very crucial event associated with periconception involves the interactions that take place between the FF and spermatozoa in the ampullar region of the oviduct. FF is a complex and dynamic biological fluid that surrounds the egg and plays an essential role in oocyte maturation and development. Therefore, the oocyte's capacity to develop and its quality may be directly impacted by the composition of the FF (Revelli et al., 2009). However, the interaction of FF does not remain confined to the female gamete (oocyte) rather it also interacts with male gametes (spermatozoa). In the process of ovulation, when a follicle bursts to release an oocyte, a part of the FF also enters the oviduct at the same time. FF is known to induce capacitation of spermatozoa and the acrosome reaction in a variety of species' spermatozoa, including bovine, equine, porcine, hamster, human, and alpaca spermatozoa (Bravo & Valdivia, 2018a; Caille et al., 2012; Leemans et al., 2015; Lenz et al., 1982). The bulk of these studies suggest that

progesterone present in the FF is responsible for enhancing spermatozoa activity and inducing the acrosome reaction (Calogero et al., 2000; Morales et al., 1992; Osman et al., 1989). FF is composed of hormones, peptides, glycosaminoglycans, glucose, albumins, and enzymes such as lactate dehydrogenases, as well as aminotransferases, cholesterol, triglycerides and EVs. Among all these, vital components albumin has been shown to act as a physiological inductor for sperm capacitation and the acrosome reactions because of its ability to induce the release of plasma membrane cholesterol for the acrosome reaction and capacitation (Huanca et al., 2017; Pacheco & Coila, 2010; Visconti & Kopf, 1998).

Furthermore, in one of our recent studies, we have demonstrated that the EVs derived from the FF as well as the FF itself alter the gene expression profiles in the epithelial cells of the bovine oviduct. This study reported that in response to FF, the upregulated genes in oviductal epithelial cells were associated with oxidative phosphorylation and thermogenesis pathways (Hasan et al., 2020). The release of energy during oxidative phosphorylation induces thermogenesis which eventually initiates the thermotaxis process and guides spermatozoa towards that site of fertilization (S. Li & Winuthayanon, 2017b; Miki & Clapham, 2013). These findings raise intriguing questions regarding the extent of influence of FF and FF-EVs on the periconception period. The importance of FF in preparing the periconceptional environment has relatively not received much attention even though FF plays an essential role in maintaining male gamete viability, nourishing the oocyte, and modulating gene expression of the oviduct.

Continued exploration into the intricate interplay between FF-spermatozoa-oviduct and the role of extracellular vesicles (EVs) in facilitating intercellular communication, has the potential to advance our understanding regarding mechanisms governing successful fertilization. Furthermore, such investigations may contribute to the development of novel diagnostic approaches and therapeutic interventions particularly in the case of couples diagnosed with idiopathic infertility.

2.7 Knowledge gaps in determining male fertility

Semen analysis is the key foundation for determining male fertility, with WHO recommendations providing standardization of procedures and reference values. From the 1980 publication of the 1st Edition of the WHO Laboratory Manual to the present 6th Edition, considerable advancements in semen testing procedures have been included (Boitrelle et al., 2021). The scientific advancements made in the understanding of spermatozoa DNA fragmentation (SDF), oxidative stress (OS), and reactive oxygen species (ROS) testing have provided additional knowledge for the prognosis of reproductive outcomes in terms of natural conception and ARTs (Agarwal et al., 2021). Nonetheless, while assessing male fertility, only the parameters of spermatozoa are analysed, while all other components of the male ejaculate are disregarded. Male ejaculate has a high quantity of EVs that promote spermatozoa function in several ways, including

boosting spermatozoa motility and regulating acrosomal activity (Poliakov et al., 2009). Exosomes in mammalian seminal plasma have been discovered to influence spermatozoa motility and mitochondrial metabolism (Sullivan & Mieusset, 2016). The epididymis in the male reproductive system plays a critical role in the maturation of spermatozoa, which depends significantly on epididymal epithelial secretions. Exosomes from the epididymis and prostate are involved in the last stages of spermatozoa maturation (Saez et al., 2003). Therefore, it is logical to assume that EV analysis may be used to evaluate the quality of a patient's spermatozoa or that EVs can be utilized to treat male infertility. A recent study suggests that EVs from fertile males have an impact on the sperm viability of non-fertile male chickens (Cordeiro et al., 2021). Hence, EVs have the potential to be considered as a promising avenue for developing non-invasive biomarkers and novel therapies for boosting reproductive success. Moreover, the ability of the spermatozoa to modulate gene expression of oviduct can be one of the assessments performed by developing *in silico* models of the oviduct.

2.8 Summary of literature review

A successful conception is not only an outcome of the union of male and female gametes, it also requires a coordinated set of cellular and molecular events occurring before fertilization. Spermatozoa and the female reproductive tract are entangled in very complex cellular communication pathways mediated by proteins and enzymes, as well as EVs. The interactions of the male gametes with the maternal tract, particularly with the oviduct, is the decision making step which determines the fate of the pregnancy. The interactions between spermatozoa and the oviduct are bidirectional whereby spermatozoa undergo many physiological changes which are necessary to initiate the process of fertilization. The oviduct also acts as a key player in the spermatozoa selection process, since it facilitates or inhibits spermatozoa transit and allows only a chosen spermatozoon to reach the oocyte. On the other hand, spermatozoa alters the transcriptome and the proteomics of the epithelial cells of the oviduct. The modulation of the oviductal gene expression is not only influenced by spermatozoa but also by FF and FF- derived EVs. Moreover, the FF boosts spermatozoa capacitation and the acrosome reaction without which the spermatozoon cannot penetrate the oocyte. All these interactions during periconception prime the maternal system for future reproductive events such as fertilization and implantation, as well as embryo development. Although EVs mediate several periconception-related processes, they are seldom used in any ART protocols. To make successful use of ART as well as for the advancements in the technologies itself, it is vital to unravel the cellular and molecular events, both pre-conception as well as post-conception, occurring in the female reproductive tract. The understanding regarding the periconception milieu can open new avenues for the treatment of couples diagnosed with idiopathic infertility.

3. AIMS OF THE PRESENT STUDY

Broadly, the main aim of the present study was to explore the intricate interplay of bovine FF derived EVs, spermatozoa and oviduct in establishing a periconception milieu.

The specific aims of the study were:

Study 1: To analyze if spermatozoa release biomolecules that could trigger gene expression changes in oviductal cells.

Study 2: To analyze if spermatozoa can induce changes in the EVs cargo derived from oviductal cells.

Study 3: To study whether bovine FF-derived EVs affect the motility, capacitation and acrosome reaction of bovine spermatozoa.

4. MATERIALS AND METHODS

4.1 Overall experimental plan

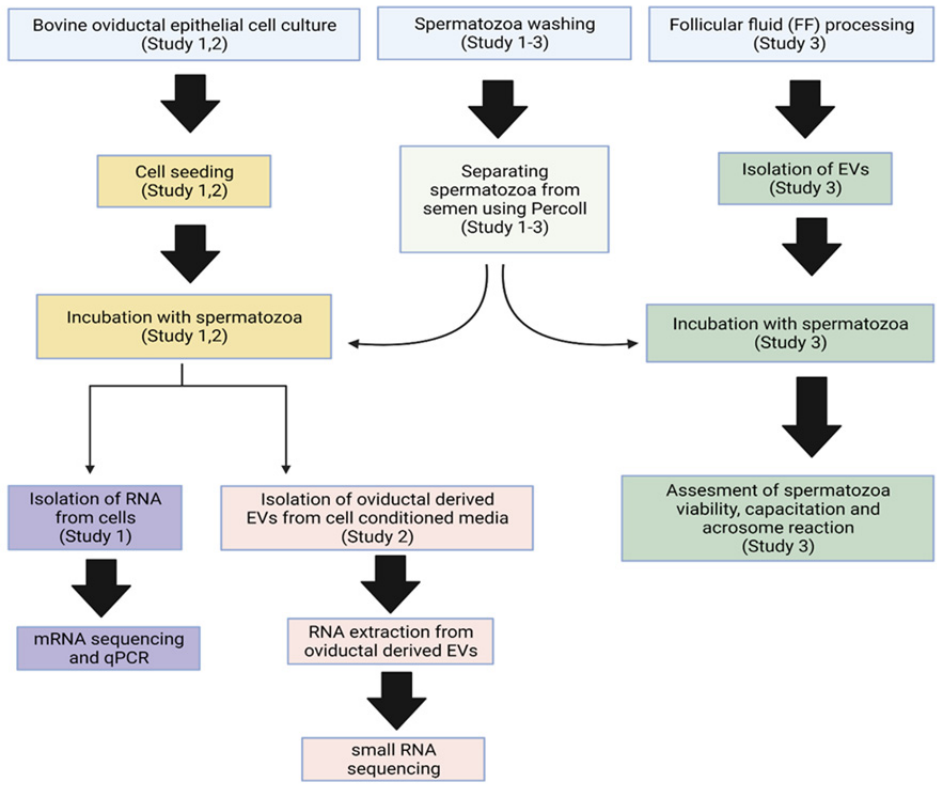

Figure 4: Flow chart depicting the overall study design

4.2 Cell culture

4.2.1 Bovine oviductal epithelial cell culture (Study 1 and 2)

Within four hours after animal slaughter, oviducts with connected ovaries were collected from the slaughterhouse and transferred to the laboratory in saline at 37˚C. Only the oviducts of the early estrous cycle (bright red corpus luteum, 0–4 days after ovulation) were chosen to extract epithelial cells (Ireland et al., 1980). Following the removal of the ovaries and connective tissue from the oviducts using a scalpel, the oviducts were washed with wash buffer-1 (DPBS mixed with Amphotericin B (1 µL/mL) and 1X Penicillin/Streptomycin (10 µL/mL)). By gently squeezing the oviduct with a sterile glass slide, the mucosa was removed and transferred to a tube containing wash buffer-1. The cells were washed three times, twice in wash buffer-1 and once in wash buffer-2 (DPBS supplemented with Amphotericin B (1 µL/mL), Penicillin/Streptomycin (10

μL/mL, and 5% FBS), and the supernatant was removed between each wash. Afterwards, the cell pellet was resuspended in culture medium (DMEM/F12 supplemented with 10% FBS, Amphotericin B (1 μL/mL), and Penicillin/ Streptomycin (10 μL/mL). The cells were cultured in T-25 flasks and left to adhere for three days without the medium being replaced, in a humidified incubator with 5% CO_2 at 38.8 °C. Subsequently, trypsinization of cells was performed and cells were seeded in a T-75 flask until they attained 80% confluency. Once the cells were confluent, the cells were trypsinized again and frozen until performing experiments. Prior to co-culturing the cells with spermatozoa, they had been split four times.

4.2.2 Analysis of epithelial cells markers using immunofluorescence (Study 1 and 2)

Sterile coverslips were placed in the cell culture plates and BOECs were grown on the coverslips. Later the fixation of the cells was performed using 4% para-formaldehyde for 15 minutes at room temperature. To permeabilize the cells, they were treated with methanol for another 10 minutes and this procedure was performed on ice. Afterwards, for 1 hour at room temperature blocking was performed using 4% normal goat serum (NGS). This step was followed by treating cells overnight with a combination of anti-Cytokeratin (C2562, 1:250, Sigma-Aldrich) and anti-Vimentin (PLA0199, 1:250, Sigma-Aldrich) primary antibodies in a blocking buffer at 4 °C. The negative control samples were treated with a blocking solution (4% NGS) devoid of any primary antibodies. After incubation with primary antibodies, the cells were incubated with secondary antibodies for 1 hour in a dark room. The secondary antibody was a combination of goat anti-mouse (Alexa Fluor 488, 1:500) and goat anti-rabbit (Alexa Fluor 594, 1:500) diluted in 4% NGS (both antibodies were from Invitrogen, Thermo Fisher Scientific, Eugene, USA). After the incubation time was over the nuclei were stained for 3 minutes with Hoechst 33342 (1:2000, Thermo Fisher Scientific) and then mounted using Fluorescence Mounting Medium (Dako, Denmark). The images were captured using a Leica DM5500B microscope coupled with a Leica DFC310 camera (Leica, Wetzlar, Germany).

4.2.3 Direct contact and non-contact co-culture models of BOECs and spermatozoa (Study 1 and 2)

Frozen BOECs were thawed and cultured in two separate petri dishes until 80% confluency. Once the aforementioned confluency was attained the cells from the isthmus and ampulla were mixed and seeded in a 12 well cell culture plate. After 24 hours, the BOECs monolayer was 80% confluent again and before adding spermatozoa, the BOECs were rinsed twice with EV-depleted sperm-TALP medium. To determine the differences in the gene expression of cells and oviduct derived EV cargo, we used two co-culture models. The first co-culture model referred to as contact co-culture, was the one where spermatozoa were

interacting directly with the BOEC monolayer. As the name suggests in non-contact co-culture the spermatozoa and BOECs were spatially separated using an insert with a pore size of 0.4 μm (Thincert cell culture insert, Greiner Bio-One GmbH, Kremsmünster, Austria). In both co-culture models, equal volumes of spermatozoa (1×10^6 spermatozoa/mL) were added. In Study 1, the mRNA sequencing co-incubation period lasted 10 hours, and for qPCR, it was 4 hours. In Study 2, the incubation period was extended to 4 hours.

4.3 Spermatozoa processing

4.3.1 Separating spermatozoa from frozen thawed semen straws and co-culturing with BOECs (Study 1–3)

In a sterile water bath maintained at 37˚C for thirty seconds, the frozen bovine semen straws were thawed. The constituents of the three straws were layered onto 4 mL of a 60% isosmotic Percoll® solution (GE Healthcare, 17–0891–02, Sweden). In order to prepare an intermediate stock of percoll solution it was diluted with 1X HEPES in a 9:1 ratio. Afterwards, the 60% percoll was made by diluting intermediate stock with EV depleted sperm TALP media. The tube containing spermatozoa layered over 60% percoll was centrifuged at 300×g for 20 minutes at room temperature, and the pellet was washed with warm EV-depleted Sperm-TALP. The sperm-TALP media included BSA (AppliChem, A1391, 0050, Germany), because of which EV depletion was carried out to reduce the amount of BSA-derived particles in the medium. This was accomplished by passing a working solution of sperm-TALP containing BSA through a 100 kDa Amicon® Ultra-15 Centrifugal Filter Unit (R9CA01172, Ireland) (Kornilov et al., 2018). After re-suspending the final pellet in 1 mL of pre-warmed EV-depleted Sperm-TALP medium, the concentration and the motility of washed spermatozoa were measured. To evaluate motility in studies I and II, washed spermatozoa were placed on a glass slide that had been warmed beforehand. Using a light microscope, the motility was examined across five distinct fields at a magnification of 40X. The final motility measurement for the sample was determined by calculating the average motility observed across these five fields.

4.3.2 Evaluating spermatozoa viability, capacitation and acrosome reaction (Study 3)

Following the manufacturer's instructions, the viability of spermatozoa was determined using the LIVE/DEAD® Viability/Cytotoxicity Kit (MP 03224, Thermo Fisher Scientific Inc., Santa Barbara, CA, USA). Briefly, 25 μL of spermatozoa solution was combined with 100 μL of a freshly prepared working solution containing 4 μM EthD-1 and 2 μM calcein. The solution was incubated for 30 minutes at room temperature. After incubation, a sample smear was formed on a microscope glass slide, sealed, and covered with a coverslip. Dead

spermatozoa exhibited a red fluorescent signal, whereas living spermatozoa displayed a green signal. A working solution of chlortetracycline (CTC) was made by dissolving 750 M of CTC–HCl (Sigma-Aldrich, Saint Louis, MO, USA) in a buffer comprising 20 mM Tris-HCl, 130 mM NaCl, and 5 mM cysteine-HCl. In a wrapped Eppendorf tube, 100 µL of spermatozoa suspension and 100 µl of CTC working solution were mixed. On a glass slide, 10 µL of this solution was combined with 12.5 % paraformaldehyde (pH 7.5). The slides were coated with coverslips and slides were analyzed using a fluorescence microscope at 400X magnification to evaluate the capacitation of spermatozoa. On average the fluorescence patterns of 250–300 spermatozoa were evaluated. The status of the acrosome reaction of spermatozoa was evaluated using FITC-PNA and EthD-1. In the tube, a mixture containing 100 µL of spermatozoa and 100 µL of EthD-1 (4 µM) was incubated for 5 minutes at 38 °C. Afterwards, the microscopic slides were prepared and the fluorescent patterns of the spermatozoa for the acrosome reaction were evaluated using a fluorescence microscope at 400X magnification, and an average of 250–300 spermatozoa per slide were analyzed.

