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MOHAMMAD MEHEDI HASAN

Characterization of follicular fluid-derived extracellular vesicles and their contribution to periconception environment

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Institute of Biomedicine and Translational Medicine, Department of Pathophysiology, Faculty of Medicine, University of Tartu, Estonia

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Supervisiors: Professor Alireza Fazeli, PhD
Professor of Clinical Genomics and Personalized medicine
Institute of Biomedicine and translational medicine
Faculty of medicine, University of Tartu, Estonia

Professor Ülle Jaakma, PhD
Institute of veterinary medicine and Animal Sciences
Estonian University of Life Sciences, Tartu, Estonia

Professor Andres Salumets, PhD
Professor of Reproductive Medicine
Institute of Clinical Medicine
Faculty of medicine, University of Tartu, Estonia

Reviewers: Prof. Reet Kurg, PhD
Professor of Molecular Biomedicine
Institute of Technology, University of Tartu, Estonia

Dr Helena Sork, PhD
Professor of Molecular Biomedicine
Institute of Technology, University of Tartu, Estonia

Opponent: Prof Gregory Lavieu, University of Paris, France

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Author's contribution to the original publications:

Study I

Design and perform the experiments; bovine oviductal epithelial cells isolation and culture. Follicular fluid isolation and extracellular vesicles purification from the follicular fluid. Co-culture of bovine oviductal epithelial cells with follicular fluid and follicular fluid derived extracellular vesicles, isolation of RNA, quality and quantity measurement of RNA. Analyzed the data and wrote the original manuscript.

Study II

Design and perform the experiments; sperm processing, isolation of extracellular vesicles from bovine follicular fluid, porcine follicular fluids, and human choriocarcinoma cell (JAr) conditioned media. Co-culture of spermatozoa with extracellular vesicles, analysis of spermatozoa viability, capacitation and acrosome reaction. Analyzed the data and wrote the original manuscript

Study III

Design and perform the experiments; isolation and characterization of extracellular vesicles from human follicular fluids. Analyzed the extracellular vesicles data and wrote the original manuscript.

*These authors contributed equally to this work

ABBREVIATIONS

AFC	antral follicle count
BFF	bovine follicular fluid
BMI	body mass index
BOEC	bovine oviductal epithelial cell
CGC	cumulus granulosa cell
COC	cumulus oocyte complex
CTC	Chlortetracycline
DE	differential gene expression
DEG	differentially expressed gene
DNA	deoxyribonucleic acid
DPBS	dulbecco phosphate-buffered saline
dsDNA	double-stranded DNA
E2	Estrogen
ESR	estrogen receptor
EthD-1	ethidium homodimer
EV	extracellular vesicle
FBS	foetal bovine serum
FDR	false discovery rate
FF	follicular fluid
FSH	follicle-stimulating hormone
GSEA	gene set enrichment analysis
hCG	human chorionic gonadotropin
HFF	human follicular fluid
ILVS	intraluminal vesicle
IUI	intrauterine insemination
IVF	*in vitro* fertilization
JAr	human choriocarcinoma cell line
K+	potassium ion
LH	luteinizing hormone
LMM	linear mixed models
MGC	mural granulosa cells
MHC	major histocompatibility complex
miRNA	microRNA
mtDNA	mitochondrial DNA
MVBS	multivesicular bodies
Na2+	sodium-ion
NOR	normal ovarian reserve
NP	Nanoparticles
NTA	nanoparticle tracking analyzer
PCOM	polycystic ovarian morphology
PCOS	polycystic ovary syndrome
pFF	porcine follicular fluid

RBP	riboprotein complexes
RNA	ribonucleic acid
RNA seq	RNA sequencing
RT	room temperature
SEC	size-exclusion chromatography
ssDNA	Single-stranded DNA
TEM	transmission electron microscopy
WB	western blot

1. INTRODUCTION

The prevalence of infertility is one of the biggest concerns in today's life and has been identified as a public health priority for several decades (Sun et al., 2019). Infertility can be defined as the inability to conceive after one year of regular unprotected intercourse, and almost 15 % of couples are infertile globally (Gerrits et al., 2017; Kurabayashi et al., 2016). Several factors are responsible for infertility, not limited to the spermatozoa (Babakhanzadeh et al., 2020) or oocyte(s) (Maddirevula et al., 2017). The preconception microenvironment (Lynch et al., 2014) and ovarian factors (Hanson et al., 2017; Vannuccini et al., 2016) also play an essential role in determining the fate of fertilization. Some of these parameters, such as the quality of spermatozoa and the preconception microenvironment, have significant room for improvement.

Follicular fluid (FF), also referred to as ovarian fluid, is known to enhance oocyte(s) maturation (Ellsworth et al., 1984), spermatozoa function (Bravo and Valdivia, 2018; Ellsworth et al., 1984; Eriksen et al., 1997) preparation of the preconception microenvironment and embryo development (Li and Winuthayanon, 2017). FF is rich in various molecules such as proteins, hormones, polysaccharides, metabolites, reactive oxygen species and antioxidants. The primary role of these molecules is to enhance oocytes and follicular maturation and ensure the protection of follicular cells from oxidative or physical damage (Ambekar et al., 2013; Bianchi et al., 2016). However, several studies reported that FF is also associated with several reproductive diseases(Butts et al., 2021; Freitas et al., 2017). Therefore, the content of FF can be used to diagnose and treat infertility-associated diseases such as polycystic ovarian syndrome (PCOS) and improve the quality and functions of male and female gametes (Ambekar et al., 2015; Tola et al., 2018). Moreover, the content of FF is also responsible for the communication between somatic and germ cells, which is vital for oocytes to acquire their competence.

In recent years extracellular vesicles (EVs) are increasingly recognized as an alternative mode of intercellular communication. EVs are membrane-bound nanosized particles and are broadly classified into three categories exosomes (40–100nm), microvesicles (100–1000nm) and apoptotic bodies (1–2 μm) (Ståhl et al., 2019; Théry et al., 2018; Yáñez-Mó et al., 2015). Despite the nano size of EVs, they are considered miniature versions of cells (Théry et al., 2009). EVs carry proteins, RNA, DNA and lipids to target cells and alter their phenotype. EVs have been isolated from almost all types of biological fluids, including FF. The EVs derived from FF play a crucial role in oocyte maturation (de Almeida Monteiro Melo Ferraz et al., 2020), fertilization (Franchi et al., 2020) and embryo development (Silveira et al., 2017).

Knowing the unique ability of FF and FF-derived EVs in reproductive physiology, there are still some fundamental knowledge gaps, especially about the role of FF EVs in the preparation of preconception microenvironment, spermatozoa functions, fertilization and embryo development. Therefore, in this

thesis, we investigated FF-derived EVs effect on gene expression changes of the oviductal epithelial cells and their subsequent function in intercellular communications and preparation of the preconception microenvironment. In addition, we also characterized and analysed the cargos derived from FF EVs in different pathophysiological conditions and their effects on the ovarian microenvironment. Furthermore, we evaluated the impact of EVs derived from different sources and species on spermatozoa viability, capacitation and acrosome reaction.

2. LITERATURE REVIEW

2.1. Reproduction

Reproduction is necessary for all living organisms and species' survival and the transmission of genetic features from one generation to the next. Without reproduction, a species will eventually become extinct. However, while the reproductive system is necessary for a species' survival, it is not necessary for the survival of an individual. Reproduction may occur in two primary ways: asexual reproduction, which requires just one parent, and sexual reproduction, which involves gametes, or sex cells, from a male and a female, which are created via the process of meiosis. In the female reproductive system, the male gamete (spermatozoa) fertilizes the female gamete (oocyte/ egg). The resulting fertilized egg is known as a zygote. The zygote develops into an embryo, which further develops into a fetus (Dey, 2010). However, fertilization is a sophisticated multi-step process, and fine-tuning of intercellular communication is essential at each step. If this communication goes astray, it might result in unsuccessful fertilization, one of the causes of which is infertility.

2.1.1. Infertility

Under the guidelines established by the World Health Organization (WHO), infertility is a disease of the reproductive tract of a male or female characterized by the inability to achieve a pregnancy after 12 months or more of regular unprotected sexual contact. According to WHO, approximately 37% of couples worldwide are diagnosed as infertile. In contrast, the percentage of infertile women was 35%, and 8% of couples was (both male and female) identified as infertile (Conception and Organization, 1992). In female infertility, polycystic ovary syndrome is the most common cause affecting more than 70 % of women (Carmina, 2004; Futterweit, 1999).

The most common identifiable female infertility is described in table 1, adapted from (Walker and Tobler, 2022).

Table 1. The most common diagnosed women's infertility.

Condition	Percentage
Ovulatory disorders	25%
Endometriosis	15%
Pelvic adhesions	12%
Tubal blockage	11%
Other tubal/uterine abnormalities	11%
Hyperprolactinemia	7%

Conversely, male infertility is usually diagnosed by identifying one or more factors, such as sperm quality issues or sperm functional parameters. Structure, functional, endocrine, genetic, or immunological defects in the male reproductive system, and sexual dysfunctions that limit semen deposition in the vaginal canal are among these factors (Schlegel et al., 2021a). In about 20% of all infertility cases, the male is solely responsible, and in another 30% to 40% is a contributing factor (Hull et al., 1985). Overall male factor infertility contributes to about 50% of all infertility cases (Leslie et al., 2022). Specific male infertility can be broadly classified based on its underlying aetiology. These include endocrine disorders (usually due to hypogonadism) at 2% to 5%, sperm transport disorders (such as vasectomy) at 5%, primary testicular defects (which include abnormal sperm parameters with no identifiable cause) at 65% to 80%, and idiopathic (where an infertile male has normal sperm and semen parameters) at 10% to 20% (Winters and Walsh, 2014). Accurate statistics are unavailable because of general under-reporting, cultural factors, and regional variations. Patients referred to a tertiary referral centre are more likely to have their condition reported. In contrast, private patients may not have their data collected (Winters and Walsh, 2014).

2.2. Gametes

The formation of the female gamete, also known as ova or eggs, is termed oogenesis. While the oogenesis (oocyte development) varies from species to species, the primary stages are similar in all species, including primordial germ cell, oogonium, primary oocyte, development and maturation of the primary oocyte, secondary oocyte and maturation of eggs (Alberts et al., 2002a). The detailed stages of oogenesis can be found below (figure 1A).

The male gamete, known as sperm (spermatozoon, spermatozoa), is derived from the Greek word Sperma, which means seed. The development process of spermatozoa is called spermatogenesis, which involves spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa (Figure 1B).

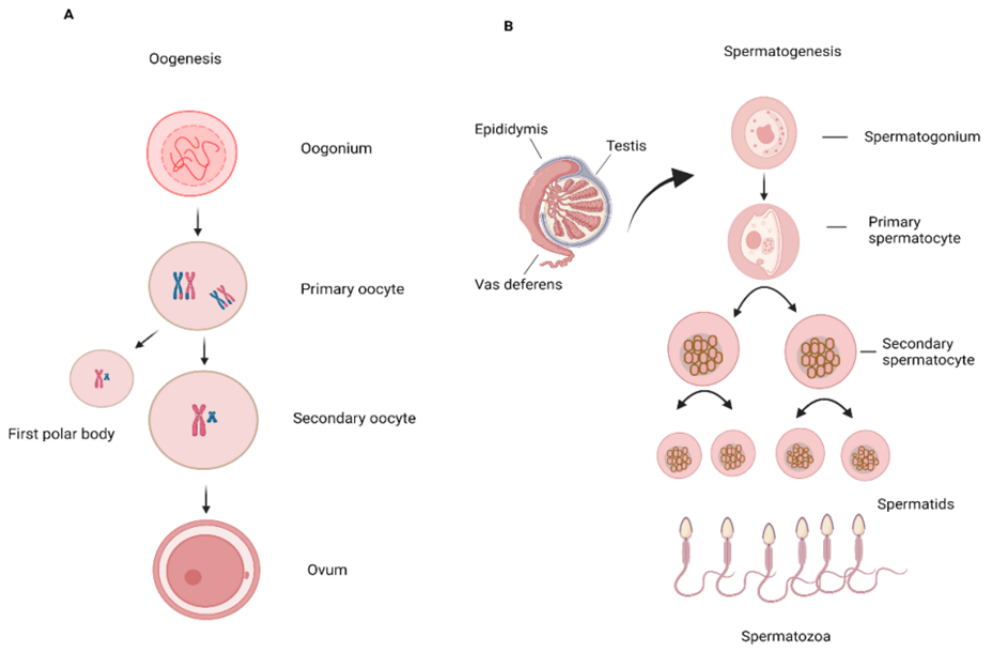

Figure 1. Development of female and male gametes. (A) The stages of oogenesis. (B) The stages of spermatogenesis.

A mature spermatozoon has three main parts (Figure 2). (A)The head contains DNA and enzymes required to penetrate the oocyte. (B) The midpiece contains combining cellular elements, microtubules, centrioles and mitochondria (the primary source of sperm energy production). Finally, the (C) long tail facilitates sperm movement (Alberts et al., 2002b). The head shape of normal sperm is oval, with a length between 3–5 µm, a width of 2–3 µm and 1.5 µm in thickness. The sperm head contains a nucleus and acrosome, covered by a nuclear membrane and metacrosomic sheath. The nucleus carries the male genetic material. However, the DNA of spermatozoa are slightly different in structure when compared with diploid cells; as a result, the DNA in sperm is more condensed, which saves space. The acrosome of sperm carries several proteolytic enzymes (trypsin, hyaluronidase, acrosin and protease). This content is released during the acrosome reaction and promotes hydrolysis in the egg's granular cells and zona pellucida. This process helps spermatozoa penetrate and merge with the egg (NELSON, 1985). The neck or midsection of the spermatozoa connects the head and tail. The midsection contains several mitochondria as spermatozoa motility is dependent on ATP generated by oxidative phosphorylation, which occurs in the mitochondrial sheath. The longest part of sperm is the sperm tail length of 40–50 µm, containing ten pairs of fibrils.

In the female reproductive tract, sperm can survive 3–5 days (Clubb, 1986; Rickard et al., 2019). However, before fertilization, spermatozoa must undergo several physical and biochemical changes, such as capacitation and acrosome reaction.

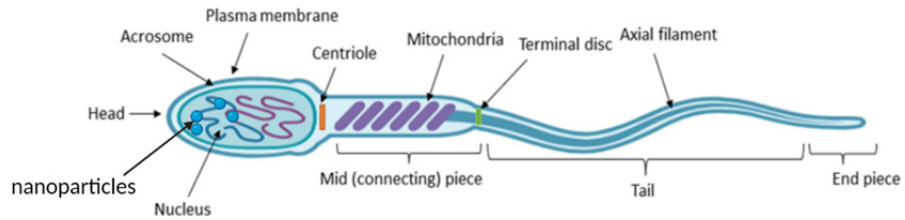

Figure 2. Illustration of a mature spermatozoon. Spermatozoa head carries a nucleus, secretory vesicles and acrosome. The neck contributes to the cell division in the embryo with the help of centrioles. The midpiece is responsible for energy supply through mitochondria, and the long tail help spermatozoa for swimming.

2.3. Periconception

The term "periconception" refers to the time 14 weeks prior to and immediately following conception (10 weeks), and it is a critical period during early development (Louis et al., 2008; Steegers-Theunissen et al., 2013). During this time, gametogenesis, organogenesis, and placental development take place. These processes are highly susceptible to epigenetic disruption, resulting in an altered profile of embryonic gene expression that lasts throughout pregnancy and childhood (Fleming et al., 2018). Any disturbance in hormonal profile, such as polycystic ovary syndrome (PCOS), including lifestyle and environmental hazards, leads to failure in conceiving (Murphy et al., 1986; Velazquez et al., 2019). PCOS is an endocrine disorder characterised by a hormonal imbalance and multiple cysts on the ovaries. The reason is unknown. PCOS is not only a physical disease but also an illness with well-described psychological difficulties caused by the many stresses of infertility and body dissatisfaction (Benson et al., 2010, 2009). Treatment of PCOS includes not limited to weight loss (Vause et al., 2010), oral pharmacologic treatment such as metformin, the combination of metformin and clomiphene (Palomba et al., 2010), oral contraceptives (Zhao et al., 2010), gonadotropin therapy (Vause et al., 2010).

2.3.1. Fertilization

Fertilization is a well-coordinated sequence of events where a male gamete merges with a female gamete. This successful fusion of maternal and paternal chromosomes creates the first form of human life, known as a zygote (a diploid cell). During sexual intercourse, millions of spermatozoa are released into the female reproductive organ (vagina). Although several thousands of spermatozoa

will die in the hostile acidic environment, many will survive due to the protective elements contained in the seminal plasma. In the following long journey, many spermatozoa die, making it easy for the remaining to swim through the mucus. When they reach the uterus, the contraction mechanism of the uterus assists their journey toward the egg. The fertilization usually occurs in the oviduct's ampulla. However, if the oocyte is not fertilized, it moves to the uterus, slowly degenerates, and is absorbed.

2.4. The gaining of fertilization capacity of the gametes

Spermatozoa and oocytes need to undergo several changes, such as maturation, in order to achieve fertilization. The cumulus-oocyte complex (COC), along with oocyte, consists of the following components; (1) An extracellular matrix known as zona-pellucida (ZP), which contains ZP1, ZP2 and ZP3 glycoproteins (2) layer of granulosa cells enriched with hyaluronic acid generally known as cumulus oophorus (Wassarman, 1999; Wassarman and Litscher, 2001). The oocytes are arrested at the prophase of meiosis II and only acquire the ability to fuse with spermatozoa when the immature oocyte resumes meiosis under the influence of luteinizing hormone (LH)surge (Zuccotti et al., 1995). Spermatozoa need to undergo several events during the maturation phase to acquire motility and fertilization potential during their journey towards the oocyte. During the processes of spermatozoa maturation, the DNA becomes more condensed via the deposition of new proteins in the nucleus. These changes make the spermatozoa head smaller and compact, essential for the parental DNA to successfully decondense in the maternal oocyte. In the female reproductive tract, sperm can survives 3–5 days (Clubb, 1986; Rickard et al., 2019). In order to acquire the fertilization capacity, mammalian spermatozoa need to undergo two post-testicular maturation steps. First maturation step, known as epididymal maturation, occurs in the male epididymis. Second maturation step, known as capacitation, occurs after the ejaculation when the semen is deposited in the female reproductive tract. The capacitation is a preliminary step for spermatozoa to acquire functional maturation, leading to an acrosome reaction. This activation phase involves changes in the plasma membrane of the spermatozoa head and enables sperm hyperactivation (Wassarman and Litscher, 2001).

Hyperactivation is the final stage for sperm activation, a high-energy phase for vigorous flagellar movement and sperm swimming capacity (Lishko et al., 2012). The capacitation process involves several chemical and functional changes. The functional coupling of signal transduction pathways regulates the initiation of the acrosome reaction in contact with ZP3. The alteration in flagellar motility is required to penetrate the ZP and develop egg fuse capacity (Evans and Florman, 2002). The events mentioned above are also responsible for altering membrane biophysical characteristics, metabolism, protein phosphorylation state, intercellular pH elevation, calcium level and membrane hyperpolarisation.

On the other hand, several other factors are involved in sperm activation *in vivo*. High-density lipoprotein, a sterol binding protein, has been identified in the Fallopian tubes, promoting the efflux of cholesterol from the sperm (Visconti et al., 1999). Nevertheless, progesterone found in Fallopian tubes, FF and the cumulus oophorus can also regulate sperm activation (Contreras and Llanos, 2001). Glucose is another vital mediator essential for capacitation. It acts as an energy source for sperm swimming and fertilizes female gamete egg/s (Goodson et al., 2012). The biochemical agents in oocyte mucous are species-specific and responsible for acrosome reaction. For example, spermatozoa from mammalian species only recognise and bind to the ZP3 glycoprotein. The concentration of O-polysaccharide, a core protein of ZP3, is vital for this aspect. Galactosyltransferase, a protein present in the anterior of the sperm head, is believed to react with o-linked oligosaccharides and trigger the acrosome reaction of sperm. Upon damage of the core protein of ZP3, the acrosome reaction can be inhibited. However, this does not affect the binding of sperm in ZP. The outer membrane of the acrosomal sac fuses with the spermatozoa plasma membrane, and acrosome content is released. The acrosomal sac enzymes help spermatozoa penetrate through the egg, eventually leading to successful fertilization.

2.5. Periconception microenvironment

It has been demonstrated that reproductive function is sensitive to physical, psychosocial, and chemical changes (Younglai et al., 2005). In the female reproductive tract, the oviduct functions as a channel that carries gametes and the embryo and offers critical nutritional, environmental, and structural support for early embryonic development, owing to its three separate structures. The infundibulum (fimbria in humans) and ampullar region of the oviduct comprise mostly ciliated epithelial cells. In contrast, the isthmus has a high number of secretory epithelial cells. The oviduct and oviductal fluid are the first environments mammalian embryos encounter at conception (Fazeli and Holt, 2016). Even though the oviduct has diverse roles to play, the role of the oviduct in reproduction is less well understood than the ovary and uterus, which have both been the subject of substantial research and are therefore rather well understood. However, oviductal function dysregulation or disturbance can lead to infertility or potentially fatal problems, such as ectopic pregnancy (Li and Winuthayanon, 2017).

2.5.1.Ovarian follicles and their maturation

A follicle's cavity or antrum development is more sophisticated than a primary epithelium. Cell-cell connections exist only between the single layer of cells lining the basal lamina in a simple epithelium, not with cells on the other side of the cavity; as a result, a potential cavity already exists between the two opposing layers of epithelial cells, even if it is not enlarged, making the structure of

the ovarian follicle unique. Before a cavity forms, it contains numerous layers of cells. Cells inside these layers, presumably, have connections with all neighbouring cells. Multiple foci of fluid initially gather during the development of preantral follicles. When they enlarge and combine more prominent centrally situated antrum forms. These fluid foci probably amass in places with fewer cell-cell interactions, or other processes enable these foci to form and merge. Cell death or apoptosis may generate cavities or lumens in the same way as blastocysts or tubes do in vitro or in vivo lumen development by endothelial cells (Coucouvanis and Martin, 1999; Meyer et al., 1997). Such occurrences may occur in preantral follicles since dead granulosa cells are seen in otherwise healthy follicles. Fluids collect in open space due to cellular death and produce osmotic pressure. While such a process may be involved in the early development of FF foci, it is unlikely to be active as the follicle progresses to the antral stage. The transition of the preantral phase to the antral phase of the follicle formation is depicted below (Figure 3).

