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KEERTHIE DISSANAYAKE

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Author's contribution to the original publications:

Study I
Developed the concept; designed the experiments; experimented for the optimum EV isolation system for single embryo derived extracellular vesicle (EV) isolation; carried out EV isolation from embryo conditioned media samples; Nanoparticle Tracking Analysis of isolated EVs; sample preparation for EV characterization by EV Array, Transmission Electron Microscopy and Scanning Electron Microscopy; Data analysis; writing the manuscript

Study II
Developed the concept: designed the experiments; isolation of EVs from embryos conditioned media; Culture of primary bovine oviductal epithelial cells, EV supplementation and RNA extraction from oviductal cells; carried out RT-qPCR; writing the manuscript

Study III
Contributed to the design of the experiments; contributed to the experiments involving JAr Spheroid formation and RT-qPCR experiments

ABBREVIATIONS

ART	assisted reproduction technology
BOEC	bovine oviductal epithelial cell
BSA	bovine serum albumin
CI	confidence interval
COCs	cumulus-oocyte complexes
CPM	counts per million
DE	differential gene expression
DPBS	Dulbecco's phosphate-buffered saline
ECM	extracellular matrix
EMMs	estimated marginal means
EMT	epithelial-mesenchymal transition
EVs	extracellular vesicles
FBS	foetal bovine serum
FDR	false discovery rate
GPCR	G-protein coupled receptor
GSEA	gene set enrichment analysis
hCG	human chorionic gonadotrophin
HEK293	human embryonic kidney cells
ICSI	intracytoplasmic sperm injection
IFN-τ	interferon tau
ISG-15	interferon-stimulated gene 15
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* embryo production
lincRNA	long intergenic non-coding RNA mRNA messenger RNA
LMM	linear mixed models
log2FC	log2 fold change
miRNA	microRNA
MX1	MX Dynamin Like GTPase 1
niPGT	non-invasive pre-implantation genetic testing
NP	nanoparticles
NTA	nanoparticle tracking analysis
OAS1Y	2'-5'-oligoadenylate synthetase 1
PA	parthenogenetic activation
PGT	preimplantation genetic testing
qPCR	quantitative polymerase chain reaction
REML	residual maximum likelihood
SEC	size-exclusion chromatography
SEM	scanning electron microscopy
SET	single embryo transfer
SOFaaci	Synthetic Oviduct Fluid with amino acids and myo-inositol

TEM	transmission electron microscopy
UC	ultracentrifugation
UF	ultrafiltration
ZP	zona pellucida

1. INTRODUCTION

Infertility is a global health problem that affects millions of couples in their reproductive age. However, its effects are not limited to the affected couples but extend to the families and society. Assisted Reproductive Technology (ART), including *in vitro* fertilization, is an important therapeutic modality for managing infertility.

Although four decades have passed since the birth of the first IVF baby, and considerable advancements in the embryo culture systems have been reported, the pregnancy and live birth rate following the ARTs are still not up to the expectations. One of the bottlenecks in this regard is identifying the best embryos with the highest implantation potential. Several morphology-based embryo grading methods and other methods based on embryo morpho-kinetics and embryo biopsy are used in clinical practice. Furthermore, many studies are ongoing on non-invasive evaluation of embryo quality and predicting transfer success. However, as all these methods have limitations, the research for identifying better or complementary tools for assessing embryo viability and predicting embryo transfer success continues.

Extracellular vesicles (EVs) are lipid bilayered nanoparticles secreted by almost all types of cells under different pathophysiological conditions. These vesicles carry a molecular cargo that includes proteins, nucleic acids including mRNA and miRNA, and lipids. They are known to be involved in mediating intercellular communication by different mechanisms and can affect the phenotype of the recipient cells. Most importantly, they can reflect and represent the pathophysiological status of their cells of origin. Thus, a biological entity such as EVs with such profound characteristics may be an excellent source of biomarkers.

Studies have shown that *in vitro* produced preimplantation embryos can release EVs to the culture media. However, previous studies have not studied the association of embryo-derived EVs with embryo quality in individual culture systems based on all embryo developmental stages. Knowing that EVs can alter the phenotype of the recipient cells, investigating the effects of embryo-derived EVs in mediating embryo-maternal communication is an exciting research area of investigation. Furthermore, such studies would give rise to indirect methods of detecting the signals sent by embryos to their outer space, hence indirectly sensing the embryo quality.