4.4 EV processing

4.4.1 Isolation of EVs derived from oviductal epithelial cells (Study 2)

The oviductal cell conditioned medium was removed and subjected to centrifugation to remove cells, cell debris, and apoptotic bodies. The initial step involved centrifugation of the sample at 500 × g for 10 minutes to separate the cellular components of conditioned media. The resulting supernatant was then subjected to a second centrifugation step at 2000 × g for 15 minutes at a temperature of 4°C. This step was performed to further isolate the undesired components e.g:-cellular debris. Subsequently, the supernatant was subjected to a third centrifugation at 10,000 × g for an additional 15 minutes to remove apoptotic bodies. The resulting supernatant was transferred to Amicon filter units (10 kDa), and the sample was concentrated until its volume reached approximately 150–200 µL. EVs were isolated using qEVsingle SEC columns (qEVsingle/70 nm by Izon Sciences, UK) according to the manufacturer's protocol, which had been previously employed by our research group (Dissanayake et al., 2020). After washing the columns, the EV-containing fractions 6–9 were combined (800 µL) and concentrated for further experiments using 10 KDa Amicon® Ultra-15 centrifugal units.

4.4.2 Collection and isolation of bovine FF derived EVs (Study 3)

The collection of follicular fluid (FF) and subsequent isolation of EVs were conducted in accordance with the protocol previously established by our

research group, with minor adjustments (Hasan et al., 2020). In summary, the ovaries were procured from a slaughterhouse and subjected to three washes with physiological saline. FF was then extracted from large follicles (>9 mm in diameter) using a vacuum pump (Minitüb GmbH, Tiefenbach, Germany). The FF collected from the follicles was pooled and processed before isolating EVs. A volume of 10 mL of FF utilized for EVS isolation was mixed with an equal amount of DPBS to reduce viscosity. The resulting mixture was then subjected to centrifugation at 300× g for 10 minutes, followed by centrifugation at 2000× g for 10 minutes to get rid of cells and cell debrii respectively. Inorder to eliminate apoptotic bodies, the sample was further centrifuged at 20,000× g for 30 minutes. The supernatant was stored in -80˚C until EVs were isolated. The isolation of EVs from the FF was accomplished using manually packed SEC columns. During this process, fractions, each consisting of 500 µL elute, were collected. Fractions 1 to 4, corresponding to the void volume, were discarded, while fractions 5 to 7 were collected and combined as the EV fractions based on a previous study published by our research group (Hasan et al., 2020).

4.4.3 Nanoparticle tracking analysis (NTA) (Study 2, 3)

The nanoparticle tracking analyzer-ZetaView® (Particle Metrix GmbH, Germany) was used as previously mentioned by our research group (Dissanayake et al., 2021), to determine the size profile and concentrations of the EVs. During the course of the measurement, the following settings were set in the machine: sensitivity 85, shutter speed 70, and frame rate 30 frames per second.

4.4.4 Transmission electron microscopy (TEM) (Study 2)

EVs were evaluated morphologically by placing a formvar/carbon coated 200 mesh grid (Agar Scientific, Stansted, UK) over a 20 µL droplet of pure, concentrated EVs for 20 minutes. The same grid was then incubated for 5 minutes with 2% uranyl acetate (Polysciences, Warrington, PA, USA) to yield contrasting EVs. The materials were air-dried, and the EV images were obtained using JEM 1400 TEM (JEOL Ltd., Tokyo, Japan, with a Morada TEM CCD camera, Olympus, Germany) equipped with a Morada TEM CCD camera.

4.4.5 Mass spectrometry (Study 2)

For the biochemical characterization of EV markers, mass spectrometry was performed. One microgram of protein was injected into an EASY-nLC 1000 device (Thermo Scientific) followed by sample elution at a rate of 250 nL/min onto a 75 m ID x 50 cm emitter-column (New Objective) filled with C18 material (3 m, 300 particles, Dr Maisch). Using nano-electrospray ionization at 2.4 kV, eluted peptides were sprayed onto a Q Exactive Plus (Thermo Fisher Scientific) quadrupole-orbitrap mass spectrometer (MS).

4.5 RNA processing

4.5.1 RNA extraction from bovine oviductal epithelial cells (Study 1) and from oviductal-derived EVs (Study 2)

The phenol and chloroform procedure was adopted to extract RNA, for which TRIzol reagent (Invitrogen) was used. The source of the RNA was adherent cells and concentrated EVs elute derived from oviductal epithelial cells. While isolating RNA from EVs, the TRIzol reagent was directly added to the EV solution whereas in the case of cells which were adherent, the conditioned media was removed, and the TRIzol reagent was added to the cell monolayer. The samples were left at room temperature for 10 minutes following which chloroform was added to 1 mL of TRIzol. After vigorous pipetting for 15 seconds, the samples were centrifuged again for 15 minutes at 12,000 x g and 4 °C. After separating the phases, the aqueous phase containing the RNA was transferred to the new tube, and the RNA was precipitated using 500 uL of isopropanol at room temperature for 20 minutes. To improve the efficiency of RNA extraction, glycogen (10 µg, UltraPureTM Glycogen, 10814-010, Thermo Fisher Scientific, Bleiswijk, Netherlands) was added to the mixture. After the precipitation step was complete, the samples were centrifuged at 18000 x g for 30 minutes at room temperature to pellet the RNA. 70% ethanol was used three times to wash the RNA pellets, and then 20 uL of nuclease-free water was used to elute the RNA. The Agilent Pico 6000 and the Qubit HS RNA kits were used to analyze RNA concentration and purity, respectively.

4.5.2 RNA sequencing mRNA sequencing (Study 1)

With minor changes, in the protocol obtained from the literature (Picelli et al., 2014) the Smart-seq2 approach was used to prepare RNA sequencing libraries. Overall, 20 ng of total RNA and 10 cycles of PCR for pre-amplification. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used and 2 µL of diluted cDNA was added to dual-index library preparation using Illumina Nextera XT DNA Sample Preparation Kit (FC-131-1024). AMPure XP beads (Beckman Coulter) were used for size selection of 200–700 bp. All samples were pooled and sequenced on Illumina NextSeq500.

4.5.3 Read quality control, alignment, and counting (Study 1)

The read quality was evaluated by FASTQC (Brown et al., 2017), and trimmomatic (Bolger et al., 2014) was used to remove low-quality reads and trim adapter sequences from reads. Reads were aligned to the reference genome using Hisat2 as the alignment tool (Kim et al., 2019). The read alignment was conducted using the default settings and with the addition of splice site information obtained from the Ensembl *B. taurus* annotation file. Using featureCounts, (Liao et al., 2014) we were able to acquire read counts at the gene level.

4.5.4 Differential expression analysis (Study 1)

The edgeR package (Robinson et al., 2010) in version 3.6.1 of R was used to perform differential expression analysis of gene-level counts. The genes that had less than 10 reads for any of the samples in a single experimental group were eliminated. The differential expression of genes was considered statistically significant if the false discovery rate (FDR) was ≤ 0.05.

4.5.5 Quantitative reverse transcription-PCR (Study 1)

cDNA was produced by combining oligo(dT) and random primers (FIREScript RT cDNA synthesis kit, Solis BioDyne, Tartu, Estonia). Each biological sample was subjected to q-PCR in triplicate and the cDNA products were amplified using the EvaGreen test kit (Solis BioDyne, Tartu, Estonia). q-PCR data were analyzed using the comparative CT technique, and the relative expression of RNA was determined using the 2-ΔΔCTmethod (Winer et al., 1999). The program Primer-BLAST was used to design the primers, and sequences spanning exon-exon junctions were given precedence in the process.

4.5.6 Small RNA library preparation and sequencing (Study 2)

Following the manufacturer's instructions, we prepared RNA libraries using a RealSeq®-AC miRNA Library Kit (Cat number 500-00012). To amplify the reverse transcription product, twenty PCR cycles were carried out. The library quality was evaluated using the Agilent DNA 1000 Kit and the Qubit dsDNA HS Assay Kit (Thermo Fisher cat Q32851). The libraries were quantified using a Qubit (High sensitivity dsDNA kit, manufacturer's procedure) and analyzed on a TapeStation 2200. (D1000 kit, protocol as per manufacturer instructions). Qubit concentration and TapeStation sizing were used to determine the molarity of libraries. Sequencing was performed on an Illumina HiSeq 2500 with onboard clustering, and a Flow Cell, 50 cycle SBS kit. The length of the read was 1x50bp, 6bp single index.

4.5.7 Read quality control, alignment, and counting (Study 2)

Read trimming was conducted using cutadapt v2.5 (REF) with the settings '-u 1 -q 20 – trim-n -m 15 -a TGGAATTCTCGGGTGCCAAGG' to trim the first base, trim N bases from the 3' end, reads with average phred33 base quality lower than 20 and shorter than 15nt were removed. The adapter sequences were also removed from the reads. The two reduced FASTQ files were then concatenated for each sample. For future analysis, we retained only reads no longer than 30nt.

4.5.8 Differential expression analysis (Study 2)

The differential expression (DE) analysis was conducted using R version 4.1 and the edgeR package version 3.36.0. Based on trended dispersions the tagwise dispersion estimates were obtained, and statistical comparisons were made using a generalized linear model. The differential expression of genes was considered statistically significant if the false discovery rate (FDR) was ≤ 0.05.

4.6 Experimental Plan

During the course of experiments, conducted in Study 1 and Study 2, the frozen-thawed BOECs from three different cows were cultured separately until they attained 80% confluency. Afterwards, the cells from three different oviducts were mixed and seeded in 12 well culture plates (100,000 cells/well). When the cells attained confluency, the direct contact (DC) and non-contact (NC) co-culture with spermatozoa was performed. The isolation of spermatozoa from semen straws was performed using 60% Percoll. These experiments were done three times on three different days using different aliquots of the same batch of primary cells each time. In the contact co-culture model, the bovine spermatozoa (1×10^6 spermatozoa/mL) were directly added to the BOECs whereas, in the non-contact co-culture model, an insert (Thincert cell culture insert, Greiner Bio-one GmbH, Kremsmunster, Austria) of 0.4 µm pore size was placed between spermatozoa and BOECs. The insert used for the experiments was made of inert materials, so there was no interference caused by it in the experiments conducted. For RNA sequencing in Study 1, thawed semen straws derived from the same ejaculate as the source of spermatozoa were used. In qPCR experiments of study 1, the spermatozoa originated from the different ejaculate of the same bull. However in case of Study 2, on each day of the experiment, spermatozoa from different bull was used. Following the incubation period in case of Study 1, the cellular RNA was isolated and subjected to mRNA sequencing. In Study 2, the EVs were isolated from the cell conditioned media using SEC. After isolating the EVs by employing SEC the size and concentration of EV elute were determined by NTA, morphology was determined by TEM, and biochemical characterization for EV markers was performed by mass spectrometry. In order to analyze the cargo of EVs from each group, the RNA was extracted from the EVs followed by library preparation and small RNA sequencing. In Study 3, The EVs from bovine FF were isolated using SEC. The experimental samples consisted of a pool of FF-EVs obtained from larger follicles. Each experiment was conducted on three separate days, and for each experiment, spermatozoa from a different bull were utilized. The different numbers of FF- EVs (1×10^6 – 1×10^9) were incubated with spermatozoa (1×10^6 spermatozoa/mL) versus control (spermatozoa only). The incubation was performed for 4 hours following which the viability, capacitation, and acrosome reaction of the spermatozoa were accessed. The

following illustration (Fig. 4) demonstrates the experimental set up for all three studies.

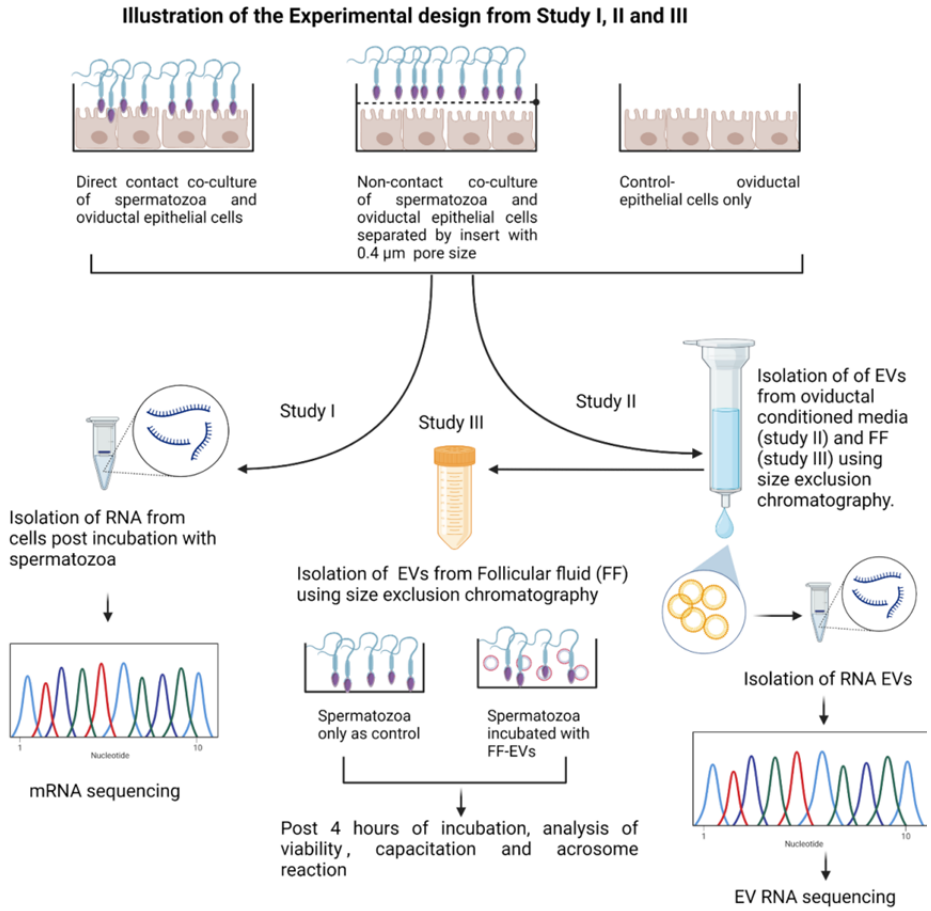

Figure 5: Schematic illustration depicting the set up of the experiments reported in the thesis

5. RESULTS

5.1 Study 1: Prior to first contact, spermatozoa trigger transcriptomic changes in bovine oviductal epithelial cells

5.1.1 Presence of epithelial markers in BOECs

Antibodies were used against cytokeratin (epithelial cell marker) and vimentin (fibroblast marker) to determine whether or not the primary cell culture that we had contained epithelial cells with the least or no contamination from fibroblasts. Staining with an anti-vimentin antibody generated a fading signal, which proved the existence of epithelial cells predominantly as cells produced a strong positive signal with an anti-cytokeratin antibody as shown in Fig. 6.