The enlargement of the follicular antrum needs remodelling of the theca interna and externa, stroma, tunica albuginea, and surface epithelium as the follicle expands. This remodelling is likely similar to that of other glands that may expand, penetrate, and even branch inside stroma by multiplying cells at their leading edges (Davies, 2002; Fata et al., 2004). Aside from the apparent necessity to rebuild the matrix and extend the theca and its vasculature, the follicle finally grows to the ovary's surface. An expansion would typically occur in the direction with the least resistance. However, since the tunica is more collagenous than the stroma, remodelling the matrix in the tunica albuginea close to the increasing follicle is necessary for follicular growth. This mechanism may be rate-limiting in big antral stages and dominant and subordinate follicles.

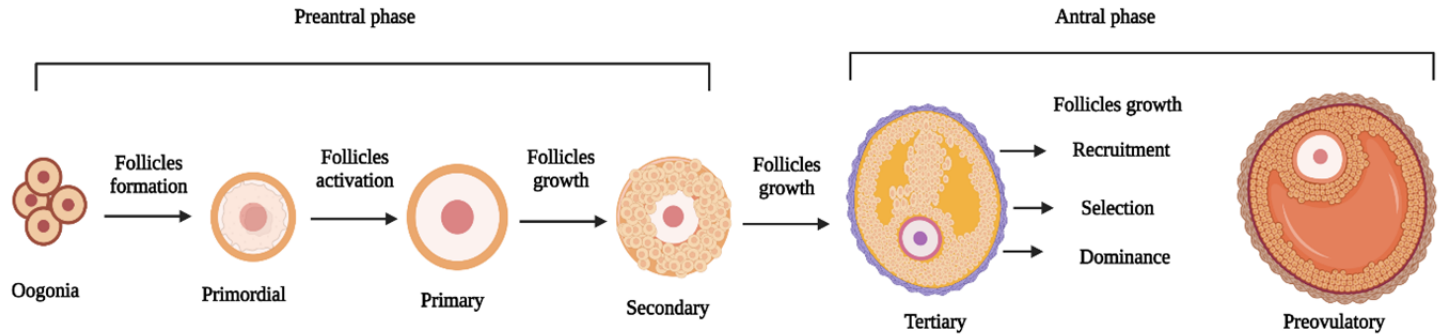

Figure 3. A schematic representation of complete follicular development: Preantral phase: formation and activation of primordial follicles and primary and secondary follicle growth. Formation of the tertiary follicle during the antral phase (antral-filled follicular fluid cavity). Follicle growth continues during the follicular waves' recruitment, selection, dominance, and preovulatory stages.

2.6. Ovarian follicular fluid

The Graffian FF is slightly viscous, straw-coloured, and has a similar pH (7.0) as plasma. FF is a complex dynamic biological fluid surrounding the developing oocyte, produced in the antral space and containing various molecules, including proteins, steroid hormones, polysaccharides, reactive oxygen species, and antioxidants. FF can be originated from the osmotic difference and the movement of fluids out of the vascular system into the tissues (Rodgers and Irving-Rodgers, 2010). These components in the FF play a crucial role in oocyte and follicle maturation, protection of follicle cells from oxidative and physical damage, ovarian physiology and steroidogenesis (Hoar, 1969). They also modulate the communication between somatic and germ cells necessary for oocyte maturation. FF was first seen as a secretion between the granulosa cell layer of growing follicles. Then, it was considered as an extracellular product of granulosa cells (Hubrecht-Laboratorium (Embryologisch Instituut), 1973). Thus, FF is an excellent tool for studying human and other animal reproduction and provides comprehensive information about the whole reproductive system. In addition, FF's dynamic nature and composition can be used as biomarkers for assisted reproductive technology.

FF is most likely produced by blood passing through the capillaries of the theca. Capillaries are uncommon in the ovarian cortex region comprising primordial follicles (Herrmann and Spanel-Borowski, 1998; van Wezel and Rodgers, 1996). They emerge as a simple network surrounding follicles during the early antral stages in most species (Jiang et al., 2002). The thecal capillaries are single-layered networks in species with small follicles; however, the network becomes much more complicated and multi-layered when the size of follicles increases (Jiang et al., 2002; Yamada et al., 1995). Interestingly when compared with lateral or basal regions, the thecal networks are not uniformed, with fewer capillaries at the apex of the follicles (Jiang et al., 2002), which leads to corresponding differences in regional blood flow (Brännström et al., 1998). As the follicle matures, the capillary networks develop and extend. Blood vasculature or blood flow is often not a limiting factor. These might, however, differ between dominant and subordinate follicles (Acosta, 2007; Berisha and Schams, 2005), differ among follicles with oocytes of different quality (Huey et al., 1999), and decline upon follicular atresia (Clark et al., 2004; Jiang et al., 2003).

The formation of FF begins with the vascularization of the theca. However, the amount of fluid accumulated during the growth of the follicles is minimal compared to the blood flow through the thecal capillaries. Therefore, the formation of FF is unlikely to depend on the degree of vascularization. However, alterations in thecal capillary blood pressure and flow could alter the fluid formation at critical times, such as ovulation.

The fluid transport from theca to the follicular antrum needs to cross the endothelium and subendothelial basal lamina (Figure 4). However, changes in the permeability of the thecal capillaries could result in oedema formation of the thecal tissue, which occurs due to LH surge (Cavender and Murdoch, 1988; Espey, 1980).

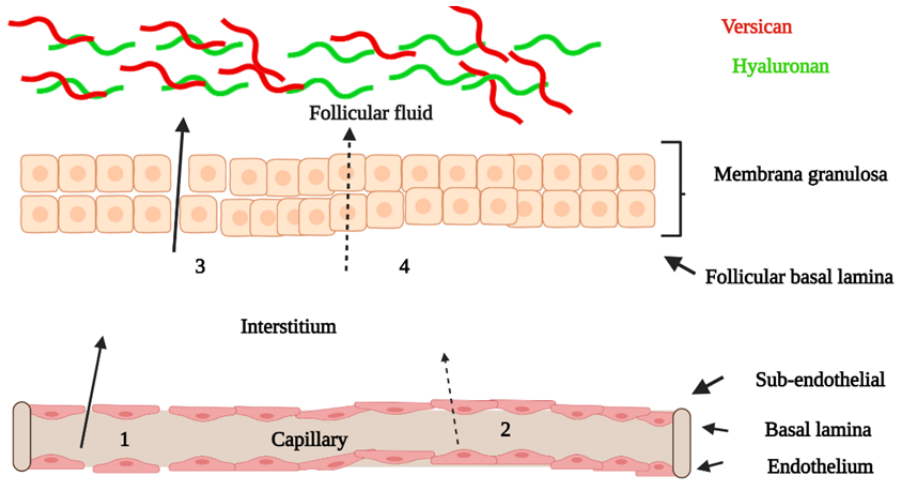

Figure 4. A schematic illustration of FF formation and the potential barriers. Subendothelial basal lamina, endothelium, interstitium, follicular basal lamina, and membrana granulosa are the main barriers where routes 1 and 3 show the fluid movement between cells and routes 2 and 4 shows the transcellular routes.

FF share similar composition to serum relating to lower -molecular-weight components. At sizes exceeding 100 kDa, the follicular basal lamina and thecal capillaries are likely the only places where the typical "blood-follicle barrier" is present (Zhou et al., 2007). It is likely that such a barrier also exists in the opposite direction. When big molecules produced by oocytes or granulosa cells cannot move through the membrana granulosa or the follicular basal lamina, an osmotic gradient may have formed. This osmotic gradient may draw fluid to the follicles' centre (Rodgers and Irving-Rodgers, 2010). Another study described the presence of large osmotically active molecules in ovarian FF (Clarke et al., 2006).

2.6.1. Role of FF in oocyte maturation and early embryo development

FF chemical composition, which includes hormones and non-hormonal components, plays an essential role in oogenesis, spermatozoa maturation, and early embryo development. The balance between these components is of utmost importance for a successful pregnancy and embryo development. Hormones like gonadotropins play an essential role in the growth and development of follicles. During IVF, a higher amount of FSH (Suchanek et al., 1988), human chorionic gonadotropin (hCG) (Ellsworth et al., 1984), and LH (Cha et al., 1986) have been reported to promote oocyte maturation which plays an essential role in enhancing the chances of fertilization.

Follicular growth is associated with a predominantly intrafollicular estrogenic environment. Estrogens (E2) are associated with the maturation of oocytes via a direct non-genomic action at the plasma membrane level (Tesarik and Mendoza, 1997) by inducing extracellular calcium influx into the cell with a specific pattern of $Ca^{2}+$ oscillations (Tesarik and Mendoza, 1997). Studies showed that higher estrogens and progesterone are associated with advanced oocyte maturation and lead to pregnancy (Botero-Ruiz et al., 1984; Lee et al., 1987; Subramanian et al., 1988; Tarlatzis et al., 1985; Teissier et al., 2000; Tesarik and Mendoza, 1997). However, a study has demonstrated that higher P concentrations can also result in multipronuclear embryos (Ben-Rafael et al., 1987). On the other hand, elevated FF androgen levels are associated with lower-quality of oocytes (Messinis and Templeton, 1987). Furthermore, data show that the lower concentration of estrogens and androgens is associated with early follicular atresia, negatively affecting fertilization and pregnancy (De Placido et al., 2005; Lisi et al., 2002).

Moreover, several studies reported that the presence of non-hormonal components in the FF, such as sodium (Na^{2+}) and potassium (K), are considerably high in human ovarian follicles (Shalgi et al., 1972). Various carbohydrates, such as seromucous and mucopolysaccharides, were found in the bovine FF (Nandedkar et al., 1992).

2.6.2. Importance of FF in regulating physiology

The dynamic structure of ovarian follicles supports oocyte maturation, ovulation, and steroid hormone synthesis. In addition, FF is involved in spermatozoa motility (Falcone et al., 1991; Getpook and Wirotkarun, 2007; Jeon et al., 2001), capacitation and acrosome reaction of spermatozoa (Bravo and Valdivia, 2018), embryo development (Revelli et al., 2009). However, studies also report that the presence of microorganisms in FF causes adverse pregnancy outcomes in IVF (Pelzer et al., 2013). In addition, hormonal imbalance of FF leads to subfertility diseases like polycystic ovarian syndrome (PCOS) (Teede et al., 2010) and endometriosis (Cordeiro et al., 2017; Regiani et al., 2015). Furthermore, the nature of the follicle's fluid-filled environment enables long-distance communication between different cell types via cell-secreted proteins (lipoproteins, riboprotein complex) and extracellular vesicles (EVs) (Fritz et al., 2016).

2.7. Extracellular vesicles

For decades, EVs have existed in various physiological environments without anybody knowing or understanding their particular roles. To be specific, EVs were observed in 1946 by Chargaff and West (Chargaff and West, 1946) as procoagulant platelet-derived particles in normal plasma and then in 1967, referred them as platelet dust by Wolf (Wolf, 1967). During the 1970–1980s, several significant independent observations were made, including plasma membrane vesicles released from rectal adenoma microvillus cells (De Broe et

al., 1975) and virus-like particles in human cell culture (Dalton, 1975). Similar particles detected in seminal plasma were termed prostasomes (Ronquist et al., 1978; Stegmayr and Ronquist, 1982). At the same time, tumour-originating membrane fragments were observed for the first time (Taylor et al., 1980). Moreover, during 1983–87, several detailed studies showed that these vesicles are also released by multivesicular bodies (MVBs) and fused with cell membrane of immature red blood cells (Harding et al., 1984; Johnstone et al., 1987; Pan and Johnstone, 1983). Later during 2006–2008, the discovery of EVs containing RNA, including mRNA, gained interest substantially as mediators of cell-cell communication (Ratajczak et al., 2006; Skog et al., 2008; Valadi et al., 2007). With advancements in knowledge and technology, EVs are now isolated and characterized from almost all body fluids and culture media.

EVs are membrane-bound nanoparticles of varying sizes. They are involved in intracellular communication (Konoshenko et al., 2018). The presence of EVs in biological fluids and their secretion from almost all types of cells have been reported by a diverse number of studies (Théry et al., 2018; Yáñez-Mó et al., 2015; Zaborowski et al., 2015). The main subtypes of EVs are apoptotic bodies, exosomes, and microvesicles (Zaborowski et al., 2015) which are different based on their mode of biogenesis, size, release pathways, content and functions (Borges et al., 2013; Yáñez-Mó et al., 2015; Zaborowski et al., 2015). The contents carried by EVs, consisting of lipids, nucleic acids, and proteins, always get the scientific community's attention to explore their roles in different research areas (Bebelman et al., 2018; Zaborowski et al., 2015). The EV subtypes exosomes and microvesicles have been identified in FF in several mammals, including humans, bovines, and equines (Diez-Fraile et al., 2014; Gabryś et al., 2022; Sohel et al., 2013a).

2.7.1. EV biogenesis

Nano-sized vesicle exosomes, typically sized between 40–100nm, are formed by a distinct mechanism of inward budding of multivesicular bodies (MVBs), as shown in (Figure 5A), which leads to the formation of intraluminal vesicles (ILVs). During early to late endosome maturation, plasma membrane releases enclosed vesicles called exosomes in the extracellular space upon fusion by the MVBs (Théry et al., 2018). On the other hand, microvesicle (100–1000nm) biogenesis differs from exosomes, as they are formed by direct buds that pinch out of the plasma membrane, as illustrated in (Figure 5A). Microvesicles are also described as "ectosomes" (Hess et al., 1999; Stein and Luzio, 1991). However, the overlapping size range of microvesicles with exosomes makes it difficult for their efficient separation. Apoptotic bodies (1–2 μm) are formed by dying cells through a number of processes, including nuclear chromatin condensation, membrane blebbing, and the disintegration of cellular content into distinct membrane-enclosed vesicles known as apoptotic bodies or apoptosomes. Apoptotic bodies are generally the largest compared to microvesicles and exosomes, as shown below (Figure 5A).

The nature of EV cell-cell interaction, including direct contact of ligand-receptor binding, has received much attention. The specific proteins and glycoproteins that are located on the surface of both the vesicle and the target cell may determine the kind of uptake process that specific populations of EVs employ (Mulcahy et al., 2014). Several EV uptake mechanisms have been reported (Figure 5B), including endocytosis and phagocytosis (Mulcahy et al., 2014).

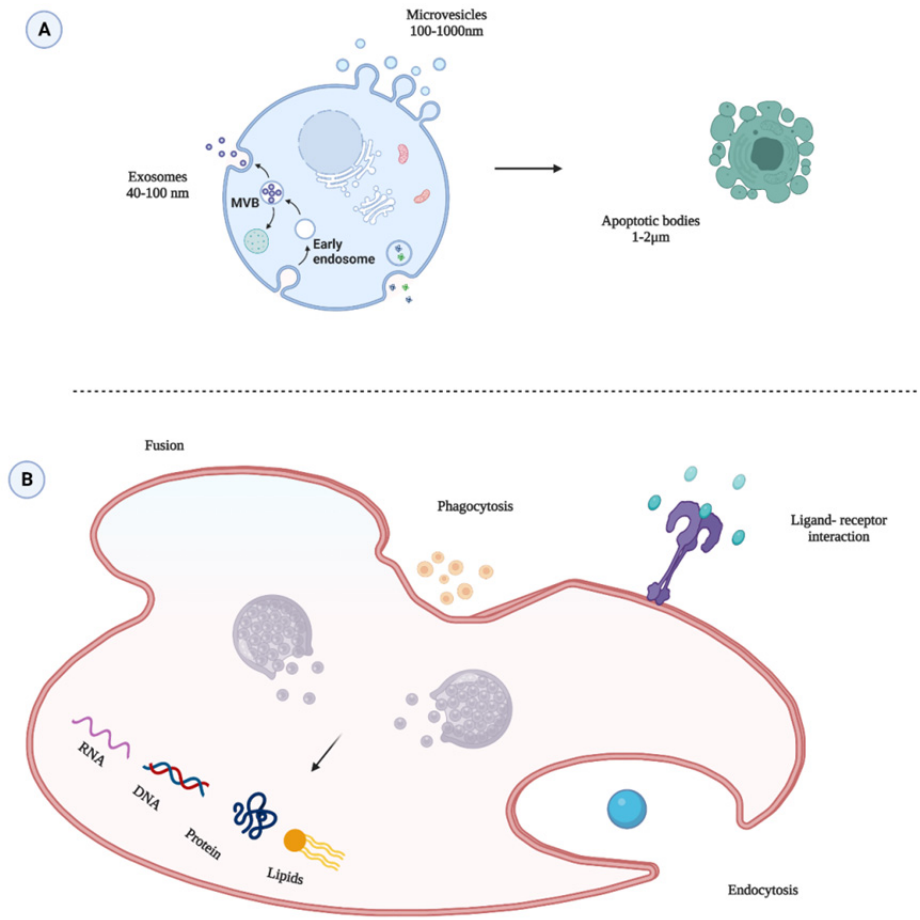

Figure 5. Schematic presentation of extracellular vesicle formation, releasing, uptake/ binding, and cargo delivery mechanism. (A) The biogenesis of microvesicles. Secreted by outward budding and fission of the plasma membrane. In contrast, late endosomes, which are created by the inward budding of the limited multivesicular body (MVB) membrane, constitutively produce exosomes. Apoptotic bodies are formed as blebs of cells that undergo apoptosis. (B) Different binding and uptake mechanisms of extracellular vesicles include fusion, endocytosis, phagocytosis, and ligand-receptor interaction.

2.7.2. EV cargo

The cargo and the uptake dynamics regulate the functional properties of EVs in intercellular communication. EVs contain various cargos, including proteins, lipids and nucleic acids.

2.7.2.1. Protein content of EVs

The protein composition and nature of EVs depend on the cell types and mode of biogenesis. Exosomes are more enriched with major histocompatibility complex (MHC) class II and tetraspanins CD63, CD37, CD53, CD81, and CD82 (Witwer et al., 2013; Wubbolts et al., 2003). However, tetraspanin proteins were initially assumed to be exosome-specific indicators, but they have been found in MVs and apoptotic bodies (Crescitelli et al., 2013; Tauro et al., 2013). On the other hand, microvesicles are enriched with post-translationally modified proteins (glycoproteins and phosphoproteins) (Doyle and Wang, 2019). In contrast, apoptotic bodies are enriched with glycosylated proteins (Borges et al., 2013; Kerr et al., 1972; Théry et al., 2001).

2.7.2.2. RNA content of EVs

RNAs can be enclosed in EVs, floated freely in the circulated form or bound in a protein complex. In 2006 the presence of functional RNA in EVs was described by Ratajczak in murine stem cell-derived EVs (Ratajczak et al., 2006). Later in 2007, the uptake capability of murine cell-derived EVs by human mast cells was described by Valadi (Valadi et al., 2007). The size of cellular RNA varies from 400–12000 nt, whereas extracellular vesicles derived mRNAs and ncRNAs, with a size of 200 nucleotides (O'Brien et al., 2020). The RNA species contained in EVs are quite diverse which include mRNA fragments (Batagov and Kurochkin, 2013), long non-coding RNA, piwi-interacting RNA, ribosomal RNA (Huang et al., 2013; Kogure et al., 2013), and fragments of vault, Y-RNA and tRNA (Bellingham et al., 2012; Nolte-'t Hoen et al., 2012). An overview of RNA species found in EV-RNA can be found in Table 2.

Table 2. Different RNA species are found in FF-derived EV.

Biological material	RNA species	Study
Bovine antral FF	small RNA (sRNA), miscellaneous RNA (miscRNA), ribosomal RNA (rRNA)	(Navakanitworakul et al., 2016)
Human FF	Piwi-Interacting RNA (PiRNA), transfer RNA(tRNA), microRNA (miRNA), Transfer RNA-derived small RNA (tsRNA)	(Hu et al., 2020)
Porcine FF	messenger RNA (mRNA), micro RNA(miRNA)	(Gad et al., 2022)
Human FF	micro RNA (miRNA)	(Martinez et al., 2018)
Equine FF	microRNA (miRNA)	(da Silveira et al., 2012, p. 1)

2.7.2.3. DNA content of EVs

Most EV research has focused mainly on the RNA or protein composition of EVs. In contrast, DNA in EVs (EV-DNA) has largely remained under-explored. The EVs have been reported to harbour single-stranded DNA (ssDNA) (Balaj et al., 2011), double-stranded DNA (dsDNA) (Thakur et al., 2014), mitochondrial DNA (mtDNA) (Guescini et al., 2009) and even viral DNA (Kahlert et al., 2014; Kouwaki et al., 2016). Studies have recently shown that specific DNAs are also packaged into EVs released by diverse cells, which are regarded as potent disease indicators for diagnosis and prognosis.

2.7.2.4. Lipid content of EVs

The lipid content of EVs has also been extensively researched in various contexts, in addition to the EV proteins and RNA (Carayon et al., 2011; Llorente et al., 2013). The lipid content generally shares characteristics with the cells of origin. However, research has revealed that particular lipids can be uniquely linked to various EV kinds. Sphingomyelin, cholesterol, ganglioside GM3, disaturated lipids, phosphatidylserine, and ceramide are lipids concentrated in EVs (Llorente et al., 2013). When compared to the composition of the cellular plasma membrane, both MVs and exosome membranes include more phosphatidylserine. The biogenesis of the various EV types, originating from the plasma membrane or the MVBs, is reflected in the variances in lipid composition between the various vesicles (Abels and Breakefield, 2016).