In this thesis, we investigated the pre-implantation embryo-derived EVs as indicators of embryo quality and their role in mediating embryo-maternal communication. Bovine embryos are considered a good model for studying human preimplantation embryonic development. Therefore, for this purpose, we opted to use *in vitro* bovine embryo culture systems. Individually cultured preimplantation bovine embryo-derived EVs were isolated from embryo conditioned media and characterized by physical and biochemical means to confirm their EV identity. Subsequently, the physical characteristics of EVs isolated from

single embryo media were compared based on the developmental stage of the embryos and their quality. The functionality of embryo-derived EVs was evaluated by supplementing embryo-derived EVs to oviductal epithelial cell cultures and analysing the differential gene expression. Here the aim was to understand the EV mediated embryo-maternal communication at the embryo-oviductal interface. Similarly, EV mediated embryo-maternal communication at the embryo-endometrial interface and its specificity was also evaluated using a human cell culture model of embryo-maternal communication.

2. LITERATURE REVIEW

2.1. Infertility and Assisted Reproduction Technology (ART)

2.1.1. Infertility

Infertility is a disorder in the male and female reproductive systems, and the World Health Organization (WHO) defines infertility as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009). It is a global public health issue affecting millions of people who are in their reproductive age. Infertility causes a significant impact on the affected families and communities. According to the predictions of the WHO, infertility will be the third-most severe global health problem in the 21st century, after cancer and cardiovascular diseases.

Infertility can arise due to various pathophysiological derangements of either the female or the male partner, or both (Carson & Kallen, 2021). In females with infertility, ovulatory disorders, tubal obstructions and endometriosis are the leading causes (Table 1). Ovarian causes attributes to ovulatory disorders, including anovulation, and accounts to approximately 25% of infertility diagnoses in women (Carson & Kallen, 2021). Polycystic ovary syndrome (PCOS) is the most common cause of anovulation and affects 70% of women with that condition. Tubal causes attributes to infertility diagnoses between 20% (Deshpande & Gupta, 2019) and 67% (Audu et al., 2009), depending on the studied population. In tubal factor infertility, either the blockage of the uterine tubes or their failure to pick up the oocyte from the pelvic cavity is observed. Endometriosis is another common cause of infertility in which endometrial-like tissues are found outside the uterine cavity. It induces chronic inflammation hence developing scar tissue and adhesions that alter the pelvic anatomy. Endometriosis was found to affect infertile women ranging from 20–50% (Tanbo & Fedorcsak, 2017). However, the extent of the effects of these factors on subfertility is variable depending on multiple factors.

Table 1. Categorization of the female factor infertility casuses – based on anatomical site

Ovarian; primarily causing ovulatory disorders	**Tubal;** primarily affecting gamete and embryo transport	**Uterine/cervical;** primarily uterine factors lead to implantation failure
Diminished ovarian reserve	Pelvic inflammatory disease	Endometriosis
Premature ovarian failure	Past ectopic pregnancies	Fibroids
Endocrine disorders (ex. PCOS, hyper and hypothyroidism	Past Tubal surgeries	Congenital uterine anomalies
	Certain STIs	Deranged cervical mucus
	Endometriosis	Infections

PCOS: polycystic ovarian syndrome, STI: sexually transmitted infections

The diagnosis of male infertility is generally made by identifying one or several factors that comprise deranged semen quality or functional parameters of sperms. Such factors include structural, functional, endocrine, genetic, or immunological defects of the male reproductive system or sexual dysfunctions limiting the deposition of semen in the vagina (Schlegel et al., 2021a). When investigated for the male factor infertility, a cause for infertility was found only in ~40–64.4% of the cases, leaving ~35.6%–60% of the men diagnosed as idiopathic infertile (Olesen et al., 2017; Punab et al., 2017). When investigating the couple together, males factor infertility, while solely responsible for 20–30% of infertility cases, overall, contributes to nearly 50% of the infertility cases indicating the significant contribution of male factors for overall infertility (Palani & Alahmar, 2020). Many different causes and risk factors can lead to the male factor infertility, and these factors can be broadly classified as congenital causes, acquired causes and idiopathic risk factors (Agarwal et al., 2021). Table 2 broadly categorizes the causes and risk factors of male infertility.