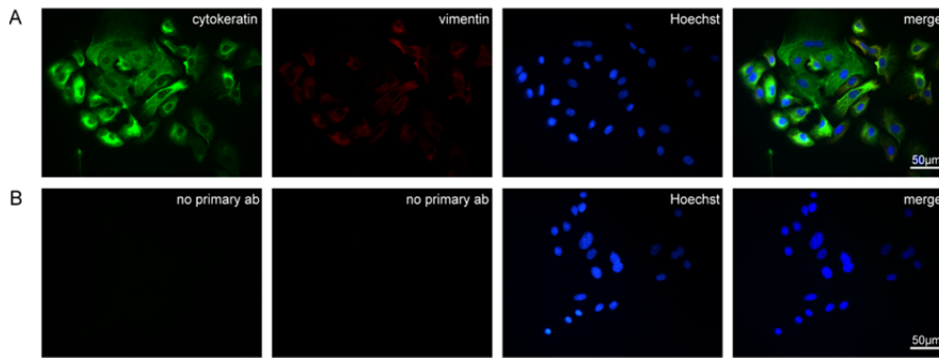

Figure 6: Expression of cytokeratin and vimentin in primary BOECs. A) Cells displayed a strong signal after incubation with an anti-cytokeratin antibody (green) and mild signal post incubation with an anti-vimentin antibody (red). B) Staining for a negative control was performed without adding primary antibodies. Hoechst (blue) was used to stain the nuclei of the oviductal epithelial cells.

5.1.2 Differential gene expression obtained from mRNA sequencing and pathway enrichment analysis (non-contact co-culture)

The differential gene expression was performed to identify the genes that exhibit significant changes in expression levels between different groups. Based on the principal component axis, we observed that the non-contact co-culture model samples were completely distinct from both control and contact co-culture model samples as depicted in Fig. 7. On the other hand, non-contact co-culture model samples displayed a significantly greater degree of noticeable intra-group variation in comparison to the other two groups. In contact co-culture, BOEC gene expression patterns there were nine genes upregulated and one gene downregulated. *DHRS3* was the most upregulated gene in response to

contact co-culture, whereas *RANBP3* was the only downregulated gene. Gene expression alterations in BOECs were surprisingly more profound after the non-contact co-culture of spermatozoa and epithelial cells of the oviduct (52 genes upregulated, and 56 genes downregulated). Both contact and non-contact co-cultures had four upregulated genes in common (*PLAU, TNFRSF11B, SCIN, DHRS3)*. Among the four commonly upregulated genes, the fold expression of *DHRS3* was higher in contact co-culture (log2FC = 1.5) as compared to non-contact co-culture (log2FC = 0.92). Interestingly, *ATF3,* a known negative regulator of *PTGS2*, was one among the other six DE genes induced by contact co-culture of oviductal cells and spermatozoa as shown in Fig. 8. Pathway over-representation analyses were performed for significantly up-regulated and down-regulated genes based on KEGG pathway annotations for B. taurus. Inorder to understand the biological relevance of the set of differentially expressed genes, pathway enrichment analysis was performed. The obtained genes were mapped with predefined pathways using database from Kyoto encyclopedia of genes and genomes (KEGG). In case of non-contact co-culture, six pathways were highly enriched among up-regulated genes, with retinol metabolism (bta00830), steroid hormone biosynthesis (bta00140), ovarian steroidogenesis (bta04913), and cytokine-cytokine receptor interaction (bta04060) being the most relevant with FDR < 0.05. In the case of downregulated genes, no significantly enriched pathways were detected.

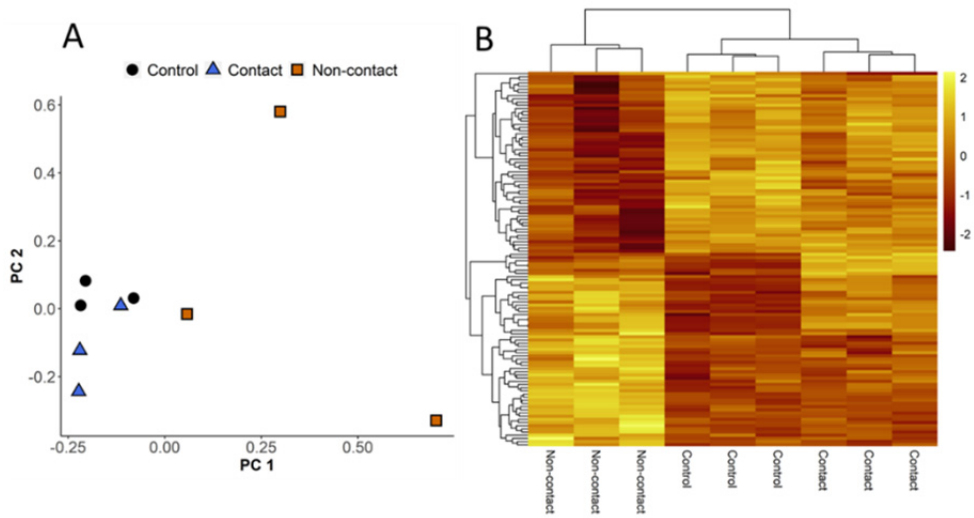

Figure 7: The gene expression profile of BOECs when incubated with spermatozoa in a direct and non-contact co-culture model A) PCA plot depicting inter and intra-sample variations based on log2 fold changes in the top 500 most differentially expressed genes in BOECs in response to spermatozoa. B) Heatmap depicting CPM values of genes differentially expressed in co-culture models and control, with lighter regions depicting higher levels of relative expression and darker regions denoting lower levels of relative expression.

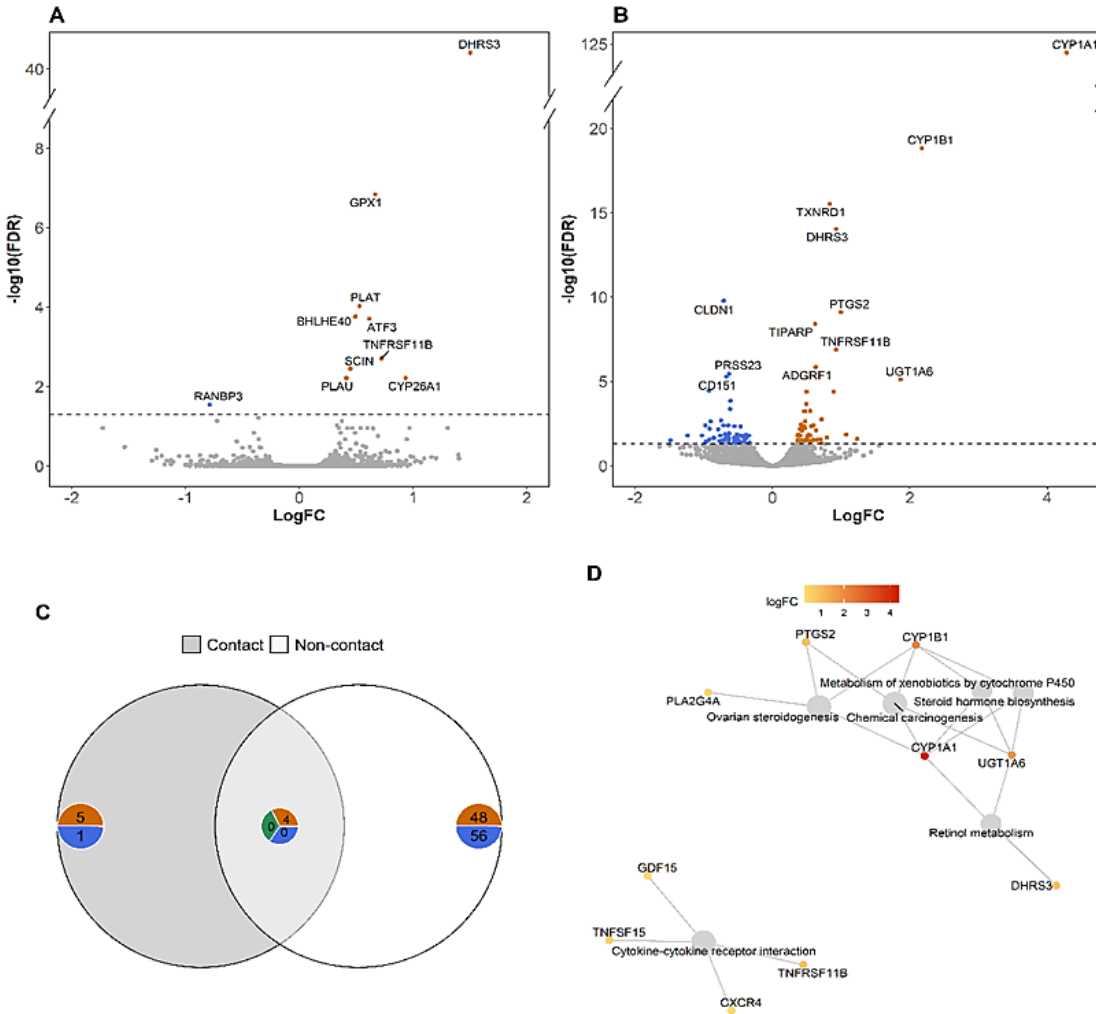

Figure 8: Volcano plots, Venn diagram and pathway enrichment analysis of differentially expressed genes in BOECs when incubated with spermatozoa in a direct and non-contact co-culture model. A) Volcano plot demonstrating the genes expressed differently in BOECs in the contact co-culture model versus control B) Differentially expressed genes in BOECs in the non-contact co-culture model when compared to control. C) Venn diagram displaying the number of genes in blue or orange backgrounds for downregulated or upregulated genes respectively, that were found common in two co-culture models. The number of contra-regulated genes is presented on the green background. D) Network diagram figure depicting the pathways that are significantly enriched among the upregulated genes in BOECs in the non-contact co-culture model.

5.1.3 Patterns of short-term gene expression influenced by contact and non-contact co-culture (qPCR)

The study conducted q-PCR analysis of four selected genes in both contact and non-contact co-culture models at different time points (30, 90, 150, and 240 minutes) to investigate the time frame in which the bovine oviductal epithelial cells (BOECs) respond to the stimuli of spermatozoa by altering gene expression, as illustrated in Fig. 9. We looked at the expression of *DHRS3* gene that was shown to be up-regulated by RNA-seq in both treatments, *CYP1B1* and *PTGS2* that was up-regulated in response to non-contact co-culture, and ATF3 that was only up-regulated in response to contact co-culture. The expression of *DHRS3* was high in both contact and non-contact co-culture but in contact co-culture, the gene expression was more pronounced, ($p = 0.02$ at 30 minutes) and a very evident increase was detected after 240 minutes had elapsed. The *CYP1B1* gene expression was prominently upregulated in response to non-contact after 90 minutes ($p < 0.001$), and after 240 minutes returned to the level of control samples. Similarly, the expression of *DHRS3* and *CYP1B1* exhibited a smaller rise in response to contact co-culture, which peaked at 150 minutes and diminished afterwards. *PTGS2* expression was elevated after 30 minutes in response to non-contact ($p= 0.003$) and remained elevated until the final time point. In contact co-culture, *PTGS2* exhibited a little rise in response to spermatozoa at 90 minutes ($p= 0.02$), but this increase was not maintained until the final time point. At the initial 30-minute time point, there was a noticeable increase in *ATF3* expression in response to both contact ($p=0.001$) and non-contact ($p=0.001$) co-culture. However, in the non-contact co-culture model, the expression of *ATF3* did not exhibit any further elevation in the following time points. On the other hand, in the contact co-culture model, the expression of *ATF3* remained significantly higher up to the 150-minute time point ($p=0.04$) before gradually decreasing.

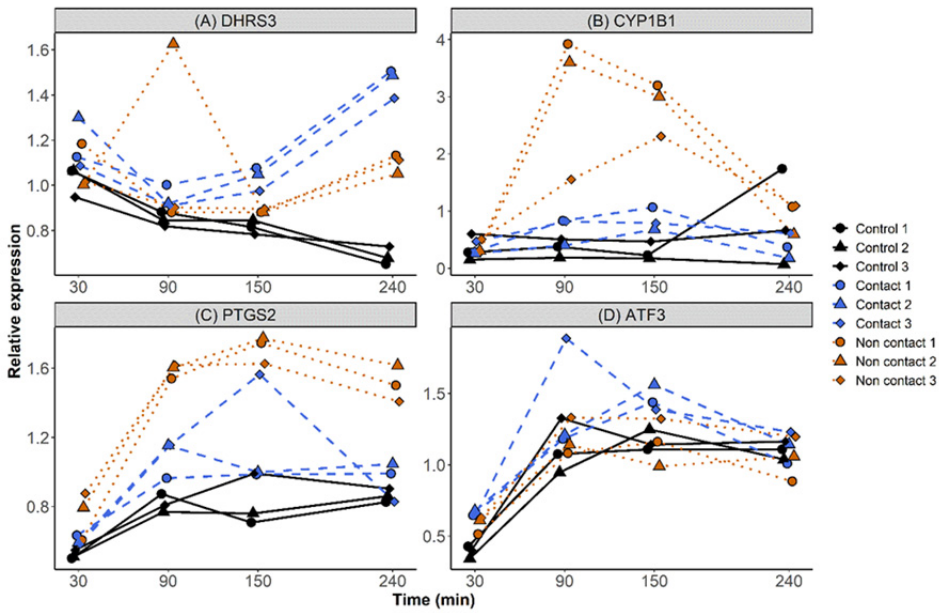

Figure 9: Short term gene expression studies: Quantitative real-time PCR-derived relative expression of four genes in BOECs in control (black, solid lines) in contact co-culture (blue, dashed lines) and non-contact co-culture (orange, dotted lines). The time is displayed on the X-axis of the graph and each point is a representation of an individual primary cell culture, with shapes denoting the experimental batches (circles: batch 1, triangles: batch 2, diamonds: batch 3). The 2-ΔΔ-CT approach was used to determine the relative expression values.

5.2 Key findings of Study 1

1) The findings of this study suggest that spermatozoa have the ability to induce changes in the transcriptomic profile of BOECs, even in the absence of direct contact, through the release of nano-scaled bioactive molecules.
2) The gene expression profile of oviductal epithelial cells exhibits substantial dependence on the nature of interaction with spermatozoa, whether it involves direct physical contact or occurs in the absence of such contact. Based on our results, the non-contact co-culture resulted in more pronounced gene expression changes compared to contact co-culture.

5.3 Study 2: Spermatozoa serves as an external trigger to change the cargo and production of oviductal epithelial EVs

5.3.1 Characterization of EVs derived from BOEC conditioned media by NTA, TEM and Mass spectrometry

The EV size obtained from all three groups (direct contact, non-contact and control) were between 75–105 nm. The total particle concentration was 2.19×10^{10}/ml $\pm5.08\times10^{9}$ for the contact co-culture, 1.55×10^{11}/ml$\pm2.01\times10^{9}$ for the non-contact co-culture group, and in the case of the control it was 5.64×10^{9}/ml $\pm1.64\times10^{10}$ (mean ± SEM; n=3). In addition, the number of EVs were higher in contact ($p<0.0001$) and non-contact co-culture system ($p= 0.0062$) compared to control and the differences were statistically significant. There were a higher number of EVs in the co-culture groups versus control samples indicating that oviductal cells released more EVs in the presence of spermatozoa as shown in Fig 10. The morphological evaluation conducted through transmission electron microscopy (TEM) indicated that the EVs obtained from both the contact and non-contact co-culture models exhibited a cup-like shape, which is commonly reported as a characteristic morphology of EVs in the scientific literature (Es-Haghi et al., 2019; Hasan et al., 2020).