2.7.3. Isolation of EVs

The success of downstream applications is contingent upon the isolation of EV. Although EVs show immense potential as a platform for developing novel treatment methods and biomarkers, significant obstacles remain. Serum and plasma include EVs derived from platelets, neutrophils, and macrophages, the release of which is likely impacted by age, infection, and inflammation. Additionally, existing separation approaches depend heavily on changes in the size and density of vesicles and on markers such as CD63 (Chen et al., 2010), which may not be exclusive to a particular kind of EV. Understanding which EVs are extracted and how to target specific EV populations may help us reach desired diagnostic or therapeutic outcomes. As a result, EV separation and purification procedures must be carefully selected according to sample types and downstream uses. The most frequently used techniques for EV isolation are included in Table 3, adapted from (Chiriacò et al., 2018; Théry et al., 2018).

Table 3. Commonly used methods for EVs isolation and their limitation

Isolation method	Principle	Advantages	Limitations
Ultracentrifugation (most widely used)	Size	Large scale	Expensive equipment and maintenance, lengthy, low yield with low purity.
Differential Centrifugation	Size	Commonly used; standardized; vesicle enrichment and protect EVs integrity	Vesicle aggregation, protein and soluble factors contamination, time-consuming and low recovery
Tangential flow filtration	Size	Large scale, high yield and purity, rapid and good integrity	Protein contaminations
Size-exclusion Chromatography (SEC)	Size	User-friendly and high yield	Low scale, time-consuming and less purity
Sucrose density gradient Centrifugation	Density	Good standard, good purity	Time-consuming and expensive equipment, low yield
Immunoaffinity capture-based techniques		Sensitive, high purity, specificity, EV subtype isolation and purity	Low yield, expensive
Combination of SEC +Differential Centrifugation	Size	User-friendly, good yield, purity and EV subtypes isolation	Time-consuming
Polymer-based Precipitation	EV precipitation using polymers altering solubility	Easy, less expensive compared to other methods and high yield	Protein contamination not suitable for large scale
Micro, nano-fluidics chips	Based on biochemical properties using acoustic, electrophoretic, and electromagnetic	Fast, inexpensive	Low yield, not suitable for large scale

2.7.4. Clinical and biomarker application of EVs

EVs are compact nanoparticles carrying proteins and genetic materials that act as physiological and pathological information sources. Body fluid-derived EVs are vesicles originating from different sources, such as cells in body fluids or the cell lining space of the extruded body fluids. The unique nature of the lipid membrane of the EVs protects their cargo from the hostile environment, for example, from degrading enzymes present in body fluids. EVs exist in nearly all types of body fluids (Théry et al., 2018). However, different body fluid EVs represent different information, such as urinary EVs comprise potential biomarkers for internal signalling for host defence and urinary tract and kidney diseases (Fang et al., 2013; Kleinjan et al., 2012). EVs derived from saliva contain tissue factors (TF) and CD26. As TF involves blood coagulation and saliva EVs induced clotting in vesicle-free plasma, EVs could be crucial mediators in mammals with less wound healing ability (Berckmans et al., 2011). EVs found in the oviduct play an essential role in sperm capacitation and fertilization (Al-Dossary et al., 2013; de Almeida Monteiro Melo Ferraz et al., 2020; Griffiths et al., 2008). Moreover, EVs derived from follicular fluid enhanced embryo development, oocyte maturation and cumulus expansion (Hasan et al., 2020; Silveira et al., 2017; Sohel et al., 2013a). On the other hand, EVs derived from synovial fluid (joint cavities membrane secreted fluid, acts as a lubricant) have a significant role in autoimmune disease mechanisms, especially for rheumatoid arthritis (Mor-Vaknin et al., 2011; Skriner et al., 2006). EVs found in bile showed involvement in cholangiocytes' regulatory mechanisms (Masyuk et al., 2013, 2010). EVs are also found in breast milk (Lässer et al., 2011). Research showed that EVs from breast milk carry miRNAs and transfer them to the baby from mother and have an essential role in developing the infant's immune system (Kosaka et al., 2010; Melnik et al., 2013; Reinhardt et al., 2012; Zhou et al., 2012).

2.7.5. Importance of EVs in reproduction

Successful pregnancy depends on several factors, including the immunological communication between mother and fetus. Soluble mediators and EVs are involved in these communication pathways in systemic and feto-maternal communications (Arck and Hecher, 2013; Oreshkova et al., 2012). A study conducted in vitro has demonstrated the transfer of embryonic-derived transcripts to the endometrium in a non-contact model. The expression of endogenous transcripts is changed as a consequence of the endometrial cells uptaking the EV-RNA derived from the embryo (Es-Haghi et al., 2019). Another study has revealed that exosomes derived from the placenta suppress T-cell activation pathways, which is essential for pregnancy (Sabapatha et al., 2006).

On the other hand, EVs play an essential role in male reproduction (semen physiology), such as transferring molecules either to sperm cells or immune cells within the female reproductive tract. During the journey to the female

reproductive tract, sperms come in contact with several types of EVs, which promote their fertilization capability by modifying their molecular composition and behaviour (Aalberts et al., 2014; Bailey, 2010; Girouard et al., 2011; Mayorga et al., 2007; Palmerini et al., 2003; Schwarz et al., 2013). EVs in seminal plasma play an essential role in fertilization by protecting the sperm cells in the hostile environment in the female genital tract and by modulating their motility and maturation. In recent years, a large number of studies have indicated the functions of EVs in sperm motility, viability, capacitation and acrosome reaction(de Almeida Monteiro Melo Ferraz et al., 2020; Foot and Kumar, 2021; Franchi et al., 2020; Martinez et al., 2018; Tamessar et al., 2021).

2.8. Summary of the literature review

EVs are new emerging tools in the area of cell-cell communication (Raposo and Stahl, 2019; Stahl and Raposo, 2019), biomarker discovery (Mathew et al., 2020; Porcelli et al., 2021) and therapeutic drug delivery (Elsharkasy et al., 2020; Herrmann et al., 2021; Keener, 2020). Despite the FF being known as a rich source of EVs (Théry et al., 2018), FF-derived EVs role in the reproductive biological perspective has got less attention. The composition of FF is an excellent tool in several aspects for reproduction and biomarker development. The role of FF in oocyte development is well studied (Benkhalifa et al., 2015). At the same time, several studies report that progesterone present in FF is responsible for enhancing the vital parameters of spermatozoa (Callaway, 2011; Calogero et al., 2000). Moreover, it is known that FF, along with oviductal fluid, can regulate the periconception environment and create a favourable environment for early embryo development; however, the role of FF in gene expression changes in the oviduct remains elusive. In addition, the impact of FF EVs on functional attributes of spermatozoa, including viability, capacitation and acrosomal reaction, remains unexplored.

Furthermore, to my knowledge, the impact of FF EVs on spermatozoa function has not been studied. Therefore, understanding the FF EV mediated communication in the cellular level of the oviductal environment, gametes maturation, and fertilization can be crucial to annotate the mechanism of successful implantation, embryo development, reproductive disease and recurrent implantation failure.

3. AIMS OF THE STUDY

The overall aim was to investigate the potential role of FF and FF EVs in preparing the periconception microenvironment for the gametes maturation.

The specific aims of the study were as follows.

1. To isolate and characterise extracellular vesicles derived from the bovine follicular fluid. Analyzing the differential expression of genes in the primary bovine oviductal epithelial cells in response to follicular fluid and follicular fluid-derived extracellular vesicles supplementation. Similarly, we also analyze how the changes in gene expression affect the preconception microenvironment, fertilization and embryo development.
2. To investigate the effects of the supplementation of extracellular vesicles isolated from different sources (bovine follicular fluid, porcine follicular fluid, and human choriocarcinoma cell line (JAr) conditioned media EVs) on sperm viability, capacitation, and acrosome reaction.
3. To isolate and characterise extracellular vesicles derived from healthy and PCOS human follicular fluid and to comprehensively analyze their cargo (miRNA) difference to unravel the contributions of PCOS in the ovarian signal disturbance.

4. MATERIALS AND METHODS

4.1. Ethics statement

The bovine FF was obtained from the slaughterhouse-derived cattle ovaries, and bovine semen samples were acquired commercially. Therefore no animals were sacrificed or used for the studies. Bovine FF and semen were used for the first and second studies. In contrast, in the 3rd study, we used human FF. The study was authorised on January 21st, 2019, by the Research Ethics Committee at the University of Tartu in Estonia, with clearance number 289/M-8. All participants provided written informed consent.

4.2. Collection of follicular fluid and mural granulosa cells (study I and II, III)

We only used bovine FF (BFF) in studies I and II. BFF were isolated from the ovaries collected from the slaughterhouse. Ovaries were washed thrice in normal saline at RT. The FF was collected from large (>9 mm), medium (6–9 mm) and small (3–5 mm) follicles using a vacuum pump. Isolated FF was centrifuged at 300 g followed by 2000 g for 10 min to remove any cells and cell debris. Finally, the processed FF was stored at −80 °C for further experiments.

In study III, we used human FF (HFF) and mural granulosa cells (MGCs); therefore, HFF and MGCs samples were collected only from the women undergoing ovarian stimulation and oocyte pick-up by ovarian puncture (OPU). Samples were collected from two different groups of women: polycystic ovary syndrome (PCOS) and fertile women. All women in this study were <40 years of age. All cellular materials present in HFF were collected from the first aspirated follicle visibly clear of blood contamination. Next, the samples were centrifuged for 10 min at 300 xg and 2000 g, respectively, to remove large cells and cell debris. The supernatant was stored at -80 ^{0}C for down-streaming experiments. Cell pellets of MGCs obtained during centrifugation (300 xg) were then lysed with QIAzol lysis reagent (QIAGEN, Hilden, Germany). The lysed samples were then stored at −80 °C until RNA extraction.

4.3. Isolation of EVs

Isolation of EVs from BFF (study I and II)

Frozen BFF was thawed and then centrifuged again at 20000×g for 30 min to remove apoptotic bodies. The supernatant was then filtered through a 0.2 µm syringe filter. Before isolation of EVs, the samples were concentrated up to 500µl. Next, EVs were isolated using a size exclusion chromatography (SEC) benchtop column (in-house). A total of 20 fractions (500 µL each) was collected. To identify the purest and highest EVs contained fraction NTA

analyzed all the fractions. The protein concentration was determined with Bradford assay using Quick Start™ Bradford Protein Assay (Bio-Rad, Berkeley, CA, USA) following the manufacturer′s protocol. Based on our analysis, fractions (5–7) were polled together, concentrated, and used for further experiments. Additionally, fractions 10–13 (non-EV1) and 14–16 (non-EV2) were collected respectively for study 2.

JAr EVs purification (study II)
EV isolation was carried out in accordance with the procedure described by Es-haghi et al. 2019. (Es-Haghi et al., 2019). First, the conditioned medium was centrifuged at 400 g for 10 minutes to remove the cells. The supernatant was then centrifuged at 4,000×g for 10 minutes, followed by 15 minutes at 20000×g, to remove cell debris and apoptotic bodies. Conditioned media was concentrated up to 500 µL. EVs were purified utilising the same SEC column as previously reported. EVs-containing fractions 7–10 (fraction size 0.5 ml) were collected, pooled, and concentrated for the downstream application.

Isolation of EVs from HFF (study III)
HFF samples were preprocessed before EV isolation. The samples were centrifuged at 300 xg and 2000 xg for 10 min, respectively, to remove cell and cell debris. The sample volume was reduced to 150 µL with Amicon® Ultra centrifugal filter units (10 kDa, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Ireland). qEVsingle/70 nm (commercially available SEC) was used to isolate EVs using the standard protocol. The nanoparticles and protein concentrations were measured in the same manner as BFF. Based on the analysis, fractions 6–9, 200 µL each and a total of 800 µL, showed the highest number of particles and the slightest protein contamination. Therefore, these Fractions were pooled together, concentrated with 10 kDa Amicon® Ultra 2 centrifugal filter units, and used for downstream experiments.

4.4. Characterization of EVs

Nanoparticle tracking analysis (study I, II and III)
Nanoparticle tracking analysis (NTA) works based on the principle of Brownian motion to analyze the size and concentration of nanoparticles (NPs). It processes data of tracking particles, acquiring them by the average sizes, modal value and size distribution. The concentration and size profile of NPs were carried out using ZetaView® nanoparticle tracking analyzer following the manufacturer's standard protocol. All the EV samples were diluted in DBPS and measured under the following settings; sensitivity-85, shutter speed-70, frame rate-30fps. Samples were measured in triplicate. (Details can be found in all studies NTA sections).

Western Blot analysis (study I, II and III)

Western blot analysis was done to detect the EVs specific positive, purity control marker of all the EVs preparation. Proteins from the EV samples were extracted using chloroform-methanol extraction. For CD63 detection, 30 µg of protein was suspended in non-reducing Laemmli buffer, and 50 µg of protein was suspended in reducing Laemmli buffer (study I, II). In contrast, 30 µg (JAr, study II) and 10 µg of protein from each sample were used (study III). Each sample was incubated for five minutes at 95 °C. Following standard protocol, protein samples were separated by 12% SDS-PAGE gel electrophoresis and transferred onto polyvinylidene difluoride membrane. The proteins were blocked for one h at room temperature. In order to detect EVs markers, samples were then incubated with diluted (5% non-fat dry milk or 5% BSA in PBS-Tween 0.05%) primary antibody (CD63, ab68418, 1:500, Abcam, Cambridge, UK, ApoA-I, sc-376818, 1:1000, Santa Cruz Biotechnology Inc., Dallas, TX, USA) for bovine FF EVs. Similarly for JAr (study II), CD63 (556019, 1:1000, BD Biosciences, NJ, USA) , CD81 (555675, 1:500, BD Biosciences, NJ, USA) , CD9 (sc-59140, 1:250, Santa Cruz Biotechnology Inc., Dallas, TX, USA). Similarly, CD63 (556019, 1:1000, BD Biosciences, San Jose, CA, USA), CD81 (555675, 1:1000, BD Biosciences), CD9 (sc-59140, 1:250, Santa Cruz Biotechnology Inc., Dallas, TX, USA) albumin (16475-1-AP, 1:10 000, Proteintech, Chicago, IL, USA), GrP94 (ADI-SPA-851-D, 1:1000, Enzo Life Sciences, Farmingdale, NY, USA), albumin (16475-1-AP, 1:10 000, Proteintech, Chicago, IL, USA) apoA-I (sc-376818, 1:1000, Santa Cruz Biotechnology Inc. for human FF EVs, study III) overnight, followed by specific secondary antibody incubation for one h at RT. After that, ImageQuant™ RT ECL™ (GE Healthcare) was used to visualize protein bands. (Details can be found in the methods section of each study).

Transmission Electron Microscopy (study I, II and III)

Purified concentrated fixed EVs (mixed with 4% paraformaldehyde) samples were analyzed by transmission electron microscopy followed the protocol described by Thery et al. 2018 (Théry et al., 2018). In brief, EVs were deposited on Formvar-carbon-coated 200 mesh copper grids (Agar Scientific, Stansted, UK) by incubating for 20 min. Next, the grid was washed with double distilled MilliQ water. Afterwards, the grid was incubated for 5 min in a mixture of 0.15M oxalic acid mixed with uranyl oxalate and methylcellulose to contrast the grid samples. Finally, the samples (grids) were analyzed using a JEM1400 transmission electron microscopy at 80kV. The image was acquired with a Morada TEM CCD camera. (Details can be found in each methods section of each study).

4.5. Cell culture

Bovine Oviductal Epithelial Cells (BOECs) isolation and culture (study I)
Oviducts with attached ovaries (stage I) were collected and transported to the laboratory within 4 hours. Before isolating BOECs, the oviducts were washed three times with saline, trimmed, and cleaned the outer tissues. A sterile glass side was used to squeeze out oviductal tissue from the ampulla to isolated BOECs. The isolated BOECs were washed twice in washing buffer by gentle centrifugation at 50 g for 2 min. The washed BOECs were cultured in a humidified atmosphere at 38.8 °C with 5% CO_2. The media was changed after 72 hours post attachment of BOECs. The confluent cells were collected and stored at -80 ^{0}C using freezing media until further experiments.

Human choriocarcinoma cell line (JAr) cell culture (study II)
Human choriocarcinoma cell line (JAr) cells were cultured, following the protocol described by Es-Haghi (Es-Haghi et al., 2019). In brief, the JAr cell line was collected from ATCC® (HTB-144 ™, Teddington, UK). RPMI 1640 media (Gibco, Scotland) with 10% FBS, 1% L-glutamine, and 1% Penicillin/ Streptomycin were used to culture the cells. The cells were incubated at 5% CO_2 at 37°C. Cells were grown until they reached 80% confluency, and the conditioned media were collected for further processing.

4.6. Immunofluorescence Staining of BOECs (study I)

BOECs grown in coverslips were fixed in 4% paraformaldehyde and permeabilized using cold methanol for 10 min at RT. Next, the cells were incubated in a blocking solution (4% goat serum in PBS), followed by anti-Cytokeratin (C2562, 1:250, Sigma-Aldrich) and anti-Vimentin Vimentin (PLA0199, 1:250, Sigma-Aldrich, USA) for 1 hour at RT. Cells used for negative control were incubated only with blocking solution without primary antibody. Before incubating with Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594, goat anti-rabbit secondary antibody cells were washed three times by DBPS. Next, cells were incubated for 45 min at RT and nuclei were counterstained with Hoechst 33,342, sealed with a coverslip, and mounted with mounting media. Images were captured with a Leica DM5500 B microscope equipped with a Leica DFC310 camera.

4.7. Preparation of EV depleted medium (study II)

EV depleted FBS was prepared using the ultrafiltration method described by Kornilov *et al.* in 2018 (Kornilov et al., 2018). Briefly, the FBS was filtered using Amicon ultra-15 centrifugal filters (100 kDa, Merck Millipore, Darmstadt, Germany) at 3,000×g for 55 min. Therefore, the filtered FBS was used as

a 10% supplementation when preparing EV depleted complete culture media described above.

4.8. Spermatozoa processing and analysis (study II)

Washing of spermatozoa (study II)
Bovine frozen semen straws were processed following the protocol described previously in our lab by Reshi et al. (Reshi et al., 2020). In brief, frozen semen straws were thawed at 37^0 C for 30 sec. The entire content was layered over 4 ml of 60% iso-osmotic Percoll® solution (GE Healthcare, 17–0891-02, Sweden) and centrifuged for 20 min at room temperature (RT), followed by washing the spermatozoa pellet with EV-depleted sperm-TALP media (In-house) (Reshi et al., 2020) for 5 min at 400 g at RT. Washed spermatozoa pellet were resuspended in prewarmed sperm-TALP media, then determined spermatozoa concentration and down streaming application.

Evaluation of spermatozoa viability (study II)
LIVE/DEAD ® Viability/Cytotoxicity Kit (MP 03224, ThermoFisher Scientific Inc., Santa Clara, CA, USA) were used to determine spermatozoa's viability by following the manufacturer's protocol. In brief, working solutions were prepared using EthD-1(4 µM) and calcein (2 µM) at a final concentration. Next, 25 µL of sperm solution were added to the working solution, appropriately mixed, followed by 25 min incubation at RT. Afterwards, a sample smear was prepared on a microscope glass slide, covered with a coverslip and sealed. Finally, the viability of spermatozoa was examined under a fluorescent microscope where green fluorescent displayed spermatozoa were counted as live and red fluorescent displayed spermatozoa were counted as dead spermatozoa.

Appraisal of sperm capacitation (study II)
The sperm capacitation process was evaluated using Chlortetracycline (CTC) following the method described by Fraser and McDermott, with slight modifications (Fraser and McDermott, 1992). A 100 µL of spermatozoa suspension were mixed with 100 µl of CTC working solution in a small 1.5 ml tube. 10 µl of CTC-spermatozoa solution were used to prepare the microscopic slides. The slides were covered with coverslips and examined for capacitation at 400x magnification using a fluorescent microscope. (The details categories of spermatozoa evaluation can be found in the study 2 method section).

Assessment of acrosomal reaction (study II)
The protocol described by Kitiyanant et al. was used to analyze the acrosomal status of spermatozoa (Kitiyanant et al., 2002). In brief, FITC-PNA labelled bull spermatozoa were categorized into four categories based on the status of the observed acrosomal reaction. The acrosomal pattern was assessed using a

fluorescence microscope at 400 x magnification. (Details can be found under methods sections of study II).

4.9. Modification of EVs surface (study II)

Trypsin-EDTA (ThermoFisher Scientific Inc., Santa Clara, CA, USA) was used to cleave EVs surface protein by following the protocol described by Skliar et al. 2017 (Skliar et al., 2017). In brief, 5 µL of 0.25 % trypsin-EDTA was mixed with 30 µL of EV suspension. The trypsin-EDTA and EVs solutions were then incubated for 20 min at 37^0C. After the incubation period, 5µL of growth media with EV depleted FBS was added to inactive the trypsin. NTA analyzed the size profile and EV concentration before and after the treatment.

4.10. Detection of progesterone concentration of FF and EVs (study II)

Chemiluminescence immunoassay-based progesterone concentration measurements in the FF and FF EV samples were conducted at the SYNLAB Eesti OÜ, Tartu, Estonia using ADVIA Centaur XP immunoassay system (Siemens Healthineers, Germany). The detection range of the progesterone assay was 0.21–60 ng/ml (0.67–190.8 nmol/l).

4.11. Supplementation of EVs

Bovine Oviductal Epithelial Cells Culture and supplementation (study I)
BOECs were in twelve well plates cultured until they reached 80% confluency. The confluent cells were treated with FF (200µL/mL) or FF-EVs (3.0×10^6/µL), whereas only BOECs served as control. The cells were collected 6 or 24h after treatment for further experiment.