Table 2. Causes/risk factors of male factor infertility

Congenital causes	Acquired causes	Idiopathic risk factors
Congenital absence of vas deferens	Varicocolele	Smoking
Anorchia/cryptorchidism	Testicular trauma	Alcoholism
Genetic causes; chromosomal aberations, monogenic, microdeletions of Y chromosome	Testicular torsion	Obesity
	Hypogonadotrophic hypogonadism	Exposure to toxins (environmental/ occupational)
Defects in hypothalamo-pitiutary-gonadal axis	Recurrent infections of the urogenital tract	Use of psychoactive drugs
Congenital obstruction of the seminal tract	External factors (heat, chemotherapy, medicines)	Advanced paternal age
Primary testicular failure	Sexual dysfunction	Psychosocial stress
	Anti-sperm antibodies	Diet

(Agarwal et al., 2021; Laan et al., 2021; Olesen et al., 2017)

A woman who has failed to achieve pregnancy after 12 months of regular unprotected sexual intercourse needs to be evaluated for infertility. As a couple can have multiple factors leading to infertility, concurrent evaluation of both partners is required (Penzias et al., 2021; Schlegel et al., 2021a). However, earlier evaluation is recommended in the following conditions; women over 35 years who failed to become pregnant after six months, women aged over 40 years, women with menstrual disorders, uterine, tubal and peritoneal disorders, stage III and IV endometriosis, and men with suspected or known infertility status (Carson & Kallen, 2021). Identifying the cause of infertility is crucial as it supports decision making on the treatment option. The initial evaluation of the partners involves general history taking (e.g., identifying the duration of the

problem and the risk factors involved) and physical examination. This initial evaluation guides subsequent testing. However, all infertility evaluations do not end up in finding a cause for infertility where both male and female partners seems apparently normal based on the findings of their evaluations. Such situations are referred to as "unexplained infertility" (Mol et al., 2018). Unexplained infertility is a diagnosis made by excluding all possible causes of infertility and may be the case in about 40% of infertile couples (Buckett & Sierra, 2019; Mol et al., 2018).

Management of infertility would demand higher financial, physical, psychological and time commitments. Table 3 summarises the investigations and treatment options for the main causes of infertility in women.

Table 3. Main causes of infertility among females, suggested investigations and treatments

	Aetiology	**Suggested investigations/testing**	**Treatment options**
Ovulatory disorders	Polycystic ovarian syndrome	TVS; free and total testosterone; DHAES; 17-OHP	PCOS: ovulation induction (if obese-weight loss)
	Thyroid disorders	TSH	Correction of the specific defect
	Hyperprolactinaemia	Prolactin	Correction of the specific defect
	Hypothalamic amenorrhoea	FSH LH Oestradiol	Hypogonadotropic hypogonadism: use pulsatile GnRH; Hypogonadotropic hypogonadism: use donor oocytes
Tubal obstruction	Endometriosis Sexually transmitted infections Hydrosalpinx Peritubal adhesions	Hysterosalpingogram Laparoscopy with chromotubation	Surgical repair; IVF/ICSI
Endometriosis	Not certain	TVS	Ovulation induction; IVF/ICSI
Diminished ovarian reserve	Ageing	AMH Antral follicle count (by USS) FSH/Oestradiol	Oocyte donation
Uterine causes	Polyps Fibroids Uterine synechiae	TVS Sonohysterogram 3D- USS, MRI	Hysteroscopic resection of the lesion

PCOS – polycystic ovarian syndrome, DHAES – Dehydroepiandrosterone; 17-OHP-17-hydroxyprogesterone, TSH – Thyroid-Stimulating Hormone, FSH – Follicle-Stimulating Hormone, LH – Luteinizing Hormone, GnRH – Gonadotropin-releasing hormone, AMH – Anti-Müllerian Hormone TVS: transvaginal ultrasound scan, MRI – Magnetic resonance imaging, USS – ultrasound scan

In terms of evaluation of men, both the American Society of Reproductive Medicine (ASRM) and the Americal Urological Association (AUA) recommend the initial assessment of the patient based on reproductive history taking, physical examination and at least two semen analysis reports, particularly when the first shows abnormal parameters (Schlegel et al., 2021a).

Semen analysis would mainly show sperm parameters such as concentration, motility and morphology, among others. Complete absence of sperms during semen analysis is referred to as azoospermia which can be either be obstructive azoospermia (OA) or non-obstructive azoospermia (NOA). Furthermore, if the sperm counts are lower than 39 million/ejaculate, it is referred to as oligozoospermia (World Health Organization, 2010). Subsequent management would rely on the findings of these initial evaluations (Table 4).