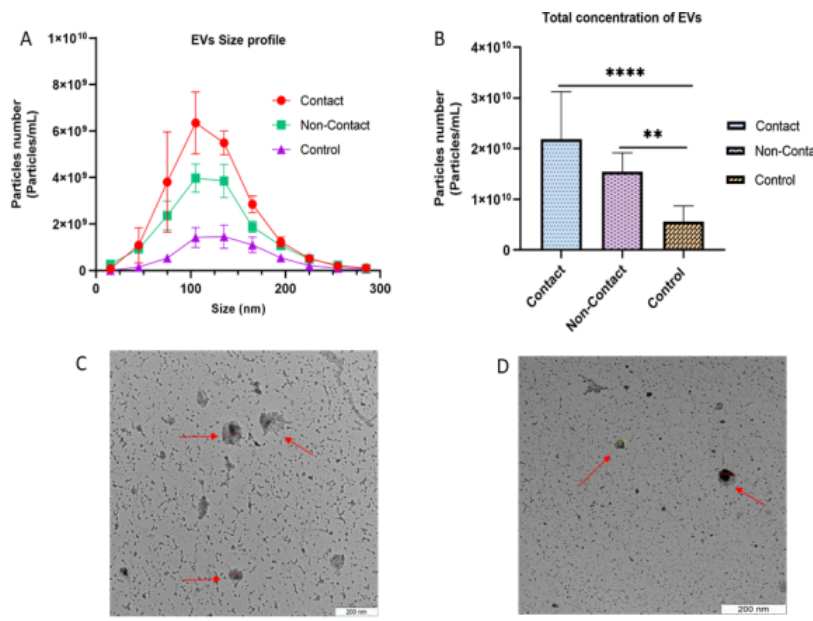

Figure 10: Characterization of EVs derived from oviductal epithelial cells (A) The size profile of the EVs where the majority fall in the range of 70–105 nm. (B) The total concentration of the EVs in all the three groups. (C) TEM imaging was performed to confirm the morphology of EVs derived from contact co-culture. (D) Morphological assessment of EVs derived from non-contact co-culture using TEM.

Through the use of mass spectrometry, it was determined that the enriched fractions included exosomal proteins and EV markers. This was determined by comparing the unpurified or neat conditioned medium to the enriched EVs elute. The list of 589 proteins reported from the mass-spectrometry analysis was cross-referenced with the top 100 reported protein lists of the public EV proteome databases ExoCarta and Vesiclepedia. Out of the top 100 EV proteins listed in the Vesiclepedia database, 76 proteins were detected in the BOEC EV protein sample, whereas from the ExoCarta database among the top 100 EV proteins, 90 proteins were detected in the BOEC EV sample as shown in Fig. 11.

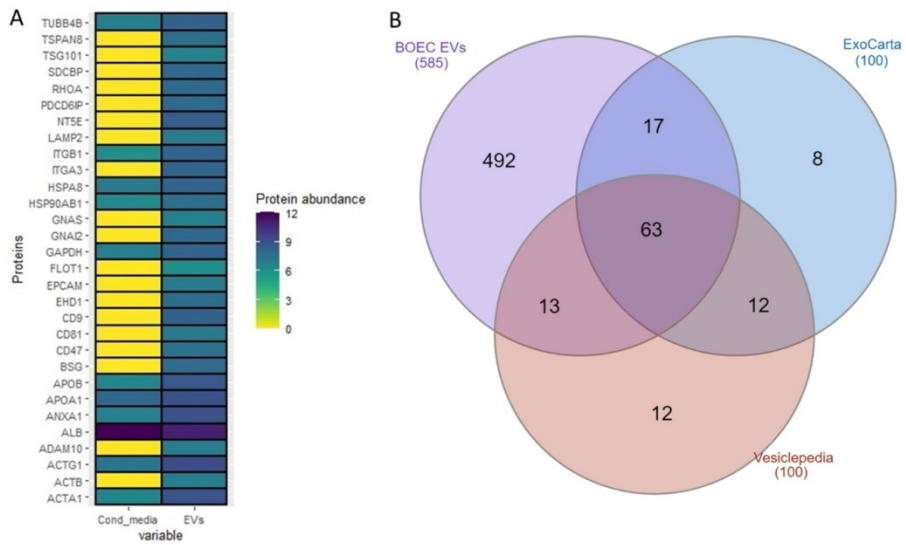

Figure 11: **Mass spectrometry analysis of EV associated marker proteins** (A) Heatmap depicting the enrichment of proteins associated with EVs versus BOEC conditioned media samples. (B) The Venn diagram displays the overlap between the proteins that were found in BOEC EVs and the top 100 proteins that were listed in the Vesiclepedia and ExoCarta databases.

5.3.2 Differential expression and pathway enrichment of mRNA fragments

Differential expression analysis of mRNA fragments derived from BOEC EVs was conducted to investigate the variations in their enrichment or depletion across different experimental groups. EV mRNA fragments (16–48 nt) derived from exonic regions of protein-coding RNA were differentially enriched. Principal component analysis revealed that the three groups (direct contact, non-contact, and control) had very little intragroup variance (3 samples per group) whereas intergroup variation was quite substantial (Fig. 12 A and B). There were 960 significantly enriched fragments and 917 significantly depleted fragments in direct contact versus control. In a comparison between the non-contact and the control group, 882 exonic mRNA fragments were considerably enriched, whereas 812 were significantly depleted. There were 878 significant enrichments and 1,000 significant depletions between direct-contact versus non-contact groups. The measure of significance was FDR < 0.05. (Fig. 12 C–E). Moreover, in order to acquire a deeper understanding of the biological ramifications associated with these mRNA fragments, gene enrichment analysis was performed on the genes from which these fragments originated. Gene set enrichment analysis (GSEA) was conducted on the differentially enriched fragmented mRNA of *Bos taurus* using gene ontology (GO) annotations. The microvesicular body-associated pathways were activated in contact cell culture versus control. Focal adhesion, collagen metabolism, actin cytoskeleton organization, and cytoskeleton protein binding are some of the pathways suppressed in direct contact co-culture group versus control (Fig. 13A). Pathways associated with the regulation, repression of innate immune responses, and defence against another organism were suppressed in non-contact co-cultures compared to the control. As compared to the control, the activated pathways in non-contact co-culture versus control were lipid localization and endosome recycling (Fig. 13B). The pathways suppressed and activated in direct vs non-contact co-cultures are shown in Fig. 13C.

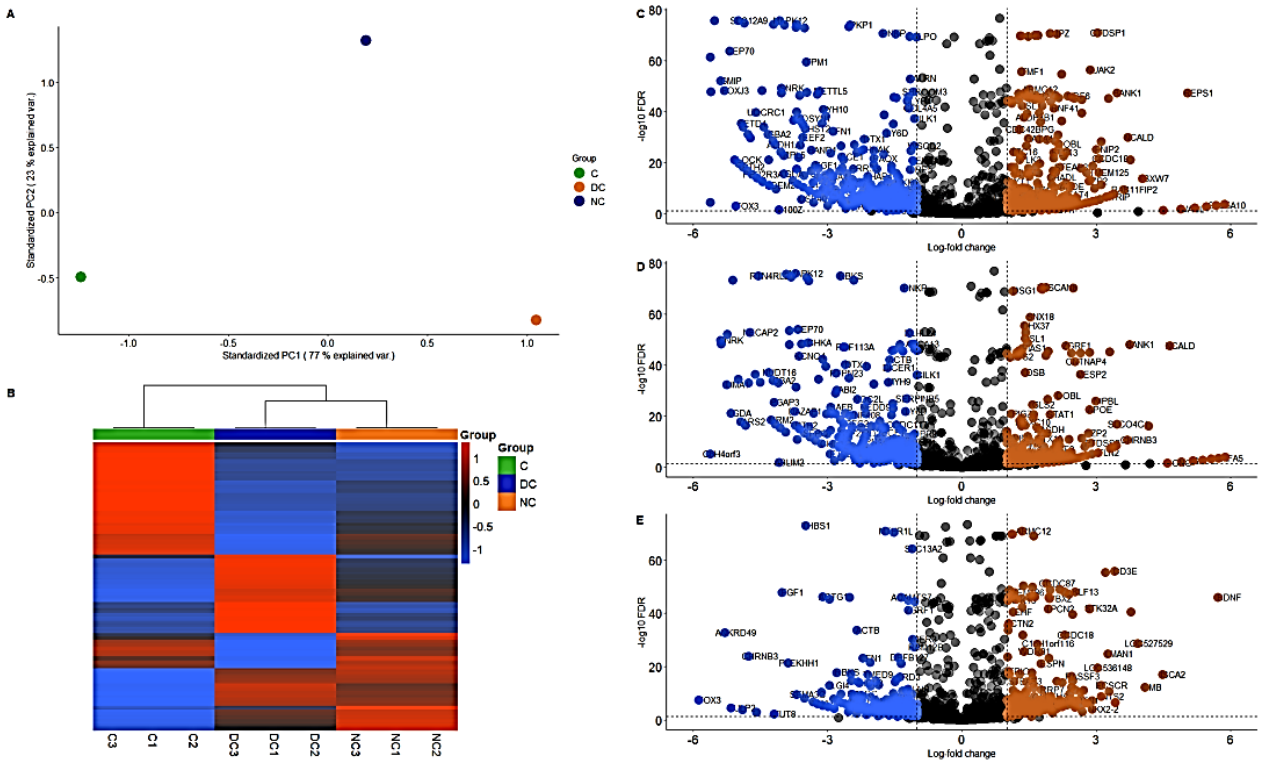

Figure 12: The mRNA profile of EVs derived from oviductal epithelial cells when incubated with spermatozoa in a direct and non-contact co-culture model. (A) PCA plot illustrating inter and intra-sample distances in EVs mRNAs derived from control (green dot), direct contact (orange dot), and non-contact (blue dot) co-culture. (B) Heatmap depicting differential expression of mRNA fragments in co-culture models versus control. The blue areas represent depleted mRNAs, whereas the red regions represent mRNAs that have been enriched. (C) Volcano plot illustrating mRNAs fragments from oviductal epithelial cells EVs in response to co-incubation with spermatozoa in direct contact versus control. (D) The volcano plot depicted the contrast between the EV mRNAs derived from non-contact co-culture versus control. (E) The contrast between the EV mRNA fragments derived from direct contact co-culture EVs and EVs derived from non-contact co-culture. The orange and blue dots in each volcano plot represent the considerable enrichment and depletion of mRNA fragments, respectively.

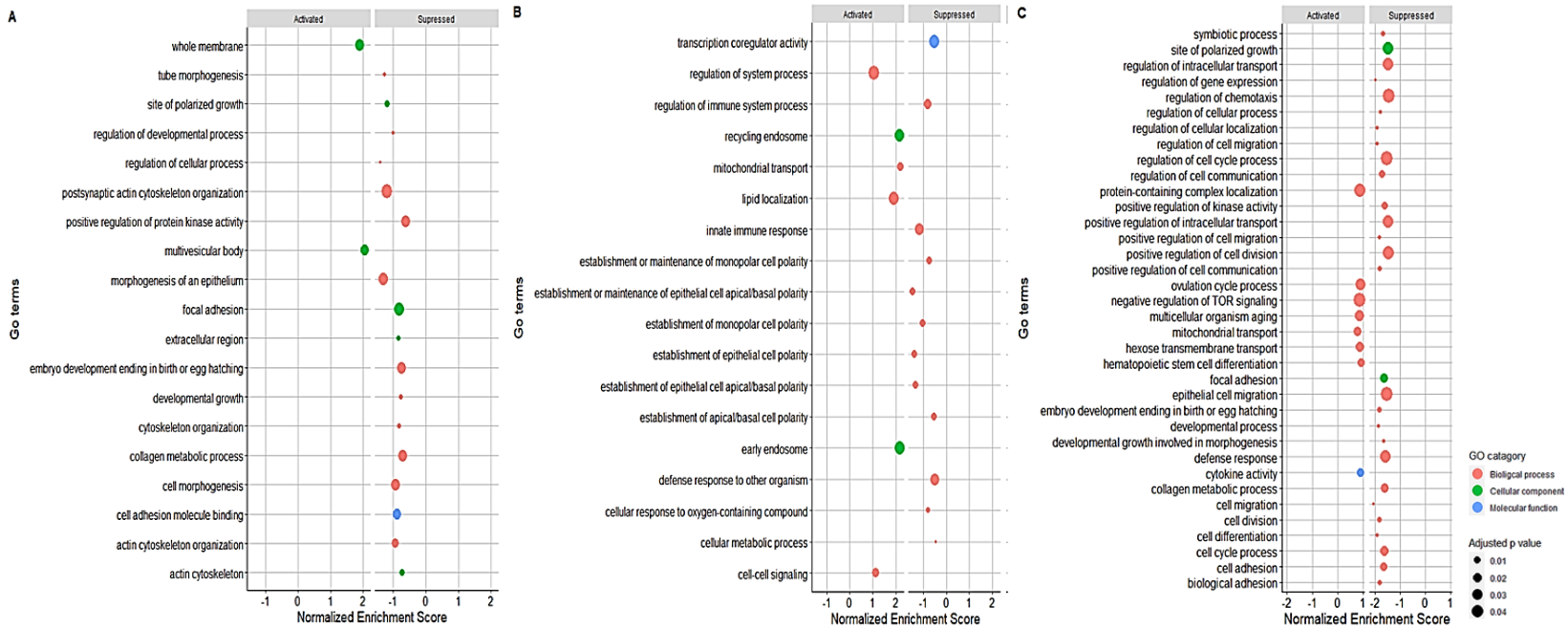

Figure 13: Biological pathways represented by EV-derived exonic mRNA fragments A) Overrepresented GO terms when oviductal epithelial cells are in direct contact with spermatozoa. B) Overrepresented GO terms when oviductal epithelial cells are in non-contact co-culture with spermatozoa. C) GO terms that are overrepresented in direct contact versus non-contact co-cultures of oviductal epithelial cells

5.3.3 Differential expression and pathway enrichment of miRNA

A differential expression analysis was performed to examine the variations in enrichment or depletion of miRNA derived from BOEC EVs in contact and non-contact co-culture system. According to the findings of the sequencing, the mi-RNAs carried by EVs in contact co-culture differ from those carried by the control group. The separation of the samples within the different groups is demonstrated by the PCA plot (Fig 14A). When compared to the control group, the direct-contact co-culture group had three miRNAs (bta-miR-100, bta-miR-191, and bta-miR-2478), enriched significantly where as two miRNAs (bta-miR-11987 and bta-miR-11980) significantly depleted. We found no differently expressed miRNAs between the non-contact co-culture group versus the control group (Fig 14 B-E). Owing to the considerable intragroup variance, an FDR of less than 0.1 was considered to evaluate statistical significance. Furthermore, we conducted GSEA based on GO of target genes linked with enriched miRNAs. Only one pathway enriched in non-contact versus control was linked with inner cell mass proliferation. Figure 15 A-B depicts the biological processes, molecular function, and cellular components of pathways associated with direct contact versus control and direct contact versus non-contact.