EVs Supplementation in sperm culture (study II)
The effects of FF EVs and non-EV fractions on spermatozoa functions (viability, capacitation, and acrosomal reaction) (study II)
Spermatozoa were incubated with either EVs or non-EVs fractions and compared to the control group for 4 hours. We included two non-EV fractions in our experiment to confirm that only EV can enhance spermatozoa viability, capacitation and acrosome reaction. The supplementation effect was analyzed at 0 and 4 hours based on the following outcomes; viability, capacitation and acrosome reaction. More details can be found in the experimental design section of study II.

Analyzing the impacts and specificity of FF EVs on sperm viability, capacitation, and spermatozoa acrosomal reaction (study II)
Spermatozoa were incubated with different EV sources for 4 hours to analyze the specificity of EVs on sperm functions. More details can be found in the experimental design section of study II.

Investigating the impact of FF EV surface modification on spermatozoa viability, capacitation, and acrosomal reaction (study II)
Spermatozoa were incubated with surface-modified EVs to analyse the surface-modified EVs effects on sperm viability, capacitation, and acrosomal reaction. More details can be found in the experimental design section of study II.

4.12. RNA Isolation, Library Preparation, sequencing and statistical analysis (Study I, III)

RNA Isolation (Study I)
Total RNA from BOECs were extracted with the TRIzol® reagent (TRIzol® reagent; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) following the manufacturer′s protocol. In addition, Qubit™ RNA HS Assay Kit (Q32852, Thermo Fisher Scientific) and Bioanalyzer were used to analyze the quantity and quality of extracted RNA.

Library Preparation for RNA-Seq (Study I)
The protocol described by Picelli with slight modifications was used for RNA seq libraries generation with Smart-seq2 (Picelli et al., 2014). In brief, twenty ng of total RNA was used for cDNA synthesis and ten cycles of PCR for pre-amplification. Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used instead of KAPA HiFi DNA polymerase in the original protocol described by Picelli. Diluted cDNA (2 µL) was used for dual index library preparation with Illumina Nextera XT DNA sample preparation Kit. We used AMPure XP beads and selected a 200–700 bp size. All samples with the same concentration were pooled into a single library and sequenced by Illumina NextSeq500 using High Output Flow Cell v2.5 (single-end, 75 bp read length). In addition, details about RNA-Seq Read Processing, Alignment, and Quantification and analysis of Differential Gene Expression can be found in the study I methods sections.

Extraction of RNA (study III)
miRNeasy Micro kit (QIAGEN) was used for miRNA extraction from isolated EVs by following the manufacturer manual except for adding 5µg glycogen per sample (Thermo Scientific) to chloroform. miRNeasy Mini kit (QIAGEN) was used for total RNA extraction from cells. In addition, a small fraction of RNA (≤200 nucleotides) was separated by the RNeasy Mini Elute Cleanup Kit (QIAGEN). We followed the standard manufacturer protocols for both total and small RNA extraction. The quality and quantity of cellular RNA samples were

evaluated on Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Small RNA Library Preparation and Sequencing (study III)
QIAseq miRNA Library Kit (QIAGEN) were used to prepare the small RNA libraries following the manufacturer's protocol. In brief, 10 ng from cellular small RNA fraction and 5 µL of RNA from EV and FF samples were initially used for the library preparation. The final libraries were separated and excised from 5% TBE gels (Bio-Rad Laboratories) after staining with 1X SYBR Gold stain (Thermo Fisher Scientific) and resuspended in 7 µL of resuspension buffer (PerkinElmer, Massachusetts, USA). Library size was measured with Agilent DNA High Sensitivity chips on the Agilent 2100 Bioanalyzer system (Agilent Technologies). Library concentrations were measured using the Qubit High Sensitivity Assay kit (Thermo Fisher Scientific) before pooling equimolar amounts. Single-end sequencing of 75 bp length was performed on NextSeq 500 platform with NextSeq 500/550 High Output Kit v2.5 (Illumina, San Diego, CA, USA).

cDNA Synthesis and RT-qPCR (study III)
Selected miRNA expression levels were validated with RT-qPCR. cDNA was prepared from small cellular RNA (30 ng) and FF and EVs small RNA (5 µL). The RT-qPCR analysis was carried out on Light Cycler 480 instrument (Roche, Basel, Switzerland). To detect miRNA expression miRCURY, LNA SYBR Green (QIAGEN) was used according to the manufacturer's protocol. The specificity of amplified PCR products was determined by melt curve analysis. miRCURY LNA miRNA PCR Assay primers were used in all reactions (QIAGEN).

4.12.1. Statistical analysis (study I, II, III)

Data analysis (study II)
The data from viability assessments, capacitation, and acrosome reaction were analysed using linear mixed models to determine the statistical significance of the observed differences between supplementation groups. The graphs and statistical significance test were done with the ggplot2 (Wickham, 2009) package in R and GraphPad prism 8.4.2. More details can be found in the methods section of study II.

miRNA Sequencing Analysis (study III)
Raw FASTQ files were filtered with Trimmomatic v 0.39 (Bolger et al., 2014) with the options of SLIDINGWINDOW:2:20. Adapter sequences (3'adapter AACTGTAGGCACCATCAAT and 5′ adapter GTTCAGAGTTCTACAGTCC GACGATC) were removed. Reads below 17 nucleotides in length were discarded. The remaining filtered and trimmed reads were counted and mapped

to the primary assembly of the human genome GRCh38 using miRDeep2 with standard settings (Friedländer et al., 2012).

Read counts from individual samples were merged using edgeR package v.3.28.1 (Robinson et al., 2010). A formed count matrix was used as input for DESeq2 v.1.26.0 (Love et al., 2014) in R version 3.6.3 for differential gene expression analysis between groups with standard options. miRNAs expressed at a low level were removed from the analysis: cut-off was set at ≥5 raw reads in 50% of samples. For visualization purposes, variance stabilizing data transformation was performed with option blind = FALSE.

The statistical significance cut-off for differentially expressed miRNAs in DESeq2 analysis was set at a false discovery rate (FDR) <0.05 when comparing three tissue types in oocyte donor samples. In addition, the cut-off for statistical significance was set at FDR <0.1 when comparing patient groups.

RT-qPCR Data Analysis (study III)

miRNA expression levels in cellular fractions were normalized for U6 snRNA and hsa-miR-132-3p. Endogenous control for FF and EV samples was hsa-miR-16-5p. All normalizations were performed according to the ΔΔCt method of relative quantification (Livak and Schmittgen, 2001). Two-tailed Student's t-test calculated statistical significance, and the significance level was set at $p < 0.05$.

miRNA Target Prediction, Gene Ontology and Over-Representation Analysis (study III)

miRDB custom prediction tool was used to find the novel miRNA(Chen and Wang, 2020). Obtained miRNA targets list was an input for gene enrichment analysis with g: Profiler (Raudvere et al., 2019), using g: GOst functional profiling tool where significance threshold was set at FDR <0.05. Results were visualized with REVIGO (Supek et al., 2011). Details can be found in study III methods sections.

Novel miRNA candidate filtering (study III)

Predicted novel miRNAs were filtered with a cut-off of miRDeep2 score >1. Remaining potential novel miRNA candidates were aligned against human transcriptome with NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), discarded if the sequences overlapped with a coding region of an annotated gene, demonstrated high similarity to other know miRNAs, were detected only in one sample or with the average occurrence in positive samples <10 raw counts. miRNA sequences with similar seed region to the potential novel miRNA were obtained from miRBase v22.1 and visualized in Jalview 2.11.1.0 (Waterhouse et al., 2009).

EV Size Profile and Concentration (study III)

To test if the sample means of the EV size profiles are non-normally distributed, 1000 samples of 1000 EVs were drawn from the NTA data of PCOS and

donors' group, their means calculated and tested using Shapiro–Wilk test. The student's t-test was then used to test if the difference between the size profile means is statistically significant. The behaviour of p-values with a smaller sample size was further analyzed by drawing 1000 random samples of size 100, 1000, 2000, and 5000 EVs each and plotting the p-value histograms. The difference in EV concentrations was tested using a two-tailed Student's t-test, and a p-value <0.05 was considered statistically significant.

5. RESULTS

5.1. Benchmarking and characterization of EVs derived from bovine and human follicular fluid (Study I, II, III)

The Minimal Information for Studies of Extracellular Vesicles (MISEV) (Théry et al., 2018) is a guideline set by the International Society for Extracellular Vesicles (ISEV). According to the guidelines, all Isolated nanoparticles should be characterised by at least 3 different methods (NTA, WB, TEM) before being labelled as extracellular vesicles. Our studies used different species of follicular fluid, cell culture medium to isolate EVs. All our isolated vesicles were characterized using NTA, WB and TEM. The details about the isolation and characterization of EVs are discussed in the following sections.

5.1.1. Isolation of extracellular vesicles from bovine and human follicular fluid using size exclusion chromatography column (SEC)

We used bovine FF to isolate EVs, which was used in our first and second studies. In contrast, we used human FF in our third study. In order to obtain the purest and high number of nanoparticles from the size-exclusion chromatography (SEC) fractions, a total of 20 fractions were collected, and particle concentration was measured using NTA. The protein concentration was also measured for all the fractions to identify the highest number of EVs with the most negligible protein contamination. Our analysis showed that fractions 5–7 (bovine) and fractions (6-9, human) were highly enriched in EVs (Figure 6A bovine, 6B human), with low or no protein contamination.

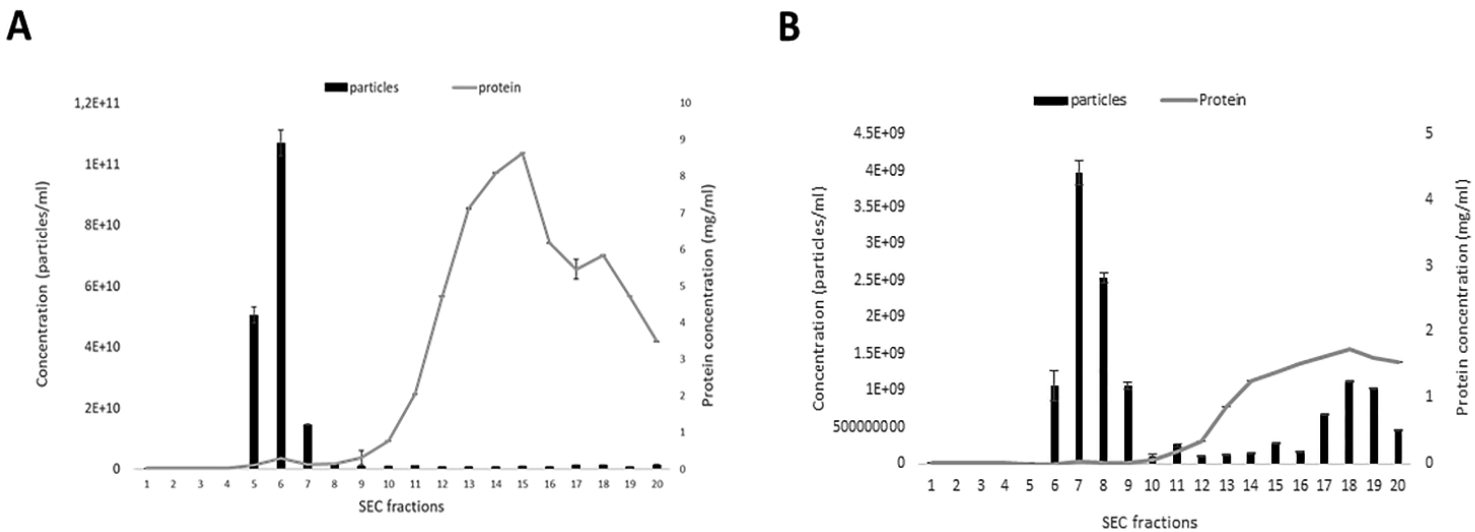

Figure 6. Benchmarking of elution profile of manually packed (in-house) and commercial (qEV single) size-exclusion chromatography (SEC). (A) Elution profile of manually packed SEC column showed that fraction 5–7 (each 500µL) suggestive of EVs with no or more negligible proteins contamination for bovine FF (Hasan et al., 2020). (B) Elution profile of commercial qEV single SEC column showed that fraction 6–9 suggestive EV fraction for human FF with less or no protein contamination. The elution volume of each fraction is 200 µl (Rooda et al., 2020).

5.1.2. Physical and biochemical characterization of extracellular vesicles

EVs isolated from bovine FF, human FF, and JAr were characterized by most standard methods (NTA, western blot (WB), transmission electron microscopy, TEM). In order to determine the size and concentrations of EVs, NTA was performed. NTA results revealed that the highest EV number across the samples is between 50–250 nm. Moreover, all the particles were under 300 nm in all samples. These are EVs size ranges as mentioned in the literature (Figure 7A bovine, JAr, and 7B human) (Raposo and Stoorvogel, 2013; Stoorvogel et al., 2002; Théry et al., 2018). WB and TEM analysis also confirms the EVs characteristic profile. WB confirms the presence of EVs positive marker CD63 and the absence of apoA-I, which was used as a negative marker in bovine FF-derived EV (Figure 7C).

Similarly, WB analysis of human FF from donor and PCOS samples showed the presence of several EV markers (CD63, CD9, CD81, Figure 7D) enriched in EVs samples. In contrast, (Grp94, apoA-1 and albumin) were absent in EVs samples while enriched in non-purified FF samples (Figure 7D). Later SEC fraction (10–14) indicates EVs' efficient separation and purity (Figure 7D). Similarly, CD63, CD81 and CD9 were enriched with purified JAr- EV while absent in JAr conditioned media (Figure 7E).TEM analysis also confirmed that the morphology of the isolated particles resembled the typical shape of EVs in the studied samples (bovine, Figure 7F, human 7G and JAr 7H).

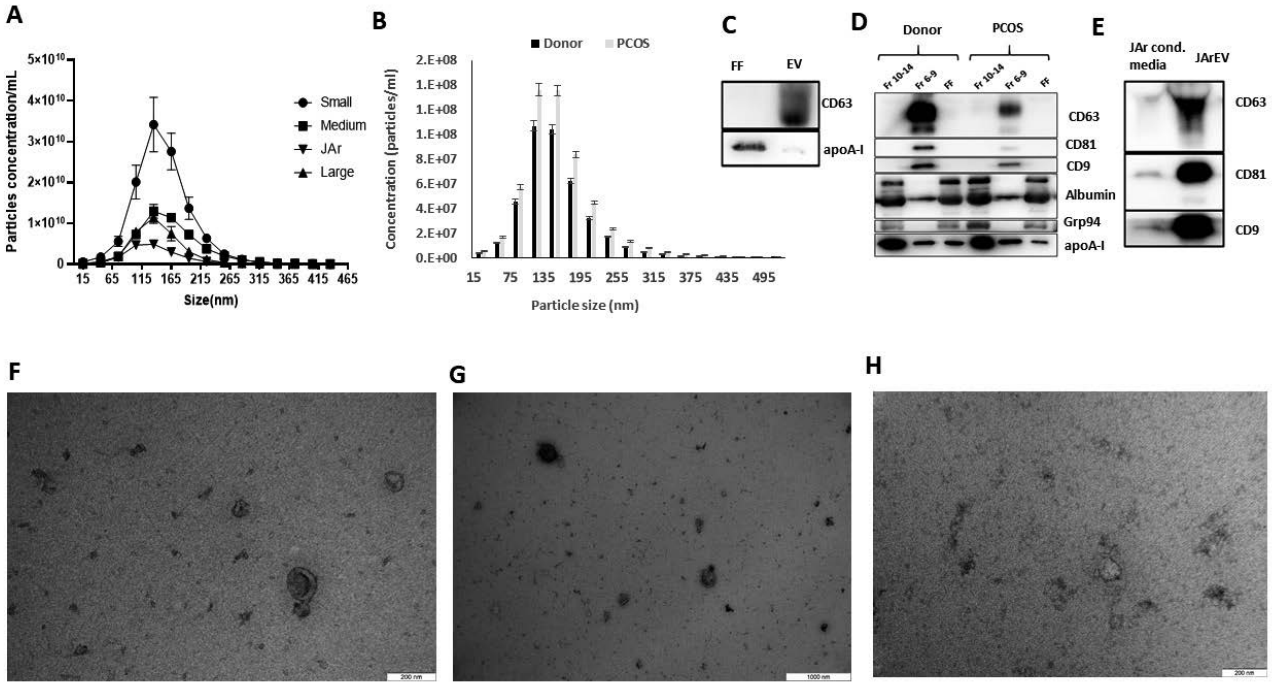

Figure 7. Biochemical and physical characterisation of EVs derived from bovine FF, human FF, and JAr conditioned media. (A) NTA analysis showed the particle size and concentration of bovine FF and JAr EVs. All the EV samples share a similar size; however, EVs derived from small bovine follicles showed the highest concentration compared with the medium, large follicles and JAr EVs. (B) Size profile of EVs isolated from PCOS and donor human FF samples. No difference was observed in size; however, the total EVs concentration in PCOS was significantly higher than in the healthy samples (mean ±SEM). (C, D, E) WB analysis of bovine, human and JAr EVs, respectively. (F, G, H) TEM analysis showed cup-shaped EVs in bovine, human, and JAr samples. The figure was adapted from (Hasan et al., 2021, 2020; Rooda et al., 2020).

5.2. Study 1 (Bovine Follicular Fluid and Extracellular Vesicles Derived from Follicular Fluid Alter the Bovine Oviductal Epithelial Cells Transcriptome)

Study design

The main objective of this study was to analyze the effect of FF and FF-derived EVs on the gene expression changes in bovine primary oviductal epithelial cells (BOECs). The BOECs were cultured in the following treatment groups, (a) BOECs+ FF, (b) BOECs+ FF EVs (c) Only BOECs as a control (Figure 8).

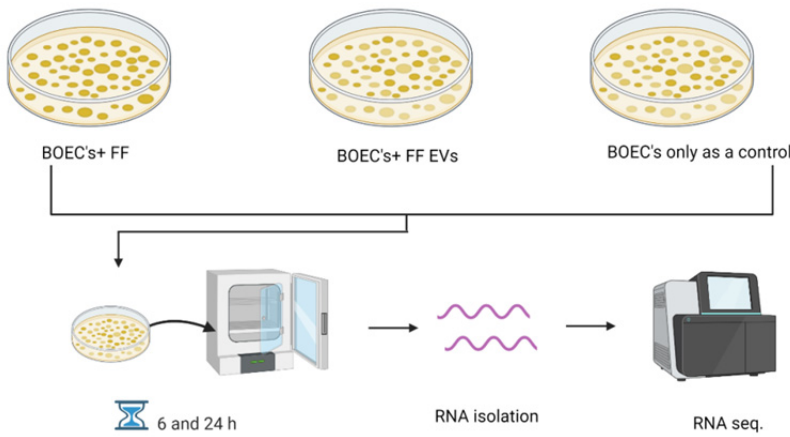

Figure 8. A schematic diagram of BOECs cocultured with FF and FF-EVs. After the incubation period (6 and 24h), RNA isolation of BOECs were done to study the gene expression changes.

5.2.1. Characterization of BOECs using immunofluorescence staining

Cytokeratins are primarily abundant in epithelial cells, while vimentin is only found in fibroblasts. We exploited this property of cells using antibodies against cytokeratin and vimentin to check the purity of our cells for the presence of epithelial or fibroblast. A strong positive signal was observed with an anti-cytokeratin antibody. In contrast, no signal was observed when cells were incubated with an anti-vimentin antibody, which confirmed the presence of epithelial cells only in BOEC culture (Figure 9).

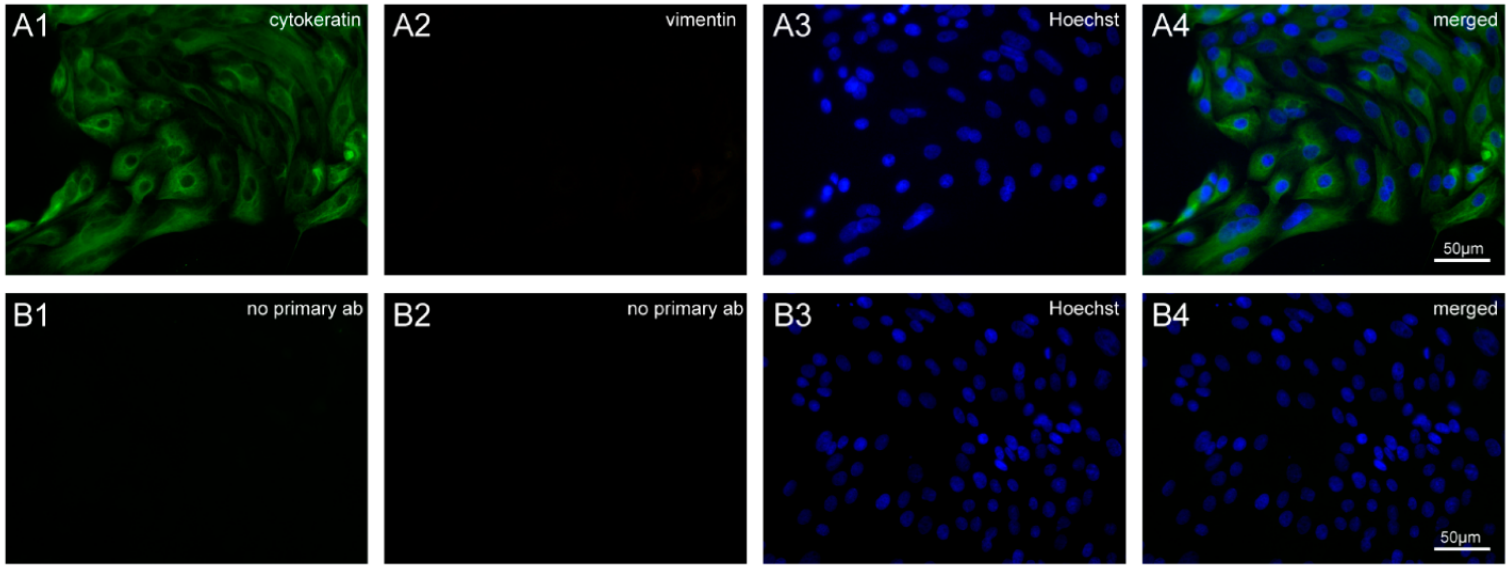

Figure 9. Characterization of BOEC by immunofluorescence staining (A1) Cells confirms the presence of cytokeratin (A2). Cells do not contain vimentin (A3) Nuclei staining with Hoechst (blue) (A4) Overlay (B1, B2) Negative control (absence of primary antibodies) (B3) Hoechst staining and (B4) Control cells overlay (Hasan et al., 2020)

5.2.2. Analysis of Differential Gene Expression

A library size of 4.9 ± 1.4 million reads (mean ± SD) per sample were yielded from the mRNA sequencing. A 99.0 ± 0.1% of the reads remained after the quality control step, which was then aligned to the B. Taurus genome assembly (ARS-UCD1.2), out of which 94.4 ± 1.9% aligned to the genome at least once. At the gene level, all read counts were summed up. After removing the genes not expressed in any experimental groups, our analysis yielded 9678 genes considered for differential expression testing. Principal component analysis (PCA) of standardized (z-score) counts per million (CPM) analysis showed that differential gene expression profile was more variable in FF-EV and control samples. At the same time, a uniform separation was observed with FF treated BOECs sample (Figure 10A). We observed more substantial changes in gene expression in FF supplemented groups in 24 hours (1022 upregulated and 976 downregulated genes) while a modest change in 6 hours FF supplemented groups (184 upregulated and 304 downregulated genes (Figure 10B).