Table 4. Further investigations, following the initial semen analysis, and treatment options for specific male factor infertility issues

Additional investigations/testing	**Treatment options**
Karyotype and Y-chromosome AZF microdeletion analysis -for NOA or severe oligozoospermia	For OA -Surgical reconstruction or epididymal or testicular sperm retrieval for ICSI
CFTR gene mutation testing -for congenital absence of vas deferens with OA	For NOA -Hormonal therapy if hormonal derangements were identified such as hypogonadotropic hypogonadism -Varicocelectomy if clinical varicocele and abnormal semen analysis findings (limited evidence) -Testicular sperm extraction (TESE) and ICSI -Sperm donation/adoption
FSH and Testosterone -for oligzoospermia/azoospermia and impaired sexual function	
Scrotal ultrasound scan -for anatomical derangements of the reproductive system	
Sperm DNA fragmentation test, anti-sperm antibody test and testicular biopsy -considered in certain situations. These are not performed routinely	

OA – obstructive azoospermia: NOA – non-obstructive azoospermia: CFTR – cystic fibrosis transmembrane conductance regulator: AZF – azoospermia factor

Overall, the treatment strategy for infertility relies on the cause, patient age, duration of infertility and patient preferences. The commonly used treatment strategy for infertility is ovulation induction followed by timed intercourse or intrauterine insemination (IUI) to fertilize the oocytes *in vivo* (Brown & Farquhar, 2016). The other strategy sequentially involves the ovarian stimulation by pharmacological means to induce mature multiple ovarian follicles, ovulation induction, oocyte aspiration, fertilizing them with sperms *in vitro* (IVF) or by intracytoplasmic sperm injection (ICSI) to produce embryo *in vitro* and subsequent transfer (Alper & Fauser, 2017). The latter *in vitro* interventions constitute assisted reproduction technologies (ARTs) in the management of infertility (Farquhar & Marjoribanks, 2018).

2.1.2. Assisted Reproduction Technology (ARTs)

International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) defined ARTs as "all treatments or procedures that include the *in vitro* handling of both human oocytes and sperms or of embryos for the purpose of establishing a pregnancy" (Zegers-Hochschild et al., 2009). Commonly used types of ARTs include IVF and ICSI (Table 5). However, artificial insemination using sperms obtained from either a woman's partner or donor sperm is not considered a type of ART.

Most ART services are used by women in the age range of 30–39. Based on the currently available data, Europe is the leading user of ARTs globally, accounting for nearly 50% of all the reported ART cycles. Approximately 940,503 treatment cycles were conducted in 39 European countries in 2017 (Gliozheni et al., 2021). In contrast, in the same year, 284,403 cycles in USA (Sunderam et al., 2020), and 82,215 cycles in Australia and New Zealand were reported. Furthermore, the number of cycles carried out in many developed countries has grown by 5–10% per annum during the last few years. Regarding the availability of ART services (cycles per million population), Nordic countries and Belgium are leading (Gliozheni et al., 2020). Furthermore, over 4% of newborns were derived from ART-based treatments in Estonia, Austria, Belgium, and Slovenia. In comparison, this figure was only slightly over 1% in the USA.

Despite many different types of oocyte insemination methods have been evolved, conventional IVF has been the most consistent and widely used method in ART practice. It is not merely adding sperms to oocyte samples but requires many different pre-requisites to be met at the time of insemination for the fertilization to occur correctly and subsequent embryo development. These factors include the presence of capacitated sperms, their subsequent acrosome reaction, the presence of mature, unaged oocytes, and optimum culture conditions (Swain, 2015). Fertilisation is the key and rate-determining step in IVF.

With tremendous developments in ARTs, including better gamete handling and the use of improved media, the fertilization rates have reached 70–80%. As a result, fertilization rates over 60% is considered a key performance indicator of ART laboratories (Embryology & Medicine, 2017). However, total fertilization failure is still a risk where the failure of fertilization of all the oocytes in a cycle takes place and is reported to occur in 3.5–20% of the IVF cycles but as low as 1–3% in ICSI cycles (Lee et al., 2017). However, IVF is a less invasive procedure and cheaper than ICSI and does not require more advanced instrumentation.