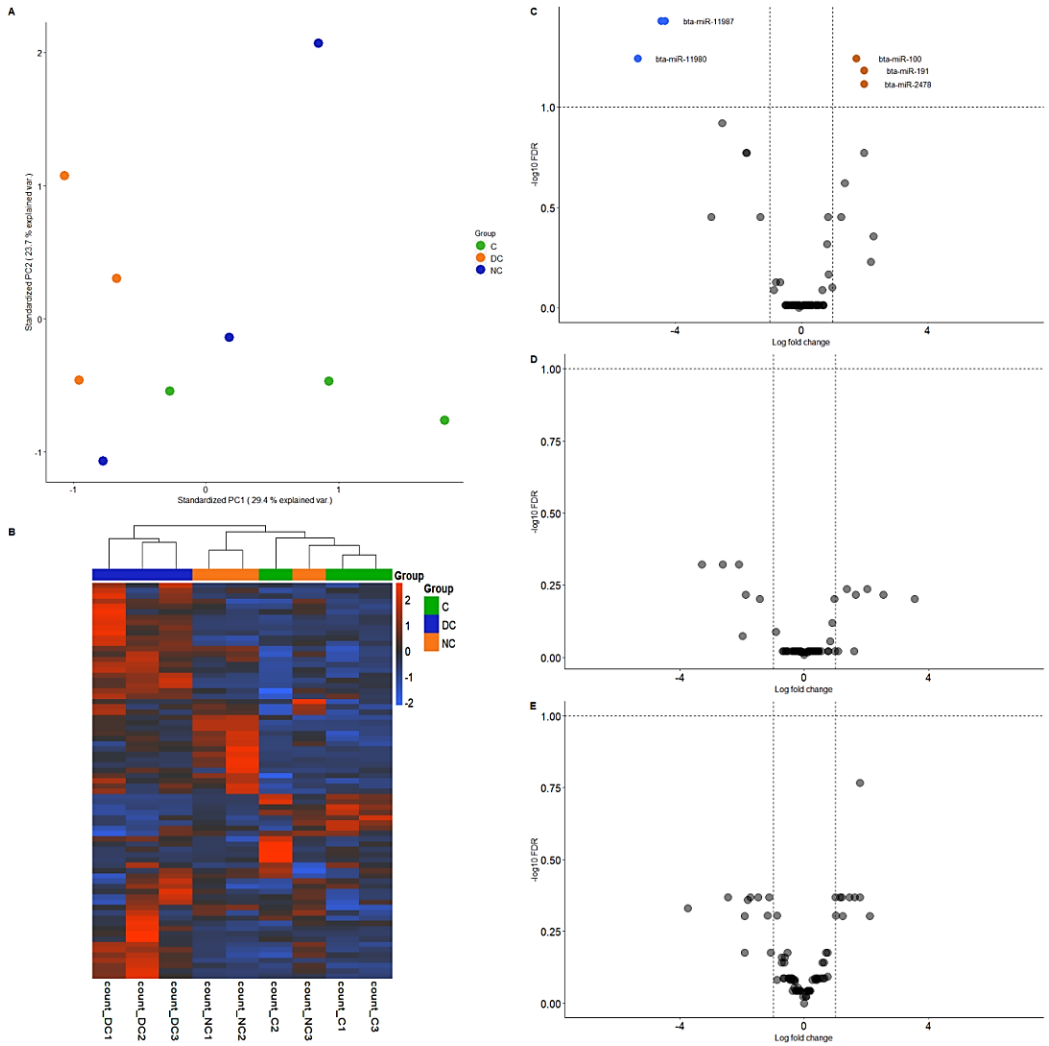

Figure 14: The miRNA profile of EVs derived from oviductal epithelial cells when incubated with spermatozoa in a direct and non-contact co-culture model. (A) Segregation of EV miRNAs derived from control (green dot), direct contact (orange dot), and non-contact (blue dot) co-culture. (B) Heatmap depicting differential expression of miRNAs in co-culture models versus control. The blue areas represent depleted miRNAs, whereas the red regions represent miRNAs that have been enriched. (C) Volcano plot illustrating miRNAs from oviductal epithelial cells EVs in direct contact versus control. The orange and blue dots in volcano plots represent the considerable enrichment and depletion of miRNAs, respectively. (D) Volcano plot showing that miRNAs from non-contact co-culture versus control had no significant differences. (E) The contrast between the EV mRNA fragments derived from direct contact co-culture EVs and EVs derived from non-contact co-culture. miRNAs having a FDR less than 0.1 were considered significantly enriched or depleted.

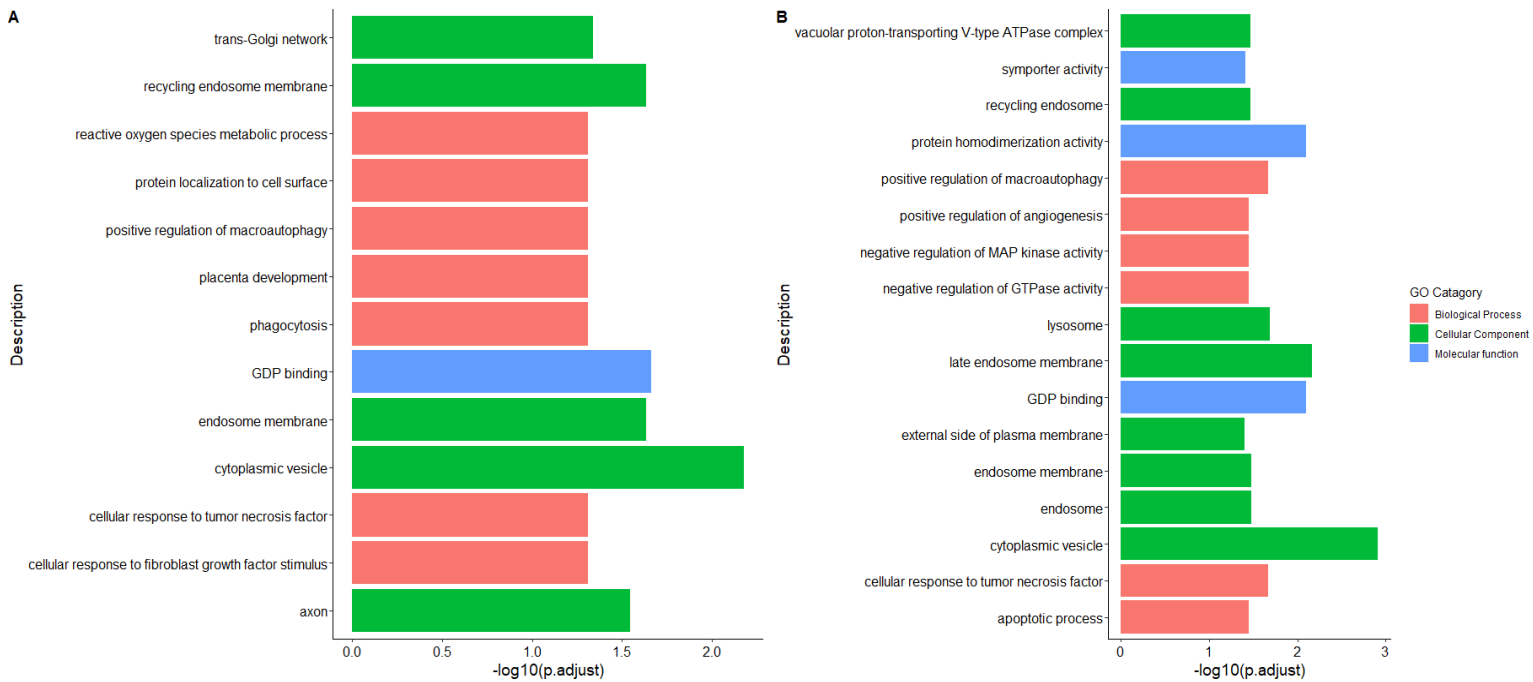

Figure 15: The GO pathway enrichment analysis: A) GO terms that are over-represented among the targets of EV miRNAs in direct contact co-culture. B) GO terms that are over-represented among the targets of EV miRNAs in non-contact versus direct contact co-cultures.

5.4 Key findings of Study 2

1) This study demonstrated that spermatozoa can alter the cargo of EVs even before establishing direct contact with the oviduct, in addition to modifying gene expression in the oviduct.
2) Contact with the spermatozoa, whether indirect or direct, induced the oviduct to release more EVs carrying distinct cargoes. However, direct interaction between the oviduct and spermatozoa boosts EV production more than indirect contact.
3) Furthermore, notable distinctions in the content of EVs were observed among the various co-culture systems involving spermatozoa and oviductal epithelial cells.

5.5 Study 3: Characterization of the changes in bovine spermatozoa viability, capacitation, and the acrosome reaction in response to different concentrations of EVs derived from bovine FF

5.5.1 Minimum concentration of FF EVs required to improve sperm viability, capacitation, and acrosomal reaction

The objective of this experiment was to determine the minimum number of EVs derived from FF that is required to have any effect on capacitation, the acrosome reaction, and viability of spermatozoa. Our results revealed that FF EVs increase spermatozoa viability, and improve capacitation, and the acrosome reaction. A minimum of 1×10^6 EVs derived from FF were sufficient to elicit the aforementioned responses in bovine spermatozoa. Furthermore, our data indicated that increasing the concentration of EVs resulted in a corresponding increase in the proportion of spermatozoa exhibiting improved viability and enhanced capacitation and the acrosome reaction. The higher rates of viability, capacitation, and the acrosome reaction were seen in the group where spermatozoa were supplemented with $1x10^9$ EVs derived from bovine FF as shown in Fig 16.

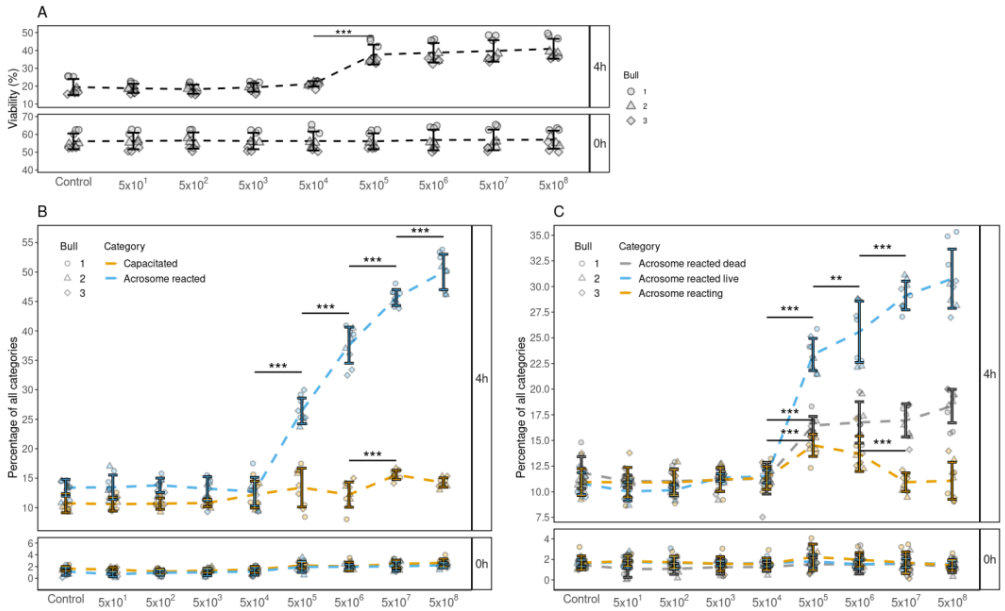

Figure 16: Analysis of bull spermatozoa motility, viability, capacitation and the acrosome reaction when incubated with different concentrations of bovine FF EVs. A) At 0 and 4 hours, the percentage of viable spermatozoa supplemented with various numbers of FF-EVs (B) Impact of different numbers of FF-EVs on the capacitation reaction of spermatozoa at two time points: 0 and 4 hours post-supplementation. (C) Different stages of acrosomal reaction at 0 and 4 hours after supplementation with FF EVs. Error bars display mean ± standard deviation (SD).

5.5.2 Combined effects of progesterone and FF EVs on sperm viability, capacitation, and the acrosome reaction

In order to evaluate if FF EVs and progesterone impart synergistic effects in boosting viability, capacitation, and the acrosome reaction of spermatozoa, the FF derived EVs were supplemented along with progesterone and the incubation was performed for 4 hours. Our findings revealed that FF EVs and progesterone (0.5 µg/µL and 1 µg/µL) both individually boost the capacitation and acrosome reaction of the spermatozoa. One anticipated finding was that spermatozoa viability was not boosted by progesterone and was much lower as compared to spermatozoa incubated with FF EVs. However, the combination of progesterone and FF EVs both had synergistic effects on vital parameters of spermatozoa. In the progesterone + FF EVs group, higher percentages of spermatozoa were capacitated and acrosome reacted as depicted in Fig. 17.

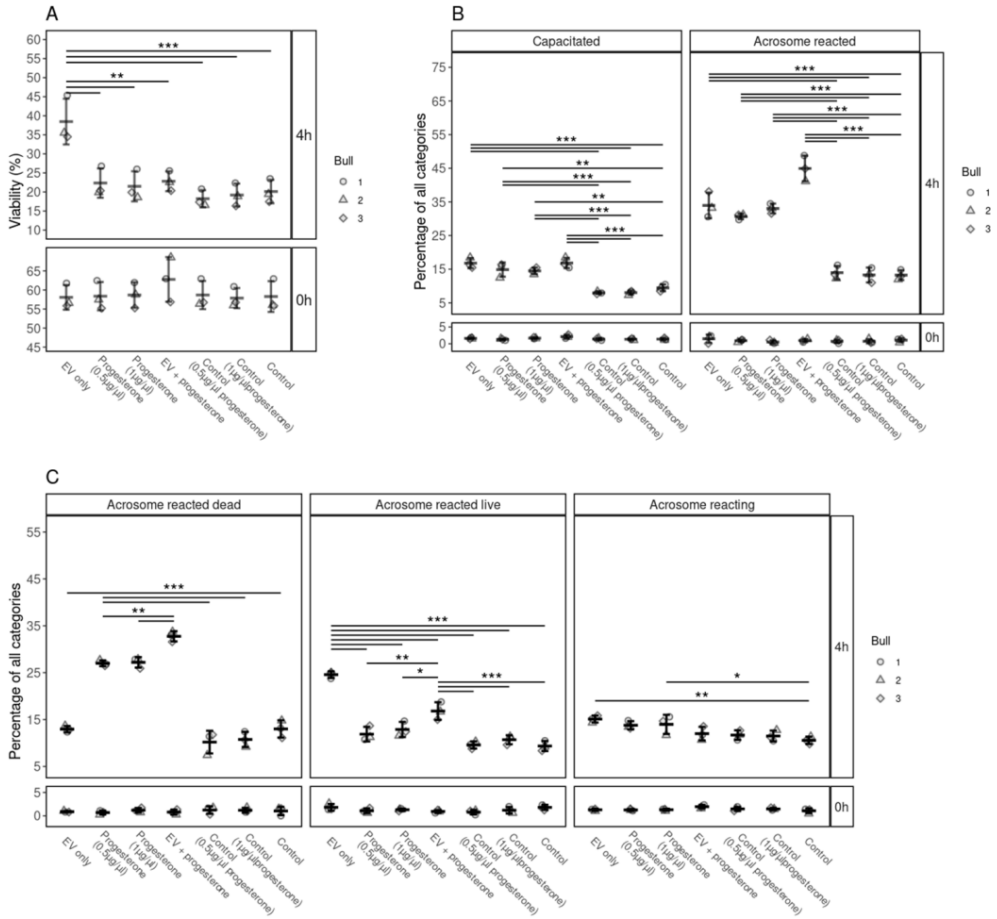

Figure 17: Viability, capacitation, and the acrosome reaction of spermatozoa after supplementation with EVs and various amounts of progesterone, as well as a combination of FF EVs and progesterone (0.5 and 1 µg/µL). (A). At 0 and 4 hours, the percentage of viable spermatozoa supplemented with FF EVs, various concentrations of progesterone + FF EVs and control. (B) At 0 and 4 hours, the percentage of spermatozoa that underwent capacitation and the acrosome reaction on supplementing FF EVs, various concentrations of progesterone + FF EVs versus control. (C) At 0 and 4 hours, the percentage of spermatozoa that have undergone the acrosome reaction on supplementing FF EVs, various concentrations of progesterone + FF EVs versus control. The mean and standard deviation are shown as error bars (SD).

5.6 Key findings of Study 3

1) The data obtained from this Study indicate that bovine follicular fluid (FF) derived extracellular vesicles (EVs) have a positive effect on capacitation, the acrosome reaction, and the viability of bovine spermatozoa, with this effect being dependent on the concentration or dose of EVs used.
2) The minimum effective threshold for FF EVs was determined to be at least 1×10^6 FF EVs/mL. This concentration was found to promote important spermatozoa parameters, including capacitation, acrosome reaction, and viability. The concentration of the spermatozoa used in the experiments was 1×10^7/mL.
3) The functional analysis showed that both FF EVs and progesterone had a positive impact on the functions of spermatozoa. However, the combined effects of FF EVs and progesterone led to a significantly higher number of spermatozoa undergoing capacitation and the acrosome reaction, indicating a synergistic effect between these two factors.
4) Interestingly, while progesterone did not have any effect on sperm viability, the use of FF-derived EVs resulted in a notable improvement in spermatozoa viability. These findings highlight the importance of FF-derived EVs in modulating the functions of spermatozoa, and suggest potential clinical applications in assisted reproductive technologies.