Surprisingly, the impact of FF-derived EVs was not statistically significant compared with FF and control groups. At the same time, only 41 genes were upregulated (Figure 10C), with no genes downregulated and no differentially expressed genes discovered after 24 hours. DIDO1 and LOC507443 were two of the genes that were elevated 6 hours after EV intake and were upregulated 6 hours after FF supplementation (Figure 10B).

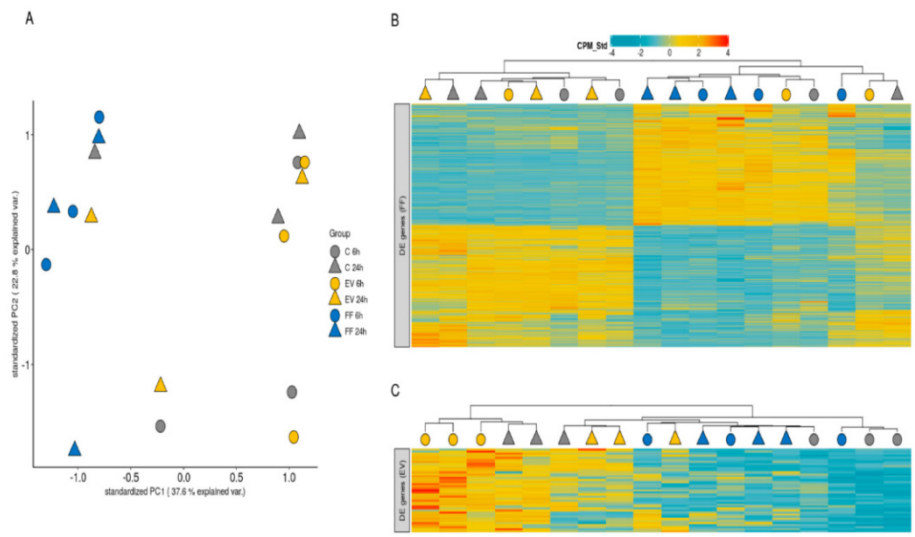

Figure 10. BOECs supplemented with FF, FF EVs, and control groups' gene expression profiles. (A) Principal Component Analysis (PCA) of all the expressed genes of BOECs in different conditions; control, supplemented with FF and supplemented with FF EVs in 6 and 24 h. (B) Heatmap analysis of differentially expressed genes in 6 or 24h of BOECs with FF treatment. (C) Heatmap analysis of differentially expressed genes in 6 or 24h of BOECs with FF-derived EVs (Hasan et al., 2020).

We detected 96 upregulated and 179 down-regulated genes in response to FF supplementation at both time points (Figure 11A). In addition, *NTS* (statistically significantly upregulated) and *CYP1A1*(statistically significantly downregulated) among other overlapping genes. Moreover, *ACKR3* was expressed in 6 h with FF and EVs treatment (Figure 11B). However, the expression differed between the groups where it was downregulated in FF and upregulated in EVs treatment.

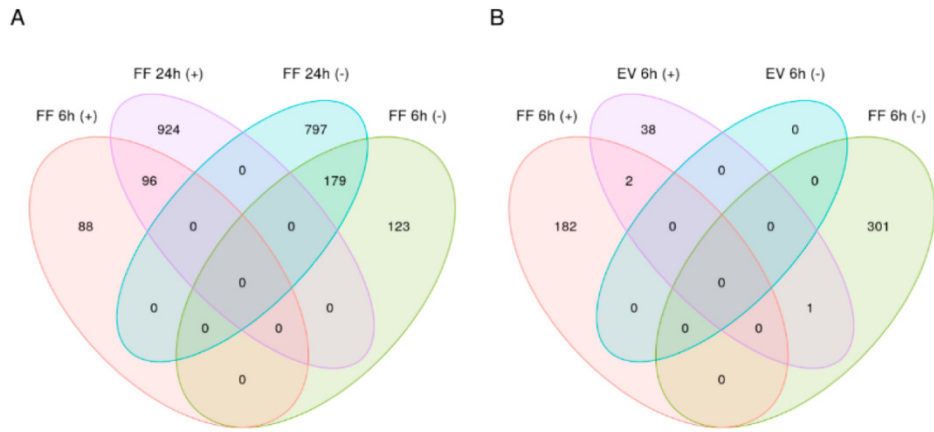

Figure 11. Venn diagram analysis of commonly found differentially expressed genes (DEGs) either in 6 or 24h with both treatment groups. (A) (DEGs) common in both time points with FF treatment. (B (DEGs) are common in both FF and EVs groups in 6 h treatment (Hasan et al., 2020).

5.2.3. Pathway Enrichment Analysis

Based on KEGG pathway annotations for B. *taurus*, Gene Set Enrichment Analysis (GSEA) of the differential expression testing data was carried out. GSEA produced two statistically significant (FDR ≤ 0.05) pathways for ribosome (bta03010, normalised enrichment score (NES) = 1.86 and FDR ≤ 0.039) and ribosome biogenesis (bta03008, normalised enrichment score (NES) = 1.93 and FDR ≤0.039) gene expression alterations at 6 hours following FF supplementation. Arachidonic acid metabolism (bta00590, normalised enrichment score (NES) = 1.82 and FDR ≤ 0.097) and ovarian steroidogenesis (bta04913, NES = 1.79 and FDR ≤ 0.097), both of which were enriched with downregulated genes in FF-supplemented BOEC samples (Data not shown). GSEA identified 50 statistically significant (FDR 0.05) pathways based on gene expression changes 24 hours after FF supplementation (Data not shown). Notably, oxidative phosphorylation (bta00190, normalised enrichment score (NES) = 1.78, FDR = 0.010) and thermogenesis (bta04714, normalised enrichment score (NES) = 1.90, FDR = 0.009) were enriched with upregulated genes, whereas the ras signalling pathway was enriched with downregulated genes (bta04014, normalised enrichment score (NES) =1.83, FDR = 0.009).

At 6 hours after EV administration, we found no statistically significant pathways. Among other non-statistically significant pathways, the tight junction pathway (bta04530) was one of the most enriched after 6 hours of EV supplementation and was enriched with upregulated genes. GSEA, on the other hand, found six enhanced pathways in both time points with EVs administration. In contrast, oxidative phosphorylation (bta00190) was enriched with down-regulated genes at 6 h and depleted with upregulated genes at 24.

Following FF supplementation, pathway over-representation analysis was carried out separately on significantly up-regulated and significantly down-regulated overlapping genes based on KEGG pathway annotations for B. *Taurus* in order to find pathways that were over-represented among genes that were differentially expressed at both time points. Eight pathways were significantly over-represented among overlapping down-regulated genes (Table 4), including ovarian steroidogenesis (bta04913, FDR = 0.026), steroid hormone biosynthesis (bta00140, FDR = 0.026), and arachidonic acid metabolism (bta00590, FDR = 0.012). No significantly over-represented pathways were found among overlapping up-regulated genes.

Table 4. KEGG pathway enrichment analysis results.

Pathway ID	Pathway name	DE genes	FDR
bta00590	Arachidonic acid metabolism	5	0.012
bta00140	Steroid hormone biosynthesis	4	0.026
bta04913	Ovarian steroidogenesis	4	0.026
bta04137	Mitophagy – animal	6	0.026
bta04145	Phagosome	8	0.026
bta04140	Autophagy – animal	8	0.033

The analysis included significantly downregulated genes (FDR ≤ 0.05) in both the 6 h and 24 h FF supplementation groups. The number of differentially expressed genes that belong to the appropriate pathway is shown in the column "differentially expressed (DE Genes)." Organ- or disease-specific pathways irrelevant to the research system were eliminated. *(Hasan et al., 2020).

5.3. Study II (Bovine Follicular Fluid Extracellular vesicles modulate the viability, capacitation and acrosome reaction of bull spermatozoa)

5.3.1. Outcomes of FF EVs and non-EVs fractions of SEC on spermatozoa viability

The viability of spermatozoa was analyzed at 0 and 4 hours with EVs and without EVs supplementation. Our analysis showed that the percentage of live spermatozoa were significantly low across all groups, from 55.05±4.91% (mean±SD) at 0 h to about 17.98±0.86% after 4 hours without EVs supplementation. The average viability of spermatozoa was significantly higher in the EVs supplemented groups (33.35±2.17, $p \leq 0.001$) than in Non-EV1, Non-EV2 and control groups (Figure 12A). Our results suggest that FF EVs support the viability of spermatozoa.

5.3.1.1. Influences of FF EVs and non-EV SEC fractions on capacitation and acrosome reaction

Spermatozoa were categorized into three groups (uncapacitated, capacitated, and acrosome reacted) based on fluorescence patterns to analyse the different stages of the capacitation process. Most spermatozoa were non-capacitated across all the groups (95.38±1.55%, data not shown) at 0 h. After four hours of incubation, most of the spermatozoa were non-viable and uncapacitated except for the EVs supplementation groups (Figure 12B). Our results showed a significantly higher ($p \leq 0.001$) number of spermatozoa were undergone capacitation reaction with EVs supplementation (19.68±4.00%) compared to the non-EV1 (SEC fraction 10–13), non-EV2 (SEC fraction 14–16), and control groups. We observed the same effects in the case of acrosome-reacted spermatozoa with EVs supplementation. The percentage of acrosome-reacted spermatozoa was significantly higher (55.83±2.56%, $p \leq 0.001$) than in other supplemented and control groups (Figure 12B).

To analyze EVs and other supplementation effects on the spermatozoa acrosome reaction, spermatozoa were categorized into four groups based on their fluorescence patterns (acrosome intact, acrosome reacting, acrosome reacted live, and acrosome reacted dead). In the beginning 0h, most spermatozoa were acrosome intact (90.57±2.59%, data not shown) in all groups. Furthermore, most spermatozoa remained similar (acrosome intact, 77.04±3.72%, data not shown) in all groups even after four hours except EVs supplemented groups. However, the percentage of acrosome-reacted dead spermatozoa (17.40±4.99%, $p \leq 0.001$) was significantly higher compared to the control, non-EV1, and non-EV2 supplemented groups. Similarly, we observed the same effect in the case of acrosome reacted live spermatozoa, where the percentage of acrosome reacted live spermatozoa in the EV supplemented group was significantly higher (25.93±2.99%, $p \leq 0.001$) compared to the control, non-EV1 and non-EV2 (Figure 12C). Thus,

our results indicated that EVs from FF increased the percentage of acrosome-reacted spermatozoa while maintaining the viability of spermatozoa.

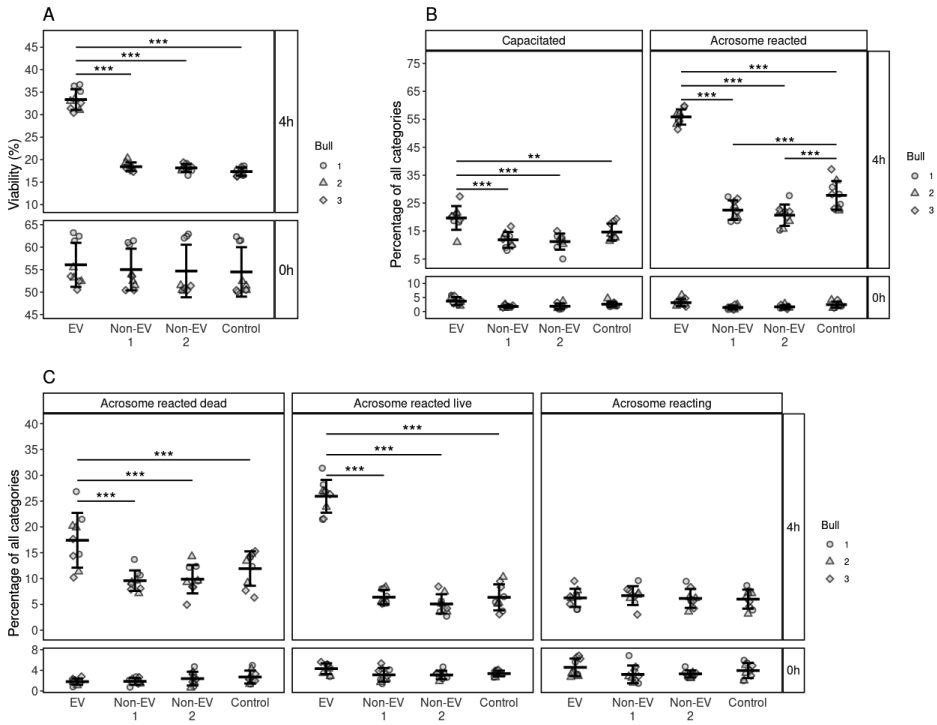

Figure 12. The impact of adding FF EV and non-EV fractions to spermatozoa on functions (viability, capacitation, and acrosome reaction). (A) Spermatozoa viability at 0 and 4 hours in response to various supplements. (B) At 0 and 4 hours after supplementation, the proportion of spermatozoa in various phases of the capacitation process. (C) At 0 and 4 hours after supplementation, the proportion of spermatozoa in various phases of acrosome reaction. The mean and standard deviation are shown by error bars (SD). Measurements of samples from several bulls are represented by different symbols. Asterisks indicate statistically significant differences between supplementation groups and correspond to the following p-values: ** p 0.01; *** p 0.001. (Hasan et al., 2021).

5.3.2. The specificity of FF EVs' effects on spermatozoa viability, capacitation, and acrosomal reaction

Our next objective was to determine EVs source (small, medium and large follicles; FF EVs) and species specificity (porcine FF EVs and JAr EVs) on sperm functions. Our results showed that despite the size of follicles where the FF EVs were collected, they all had a similar effect on sperm functions. We did not observe any effect of EVs collected from porcine FF EVs and JAr EVs on bovine spermatozoa function (figure 13A-13C).

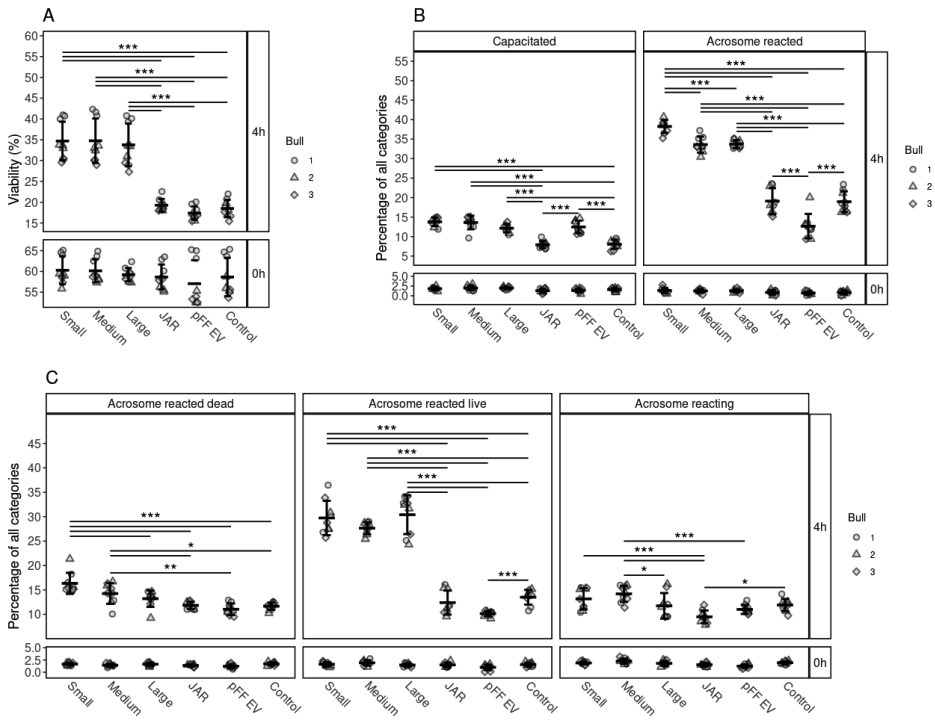

Figure 13. The effects of EV supplementation from various cellular sources on spermatozoa viability, capacitation, and acrosomal reaction. (A) Live spermatozoa percentages following supplementation with EVs from small, medium, large follicles, JAr EVs, pFF EVs, and control at 0 and 4 hours. (B) Spermatozoa percentages at various phases of the capacitation response, at 0 and 4 hours following supplementation with EVs from various sources. (C) Spermatozoa percentages at various phases of the acrosomal response following supplementation with EV from various sources at 0 and 4 hours. The mean and standard deviation are shown by error bars (SD). Measurements of samples from several bulls are represented by different symbols. Asterisks indicate statistically significant differences across supplementation groups and correspond to the following p-values: * 0.05; ** 0.01; *** 0.001. P-values in the same category are annotated with double lines. Asterisks above the top line also apply to the bottom line in this circumstance. The contrasting groups are shown by the endpoints of the lines. (Hasan et al., 2021).

5.3.3. The influence of surface-modified FF EVs on spermatozoa function (viability, capacitation, and acrosomal reaction)

The purpose of this study was to see how trypsin-treated surface-modified EVs affected sperm functions. According to our findings, EVs no longer promote sperm viability (Figure 14A). We also discovered that trypsin-treated EVs lost their ability to promote capacitation and the acrosome response (Figure 14B & 14C). Furthermore, we discovered a significant decrease in the overall number

of EVs after trypsin treatment (Figure 14D) and a decrease in the number of large-sized EVs after trypsin treatment (Figure 14E). Protein concentration was reduced by approximately 82 percent after trypsin treatment (1,017 mg/ml before and 0.185 mg/ml after, data not shown).

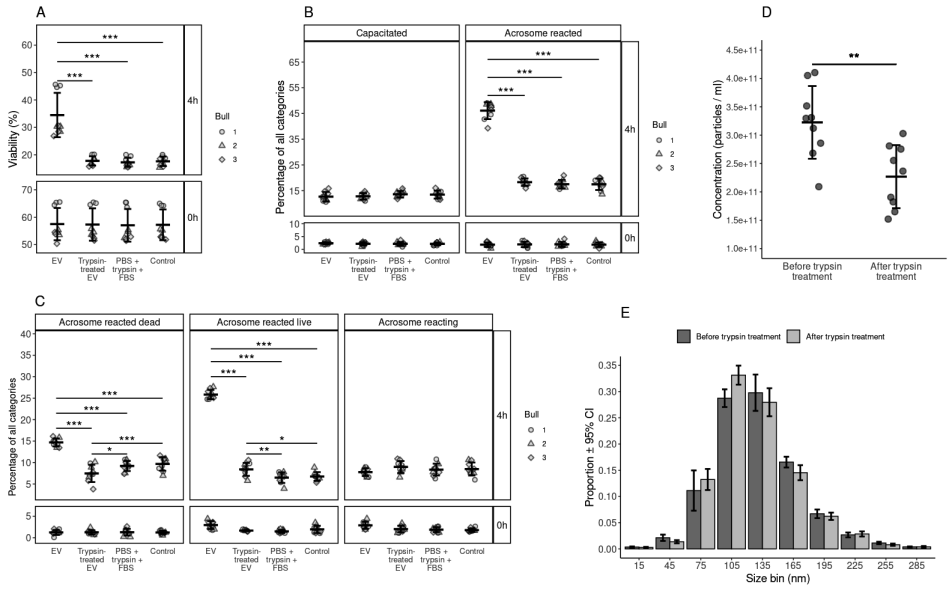

Figure 14. Incubating bovine spermatozoa with trypsin-treated, non-treated, and PBS + trypsin + FBS EVs investigated their functional characteristics. (A) The percentage of live spermatozoa at 0 and 4 hours after treatment. (B) Various stages of capacitation response of Spermatozoa at 0 and 4 hours after supplementation. (C) Spermatozoa percentages at various stages of the acrosomal response at 0 and 4 hours. (D) The concentration of nanoparticles in EV samples before and after trypsin treatment. (E) Distribution of nanoparticle size in EV samples before and after trypsin treatment. The error bars, with the exception of subfigure E, show the mean standard deviation (SD). Where relevant, various symbols denote measurements of samples from different bulls. Asterisks denote statistically significant differences and are related to p-values in the following way: * p 0.05, ** p 0.01; *** p 0.001(Hasan et al., 2021).

5.3.4. Progesterone concentration in FF and FF EVs

The progesterone concentration in FF and FF EVs was determined using a Chemiluminescence immunoassay system to identify if FF EVs have any progesterone. While progesterone was detected in the FF, it was not detectable in FF EVs (Table 5).