ICSI was introduced to treat couples with male factor infertility (Halliday, 2012). Although ICSI per se does not correct the cause/s that leads to male factor infertility, it has shown to be very effective when natural conception does not occur due to male factor infertility. This procedure can overcome the limitations of the quality of the sperm samples due to lower concentration, motility and morphology as long as viable sperms to inject into the oocytes exist (Schlegel et al., 2021b). In the case of male factor infertility, ICSI has been

recommended for azoospermia, severe oligoasthenoteratozoospermia (OATs), cryptozoospermia, absolute asthenozoospermia and sperm DNA fragmentation, among others (Esteves et al., 2018). However, ICSI has been increasingly used for treating many couples with non-male factor infertility (Quaas, 2021). Unexplained infertility, poor-quality oocytes, advanced maternal age and preimplantation genetic testing are examples of such situations where ICSI is performed. Recently, a study by Song et al. showed that ICSI procedure, compared to conventional IVF, does not increase the live birth rate in couples with unexplained infertility (Song et al., 2021). Lee et al. compared the clinical outcomes between IVF and ICSI in split insemination cycles. They did observe increased total fertilization failure in IVF compared to ICSI. However, embryo quality before transfer or pregnancy outcomes did not differ between the ICSI and IVF (Lee et al., 2017). Therefore, regular use of ICSI for all couples with infertility, irrespective of the cause, is not recommended.

Based on ARTs, nearly 9 million babies have been born globally during the past four decades (Mandal, 2018). However, despite recent advances in ARTs, the quality of the *in vitro* produced embryos is lower than their *in vivo* counterparts (Ferré et al., 2020). Furthermore, the success of ARTs is not up to the expectations. It is reported that the success of ARTs is about 30% for IVF and 28% for ICSI in Europe (De Geyter et al., 2018). Hence, more research seems to be needed to optimize the current strategies of ARTs.

The production of embryos *in vitro* by ART in human and animals have multiple applications in addition to treating human infertility. They include research in preimplantation embryonic development, improving domestic animals' reproductive efficiency in the livestock industry, and conserving rare animals and biodiversity (Choudhary et al., 2016; Niakan et al., 2012).

The most researched methods of improving the ART success are the development of embryo culture systems (Castillo et al., 2020), developing strategies to evaluate the embryo quality and predicting the best *in vitro* produced embryo for uterine transfer (Mizuno et al., 2021), and assess the endometrial receptivity (Robert, 2020). Altogether, the findings of these studies would facilitate the elective single embryo transfers (eSET), thus preventing the complications of multiple gestations and reducing the time to pregnancy.

Table 5. Different modes of ARTs and their indications

ART procedure	Description	Indications
In vitro fertilization (IVF) (Siristatidis et al., 2021)	Involves ovulation induction, oocyte aspiration, oocyte fertilization using sperms, culture of the resulting zygote *in vitro* and embryo transfer.	As mentioned in table 3.
Intracytoplasmic sperm injection (ICSI) (Farhi et al., 2019)	Involves the injection of a single sperm to the oocyte, culture of the resulting zygote *in vitro,* and embryo transfer.	As mentioned in table 4. Male factor infertility. Failed previous IVF However, currently used for many non-male factor infertility treatments (Quaas, 2021).
Gamete intrafallopian transfer (GIFT) (Klonoff-Cohen et al., 2003)	involves oocyte aspiration and, along with sperms, transferring to the fallopian tube by laparoscopic surgery.	Natural alternative to IVF; for those who do not wish to get fertilized outside the body (religious reasons). Rarely used nowadays.
Zygote intrafallopian transfer (ZIFT) (Weissman et al., 2013)	The embryo is produced *in vitro*, and at the zygote stage, transferred to the fallopian tube laparoscopically.	Used in situations where gamete transfer is not possible through the cervix. No longer used
Third-party ART (Ardakani et al., 2021)	When conventional ART fails, rely on a third party to get pregnant. Involves either or in a combination of sperm donation, oocyte donation, embryo donation or surrogacy	Depends on the cause of infertility of the couple.

2.1.3. Evaluation of embryo quality

Development of the embryo culture systems has led to increased implantation and pregnancy rates as well as paved the way to culture *in vitro* produced human embryos up to day 6 and in other mammalian embryos, such as bovine, up to day 14 (MacHado et al., 2013). While these advances have enabled the production of multiple embryos in a given cycle, it has also facilitated the transfer of embryos to patients at any stage of pre-implantation embryonic development. From any cohort of embryos produced *in vitro,* in a given cycle, selecting the embryo having the highest developmental potential is of utmost significance. Since the beginning of IVF technology, identifying the optimum embryo for uterine transfer in human and animal-assisted reproduction was recognized as one way to improve the assisted reproductive outcome.