6. DISCUSSION

The broad aim of this thesis was to investigate the involvement of spermatozoa, oviduct, FF and EVs in establishing a periconception milieu which plays a significant role in mediating several reproductive processes. These objectives were accomplished by using *in vitro* primary cell culture of bovine oviductal epithelial cells, bovine spermatozoa, and bovine FF as models. Through experimentation, it was observed that direct contact between spermatozoa and oviductal epithelial cells is not required to induce changes in the gene expression profile of the oviductal cells. The differentially expressed genes varied between contact and non-contact co-culture of spermatozoa and oviductal epithelial cells. This finding led us to hypothesize that spermatozoa release some biomolecules that are capable of inducing changes in the gene expression profile of the recipient oviductal cells. Important pathways including retinol metabolism and ovarian steroidogenesis were significantly enriched in the non-contact co-culture of spermatozoa and oviduct which are important in terms of fertilization and early embryo development. Apart from studying the alterations in gene expression of oviductal epithelial cells, we also studied the changes in the cargo of oviduct derived EVs in response to spermatozoa in a contact and non-contact co-culture. We demonstrated that EVs derived from oviductal cells carry distinct cargoes in the form of diverse species of RNA when incubated with spermatozoa in different co-culture models. Additionally, variations in cargo were also observed in oviduct derived EVs isolated from direct contact and non-contact co-culture. The gene set enrichment analysis revealed that some of the pathways associated with the target genes of EV derived RNA in response to spermatozoa were essential in terms of early embryo development, immune suppression and spermatozoa survival. Furthermore, we also studied the effects of FF derived EVs on the vital functions of spermatozoa which included viability, capacitation, and the acrosome reaction. The findings obtained suggest that FF EVs improved the viability, capacitation, and acrosome reaction of frozen thawed bovine spermatozoa in a dose/concentration manner. Collectively, these findings suggest the existence of a symbiotic relationship between spermatozoa and the oviduct. The presence of FF in the oviduct facilitates the functional parameters of spermatozoa that are crucial for fertilization. In exchange, the spermatozoa modify the gene expression and EV cargo of the oviductal epithelial cells, and the pathways identified in both studies suggest their significance in spermatozoa survival, and early embryo development.

In the instance of IVF, periconception interactions are ignored, yet a viable pregnancy is nevertheless achieved. However, the technology of IVF is not entirely efficient because of recurrent implantation failure (RIF) in several cases (Bashiri et al., 2018; Coughlan et al., 2014). Another challenging situation associated with IVF is the increased possibility of imprinting defects and epigenetic alterations in IVF-conceived children. After fertilization *in vivo,* the early embryo during its passage through the oviduct undergoes DNA methyla-

tion reprogramming (Hattori et al., 2019). These epigenetic alterations are influenced by environmental factors, including the oviductal milieu (Canovas et al., 2017). There are several commercial culture systems for IVF, but none are as intricate and dynamic as oviduct fluid and the concentration of certain vital components in culture media can affect embryo development. Consequently, there is a need to modify the protocols utilized in ARTs for better outcomes.

The studies conducted within this thesis pertaining to extracellular vesicles (EVs) adhere to the guidelines specified by the ISEV (Théry et al., 2018b). In Study 2, a comprehensive characterization of EVs derived from BOEC conditioned media was conducted, encompassing an assessment of their size, morphology, and the presence of EV markers. Correspondingly, in Study 3, the FF-EVs used were previously subjected to characterization based on identical parameters in a research publication authored by our research group (Hasan et al., 2020).The experimentation conducted in this thesis with an *in vitro* model revealed that spermatozoa not only alter gene expression of the oviduct but also the cargo of EVs derived from them. Surprisingly in both cases, the changes take place without spermatozoa maintaining direct contact with the oviduct epithelial cells. Although only one spermatozoon is required to fertilize the egg, we deduced that the millions of spermatozoa traversing through the maternal reproductive tract are there because not all the spermatozoa are destined to fertilize the egg. Many of them may die due to the hostile environment in the uterus while a few thousand reach the oviduct and trigger changes in the oviduct. There is an active two-way dialogue existing between spermatozoa-oviduct and the mediators of this dialogue emitting from the oviduct appear to be EVs. Our results might provide valuable insights for a better understanding of EVs as potential modulators and facilitators of sperm-oviduct and embryo-maternal interactions and their implications for improved fertility therapies in the future.

6.1 Alterations in gene expression of oviductal epithelial cells in response to spermatozoa arrival

The alterations in the transcriptome profile of oviductal epithelial cells as a result of interaction with spermatozoa have been the subject of a significant amount of research and are well documented (Almiñana et al., 2014; Fazeli et al., 2004; Kodithuwakku et al., 2007a). The significance of these modifications and the interaction mechanism between spermatozoa-oviduct remains relatively unclear. We aimed to explore whether there is a possibility of distant communication via some mediators between the oviduct and spermatozoa. Two co-culture models were utilized in this study: a contact co-culture model where spermatozoa directly interact with BOECs, and a non-contact co-culture model where a 0.4 μm pore size insert is placed between the spermatozoa and BOECs. After incubation for 10 hours, the mRNA sequencing results revealed the gene expression changes in both co-culture models versus control. This suggests that

spermatozoa prior to mediating direct contact with oviductal cells, signal the maternal tract via some biomolecules that cause changes in gene expression of the oviduct. Not only were spermatozoa capable of eliciting gene expression changes in BOECs in the absence of direct physical contact, but the magnitude of gene expression changes generated by spermatozoa in non-contact co-culture was greater than that induced by direct physical contact. The responses that were induced by direct contact may be more immediate than the responses that are induced by non-contact, which is one hypothesis that could explain the greater magnitude of differentially expressed genes in non-contact co-culture systems versus contact co-culture after 10 hours of incubation. According to the findings of the RNAseq experiment, the contact co-culture did not affect the expression levels of *CYP1B1* and *PTGS2*; however, during the 4-hour observation period of the qPCR experiment their levels did rise in response to the contact co-culture. A transcription factor *ATF3*, which was upregulated in response to contact culture but not non-contact culture, is known to have negative regulatory effects on the expression of *PTGS2* during acute inflammation (Hellmann et al., 2015). This explains the expression of *PTGS2* being lower in contact co-culture due to the high expression of *ATF3*. However, in the qPCR timeline experiment after 90 minutes a mild significant increase in the expression of *PTGS2* was observed compared to the control. Previous research has also shown that the expression of *PTGS2* is increased in bovine oviductal epithelial cells when incubated with spermatozoa in a contact culture. In addition, the study suggests that *PTGS2* might increase oviductal ciliary motility and speed the transit of spermatozoa towards the oocyte (Kodithuwakku et al., 2007b).

The qPCR experiments revealed that *DHRS3* was considerably upregulated in both co-culture systems within a short period. In addition, pathway enrichment analysis identified the retinol metabolism pathway as the enriched pathway with upregulated genes in non-contact co-culture. *DHRS3* is one of the major participants in this pathway that regulates the synthesis of retinoic acid and is essential for early embryo patterning (Kam et al., 2013). Ovarian steroidogenesis was also identified as one of the considerably enriched pathways, which is relevant since there is evidence that a steroid-rich environment promotes oocyte fertilization. A study in pigs revealed the ability of the oviduct to release steroids and it was hypothesized that the oviduct may have a role in regulating the early stages of pregnancy (Martyniak et al., 2018; Yoshimura et al., 1986). The process of fertilization and early embryonic development is supported by the secretion of oviductal factors, which are regulated by the action of oviductal steroids. (Ballester et al., 2014; S. Li & Winuthayanon, 2017b). The significance of these pathways, which were enriched in response to spermatozoa, is still unclear; nonetheless, the general consensus implies that these pathways could have some part to play in early embryo development and fertilization. This study provides a basic concept of temporal gene expression variations, and as we did not bridge the gap between 4-hour responses (qPCR) and 10-hour responses (RNAseq), the degree to which short-term responses

vary from long-term responses is still unknown. The results of our research, taken as a whole, provide novel insights into the communication that occurs between spermatozoa and the cells of the oviduct. Our findings demonstrated for the very first time that contact between spermatozoa and oviductal epithelial cells is not required to induce changes in the gene expression profile of the oviductal cells. This leads us to hypothesize that spermatozoa release signals that are capable of inducing alterations in the gene expression profile of the recipient oviductal cells. The transmission of these signals could be facilitated by cell-free nucleic acids or cell-free proteins, including those that are transported via EVs. Some studies have also demonstrated that proteins as well as exosomes from seminal plasma remain attached to the surface of spermatozoa and thus might influence their interaction with the maternal tract (Kasvandik et al., 2015; Samanta et al., 2018). The findings from our study also demonstrated that gene expression levels are highly dependent on the type of co-culture system. This implies the existence of molecular signals released by the spermatozoa which are capable of evoking functional responses in the maternal tract. Nevertheless, it is unknown whether the EV cargo produced from the oviductal epithelial cells also changes as a consequence of the arrival of spermatozoa.

6.2 Alterations in the EV cargo derived from oviductal epithelial cells in response to spermatozoa prior to its arrival

There is a growing body of literature suggesting EVs as a new realm in mediating intercellular communication due to their capacity of shuttling cargo from one cell to another cell, both in prokaryotes and eukaryotes (Yáñez-Mó et al., 2015). Overall in reproductive physiology, a plethora of studies have demonstrated the role of EVs in facilitating various reproductive events which include functional maturation of spermatozoa, fertilization, implantation, and maintenance of pregnancy (Bravo & Valdivia, 2018b; Dissanayake, Nõmm, et al., 2021; Es-Haghi et al., 2019; S. Li & Winuthayanon, 2017a). The EVs derived from the oviduct have been shown to improve the fertilizing ability of spermatozoa, embryo development, and increase the efficiency of embryo transfer by increasing the birth rate (Al-Dossary et al., 2013; Almiñana et al., 2017; Bathala et al., 2018). Furthermore, the gene expression of the oviduct is significantly altered on interaction with spermatozoa and our previous study also corroborates with this finding (Almiñana et al., 2014; Fazeli et al., 2004; Kodithuwakku et al., 2007a). However, our previous study also demonstrated that spermatozoa can communicate with the oviductal epithelial cells remotely via bioactive molecules and alter the gene expression of the oviduct. The influence of spermatozoa on the cargo of oviduct-derived EVs is not known and this study examined the changes in EV production and RNA cargo when bovine oviductal

epithelial cells were co-cultured with spermatozoa in a contact and non-contact co-culture system.

The results from this study revealed an increased number of EVs in both co-culture models versus control. Our results indicate that the presence of spermatozoa in co-culture with oviductal cells led to an increase in the production and release of extracellular vesicles (EVs). Additionally, the EVs derived from BOECs in response to spermatozoa had diverse cargoes in the form of fragmented mRNAs and miRNAs. Surprisingly, the cargo derived from BOECs EVs from direct contact was different as compared to the non-contact co-culture of spermatozoa and BOECs. This suggested that non-contact and direct contact between spermatozoa-oviductal epithelial cells induced distinct responses. Despite observing a higher number of EVs in direct contact co-culture, our study found that spermatozoa stimulated the production and release of EVs from oviductal cells even in a non-contact co-culture system. The increase in the number of EVs derived from co-culture models may be attributed to the distinct cargo loaded in oviductal derived EVs on the arrival of spermatozoa.

The GSEA with *Bos taurus* GO term annotations revealed the role of the miRNAs in the biological processes linked with phagocytosis, positive regulation of macro autophagy, cellular responses to tumour necrosis factor (TNF) and fibroblast growth factor (FGF), and placenta development in the case of direct contact versus control. According to a published study, autophagy pathways are active throughout the early stages of human embryogenesis and are crucial to the development of the organism (S. Song et al., 2022). Additionally, a study has demonstrated the localisation of FGF during early pregnancy in the rat oviduct (Alan & Liman, 2021). FGFs play a critical role in the development of the embryo by regulating cellular migration, differentiation, and proliferation (Cotton et al., 2008). Furthermore, a study in cows has revealed that the interaction between spermatozoa and oviductal epithelial cells controls TNF production and phagocytosis, which promotes spermatozoa survival in the oviduct (Yousef et al., 2016). These results suggest that spermatozoa induce the oviduct to produce EVs that act as the main players of the cross-talk between oviduct-spermatozoa.

The analysis revealed a substantial amount of reads aligned with protein-coding genes were between 16 – 48 bases in length, indicating a highly fragmented population of “EV-derived mRNA”. Multiple studies have reported the presence of fragmented RNA species in EVs and their potential functions (O’Brien et al., 2020; Prieto-Vila et al., 2021). We suggest that fragmented EV RNA may be utilized as an analogue for the cellular transcriptome and acts as a snapshot revealing the physiological state of the cells from where EVs are derived. The differential enrichment analysis was conducted on the EV RNA reads which mapped to the exonic regions of the bovine species. The genes associated with mRNA from EVs isolated from non-contact co-culture were associated with suppression and regulation of the immune system. Several investigations conducted on cattle have shown that spermatozoa-oviduct binding induces anti-inflammatory responses that promote spermatozoa survival

in the oviduct (Almiñana et al., 2014; Marey et al., 2016). Consequently, this offers crucial insights into the function that EVs may play a role in the regulation of the immune system during spermatozoa-oviduct interactions. This result suggests that spermatozoa signals oviductal cells before their arrival, stimulating the oviduct to produce EVs carrying mRNA that promotes suppression of the immune system. There is a possibility that the oviductal EVs containing immune-suppressing cargo trigger autocrine signalling, which protects the spermatozoa from being attacked by the immune system. In addition, research has uncovered a parallel mechanism protecting the embryo in the female reproductive tract. To escape rejection by the mother's immune system, a semi-allogenic embryo releases biomolecules that reduce inflammation in the bovine oviduct (Maillo et al., 2015; Talukder et al., 2018). Three to four days are the maximum amount of time spermatozoa remain alive in the mammalian oviduct, and this period coincides with the earliest stages of embryo development in the oviduct (Camara Pirez et al., 2020; S. Li & Winuthayanon, 2017c). This strengthens the idea that suppression of the immune system is a result of collaborative co-action by the early embryo and spermatozoa. They (embryo-spermatozoa) stimulate the oviduct to produce EVs with cargo that mediates the processes associated with immune responses which might help them to evade the immune system. Surprisingly the direct contact co-culture EV mRNA was enriched for genes involved in the suppression of collagen metabolism and focal adhesion pathways. Attachment of the embryo to the endometrium is required for successful implantation, and adhesion molecules are essential for initiating this process. The endometrium undergoes a series of cytoskeletal rearrangement processes and the expression of adhesion molecules is essential for embryo attachment (Achache & Revel, 2006). Since the oviduct is not the site where the embryo must attach, it is plausible that suppressing these pathways prevents ectopic pregnancy. This may possibly be the cause of the relatively few occurrences of ectopic pregnancy in cows (Corpa, 2006). Therefore, it may be inferred that spermatozoa interact with the oviduct and cause it to repress these pathways. These findings also imply that the increase in the number of EVs occurs with the arrival of spermatozoa, which stimulates oviductal cells to release EVs packed with specific cargo.

Combining these findings raises the notion that spermatozoa serve as an external cue, stimulating oviductal epithelial cells to release EVs with particular cargoes that are critical for both spermatozoa survival and early embryo development. It would also be interesting to investigate if spermatozoa from bulls with poor fertility indices may cause similar responses in the oviduct. The role of the oviduct and spermatozoa is not limited to fertilization, but precise oviduct-spermatozoa communication is necessary for a healthy pregnancy (S. Li & Winuthayanon, 2017c). Our research may provide valuable information on the potential function of EVs as regulators and facilitators of spermatozoa-oviduct and embryo-maternal interactions, which may improve the current fertility treatments. The modifications that take place during the periconception period are not only limited to changes in gene expression of oviduct or oviduct

derived EVs in response to spermatozoa. The interactions are bidirectional as the oviduct derived EVs and FF entering the oviduct boosts the viability, capacitation and acrosome reaction of the spermatozoa. However, the impact of FF derived EVs on the vital functions of spermatozoa remains elusive.