Table 5. The concentration of progesterone in FF and FF-derived EV samples.

Sample type	Concentration (nmol/L)
FF (small)	9,63 nmol/l
FF (medium)	6,64 nmol/l
FF (large)	7,64 nmol/l
FF EV (small)	<0,67 nmol/l
FF EV (medium)	<0,67 nmol/l
FF EV (large)	<0,67 nmol/l

Chemiluminescence – immunoassay-based measurement of the concentrations of progesterone using ADVIA Centaur XP immunoassay system (Siemens Healthineers, Germany). The measuring range of the progesterone was 0.21–60 ng/ml (0.67–190.8 nmol/l) (Hasan et al., 2021).

5.4. Study III (Cellular, Extracellular and Extracellular Vesicular miRNA Profiles of Pre-Ovulatory Follicles Indicate Signaling Disturbances in Polycystic Ovaries)

5.4.1. Sample collection and processing

We aimed to study the intrafollicular communication difference between healthy and PCOS ovaries. The study was modelled by analysing materials from 3 distinct sources collected from the same follicles. Small RNA from mural granulosa cells in the follicular fluid (MGC), all cell-free small RNA populations from the follicular fluid (FF), and small RNA in EVs purified from the follicular fluid (EV). The sample collection and purification outline is below (Figure 15).

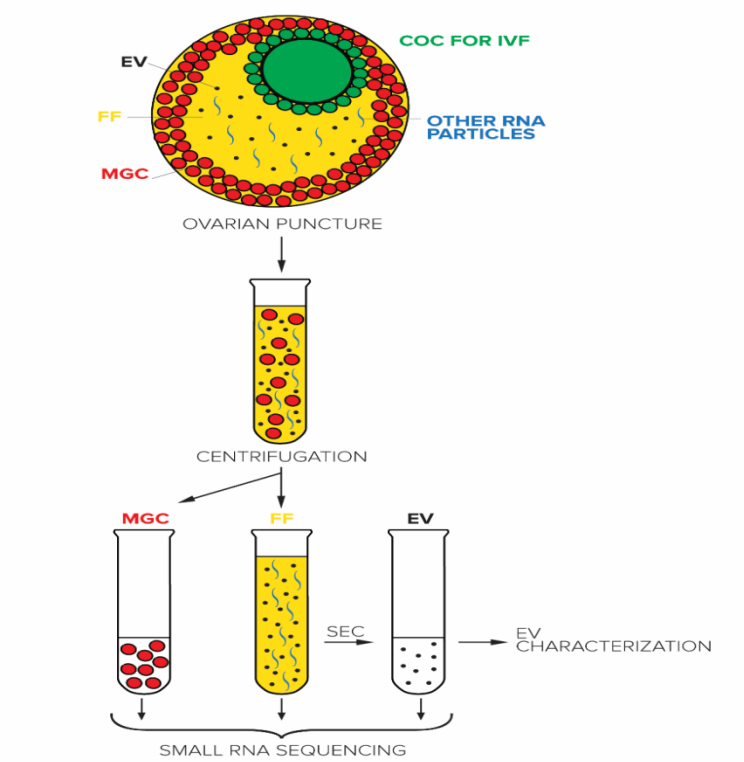

Figure 15. Sample collection and processing procedure of human pre-ovulatory follicles. Cumulus-oocyte complex marked as COC, extracellular vesicles marked as EV and follicular fluid marked as FF in the illustration. Similarly, IVF – *in vitro* fertilization, MGC – mural granulosa cells, SEC – size-exclusion chromatography (Rooda et al., 2020).

Three different types of samples were collected for EVs isolation and characterization. Similarly, all three sample types were also used for RNA sequencing and real-time quantitative PCR (RT-qPCR). The analysed sample details based on each method type are below (Table 6).

Table 6: Study participant details (Rooda et al., 2020).

		Age (mean, years)	SD (years)	BMI (mean, kg/m^2)	SD (kg/m^2)
Extracellular vesicle characterization:					
PCOS	n=15	32.7	4.3	23.5	3.1
Oocyte donors	n=15	25.8	3.2	22.3	2.8
p-value		<0.001		0.313	
Small RNA sequencing:					
PCOS	n=7	34	4.8	22.7	2.6
Oocyte donors	n=8	26.9	2.2	22.7	3.6
p-value		0.002		0.999	
miRNA expression validation with RT-qPCR:					
PCOS	n=15	32.7	4.3	23.5	3.1
Controls	n=16	33	4	23	3
p-value		0.626		0.831	

5.4.2. MiRNAs in granulosa cells, cell-free follicular fluid and extracellular vesicles

One thousand five hundred twenty-five unique miRNAs were detected at least once from all investigated samples: 658 from EV, 1060 from FF, and 1381 from MGC. In addition, all sample types share a large proportion of the most abundant miRNAs among the twenty most prevalent sequences across all sample types (EV and FF share 15, FF and MGC share 8 out of 20).

Our clustering analysis showed differences between sample types according to their miRNA content (Figures 16A and 16B). Compared to MGC samples, EV and FF samples were clustered closer to each other as EV samples are a sub-compartment purified from the corresponding FF samples.

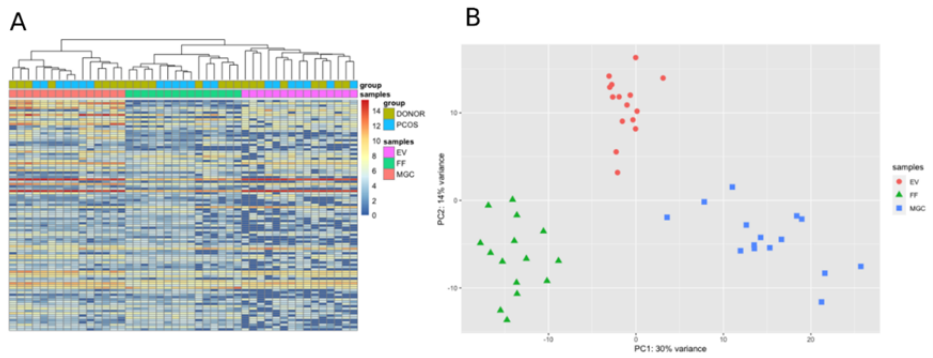

Figure 16. Cluster and principal component analysis of FF, EVs and MGC samples. (A) Hierarchical clustering (DESeq2 normalized counts on the $\log_2$ scale) of the top 100 most variable miRNAs across all samples. (B) Principal component analysis of FF, EV and MGC samples.
* MGC – granulosa cells, FF – cell-free follicular fluid, EV – extracellular vesicles, PCOS – polycystic ovary syndrome (Rooda et al., 2020).

5.4.3. Differences in miRNA expression in PCOS patients and oocyte donors

A comparison of samples between patient groups revealed that 30 miRNA in MGC and ten miRNAs in FF were differentially expressed between PCOS patients and oocyte donors (FDR <0.1, Figure 17A). However, due to the higher variation of miRNA expression in EV samples in both groups (Figure 17B), no miRNAs reached the same FDR cut-off level. Nevertheless, seven miRNAs were differentially expressed between the two groups in EV samples without considering the FDR (p-value <0.05, Figure 17C).

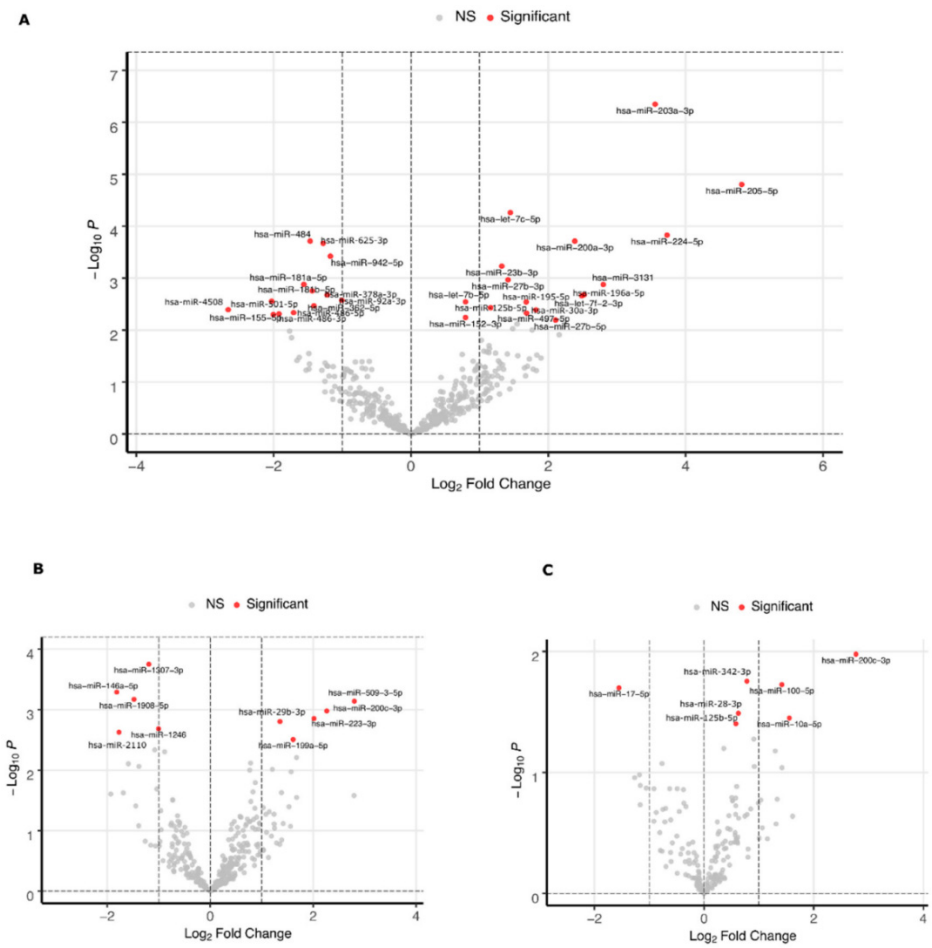

Figure 17. Differentially expressed miRNAs in mural granulosa cell samples of oocyte donor and PCOS patients. A. follicular fluid samples (cell-free). B. Extracellular vesicles samples and C. Statistical significance cut-off is FDR < 0.1 (A and B) or p < 0.05 (Rooda et al., 2020).

RT-qPCR validated differentially expressed miRNAs. Validation was performed for miRNAs with the highest fold change and that have been previously related to ovarian functions (Table 7). miRNA expression validation results by RT-qPCR confirmed the exact directional change for most miRNAs observed by sequencing.

Table 7. Differentially expressed miRNAs between polycystic ovary syndrome (PCOS) patients and oocyte donor samples were used for validation studies.

A. MGC PCOS vs donors:		
miRNA	Log_2 Fold Change	Presently available information of miRNA role in ovary
hsa-miR-205-5p	4,82	miRNA expression is upregulated in MI oocytes upon IGF-1 treatment (Xiao et al., 2014).
hsa-miR-203a-3p	3,56	Expression levels are higher in young women with normal ovarian reserve granulosa cells than in those with diminished ovarian reserve(Woo et al., 2017).
hsa-miR-196a-5p	2,49	miRNA expression is detectable in bovine granulosa cells on day three but not on day 7 of the estrous cycle (Salilew-Wondim et al., 2014).
hsa-let-7c-5p	1,45	Expressed higher in human CGC compared to MGC cells. miRNA expression is decreased in granulosa cells of early and progressive atretic follicles and a case of POF syndrome (measured from plasma)(Zhang et al., 2019).
hsa-miR-181a-5p	-1,56	Expressed higher in human CGC compared to MGC(Velthut-Meikas et al., 2013). In mice, granulosa cells miR-181a-5p target ACVR2A (Activin Receptor IIA) and inhibit granulosa cell proliferation(Q. Zhang et al., 2013). In oxidative stress conditions, miRNA expression is upregulated in mouse granulosa cells and mediates granulosa cell apoptosis(M. Zhang et al., 2017)
B. **FF PCOS vs donors:**		
miRNA	Log_2 Fold Change	miRNA role in ovary
hsa-miR-509-3-5p	2,80	Expression is higher in PCOS patients' FF than controls(Butler et al., 2019).
hsa-miR-200c-3p	2,26	Expression is higher in granulosa cells from PCOS patients than in control samples(He et al. 2018) and in FF samples of PCOS patients(Yao et al., 2018).
hsa-miR-223-3p	2,01618337	EVs obtained from FF show expression of hsa-miR-223-3p(Martinez et al., 2018). miRNA expression is decreased in cumulus cells of PCOS patients(Yao et al., 2018).
hsa-miR-146a-5p	-1,81	Expression is higher in human MGC samples compared to CGC(Daniela et al., 2019).

C. **EV PCOS vs donors:**		
miRNA	Log_2 Fold Change	miRNA role in ovary
hsa-miR-200c-3p	2,77	Expression is higher in granulosa cells from PCOS patients than in control samples (He et al. 2018) and FF obtained from PCOS patients(Yao et al., 2018).
hsa-miR-100-5p	1,42	Associated with cell proliferation regulation. Downregulated in young women with diminished ovarian reserve compared to normal ovarian reserve (Woo et al., 2018).
hsa-miR-17-5p	-1,55	Compared to controls, expression is downregulated in granulosa cells and FF of PCOS women (Liu et al., 2020a). miRNA expression is detected in EVs obtained from FF(Martinez et al., 2018).

MGC – mural granulosa cells, FF – follicular fluid, EV – extracellular vesicles, and CGC – cumulus granulosa cells (Rooda et al., 2020).

5.4.4. Distinctive functions are dysregulated in each analysed follicular compartment of PCOS patients

Because one miRNA can target multiple genes and one gene can be targeted by multiple miRNAs, we wanted to see if the differentially expressed miRNAs changed any common pathways in PCOS patients. Reactome pathway over-representation was performed in each sample type for all differentially expressed miRNAs based on RNA sequencing. Separate lists demonstrating higher and lower miRNA expression levels in the PCOS group compared to controls were examined.

Significantly upregulated miRNAs in the PCOS group regulate 20 pathways in MGC, 25 in FF, and 13 in EV. Most over-represented terms in MGC are transcription regulation and cell cycle pathways. Furthermore, in the MGC of PCOS patients, miRNAs that regulate oestrogen receptor (ESR) signalling and nuclear receptors, in general, were more abundant. Several signal transduction pathways are regulated by over-represented miRNAs in FF: AKT, TGF-beta, and apoptosis and protein modification by SUMOylation were the most frequently used terms. The IGF1R signalling pathways were the most common terms for miRNA-s that were significantly more abundant in the EVs of PCOS women.

Furthermore, miRNAs up-regulated in PCOS women's MGC and FF samples target common immune system pathways that are not predicted targets for miRNAs in EVs.

In the pathway "Cellular responses to external stimuli," miRNAs expressed at higher/lower levels in the MGC of PCOS patients are involved. In the PCOS group, there was no over-representation of pathways for miRNAs with low abundance in FF and EV (Figure 18).

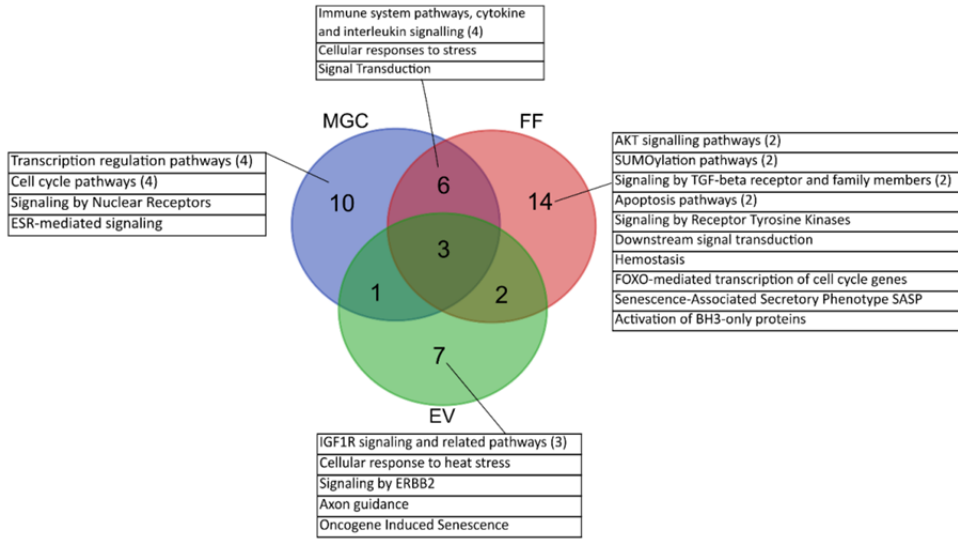

Figure 18. List of over-represented reactome pathways for miRNAs expressed at higher/lower levels in PCOS patients than in the fertile control group in each sample type. Numbers in brackets refer to combined pathways with a similar outcome. MGC – mural granulosa cells, FF – cell-free follicular fluid, EV – extracellular vesicles (Rooda et al., 2020).

5.4.5. Potential novel miRNA as a marker for follicular EVs

Data from RNA sequencing can be used to predict novel, unannotated miRNAs. We propose one potential new miRNA (mature sequence: CCUGGG CAUGGGACUGG, predicted stem-loop sequence in Figure 19A) that was expressed in 12 different patients and in all three sample types after filtering candidate novel miRNA sequences suggested by the miRDeep2 algorithm (EV, FF and MGC). It was found most frequently in EV samples (nine EV samples, four FF samples, and five MGC samples), with significantly higher expression levels than in FF and MGC samples (Figure 19B). Furthermore, RT-qPCR validation revealed significantly higher levels of this sequence in EVs than in FF samples (Figure 19B). In any of the sample types, the expression levels of the novel miRNA did not differ between PCOS and the donor group.

A similar seed sequence (nucleotides 2–8 from the 5′ end) is shared by five previously annotated miRNAs and the potential novel miRNA (Figure 19C). miRDB predicted 1430 potential targets for the novel miRNA, which were then enriched into 82 terms in the biological processes ontology domain (FDR < 0.05). Semantic similarity analysis further narrowed these down to three major categories: cell communication regulation, cell junction organisation, and nervous system development.

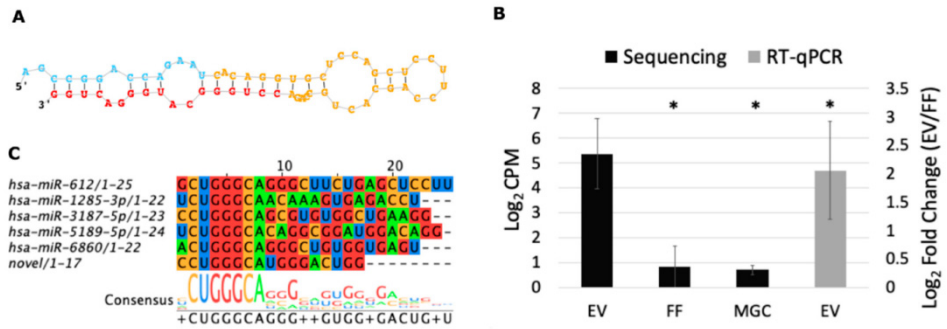

Figure 19. Detected novel miRNA by small RNA sequencing of single follicle components. (A) Stem-loop sequence predicted by miRDeep2. (B) The expression level of novel miRNA in extracellular vesicles (EV), follicular fluid (FF) and granulosa cells (MGC). (C) Alignment of novel miRNA with previously annotated miRNAs with similar seed sequences. * $p < 0.05$, Student's t-test (Rooda et al., 2020).

6. DISCUSSION

6.1. FF and FF EVs in gene expression and embryo development (study I)

The oviduct plays a crucial role in early reproductive events such as the transport of oocytes, fertilization and embryo development. Over the past few decades, the knowledge of oviductal biology has seen steady development, providing us more insight into the importance of a favourable oviductal environment for successful embryo development. Furthermore, the embryo and maternal communication have a long-term effect on maternal recognition of pregnancy, implantation, offspring and maternal phenotype (Diskin and Morris, 2008; Fazeli, 2008). The oviduct is responsible for hosting gametes. The embryo seems to be an unimposing organ. However, the oviduct processes a series of complex events that need to be precisely initiated and completed. Therefore, small changes in the molecular level could alter the oviduct's environment, leading to failure in pregnancy. As a result, the gene expression changes of oviductal epithelial cells in response to FF and FF-derived EVs and their effect on fertilization and early embryo development were investigated in this study.

Differential gene expression analysis of bovine oviductal epithelial cells (BOECs) with bovine FF and FF EVs coculture revealed that both FF and FF EVs could induce transcriptomic changes BOECs. Furthermore, these changes are distinguishable between FF and FF-derived EVs regarding differentially expressed gene number and incubation duration. We observed a more prominent effect in 24 hours than six hours when BOECs were treated with FF. However, we observed the opposite effect in the FF EVs treatment, where effects were more prominent in six hours than in 24h. The prominent effects of FF compared to FF EVs could be hormonal, non-hormonal, and metabolic components in FF that may not be present in FF-EVs. The effects observed from FF-derived EVs in 6h were independent and did not correlate with FF treatment as there were no common genes upregulations between them. There was no effect in 24h EVs supplementation because FF-derived EVs may have a short lifetime. Their effects were visible in the early incubation time (6h) and faded away during longer incubation times. Future studies about EVs effect on BOECs for short incubation time, such as between 1–6h or 6–24h, several time points could give us a more precise answer if FF-derived EVs have a short-lived effect on BOECs.

It is well known that several factors such as spermatozoa (Fazeli, 2008, p. 201), oocytes (Lee et al., 2017), the embryo (Geisert and Schmitt, 2002), even the oviduct´s own fluid (Guérin et al., 2001) can alter the transcriptome of the oviduct. However, our study demonstrates that FF-derived EVs and FF itself can alter the gene expression of BOECs, which may create a favourable

environment for sperm survival and fertilization in harsh conditions of the oviduct environment.

KEGG pathway analysis of 24 h FF treated differentially expressed genes (upregulated) suggests that thermogenesis and oxidative phosphorylation were significantly enriched among other pathways. Oviductal cells' proper functions are regulated by oxidative phosphorylation, which plays a vital role in ATP production. Furthermore, the energy generated by oxidative phosphorylation may activate thermogenesis pathways that generate heat and the sperm thermotaxis process. Thermotaxis is a unique process where sperm follow the heat gradients and leads spermatozoa to the fertilization site (Li and Winuthayanon, 2017; Miki and Clapham, 2013). Based on this evidence, we can postulate that induction of oxidative phosphorylation and thermogenesis pathways FF guides spermatozoa to the fertilization site.

Similarly, GSEA also revealed that biosynthesis of amino acids (24 h FF treatment) and the tight junction pathway (6h EVs treatment) were enriched with upregulated genes. In the oviduct, after fertilization-embryo usually stays 3–4 days (Croxatto, 2002). During the stay in the oviduct, the embryo needs nutrients and lipids; pyruvate, lactate and amino acids are the primary embryo nutrients (Nichol et al., 1992; Nieder and Corder, 1983). Therefore, FF and FF EVs may contribute to early embryo nutrient supplements and support embryo development by inducing these pathways. Moreover, several studies showed that oviductal epithelial cells are involved in nutrient transfer, while tight junction between epithelial cells plays a vital role in this process (Ballard et al., 1995; Eckert and Fleming, 2008).

Our analysis revealed that some pathways were over-represented among the downregulated genes in both 6 and 24 h with FF treatment. Steroid hormone biosynthesis and arachidonic acid metabolism were more prominent than other downregulated genes. Arachidonic acid oxygenation can produce several metabolites known for immune reaction modulations (Hanna and Hafez, 2018; Samuelsson, 1991). In the female reproductive organ, spermatozoa travel a long distance to reach the fertilization site (fallopian tubes). They may register as a nonself entity (pathogen). Hence, it is of utmost importance to modulate the local immune system, which could enable the safe passage of spermatozoa during the journey toward the fertilization sites (Hansen, 2011).

Along with other factors, a proper balance of steroid hormones such as estradiol and progesterone plays a vital role in oogenesis, fertilization, and successful motherhood. An optimal environment with decreased estradiol concentration is required to transport a COC from the infundibulum to the fertilization site. However, studies suggest that the steroid hormone level can fluctuate before and after fertilization (Binelli et al., 2018; Wood et al., 2007). Therefore, evidence from these studies indicates that FF may assist COC transportation by inducing downregulation of the steroid hormone biosynthesis pathway. Another study confirmed that FF's estradiol could modulate the gene expression related to inflammation and DNA damage in BOEC (Palma-Vera et al., 2017).

Nevertheless, some genes with proven function in reproduction were significantly upregulated in 24 h FF treatment but did not represent any significant pathways. Prostaglandin E synthase 2 (PTGGES2) was an upregulated gene known to regulate oviductal muscular activity (Lyons et al., 2006). Prostaglandins promote spermatozoa to reach the fertilization site and guide the embryo to reach the uterus from the oviduct by enhancing the contractions of the oviductal smooth muscle cells (Harper et al., 1980; Kodithuwakku et al., 2007; Lyons et al., 2006). The most significant upregulated genes with FF treatment were in both 6 and 24-h neurotensin (NTS). Several studies in mice and Japanese black cattle reported that NTS is involved in sperm capacitation and acrosome reaction (Hiradate et al., 2014; Umezu et al., 2016). Sperm capacitation and acrosome reaction are vital for fertilization, suggesting that, among other functions of FF in the oviduct, it enhances fertilization by upregulating NTS. Despite FF's other roles, its role in preparing a periconceptional environment has been less studied; hence our study has addressed a fundamental knowledge gap. This knowledge could help us understand the fine tunes of the oviductal environment to ensure that the embryo receives the proper developmental signal and nutrients, helps embryos overcome environmental stress and protects embryos from the reproductive tract immune system.

6.2. Role of EVs in spermatozoa functions (study II)

The ability of FF to enhance sperm motility, capacitation and acrosome reaction is well established (Bravo and Valdivia, 2018; El-Shahat et al., 2018; Falcone et al., 1991; McNutt and Killian, 1991; Yao et al., 2000). Furthermore, previous research has shown that progesterone is the FF's main active component, which regulates spermatozoa's functional properties, such as capacitation and acrosome reaction (Jeon et al., 2001). Interestingly, the current investigation demonstrated that sperm viability is maintained by EVs isolated from the bovine FF. In addition, we showed that FF EVs could induce capacitation and enhance the life span of spermatozoa after the acrosome reaction. However, the EVs active components that mediate these effects are unlikely to be progesterone, as it was not detectable in FF EVs. Therefore, current data shows that some components utilizing the surface proteome or molecular cargo of FF EVs, other than progesterone, also play a vital role in modulating EVs functional properties, affecting spermatozoa function.

Several studies reported that during ovulation FF enters into the oviduct along with oocytes and affects the vital parameters of spermatozoa function (Hansen et al., 1991; Hunter et al., 1999); Based on our current study, we can hypothesize that FF EVs mediate communication between ovarian somatic cells involved in folliculogenesis and male germ cells, i.e. spermatozoa, and influence their fundamental functional properties. This new dimension of female-male communication has been wholly overlooked thus far. Exploring these modes of communication provides a deeper understanding of the mother-child communication and cooperation during the early stages of reproduction,

particularly the molecular mechanisms underlying events like sperm competition. Sperm competition is the repercussion of polyandry. The competition arises between ejaculates of different males to fertilize a particular oocyte. The FF EVs interact with spermatozoa prior to fertilization.

The overall aim of this study was to identify whether FF EVs support vital functional properties of spermatozoa. Our results showed that FF EVs could maintain sperm viability and induce sperm capacitation and sperm acrosome reaction. Furthermore, these effects are specific to FF EVs. While EVs obtained from porcine FF and JAr conditioned media neither maintain sperm viability nor induce capacitation or acrosome reaction. This is a critical aspect of the interaction of EVs with their responding cells.

The specificity and mode of action of bovine FF EVs in transferring the required signals to bull spermatozoa could be explained by the different proteins and cargo carried by EVs from different sources (Cai et al., 2020; Doyle and Wang, 2019; Murdica et al., 2019). Furthermore, we observed that non-EV fractions of FF obtained via SEC did not affect spermatozoa's key functional properties. As a result, the non-EV fractions may lack specific proteins or biomolecules that do not have the desired positive effects on spermatozoa; hence, FF EVs are the main contributors to enhancing the studied critical functional parameters of sperm.

Only hyperactive and capacitated spermatozoa can penetrate the oocyte's zona pellucida and fuse the egg plasma membrane during fertilization. These processes require additional energy because they involve substrate reactions (Ferramosca and Zara, 2014; Murphy et al., 1986). Furthermore, several studies suggest EVs are involved in enzyme kinetics and act as enzyme carriers (Gerth et al., 2019; Margolis and Sadovsky, 2019). Therefore, it is possible that FF EVs may transport enzymes that improve spermatozoa viability, capacitation, and acrosomal reaction.

Based on the size of the ovarian follicle, the concentration and molecular cargo of FF EVs may vary (Navakanitworakul et al., 2016). Thus, a stage-specific, EV-mediated, intercellular communication must occur during the process of antral follicle development. The size of the ovarian follicle is thought to be related to the oocyte's competence (Wirleitner et al., 2018) and future embryo development (Nivet et al., 2016) during the assisted reproduction process. In another study, Wirleitner et al. (Wirleitner et al., 2018) demonstrated that the live birth rate of blastocysts derived from oocytes aspirated from small (<1 mm) follicles are higher than that of blastocysts derived from medium (1–6 mm) or large (> 6 mm) follicles.

While these differences could be attributed to differences in FF EVs in different-sized follicles, similar differences in spermatozoa critical functions would also be observed. To test this hypothesis, EVs were isolated from the FF of different sized follicles: small, medium, and large, and their consequent effect on various functional parameters of spermatozoa were analyzed. Our result showed that all size follicle's EVs positively affect spermatozoa functions. However, a higher percentage of spermatozoa underwent acrosome

reactions with small-sized follicles EVs. This was expected as the particle concentration observed in FF EVs collected from smaller follicles contained a higher number of EVs than those from large and medium-sized follicles. This also points out that a higher number of FF EVs, which collectively contain a high number of essential inducers (proteins/cargo), are required to induce the acrosome reaction in spermatozoa. However, the viability was maintained, and spermatozoa capacitation was enhanced even at lower concentrations of FF EVs (Data not shown).

Finally, we aimed to understand the possible mechanism of EV-mediated signalling involved in these processes. Using trypsin, we modified the surface of FF EVs, causing the breakdown of some proteins on the EV surface. Interestingly, trypsin-treated FF EVs lost their ability to induce capacitation, acrosome reaction, and spermatozoa viability. This result could have several explanations: either the membrane proteins involved in EV uptake by spermatozoa were destroyed, or trypsin-treated FF EVs lost their ability to fuse with the sperm plasma membrane. More research is needed to fully comprehend the mechanism of action of FF EVs on spermatozoa functions.

The role of FF EVs in enhancing the functional parameters of spermatozoa has never been explored in detail, and these new findings address a fundamental knowledge gap. It has previously been demonstrated that incubating human spermatozoa with human FF increased the pregnancy rate in patients undergoing intrauterine insemination (IUI) (Blumenfeld and Nahhas, 1989) and *in vitro* fertilization IVF (Ghetler et al., 1990). Our findings indicate that FF EVs improve the key functions of frozen-thawed spermatozoa. More information about FF EVs' cargo/surface proteins would be extremely useful in improving current ART protocols, which could eventually lead to successful fertilization. More research is necessary to identify the FF EV cargo/surface composition that is causing these changes in spermatozoa functional attributes. This is a novel aspect of evolutionary biology, especially in the interaction between sexes context, as FF originated from female follicular somatic cells and oocytes; hence it is possible they started the interaction with spermatozoa long before the male and female gametes met each other. Therefore FF and FF EVs are probably involved in sperm functions and cryptic female choice. This information will help us better understand molecular events between sexes during the earlier stages of reproduction.

6.3. microRNA expression differences in the ovaries of PCOS women (study III)

Intercellular communication is crucial for cellular and organismal homeostasis in higher mammals. This is because cells are required constant communication between them to maintain the body's vital functions. Some of the critical mediators of this cell-cell communication are cell-free RBPs and nanoparticles, including EVs containing molecules from many different biotypes of RNA

(Fritz et al., 2016). Moreover, all body fluids, including FF, are rich in EVs and cell-free nucleic acids (Théry et al., 2018). However, EVs complex and heterogeneous nature, especially their subtypes exosome and microvesicles, share an overlapping size range that imposes difficulties in their efficient separation and purification (Doyle and Wang, 2019). Therefore the isolations methods must be chosen wisely based on sample sources and types (Théry et al., 2018).

Furthermore, each method has limitations regarding the yield and purity of isolated EVs and other RNA-containing particles. Combining several methods always gives more advantages (data not shown); therefore, we used SEC with differential centrifugation methods in this study, which helped us recover a high yield and pure EVs (Benedikter et al., 2017; Böing et al., 2014). The role of EVs in intracellular communication in different pathophysiological conditions is already established (Simon et al., 2018). For example, a recent study presented a higher blood plasma concentration of platelet-derived microparticles in PCOS patients than in healthy women (Koiou et al., 2013). Our study also detected significantly more EVs in PCOS women's FF than oocyte donors with normal ovarian morphology.

In this current research, we have analysed matched cellular and extracellular small RNA profiles from individual pre-ovulatory follicles by whole-genome small RNA sequencing. Moreover, we analysed small RNA content considering two factors; small RNA carried by EVs and small cell-free RNA floated in FF. While individual studies about the RNA content of human MGC(Velthut-Meikas et al., 2013), FF, and EVs (Sang et al., 2013), have already been reported; a competitive combination study of these three closely related biological components is unique in this study which gives us more accurate and reliable information at the same time. In our analysis, we observed a distinguishable difference between the miRNA profile of our three sample types, which indicates that the release mechanisms of EVs and non-EVs miRNA are different and may have different functional purposes. Several other studies also observed the same effects in plasma samples (Endzeliņš et al., 2017; Min et al., 2019), supporting our current finding.

Many sorting mechanisms have been proposed for loading miRNAs into EVs: sequence characteristics, post-transcriptional modifications, subcellular location, and intracellular concentration have all been shown to influence miRNA segregation into EVs (Fritz et al., 2016). Moreover, EVs' heterogeneous nature also influences the nucleic acid content (Jeppesen et al., 2019). Compared with other macromolecular complexes, such as non-vesicular RBPs, a large amount of small RNA is secreted outside of EVs; however, the mechanism is still unknown by which protein interacts with miRNAs before their secretion (Fritz et al., 2016). The study suggests that despite which particular protein forms the complex, miRNA export by these pathways is an energy-dependent process (Fritz et al., 2016).

Our analysis showed that 172 miRNAs were commonly present across all the sample types suggesting that miRNAs are secreted non-selectively from cells via EVs and/or non-vesicular RBP pathways. On the other hand, 113

miRNAs were only present in cells or FF, indicating that these miRNAs are secreted out of cells in RBP. Similarly, 175 miRNAs were observed in the EVs samples. Over-representation analysis between EV and FF miRNA revealed that miRNAs present in EVs are packed with a specific molecular signal. A study by Baglio revealed that the cargo types loaded into EVs are unique, not random events (Baglio et al., 2015). In this study, the author confirms that EVs derived from bone marrow and adipose mesenchymal stem cells carry specific small RNA molecules linked to their differentiation status.

Moreover, several other studies in bovine and equine showed that EVs derived from FF were uptaken by granulosa cells and resulted in gene alteration involved in follicles development (da Silveira et al., 2012; Hung et al., 2017). Furthermore, changes in the expression of some miRNAs in follicular EVs have been observed between follicles with different oocyte maturation stages (Sohel et al., 2013b). These studies indicate that EVs in follicular fluid most likely play important regulatory roles. The miRNA presented in EVs may follow specific Reactome terms, “Signaling by Nuclear Receptors”, “PTEN Regulation”, “ESR-mediated signalling”, and more. Estrogen receptors are tagged as the nuclear receptor family. The nuclear receptor with androgen receptor has known functions in follicles development and ovulation (Astapova et al., 2019; Tang et al., 2019).

On the other hand, PTEN is known for follicle growth and activation. A high level of PTEN is reported for poor oocyte competence (Andrade et al., 2017; McLaughlin et al., 2014). The miRNA profile analysis from fertile female samples may follow specific miRNA segregation with several downstream pathways. In contrast, the selection of miRNAs for secretion by non-EVs mediated pathways that tend to casual events in the follicles. It is debatable whether all the miRNAs present in the extracellular space are involved in intracellular signalling and the threshold of EV, protein-bound miRNAs in any physiological functions. A study by Chevillet reveals that every single EV only contain less than one copy of miRNAs (Chevillet et al., 2014), while during sequencing experiments, small RNA cargo is analysed in bulk; therefore, it is still unknown whether all EVs contain the same quantity of miRNA or they follow certain cargo distributions manner in different EVs population. Therefore a specific separation of miRNA molecules may significantly increase the copy number of individual miRNAs in each EV population (Chevillet et al., 2014).

The primary goal of this study was to determine whether PCOS affects ovarian miRNA expression, secretion, or segregation into EVs. We discovered that PCOS affects each follicular compartment differently, as the miRNA profiles in MGC, FF, and EV differ from those in the fertile ovary. As a result, PCOS affects separate pathways in MGC and potential recipient cells for extracellular miRNA in the FF and EV.

Studies reported that several miRNAs were differently expressed in FF and granulosa cells of fertile and PCOS women (Butler et al., 2019; Cirillo et al., 2019). Our study found the highest number of differences in miRNA expression from cellular material. The EV samples diverged the least between the two

groups at the single follicle level. However, some differences could be detected without multiple testing adjustments. Fewer differences in EV may be due to the more complex processing of EV samples prior to RNA extraction, which may affect the results.

Our study detects the highest differences in miRNA expression profile from the cellular material samples. We observed numerous miRNAs that were differentially expressed between the patient groups but were not previously affiliated with PCOS but are known to regulate gene expression in follicles or other ovary-related disorders. For example, hsa-miR-224-5p (Yao et al., 2010, p. 4), which was up-regulated in the MGC of PCOS women in our study, inhibits SMAD4, which is involved in the regulation of granulosa cell apoptosis(Du et al., 2020). It has been demonstrated that hsa-miR-203a-3p, hsa-miR-195-5p, hsa-miR-486-3p, and hsa-miR-484 levels are altered in the granulosa cells of women with diminished ovarian reserve (Woo et al., 2017). Interestingly, all four miRNAs referenced above are expressed in the MGC of our PCOS group in the same pattern as in normal ovarian reserve (NOR) patients: hsa-miR-203-3p and hsa-miR-195-5p are both more abundant in NOR and our PCOS group, whereas hsa-miR-486-3p and hsa-miR-484 are less abundant in both NOR and our PCOS samples. PCOS women have a slower age-related decline in antral follicle count (AFC) compared to non-PCOS patients (Wiser et al., 2013), and AFC is considered a reliable marker for evaluating ovarian reserve (Ferraretti et al., 2011). A slower decline in AFC in PCOS women may also account for our miRNA expression findings. hsa-miR-486-5p (Shi et al., 2015) expression differences in MGC have previously been linked with PCOS, particularly in comparison to women undergoing IVF due to male factor infertility, with the same directional expression as in our study. Furthermore, hsa-miR-200a-3p (Xu et al., 2015) and hsa-miR-30a-3p(Yao et al., 2018) have previously been linked to PCOS, but somewhat in different follicular compartments. These studies found that hsa-miR-200a-3p in cumulus granulosa cells and hsa-miR-30a-3p in FF were less abundant in PCOS women, which contradicts our findings. Differential expression of hsa-miR-509-3-5p and hsa-miR-200c-3p (Butler et al., 2019; He et al., 2018) in FF has previously been linked to PCOS, with mutual expression direction consistent with our findings. Hsa-miR-1307-3p (Li et al., 2015) and hsa-miR-223-3p (Xu et al., 2015) are also altered in PCOS patients' cumulus granulosa cells, but in the opposite direction as ours. These comparisons show how miRNA expression disturbances vary based on the cellular environment. When the samples were collected, cumulus granulosa cells had differentiated from MGC. They showed significant differences in gene expression and post-transcriptional regulation patterns (Kõks et al., 2010; Velthut-Meikas et al., 2013). Hsa-miR-200c-3p (Butler et al., 2019; He et al., 2018) and hsa-miR-17-5p (Liu et al., 2020b) have been altered in PCOS women using EV samples, which is consistent with our findings.

Our findings unequivocally show that, depending on the types of sample under investigation, the consequences of PCOS-related variations in miRNA

expression result in various molecular outcomes. For instance, non-EV-mediated RNA secretion and the cellular compartment of cytokine-mediated signalling were both impacted. However, EV-mediated signalling may impact the IGF1R pathways in PCOS patients. These findings highlight how crucial it is to examine the follicle as a whole in order to comprehend intercellular signalling and potential molecular abnormalities in the PCOS ovary.

6.4. Future perspectives

We successfully isolated and characterized EVs derived from bovine FF in the first study. Moreover, we also identified the effects of FF and FF EVs on the gene expression of oviductal epithelial cells (BOECs), where both FF and FF EVs altered the gene expression BOECs. However, a more robust and time-dependent effect was observed with FF, whereas a mild effect was observed with EVs. Therefore, it is essential to study the content of EVs in more wide ranges, such as their protein molecule types and cargo contents, and identify the signalling mechanisms triggering gene expression changes in response to FF EVs. Furthermore, a more targeted experiment is necessary to analyze if the transcriptional responses of the FF or FF EVs could translate to phenotypic aspects in the reproduction. A further study could assess the effects of FF-derived EVs on primary cell types other than oviduct to study if the effects caused are different or specific to oviductal epithelial cells. Furthermore, the species specificity could also be studied by analysing gene expression changes in oviductal epithelial cells of one species when incubated with FF derived from another species. Moreover, the changes we observed in vitro may be replicated in vivo, implying that these changes may help to create a favourable micro-environment for sperm survival, fertilization, and embryo development.

The second study explored the effect of different sources and species of FF EVs on spermatozoa viability, capacitation and acrosome reactions. We successfully isolated and characterised FF EVs from different sizes of follicles from the bovine ovary, porcine FF, and JAr conditioned media. We observed the highest concentration of EVs in small follicles compared to medium and large follicles derived EVs. Furthermore, our results suggested that a higher percentage of spermatozoa were undergoing capacitation and acrosome reaction incubated with FF EVs derived from small follicles of the bovine ovary. Hence there is a need to understand the differences in the components of FF present in the follicles of various sizes and the internal cues that cause the accumulation of these components in the small follicles, which dilutes over time in larger follicles.

In contrast, no effects were observed from porcine FF and JAr EV. These results imply that the effects observed by FF EVs are specific to the source of FF and the species from which FF EVs are derived. A more detailed study regarding the FF EVs cargo, surface proteins and the mechanism underlying these effects of EVs on spermatozoa is required. Moreover, it would be

intriguing to study if age, diet, alcohol, and drug addiction alter FF-derived EVs' cargo and its effects on spermatozoa. The successful translation of this knowledge could be exploited for various therapeutic interventions.

Our third study identifies FF EVs derived from PCOS patients that carry particular types of miRNA which contribute to ovarian disturbances. Different pathophysiological conditions alter the ovarian follicular environments. Therefore, it is possible to detect these changes by studying different follicular compartments such as MGC, FF, FF derived EVs and the changes in intercellular communication mediated by FF EVs. However, a more robust and specific study is required to identify how these cargos are changed and their potential for future clinical prospects.