6.3 Alterations in the bovine spermatozoa viability, capacitation and acrosome reaction in response to bovine FF EVs in a dose/concentration-dependent manner

Males deposit a significant number of spermatozoa in the female reproductive canal after sexual intercourse. Prior to fertilization spermatozoa undergo a considerable number of physical and biomolecular changes, including hyperactivation, capacitation, and the acrosome reaction, to fertilize the egg. Since the egg is present in the ampullar region of the oviduct and is immotile, so spermatozoa are required to move to the upper region of the oviduct which is closer to the ovary. Moreover, a study has revealed that approximately 0.5% of the FF from the ruptured follicle reaches the oviduct along with the ovum during ovulation (Hansen et al., 1991b). Multiple studies in several mammalian species have demonstrated that FF improves spermatozoa capacitation and the acrosome reaction (Funahashi & Day, 1993; Thérien et al., 2005; Yanagimachi, 1969). Several studies have shown that progesterone present in the FF is responsible for boosting the key functions of spermatozoa which includes capacitation and the acrosome reaction (Calogero et al., 2000; Morales et al., 1989; Osman et al., 1989). Furthermore, previously a study has reported that FF-derived EVs positively impact the cumulus expansion of the oocyte (Hung et al., 2015). Hence, it would be reasonable to postulate that FF EVs may affect the vital functions of male gamete i.e. spermatozoa. In this work, we demonstrated that bovine FF-derived EVs enhance viability, capacitation and the acrosome reaction of the spermatozoa in a dose/concentration-dependent manner.

The findings indicate that a minimal EV concentration of 5×10^5 particles/ 500 µL was enough to elicit substantial alterations in the aforementioned functional characteristics of spermatozoa. Based on our calculations it seems that one EV particle is adequate to maintain the critical functional parameters of one spermatozoon. We observed a dose-dependent influence of EVs on spermatozoa capacitation and the acrosome reaction that exhibited striking similarities to enzyme–substrate reaction patterns. The effects on viability, capacitation, and the acrosome reaction did not improve any further after a particular concentration of FF EVs. The curve reached a plateau which points to the possibility that EVs might be functioning as enzymatic catalysts during these processes. Multiple studies have already shown that EVs are involved in enzyme kinetics and transport enzymes (Gerth et al., 2019; Margolis & Sadovsky, 2019). Thus, it can be suggested that FF-derived EVs might carry enzymes as a part of their

cargo that eventually influences the viability, induction of capacitation, and the acrosome reaction of spermatozoa. As previously mentioned, progesterone follows the same trend as that of FF EVs in influencing the functional parameters of spermatozoa. Hence, we conducted an experiment to study the synergistic effects of progesterone and FF EVs on the vital functions of spermatozoa. The results from these experiments revealed that both FF EVs and progesterone boosted the capacitation and acrosome reaction of spermatozoa independently. However, a higher percentage of spermatozoa underwent capacitation and the acrosome reaction under the cumulative effect of progesterone and FF EVs. This result is in corroboration with a finding from the literature, which shows that progesterone and FF had a synergistic impact on zona pellucida-induced acrosomal response (Schuffner et al., 2002). The remarkable finding was that FF EVs were more effective at maintaining spermatozoa viability as compared to progesterone. Therefore, we can deduce that *in vivo* progesterone and FF EVs might work in a synergistic way that eventually leads to improving the key parameters of spermatozoa, which are very essential for fertilization.

7. FUTURE PERSPECTIVES

In summary, the findings presented in this thesis suggest that the oviduct is not merely a conduit for spermatozoa or a site for fertilization, but rather a complex and dynamic organ where various factors, including gametes and FF, interact through a well-orchestrated sequence of events leading to the commencement of new life. The critical role played by EVs in mediating this communication underscores their significance in reproductive biology. The studies discussed here offer several avenues for future investigation:

1) **Characterization of biomolecules released from spermatozoa**: The outcomes of our research demonstrated that bioactive nanoparticles discharged from spermatozoa can trigger alterations in gene expression within the oviduct. Nevertheless, the exact composition and nature of these biomolecules still remain unknown. Therefore, it is imperative to conduct additional investigations to identify and characterize the specific biomolecules released from spermatozoa.

2) **Determining the specificity of responses triggered by biomolecules released from spermatozoa:** It would be intriguing to investigate whether the biomolecules released from spermatozoa evoke the same responses in any other non-reproductive cells as oviductal epithelial cells, upon the arrival of spermatozoa.

3) **Effect of diet, age, and drugs on the nanoparticles released from spermatozoa:** The effect of various factors e.g. age, diet, drug addiction, as well as other environmental hazardous exposure, on these biomolecules must be investigated. Moreover, it would be interesting to investigate if spermatozoa from a male with idiopathic fertility are capable of eliciting similar gene expression changes in the oviduct and with the same magnitude as in normal healthier individuals.

4) **Change in the proteome of oviductal epithelial cells in non-contact coculture:** Multiple studies have reported the changes in the proteins released from the oviduct in response to spermatozoa. However, it would be interesting to study the changes in protein composition when the spermatozoa are not in direct contact with spermatozoa.

5) **Detailed information on diverse cargoes present in EVs:** We have only analysed the changes in the small non-coding RNA present in oviductal EVs in response to spermatozoa. However, the cargo present in the EVs is diverse, containing DNA, proteins and lipids. The changes in these cargoes in response to spermatozoa must be considered for future studies.

6) **Detailed assessment of male ejaculate:** While accessing the semen, the main importance is given to the spermatozoon as it is the carrier of genomic information. However, the other contents of the ejaculate including EVs must be analysed as well because they play an important role in maintaining the viability and other functions of spermatozoa. Moreover, it would be interesting to investigate if spermatozoa from a male with idiopathic fertility are capable of eliciting similar gene expression changes in the oviduct and with the same magnitude as in normal healthier individuals. It would be intriguing to analyse the effects of seminal plasma EVs from fertile males on the viability of the spermatozoa of the infertile male.

7) **Analyzing the cargo and surface proteins of FF-EVs:** To enhance the present ART techniques that may finally result in successful fertilization, further knowledge on the cargo/surface proteins of FF EVs would be of utmost relevance. Further investigation is necessary to determine the components of the FF EV cargo and surface that are changing the spermatozoa functional characteristics. When employing assisted reproductive technologies, the findings may be used in therapeutic interventions.

8. CONCLUSION

Study 1: According to the findings of this study, direct contact between spermatozoa and oviductal epithelial cells is not required to induce changes in the transcriptomic profile of the oviductal cells. The spermatozoa are capable of communicating with the oviductal epithelial cells remotely by releasing biomolecules that are capable of modifying the gene expression profile of the recipient oviductal cells.

Study 2: According to the findings of this study, the spermatozoa act as an external signal and without establishing direct contact with the oviductal epithelial cells, induces them to ramp up their production of EVs. In addition to this, the oviductal epithelial cells release EVs with distinct cargo in response to spermatozoa in a contact and non-contact co-culture. Therefore, not only is the gene expression of oviductal epithelial cells modified but also the cargo of EVs released by them is modified before spermatozoa mediate direct contact with them.

The findings of Study 1 and II demonstrated that spermatozoa alter EV cargo and the transcriptome of oviductal epithelial cells via a distant mode of communication. These pathways associated with changes in gene expression and EV cargo are reported to be essential for the survival of spermatozoa, fertilization, and early embryo development.

Study 3: According to the findings of this study, FF EVs in a dose dependent manner improve the essential functional aspects of spermatozoa which includes viability, capacitation and acrosome reaction in a dose-dependent manner. Apart from this FF EVs and progesterone work synergistically to enhance the capacitation and acrosome reaction of spermatozoa but FF EVs were more competent than progesterone in maintaining the viability of spermatozoa.

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SUMMARY IN ESTONIAN

Emaka ja spermatosoidide ekstratsellulaarsete vesiikulide suhtluse iseloomustus eostamiseelsel perioodil

Sisukirjeldus

Viljastamiseelse ja -järgsete sündmuste keeruliste molekulaarsete mehhanismide uurimine on vajalik, et luua viljatuse ravimiseks uusi reproduktiivtehnoloogilisi võimalusi. Just need teadmised molekulaarsetes mehhanismides on juhtinud viimase kümnendi tehnoloogilisi edusamme kuntslikus reproduktiivtehnoloogias (ART) ja viinud erinevate tööriistade innovatsioonini. Küll on seni ART-s vähe arvesse võetud perikontseptsiooni perioodi. Olgugi, et viljatusravi tulemused ühe embrüo siirdamisel on aastate jooksul paranenud, on ART-ga saavutatud rasedused emale ja lootele endiselt suure terviseriskiga.

Enne viljastamist mängivad meessugurakkude (spermatosoidid) ja munajuharakkude vahelised raku- ja molekulaarsed mõjud olulist rolli parima spermi valikul munaraku viljastamiseks. Spermatosoidid suhtlevad munajuha epiteelirakkudega peamiselt seondudes pinnaretseptoritega, mis mõjutavad nende geeniekspressiooni. Uuringud on näidanud, et isaste hiirtega paaritatud emaste hiirte munajuharakkude adrenomedulliini ja prostaglandiini endoperoksitaasi süntaas 2 geenide transkriptoom oli üles reguleeritud võrreldes emaste hiirtega, keda paaritati viljatute (spermatogenees peatunud) isaste hiirtega. See tulemus näitab, et munajuha geeniekspressiooni esilekutsumiseks on vajalik elujõulisi spermatosoide munajuhas, kuid nende tulemuste asjakohasust teistele liikidele on vähe uuritud. Lisaks on puudujääke teadmistes spermatosoidide ja munajuha epiteelrakkude vahelistest suhtlusmehhanismidest, mis viivad munajuha geeniekspresiooni muutusteni. Seni on kirjeldatud vaid mõningaid sperma ja munajuha interaktsioone vahendavaid sekreteerivaid valke ja retseprtoreid, mis hõlbustavad isaste sugurakkude ja emaste suguelundite vahelist rakusuhtlust.

Lisaks munajuhade epiteelirakkude sekreteeritud valkudele võib neid interaktsioone vahendada veel üks klass sõnumitoojaid: rakuvälised ehk ekstratsellulaarsed vesiikulid (EV-d). EV-d on heterogeensed lipiidikihtidega ümbritsetud nanoosakesed, mis vahendavad rakkudevahelist suhtlust. Munajuhaepiteeli EV-d mängivad olulist rolli toetamaks spermatosoidide liikuvust ja elujõulisust ning suhtlevad ka naissoost sugurakkudega (ootsüüdiga). Eelnevad uuringud on näidanud, et koerte munajuha mikrovesiikulid aitavad ootsüütidel küpseda. Munaraku vabanemisel folliikulist satuvad osad follikulaarvedeliku (FF) EV-d munajuhasse, mis mõjutavad munajuha transkriptoomi ja kutsuvad esile spermatosoidide kapatsitatsiooni- ja akrosomaalreaktsiooni. Küll ei teata FF-i EV-de mõju ulatust kirjeldatud spermi arengumuutustele.

Eelnevad uuringud on näidanud, et spermatosoidide ja munajuha kahepoolset suhtlust vahendavatel EV-del on roll rakkudevahelistes suhtluses. See tähendab, et spermatosoidid mõjutavad munajuha transkriptoomi ning munajuha EV-d muudavad spermatosoidide elutähtsaid funktsioone. Selle suhtlusprotsessi

kõrvalekalle normaalsest füsioloogilisest olukorrast võib aga põhjustada viljatust, mille molekulaarseid muutuseid võib potentsiaalselt kasutada viljatuse diagnoosimiseks ja raviks. Seetõttu käesolev uurimistöö käsitlebki spermatosoidide ja munajuha epiteelrakkude EV-de vahendatud suhtlusprotsessist tingitud munajuha geenieksрessioni muutuseid. Lisaks uuritakse munajuha EV-de sisu ja tootmist vastusena spermatosoididele ning kas mõju spermatosoidide elutähtsatele funktsioonidele on sõltuvuses FF-i EV-de hulgast.

Eesmärgid

Üldine töö eesmärk on uurida spermatosoidide interaktsiooni emaka suguteede epiteelrakkudega ja sellest tingitud munajuha genoomi ekspressioonimuutusi, mis on vajalikud viljastumisprotsessiks. Selle eesmärgi saavutamiseks tehti kahesuguseid uuringuid: analüüsiti otsest või kaudset spermatosoidide ja munajuha rakkude interaktsioonist tingitud geenieksрessiooni. Lisaks uuriti munajuha EV-de sisu ja tootmise muutusi vastusena spermatosoididele ning FF EV-de mõju spermatosoidide elujõulisusele, kapatsitatsioonile ja akrosomaalreaktsioonile.

Konkreetsed teadustöö eesmärgid:

- Uuring 1: analüüsida kas spermatosoidid vabastavad biomolekule, mis võivad vallandada geenieksрessiooni muutusi munajuharakkudes.
- Uuring 2: analüüsida kas spermatosoidid võivad esile kutsuda muutusi munajuha EV-de sisus.
- Uuring 3: uurida kas veise FF-i EV-d mõjutavad veise spermatosoidide elujõulisust, kapasitatsiooni ja akrosomaalreaktsiooni.

Materjalid ja meetodid

Munajuhad koos munasarjadega hangiti tapamajast ning transporditi nelja tunni jooksul 37℃ füsioloogilises lahuses laborisse. Ainult varase innatsükliga lehmade munajuhad (helepunane *corpus hemorrhagicum*, 0–4 päeva pärast ovulatsiooni) valiti epiteelrakkude eraldamiseks. Kasutades immunoflorestsents-mikroskoopia metoodikat kinnitati, et eraldatud rakkudel on epiteelrakkude markerid. Veise munajuha epiteelrakud (BOEC) külmutati kuni katsete läbiviimiseni. Katseteks sulatati BOEC ja kasvatati petritassides kuni 80% tassist on kaetud rakkudega, mis pandi edasi kasvama 12 süvendiga rakukultuuriplaadile. BOEC-i monokultuurikiht kattis 80% plaati 24 tunni pärast kasvatamist, mida edasisteks katseteks loputati kaks korda vesiikulivaba sperma-TALP söötmes. Samal ajal sulatati külmutatud veisespermakõrred steriilses 37℃ vesivannis. Kolme kõrre sisu kanti 4 ml 60% Percolli® lahusesse kust lisati spermaga koos kultiveerimiskatsetesse võrdsed kogused 1 x 10^6 spermi/ml.

Munajuha rakkude ja nende EV-de sisu geenieksрessiooni erinevuste analüüsimiseks kasutati kahte kultuurimudelit. Esimeses kontakt-kultuurimudelis

kasvatati BOEC-i monokihti koos spermatosoididega. Teises mittekontakt-kultuurimudelis kasvatati BOEC-i monokihti kasutades 0,4 µm poorisuurustega eraldust spermatosoididest. Esimeses uuringus inkubeeriti BOEC-i spermidega 10 tundi mRNA sekveneerimiseks epiteelrakkudest ja neli tundi qPCR-iks. Teises uuringus inkubeeriti kultuuri kokku neli tundi, mille möödudes eraldati EV-d rakukasvusöötmest suurusvälistuskromatograafia (SEC) meetodil. Nanoosakeste jälgimisanalüsaatori ZetaView®-ga mõõdeti EV-de suurusprofiil ja kontsentratsioon. EV-de morfoloogiat hinnati ülekandeelektronmikroskoopiaga (TEM). Munajuha EV-de biokeemiline iseloomustamine viidi läbi massispektromeetriaga. TRIzol reagenti ja RealSeq®-AC miRNA raamatukogu komplekti kasutati EV-de RNA eraldamiseks, mille ettevalmistust kvantifitseeriti kasutades Qubiti analüsaatori (kõrge tundlikkusega dsDNA komplekt, tootja protseduur) ja analüüsiti Tapestation 2200-ga. Sekveneerimine viidi läbi Illumina HiSeq 2500-ga ja Flow Cell 50-tsüklilise SBS-komplektiga.