7. CONCLUSIONS

1. The observation of study I indicates that FF and FF EVs cause gene expression changes in bovine oviductal epithelial cells.
2. The overall findings of study II demonstrated that FF EVs play a vital role in boosting sperm viability and enhancing sperm capacitation and acrosome reactions. Moreover, these observed effects are specific to FF EVs.
3. The overall finding of study III demonstrated that EVs derived from FF of PCOS patients carry particular types of miRNA, which contribute to the ovarian environment disturbance**.**

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SUMMARY IN ESTONIAN

Follikulaarsest vedelikust pärinevate ekstratsellulaarsete vesiikulite iseloomustus ja nende panus perikonseptsiooni keskkonnas

Sisukirjeldus

Viljatuse levimus on tänapäeval üheks suurimaks murekohaks. Seda on juba mitu aastakümmet peetud rahvatervise prioriteediks. Viljatust võib määratleda kui paari võimetust rasestuda ühe aasta jooksul isegi pärast sagedast kaitsmata seksuaalvahekorda. Maailmas on hinnanguliselt 180 miljonit viljatut inimest. Maailma Terviseorganisatsiooni prognooside kohaselt on viljatus 21. sajandil suuruselt kolmas ülemaailmne terviseprobleem, mis jääb alla ainult vähi ja südame-veresoonkonna haigustele. Abistav reproduktiivtehnoloogia (*Assisted Reproductive Technology* – ART), sealhulgas *in vitro* viljastamine, on oluline terapeutiline meetod viljatuse ravis. ART edukuse määr ei ole aga endiselt kõrgel tasemel. Üks peamisi põhjuseid võib olla sugurakkude kvaliteedi tõstmise ebaõnnestumine ning ka ebasobiv implatatsioonieelne keskkond. Follikulaarne vedelik (*follicular fluid* – FF) on munasarjavedelik, mis soodustab munaraku küpsemist, spermatosoidide funktsioone, perikonseptsiooni keskkonna ettevalmistamist ning ka embrüo arengut. Seetõttu saab FF kasutada viljatusega seotud haiguste (näiteks PCOS) diagnoosimiseks ja raviks ning lisaks ka meeste ja naiste sugurakkude kvaliteedi ja funktsioonide parandamiseks.

Ekstratsellulaarsed vesiikulid (EV-d) on viimastel aastatel üha enam tunnustatud kui alternatiivne rakkudevahelise suhtluse viis. EV-d on membraaniga ümbritsetud nanosuuruses osakesed, mis jagunevad kolme klassi: eksosoomid (40–100 nm), mikrovesiikulid (100–1000 nm) ja apoptootilised kehad (1–2 μm). Vaatamata nanosuurusele arvatakse, et EV-d on rakkude miniatuursed versioonid. EV-d viivad sihtrakkudesse valke, RNA-d, DNA-d ja lipiide, muutes nende fenotüüpi. EV-d on eraldatud peaaegu igat tüüpi bioloogilistest vedelikest, sealhulgas FF-st. FF-st pärinevad EV-d on hädavajalikud munarakkude küpsemiseks, viljastumiseks ja embrüo arenguks.

Käesolevas doktoritöös uurisime FF EV-de rolli prekonseptsiooni mikrokeskkonna ettevalmistamisel, selle mõju spermatosoidide elutähtsatele funktsioonidele ning ka FF-ist saadud EV-de kaasaskantavate komponentide muutusi PCOS-i patsientidel võrreldes tervete inimestega. Seetõttu uurisime ka FF EV-de mõju munajuhade epiteelirakkude geeniekspressiooni muutustele ja nende edasisele funktsioonile rakkudevahelises suhtluses ja eelkontseptsiooni mikrokeskkonna ettevalmistamisel.

Meie esimeses uuringus kogusime veiste FF-i ja veiste munajuhade epiteelirakke (BOEC). EV-d isoleeriti veiste FF-st ning teostati proovide biokeemiline iseloomustus, et kinnitada EV-de olemasolu. BOEC-i ja EV-sid kultiveeriti koos, et analüüsida FF EV-de mõju geeniekspressiooni muutustele, mis võivad

aidata kaasa perikonseptsiooni mikrokeskkonna ettevalmistamisele. Samamoodi hindasime teises uuringus erinevatest allikatest ja liikidest saadud EV-de rolli spermatosoidide elujõulisusele, kapatsitatsioonile ja akrosoomireaktsioonile. Lisaks iseloomustasime ja analüüsisime kolmandas uuringus, kuidas EV-de kaasaskantavad komponendid muutuvad erinevates patofüsioloogilistes tingimustes (PCOS) ja EV-de mõju munasarjade mikrokeskkonnale.

Doktoritöö eesmärgid

Töö üldine eesmärk oli uurida FF-i ja FF EV-de potentsiaalset rolli sugurakkude küpsemises, viljastumises ja embrüo implantatsioonieelse mikrokeskkonna ettevalmistamisel.

Doktoritöö täpsed eesmärgid olid järgmised:

1. Eraldada ja iseloomustada veise FF-st pärinevaid EV-sid ning analüüsida geenide erinevat ekspressiooni veise primaarsetes munajuha epiteelirakkudes vastusena FF-le ja FF-st saadud EV-de lisamisele. Analüüsime ka seda, kuidas geenikspressiooni muutused võivad mõjutada perikonseptsiooni mikrokeskkonda, viljastumist ja embrüo arengut.
2. Uurida erinevatest organismidest (veise FF, sea FF ja JAr rakud) eraldatud EV-de lisamise mõju spermatosoidide elujõulisusele, kapatsitatsioonile ja akrosoomireaktsioonile.
3. Eraldada ja iseloomustada tervetest ja PCOS-i põdevate inimeste FF-st saadud EV-sid ning analüüsida nende kaasaskantavate komponentide, miRNAde, erinevusi kasutades kõikehõlmavaid genoomi meetodeid (*comprehensive genomic manner*), et selgitada välja PCOS-i rolli munasarjade signaali häiretesse.

Materjalid ja metoodid

Esimeses uuringus kogusime tapamajast veiste munajuhasid (Holstein) koos munasarjadega, et isoleerida veiste munajuhade epiteelirakud (*bovine oviductal epithelial cells* – BOEC). Katse jaoks koguti ainult 1. staadium (0–4 päeva pärast ovulatsiooni) munajuhasid. BOEC-d pigistati õrnalt munajuha koest välja, pesti ja seejärel kultiveeriti edasisteks katseteks. Samamoodi koguti FF-i tapamaja materjalidest EV-de isoleerimiseks. FF EV-d eraldati käsitsi täidetud suuruseralduskromatograafia kolonnide abil. Eraldatud EV-d iseloomustati nanoosakeste jälgimise analüüsi (*nanoparticle tracking analysis* – NTA), Western blot (WB) ja transmissioonelektronmikroskoopia (TEM) abil. Analüüsiti FF-i ja FF-ist saadud EV-de mõju BOEC-de geenikspressioonile. BOEC rakukultuure inkubeeriti 6 ja 24 tundi kolmes erinevates rühmades – koos FF, FF-ist saadud EV-d ja kontrollrühm. Pärast vastavaid inkubatsiooniperioode eraldati kogu BOEC RNA ja kasutati RNA sekveneerimiseks.

Teises uuringus eraldati FF väikestest (läbimõõt 2–6 mm), keskmisest (6–9 mm läbimõõduga) ja suurtest folliikulitest (läbimõõt >9 mm) EV-de isoleerimiseks. EV-de isoleerimiseks koguti ka sea FF ja JAr konditsioneeritud

söödet. Sarnaselt esimese uuringuga järgisime EV-de isoleerimiseks ja iseloomustamiseks sama protokolli. Analüüsiti EV-de mõju spermatosoidide elujõulisusele, kapatsitatsioonile ja akrosoomireaktsioonile. Veiste spermatosoididele lisati erinevatest organismidest pärinevaid EV-sid ja inkubeeriti 0 ja 4 tundi. Pärast inkubatsiooni analüüsiti EV-de mõju spermatosoidide elujõulisuse, kapatsitatsiooni ja akrosoomi reaktsiooni muutuste järgi. Arvutati ka antud omaduste protsentuaalsed muutused.

Kolmandas uuringus kogusime inimese FF-i PCOS-i patsientidelt ja viljakatelt naistelt. EV-d eraldati qEVsingle® suuruseralduskromatograafia kolonnide abil ja isoleeritud EV-sid iseloomustati nagu eelmistes katsetes. PCOS-i patsientide ja viljakate naiste FF-st saadud EV-de miRNA-de erinevuste analüüsimiseks teostati miRNA eraldamine miRNeasy Micro komplekti (QIAGEN) abil. Diferentsiaalse miRNA ekspressioonianalüüsi tulemuste põhjal viidi läbi täiendav valideerimine, kasutades RT-qPCR-i.

Tulemused

Esimeses uuringus kinnitasid EV iseloomustamise meetodid EV esinemist veiste FF-is. NTA analüüs kinnitas, et veiste FF-st eraldatud EV-d olid alla 350 nm, mis on EV-de suurus. Samamoodi näitas WB analüüs, et isoleeritud EV-d olid CD63 EV markeri suhtes tugevalt positiivsed. Samal ajal täheldati apoA-I puhtusemarkeriga kerget signaali. WB analüüs kinnitab FF-st saadud puhaste EV-de olemasolu. Veelgi enam, TEM-analüüs kinnitas tassikujuliste EV-de olemasolu prepareeritud EV proovides.

RNAseq analüüs näitas, et nii FF kui ka FF EV-d suutsid muuta BOEC-de geeniekspressiooni. Geeniekspressiooni profiil näitas, et FF-raviga ekspresseeriti erinevalt mõlemal ajahetkel 9678 geeni. Samal ajal ekspresseeriti erinevalt FF EV-raviga ainult 41 geeni. Huvitaval kombel näitas meie analüüs, et enamik geene ekspresseeriti 24-tunnise FF-raviga. *Pathway enrichment* analüüs kinnitas 4 statistiliselt olulist (FDR $\leq$ 0,05) rada: ribosoomide ja ribosoomide biogenees, arahhidoonhappe metabolism ja munasarjade steroidogenees, mille FDR oli $\leq$ 0,1 6-tunnise FF-raviga.

Lisaks andis GSEA tulemuseks 50 statistiliselt olulist (FDR $\leq$ 0,05) rada 24-tunnise FF-ravi ajal. Eelkõige rikastati oksüdatiivset fosforüülimist ja termogeneesi ülesreguleeritud geenidega. Seevastu Ras signalisatsioonirada oli rikastatud allareguleeritud geenidega. Kuid GSEA ei näidanud FF EV-de ravimisel statistiliselt olulisi radu. Need tulemused viitavad sellele, et BOEC-de geeniekspressioon sõltub ajafaktorist ning lisatud komponentidest.

Teises uuringus kirjeldati ka EV-sid, et kinnitada nende füüsikalisi ja biokeemilisi omadusi. NTA analüüs näitas suuremate EV-de populatsiooni olemasolu kõigis proovides. Me ei täheldanud EV-de suurusprofiili erinevusi erinevatest organismidest pärinevate EV-de puhul. Siiski kontsentratsiooni osas täheldasime, et väikeste folliikulite vedelikust eraldatud EV-d sisaldavad rohkem EV-sid võrreldes keskmiste ja suurte folliikulite vedelikust pärinevate EV-dega. Me täheldasime samu tulemusi sigade FF EV-de ja JAr EV-de osas.

WB analüüs kinnitas positiivsete EV-markerite olemasolu kõigis EV-de proovides. TEM-analüüs näitas ka kõigis proovides esinevaid tassikujulisi EV-sid.

Nende organismide põhjal täheldasime EV-de erinevat mõju spermatosoidide elujõulisusele, kapatsitatsioonile ja akrosoomireaktsioonidele. Vaatamata folliikulite suuruse erinevusele suutsid kõik FF EV-d suurendada elujõulisust, kapatsitatsiooni ja akrosoomireaktsiooni. Siiski täheldati väikestest folliikulitest pärinevate EV-de puhul suuremat mõju kui keskmiste ja suurete folliikulite puhul. Samal ajal ei täheldatud sigade FF EV-del ja JAr EV-del mingeid muudatusi. Need tulemused viitavad sellele, et ainult FF EV-d võivad spermatosoidide funktsioone moduleerida.

Kolmandas uuringus iseloomustasime inimese FF-st pärinevaid EV-sid. NTA analüüs ei näidanud EV suuruses erinevusi. Siiski näitas see olulist erinevust EV kontsentratsiooni osas. PCOS-i patsientide FF EV-d sisaldavad suuremat arvu EV-sid kui viljakate naiste FF EV-d. WB analüüs näitas, et mõlemal on positiivsed EV-markerid (CD63, CD81, CD9) ja puuduvad albumiini, Grp94 ja apoA-1 puhtuse kontrollmarkerid. WB kinnitas, et meie isoleeritud EV-d olid puhtad ja valkude vabad. Samamoodi kinnitas TEM-analüüs tassikujuliste EV-de olemasolu.

FF, FF EV-de ja granuloosrakkude (*mural granulosa cells* – MGC) miRNA-de analüüs tuvastas 1525 ainulaadset miRNA-d. Kõik proovitüübid omavad suure osa kõige olulisematest miRNA-dest ja seda isegi 20 kõige enam esindatud järjestuse hulgas (EV ja FF jagavad 15, FF ja MGC jagavad kaheksat 20-st). Põhikomponentide analüüs (PCA) näitas, et kõik proovid olid rühmitatud eraldi. FF ja MGC proovide võrdlus andis tulemuseks 159 erinevalt ekspresseeritud miRNA-d (FDR < 0,05). Võrdluseks näitas EV ja FF proovide DE analüüs 135 miRNA statistiliselt olulist ekspressiooni (FDR < 0,05).

Huvitaval kombel ekspresseeriti kõigis rühmades ühiselt 93 miRNA-d. Need tulemused näitasid, et miRNA ekspressioonimuutused analüüsitud osade vahel viitavad erinevatele sekretsioonimehhanismidele: pidevalt suureneva tasemega miRNA-d on tõenäolisemalt pakitud spetsiifiliselt EV-desse. Seevastu FF-s suurima arvukusega miRNA-d sekreteeritakse tõenäolisemalt teistes makromolekulaarsetes kompleksides rakuvälisesse ruumi. Samuti valideerisime RT-qPCR abil 13 erinevalt ekspresseeritud miRNA-d. Meie analüüs pakkus välja ka ühe potentsiaalse uue miRNA järjestuse (CCUGGGCAUGGGACUGG) miRDeep2 algoritmi järgi.

Arutelu

See projekt viidi läbi selleks, et uurida FF-st pärinevate EV-de potentsiaalset rolli reproduktiivhaiguste terapeutise ja diagnostilise biomarkerina ning kuidas muutuvad EV-de molekulaarsed ja füüsikalised omadused erinevate organismide ja patofüsioloogiliste seisundite korral.

Meie uuringutes FF-is EV-de olemasolu kinnitamine võimaldab edasi uurida nende rolli sugurakkude kvaliteedi parandamisel, mikrokeskkonna ettevalmistamisel ja edukal implantatsioonieelsel embrüo arengus. Erinevatest allikatest

(väikesete, keskmiste ja suurte folliikulite FF) ja liikidest (inimene, veis ja siga) eraldatud EV-de suurus ja kontsentratsioon annavad meile ülevaate, et erinevatel EV-del võivad olla erinevad kaasaskantavad komponendid ning võib olla ka erinev mõju sugurakkude funktsioonidele ja perikontseptsiooni mikrokeskkonnale. Meie uuringud näitasid, et veiste väikeste folliikulite EV-de kontsentratsioon on suurem kui keskmiste ja suurte veisefolliikulite puhul. Samuti on PCOS-i patsientide FF EV-de kontsentratsioon kõrgem kui viljakate naiste FF EV-del.

Meie uuringutes ilmnesid erinevused EV kaasaskantavate komponentide osas kaudselt. Meie esimese uuringu eesmärk oli uurida veiste munajuhade epiteelirakkude (BOEC) transkriptsioonilisi muutusi vastusena veiste FF EV-de ja FF-iga täiendamisele. Tulemused näitasid, et BOEC-de transkriptsioonilised muutused olid rühmade vahel täiesti erinevad. Need muutused mõjutavad positiivselt implantatsioonieelse mikrokeskkonna ettevalmistamist.

Teises uuringus uurisime, kuidas erinevad EV-de allikad ja liigid mõjutavad veiste spermatosoidide elujõulisust, kapatsitatsiooni ja akrosoomireaktsiooni. Meie uuring näitas, et väikestest veiste folliikulitest pärinevad EV-d mõjutavad veise spermatosoidide elujõulisust, kapatsitatsiooni ja akrosoomireaktsiooni rohkem kui keskmistest ja suurtest folliikulitest pärinevad EV-d.

Lisaks kinnitasid meie tulemused, et need mõjud on liigispetsiifilised, kuna sigade FF EV-d ja JAr EV-d ei mõjuta spermatosoidide funktsioone. Siiski on vaja täiendavaid uuringuid selle testimiseks inimproovidega, et uurida praeguste leidude järjepidevust liikide lõikes. Sellisel juhul oleks tulemustel suurem mõju nende leidude ülekandmisel inimese ART praktikasse.

Meie teise uuringu tulemused viivad meid võimaluseni, et EV-de kaasaskantavad komponendid muutuvad sõltuvalt nende päritolust ja patofüsioloogilistest tingimustest. Seetõttu uurisime oma viimases uuringus, kuidas EV-de kaasaskantavad komponendid muutuvad erinevates patofüsioloogilistes tingimustes (PCOS ja viljakad naised) ja nende panust munasarjade signaali häiretesse. miRNA-de analüüs näitas, et PCOS-i naistelt pärinevad EV-d kannavad erinevat miRNA-lasti võrreldes tervete naiste FF-st saadud EV-dega. Meie tulemused kinnitasid ka uudse miRNA, mida kandsid PCOS-i naisetelt saadud FF EV-d. Antud miRNAd võiks kasutada PCOS-i diagnoosimise potentsiaalse biomarkerina.

Kokkuvõte

Kokkuvõtteks kinnitasid meie uuringud EV-de olemasolu FF-is ja nende mõju sugurakkude küpsemisele, funktsionaalsuse paranemisele ja osalemisele perikonseptsiooni mikrokeskkonna ettevalmistamisel. Meie tulemused näitasid, et FF EV-d võivad muuta BOEC-de geeniekspressiooni, mis mõjutab perikonseptsiooni mikrokeskkonda, mis omakorda võib aidata viljastumist ja embrüo arengut. Lisaks toetavad meie tulemused hüpoteesi, et erinevad EV allikad ja liigid kannavad erinevaid komponente ja neil on erinev mõju spermatosoidide funktsioonidele. EV kantavad komponendid varieeruvad sõltuvalt patofüsioloogilisest olukorrast, mis viitab võimalusele, et EVsid saab kasutada diagnostiliste biomarkeritena.

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PUBLICATIONS

CURRICULUM VITAE

Name:	Mohammad Mehedi Hasan
Date of birth:	7th of May 1992
Address:	Institute of Biomedicine and Translational Medicine University of Tartu,Ravila 14B, 50411, Tartu, Estonia
E-mail:	mehedi.hasan@ut.ee, rajibmehedi3@gmail.com
Telephone:	+44(0)7544856584

Tertiary education

2018–to date	PhD (Medicine), Faculty of Medicine, University of Tartu, Estonia
2016–2017	Master of Pharmacy (M.pharm), Department of Pharmacy, State University of Bangladesh
2012–2016	Bachelor of Pharmacy (B.Pharm), Department of Pharmacy, Primeasia University of Bangladesh

Employment

2022–to date	Research fellow, Institute for Women's Health, Maternal and Fetal Medicine Department, University College London (UCL), London, UK
2021–2022	Specialist, Estonian University of Life Sciences, Tartu, Estonia
2018–2021	Specialist, University of Tartu, Tartu, Estonia
2017–2018	Production Pharmacist, Drug International Ltd, Dhaka, Bangladesh
2017–2017	Scientific officer, SUNMAN-BIRDEM Pharma Ltd, Dhaka, Bangladesh

Membership in professional organizations

Member of the Society of Reproduction and Fertility, UK
Member of Uk society for extracellular vesicles, UK

List of publications

1. Hasan, Mohammad Mehedi, Janeli Viil, Freddy Lättekivi, James Ord, Qurat Ul Ain Reshi, Kersti Jääger, Agne Velthut-Meikas et al. "Bovine follicular fluid and extracellular vesicles derived from follicular fluid alter the bovine oviductal epithelial cells transcriptome." International journal of molecular sciences 21, no. 15 (2020): 5365.
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ELULOOKIRJELDUS

Nimi: Mohammad Mehedi Hasan
Sünniaeg: 7th of May 1992
Aadress: Bio- ja siirdemeditsiini instituut,
Tartu Ülikool, Ravila 14B, 50411 Tartu, Eesti
E-post: mehedi.hasan@ut.ee, rajibmehedi3@gmail.com
Telefon: +44(0)7544856584

Hariduskäik

2018–	PhD (Meditsiin), Arstiteaduskond, Tartu Ülikool, Eesti
2016–2017	Farmaatsia magister (M.pharm), Farmaatsia instituut, Bangladeshi osariigi ülikool, Dhaka, Bangladesh
2012–2016	Farmaatsia bakalaureus (B.Pharm), Farmaatsia instituut, Primeasia ülikool, Dhaka, Bangladesh

Töökogemus

2022–	Teadur, Naiste Tervise Instituut, Emade ja Loote Meditsiini osakond, University College London (UCL), London, UK
2021–2022	Spetsialist, Eesti Maaülikool, Tartu, Eesti
2018–2021	Spetsialist, Tartu Ülikool, Tartu, Eesti
2017–2018	Tootmisapteeker, Drug International Ltd, Dhaka, Bangladesh
2017–2017	Teadusametnik, SUNMAN-BIRDEM Pharma Ltd, Dhaka, Bangladesh

Liikmelisus

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