Kolmandas uuringus eraldati veise spermatosoidid kasutades 60% perkolli lahust. Spermatosoidide elujõulisust hinnati kasutades 2 µM kaltseiini ja 4 µM etiidiumhomodimeer-1, mille tulemusel surnud spermatosoidid fluorestseerusid punaselt ja elusad roheliselt. Veise spermatosoidide kapatsitatsiooni hindamiseks kasutati kloortetratsükliini (CTC) töölahust ja akrosomaalreaktsiooni staatust hinnati FITC-PNA ja EthD-1 meetodil. Tulemused visualiseeriti objektiklaasil fluorestsentsmikroskoobiga 400X suurendusel. Keskmiselt hinnati 250-300 spermatosoidi slaidi kohta.

Tulemused

Esimeses uuring näitas, et spermatosoidid kutsuvad esile transkriptoomilisi muutusi BOEC-des, enne kui spermid loovad nendega otsese kontakti nanoskaala bioaktiivsete molekulidega. Geeniekspressiooni muutused aga sõltuvad spermatosoidide ja munajuha epiteelrakkude vahelise kontakti olemasolust. Kontaktkultuuri uuringus tuvastati BOEC-i üheksa ülesreguleeritud ja ühe allareguleeritud geeni. Kõige ülesreguleerituim geen oli DHRS3 ja ainuke allareguleeritud oli RANBP3. Geeniekspressiooni muutused BOEC-des olid suuremad aga mittekontakt-kultuuris, kus 52 geeni olid ülesreguleeritud ja 56 allareguleeritud. Mõlemas kultuurisüsteemis olid neli ühesugust geeni ülesreguleeritud (PLAU, TNFRSF11B, SCIN, DHRS3). Neist DHRS3 ekspressioon oli kõrgem kontakt-kultuuris (log2FC = 1,5) kui mittekontakt-kultuuris (log2FC = 0,92). Huvitav tähelepanek oli see, et ATF3 geen, teadaolev PTGS2 negatiivne regulaator, oli üks kuuest statistiliselt erinevalt ekspresseeritud geenist, mille muutus oli tingitud munajuharakkude ja spermatosoidide koos kultiveerimisel, nagu on näha Joonisel 8. Rajaanalüüs *Bos tauruse* KEGG annotatsioonidel teostati statistiliselt erinevalt üles- ja allareguleeritud geenidel, et tuvastada radu, mis on esindatud mittekontakt-kultuuri geenidel. Uuringus tuvastati kuus olulist rikastunud rada (FDR < 0,05): retinooli metabolism (bta00830), steroidhormooni biosüntees (bta00140), munasarjade steroidogenees (bta04913) ja

tsütokiini-tsütokiini retseptori interaktsioon (bta04060). Allareguleeritud geenidega ei tuvastatud oluliselt rikastunud radu.

Nelja huvipakkuvat geeni (DHRS3, CYP1B1, PTGS2, ATF3) uuriti qPCR analüüsiga. Geenide ekspressiooni mõõdeti pärast 30-, 90-, 150- ja 240-minutilist kultiveerimist, et mõista kui kiiresti reageerivad BOEC-d spermatosoidide stiimulitele ja muudavad oma geeniekspressiooni. DHRS3 ekspressioon oli kõrge mõlemas kultuurimudelis, kuid kontakt-kultuuris oli geeni ekspressioon kõrgem ($p = 0,02$; 30 min) ja silmnähtavalt kõrge 240 min pärast kultiveerimist. CYP1B1 geeniekspressioon oli ülesreguleeritud mittekontakt-kultuuris 90 min pärast kultiveerimist ($p < 0,001$) ja langes kontroll-prooviga samale tasemele 90 min pärast kultivatsiooni. Mõlema geeni, DHRS3 ja CYP1B1, ekspressiooni väiksemat tõusu oli märgata kontakt-kultuuris, mis saavutas haripunkti 150 min kultiveerimisel ja siis langes. PTGS2 ekspressioon tõusis 30 min pärast mittekontakt-kultuuri kultiveerimist ($p = 0,003$) ja püsis sarnaselt katse lõpuni. Kontakt-kultuuris mõõdetud PTGS2 ekspressioon tõusis 90 min pärast kultiveerimist ($p = 0,02$), kuid selle tõus ei säilinud sarnasel tasemel katse lõpuni.

Teises uuringus täheldati, et munajuha otsesel või kaudsel kokkupuutel spermatosoididega ajendas see munajuharakke vabastama rohkem EV-sid. Küll oli otsesel kontaktil EV-de kontsentratsioon söötmes suurem kui kaudsel kontaktil. Lisaks erinevatele EV-de arvudele söötmes, täheldati EV-de sisumuutusi olenevalt kultuurimudelist. Valku kodeeriva RNA eksoonilise regiooni miRNA fragmendid (16-48 nt) olid EV-des erinevalt rikastunud. Kontakt-kultuuris oli 960 oluliselt rikastunud fragmenti ja 917 ammendunud fragmenti ning mittekontaktkultuuris oli 882 rikastunud ja 812 ammendunud fragmenti võrreldes kontroll-kultuuriga. Võrreldes aga kontakt- ja mittekontakt-kultuure tuvastati 878 olulist rikastunud ja 1000 ammendunud fragmenti ($FDR < 0,005$). Geenikomplekti rikastamise analüüsil (GSEA), kus kasutati *Bos tauruse* erinevalt rikastunud fragmenteeritud mRNA geeniontoloogia (GO) annotatsioone, tuvastati mikrovesiikulitega seotud radade aktivatsiooni kontakt-kultuuris võrreldes kontroll-kultuuriga. Lisaks olid fokaalse adhesiooni, kollageeni metabolismi, aktiinitsütoskeleti organisatsiooni ja tsütoskeleti valgu sidumise rajad deaktiveeritud kontakt-kultuuris võrreldes kontroll-kultuuriga. Rajad, mis olid seotud regulatsiooni funktsioonidega, kaasasündinud immuunvastuse repressiooniga ja immuunvastusega teisele organismile, olid deaktiveeritud mitte-kontaktkultuuris võrreldes kontroll-kultuuriga.

Sekveneerimisanalüüsil selgus, et EV-de miRNA-de ekspressioon oli kontaktkultuuris erinev kontroll-kultuuriga: kolm miRNA-d olid oluliselt rikastunud (bta-miR-100, bta-miR-191 ja bta-miR-2478) ning kaks miRNA-d olid ammendunud (bta-miR-11987 and bta-miR-11980) kontakt-kultuuris. Küll aga ei olnud erinevusi mittekontakt-kultuuri ja kontroll-kultuuri miRNA ekspressioonis. Märkimisväärse rühmasisese dispersiooni tõttu kasutati statistilise olulise määramiseks $FDR < 0,1$. Teostatud GSEA rikastunud miRNA-dega tuvastati ainult raku sisemise massi proliferatsiooni raja, mis oli rikastunud mittekontakt-kultuuris võrreldes kontroll-kultuuriga. Joonisel 15A-B on kujutatud bioloogilisi protsesse, molekulaarseid funktsioone ja rakulisi komponente, mis olid seotud

kontakt-kultuuri vs kontroll-kultuuriga ja kontakt-kultuuri vs mittekontakt-kultuuriga.

Kolmandas uuringus näidati, et FF EV-d suurendavad spermatosoidide elujõulisust, kapatsitatsiooni ja akrosomaalreaktsiooni. Antud spermatosoidide muutuste esilekutsumiseks piisas 1 x 10^6 FF EV-dest. Suurendades EV-de kontsentratsiooni söötmes, suurenes ka spermatosoidide proportsioonid, mis läbisid kapatsitatsiooni ja akrosomaalreaktsiooni ning olid elujõulised. Kõige suurem spermatosoidide proportsioon, mis läbisid eelnevalt nimetatud muutused, toimus 1 x 10^9 FF EV-de kontsentratsiooni mõjul. Lisaks leiti, et FF EV-del ja progesteroonil (0,5 µg/µl ja 1 µg/µl) on sünergistlik mõju spermatosoidide elujõulisusele, kapatsitatsiooni ja akrosomaalreaktsioonile, kus oli suurim proportsioon viimast kahte mainitud sündmust läbinud spermatosoide. Individuaalselt hinnates FF EV-de ja progesterooni mõju parameetritele selgus, et FF EV-d ja progesteroon suurendavad kapatsitatsiooni ja akrosomaalreaktsiooni, kuid progesteroon ei suurendanud spermatosoidide elujõulisust vaid oli madalam kui FF EV-dega spermatosoide inkubeerides.

Arutelu

Palju teadustöid on avaldatud munajuha epiteelrakkude transkriptoomiprofiili muutustest, mis on tingitud spermatosoididega suhtlusest. Küll on ebaselge nende muutuste tähtsus viljakuses ning spermatosoidide ja munajuha vahelise koostoime mehhanism. Antud teadustöö eesmärk oli uurida munajuha ja spermatosoidide vahelist suhtlust kahes erinevas kultuurimudelis: kontakt-kultuurimudelis, milles on spermatosoididel otsene kontakt BOEC-dega, ja mittekontakt-kultuurimudelis, milles spermatosoidid olid eraldatud BOEC monokultuurist kasutades 0,4 µm suuruste pooridega eraldust. Mõlemas mudelis inkubeeriti kultuuri 10 tundi ja sekveneeriti mRNA geenieksрessioon, kus võrreldi mõlemas kultuurimudelis geenieksрesiooni muutuseid kontroll-kultuuriga.

Uuringus selgus, et spermatosoidid põhjustavad geenieksрesiooni muutuseid enne munajuha rakkudeni jõudmist, mis on vahendatud teatud biomolekulidega. Mitte ainult ei suuda spermatosoidid esile kutsuda neid munajuha muutuseid kontaktivabalt, vaid mittekontakt-kultuuris olid geenieksрesiooni muutused suuremad kui kontakt-kultuuris. See tähendab, et vastavalt kontaktitüübile kutsub spermatosoidid esile selgelt erinevaid rakuvastuseid. Eraldades BOEC-i EV-d, siis tuvastati EV-de sisus fragmenteerunud mRNA-d ja miRNA-d, mille ekspressioonis oli erinevusi kontakt- ja mittekontakt-kultuuride vahel. EV-de sisu uuringud näitasid, et munajuha EV mRNA ja miRNA muutused olid funktsioonides, mis on olulised raseduse tekkimiseks ja säilitamiseks. Seetõttu on spermatosoidide stimulatsioon munajuha rakkudele ning nende EV-de sisumuutused kriitilise tähtsusega nii spermatosoidide ellujäämiseks kui ka varajase embrüo arenguks. Lisaks spermatosoidide põhjustatud geenieksрesiooni muutustele oli mõlemas kultuurimudelis munajuha EV-de kontsentratsioonis erinevused võrreldes kontroll-kultuuriga, kus EV-de kontsentratsioon oli suurem

kultuurimudelites võrreldes kontroll-kultuuriga. Võrreldes aga kultuurimudeleid, siis kontakt-kultuuris oli EV-de arv suurem. Olenemata, et kontakt-kultuuris suutsid spermatosoidid stimuleerida munajuharakke rohkem EV-sid tootma, siis antud tulemus näitas, et EV-de tootmiseks ei ole otsest kontakti rakkudel vaja. EV-de vabanemiseks rakkudes on vaja vaid spermatosoidide signaale, stimuleerides munajuha epiteelrakke vabastama EV-sid kindla sisuga vastavalt spermatosoidi vastusest.

Antud uurimistöös selgus, et progesterooni lisamisel spermatosoididele suureneb elujõulisus, kapatsitatsiooni ja akrosomaalreaktsiooni läbinud spermatosoidide proportsioon. Kui lisati veise FF EV-de spermatosoididele, siis sõltuvalt EV-de kontsentratsioonist suutsid EV-d sarnaselt ensüümi-substraadi reaktsioonimustrile suurendada spermatosoidide funktsionaalseid parameetreid. See tähendas, et nii FF EV-d kui ka progesteroon suudavad individuaalselt parendada spermatosoidide funktsioone. Küll FF EV-d olid efektiivsemad säilitamaks spermatosoidide elujõulisust kui progesteroon. Lisades nii progesterooni kui ka FF EV-sid spermatosoididele avaldasid mõlemad sünergistlikku mõju, mis suurendas kapatsitatsiooni ja akrosomaalreaktsiooni läbinud spermatosoidide proportsiooni kui kumbki eraldi. Sellest järeldub, et pigem progesterooni ja FF EV-de koos kasutamine kui eraldi võiks olla üheks viljatusravi viisiks *in vivo,* parendamaks spermatosoidide viljastumisprotsessis olulisi parameetreid.

Kokkuvõte

Olgugi, et munaraku viljastamiseks on vaja ühte spermatosoidi, aitavad miljonid spermatosoidid, mis liiguvad läbi emakakanali, kutsuda esile vajalikke emakarakkude muutuseid implantatsiooniks. Paljud spermatosoidid surevad emakakanalis ja ei ole määratud munarakku viljastama, mis on tingitud emaka vaenulikust keskkonnast. Kuid tuhanded spermatosoidid jõuavad sellegi poolest munajuhasse, kus toimub munaraku viljastumine. Spermatosoidide ja munajuha epiteelrakkude vahel toimub aktiivne kahepoolne dialoog, mida vahendavad lisaks raku vabadele signaalmolekulidele ka EV-d. Käesolevas teadustöös läbiviidud *in vitro* katsed näitasid, et spermatosoidid ei mõjuta ainult munajuha geeniekspresiooni, vaid ka munajuha EV-de kontsentratsiooni ja sisu. Üllataval kombel ei vaja spermatosoidid munajuha epiteelrakkudega otsekontakti, et kutsuda esile kirjeldatud muutuseid, piisab vaid EV-de vahendatud suhtlusest. Töö tulemused annavad väärtusliku teavet EV-de kui sperma ja munajuha ning embrüo ja emaka suhtlusvahenditest, mida saab tulevikus kasutada, et arendada uudseid viljatusravi tehnoloogiaid.

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PUBLICATIONS

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List of publications

1. Hasan MM, Viil J, Lättekivi F, Ord J, **Reshi QUA**, Jääger K, Velthut-Meikas A, Andronowska A, Jaakma Ü, Salumets A, Fazeli A. Bovine Follicular Fluid and Extracellular Vesicles Derived from Follicular Fluid Alter the Bovine Oviductal Epithelial Cells Transcriptome. International Journal of Molecular Sciences. 2020
2. **Reshi, Qurat Ul Ain**, Janeli Viil, James Ord, Freddy Lättekivi, Kasun Godakumara, Mohammad Mehedi Hasan, Monika Nõmm et al. "Spermatozoa induce transcriptomic alterations in bovine oviductal epithelial cells prior to initial contact." Journal of cell communication and signaling 14, no. 4 (2020): 439–451.
3. Dissanayake, K., Nõmm, M., Lättekivi, F. et al. Oviduct as a sensor of embryo quality: deciphering the extracellular vesicle (EV)-mediated embryo-maternal dialogue. J Mol Med 99, 685–697 (2021)
4. Hasan MM, **Reshi QUA**, Lättekivi F, Viil J, Godakumara K, Dissanayake K, Andronowska A, Jaakma Ü, Fazeli A. Bovine Follicular Fluid Derived Extracellular Vesicles Modulate the Viability, Capacitation and Acrosome Reaction of Bull Spermatozoa. Biology. 2021.

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1. Reshi, Qurat Ul Ain, Mohammad Mehedi Hasan, Keerthie Dissanayake, and Alireza Fazeli. "Isolation of Extracellular Vesicles (EVs) Using Benchtop Size Exclusion Chromatography (SEC) Columns." Next Generation Culture Platforms for Reliable In Vitro Models: Methods and Protocols (2021): 201–206.

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Raamatu peatükid

1. Reshi, Qurat Ul Ain, Mohammad Mehedi Hasan, Keerthie Dissanayake, and Alireza Fazeli. “Isolation of Extracellular Vesicles (EVs) Using Benchtop Size Exclusion Chromatography (SEC) Columns.” Next Generation Culture Platforms for Reliable In Vitro Models: Methods and Protocols (2021): 201–206.

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