Furthermore, evaluating embryo quality allows the ranking of the rest of the embryos that are cryopreserved to ensure, if another embryo transfer is needed, the next best embryo is transferred (Gardner et al., 2015). Transferring a single

embryo (SET) to the mother instead of multiple embryos minimize the potential complications of multiple pregnancies such as miscarriages, gestational hypertension and diabetes, and preterm labour (Lee et al., 2016). Many embryo quality evaluation methods have been developed or under extensive research mainly based on direct embryonic parameters such as embryo morphology. While morphology-based embryo evaluation is the most commonly used method (Gardner & Balaban, 2016), morphokinetics based embryo evaluation using time-lapse microscopy (Sayed et al., 2020) and pre-implantation genetic testing (Harris et al., 2021) are used to variable extents in ART laboratories. A big focus is currently on the embryo conditioned media to identify a potential biomarker of embryo quality and predict embryo transfer success (Zmuidinaite et al., 2021).

2.1.3.1. Morphology based embryo evaluation

The classical method of evaluating the *in vitro* produced embryos, both in human and other mammalian species, has been based on embryo morphology (Figure 1) (Bó & Mapletoft, 2013; Fabozzi et al., 2016). This recognizes the embryo morphological quality and the development rate of the embryos. Over the years, many embryo grading systems have been developed to evaluate embryos' development, quality, and viability (Gardner & Balaban, 2016). However, considering the highly dynamic nature of embryos in terms of their development during the preimplantation period, such grading has become very challenging.

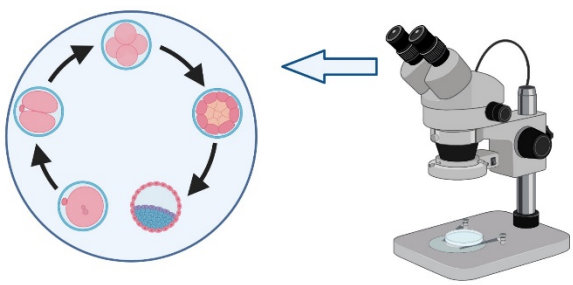

Figure 1. Morphology based evaluation of embryos. Embryo morphological quality and their development rates are assesed using this method. Created with BioRender.com

Human embryos are generally graded at day 2–3, day 4 and day 5 that corresponds to cleavage stage, morula stage, and blastocyst stage, respectively. Parameters considered to classify good quality embryos during morphological evaluation are: blastomere number; symmetry of the blastomeres; multinucleation; cleaving to two-cell stage; cellular fragments; extent of expansion of the blastocoele cavity, and the number of cells and cohesiveness of inner cell mass (ICM) and trophectoderm (TE).

Transfer of the cleavage stage (day 2 or 3) embryos had been the desired approach in the past (De Croo et al., 2020). However, this was changed gradually with the advancement of the embryo culture systems, where more advanced embryos in their blastocyst stage were transferred. The embryo evaluation at the cleavage stage is mainly based on the cell number, degree of fragmentation, multinucleation, and blastomeres' quality in terms of their size. Several professional societies such as Society for Assisted Reproductive Technology (SART) (Racowsky et al., 2010), Alpha Scientists in Reproductive Medicine, and the European Society for Human Reproduction and Embryology (ESHRE) (Balaban et al., 2011) have developed different cleavage-stage embryo grading. They are currently being used in ART practice. Table 6 illustrates the cleavage stage embryo evaluation method developed at the Istanbul consensus workshop on embryo assessment (Balaban et al., 2011). This is the joint consensus prepared by Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. In this assessment, the cell number varies depending on the evaluation day: 4 cells on Day 2 and 8 cells on Day 3.

Table 6. Day 2–3 assessment (Cleavage stage)

Grade	Rating	Description
1	Good	10% fragmentation Stage-specific cell size No multinucleation
2	Fair	10–25% fragmentation Stage-specific cell size for the majority of cells No evidence of multinucleation
3	Poor	Severe fragmentation (>25%) Cell size not stage-specific Evidence of multinucleation

Considered parameters are other than the cell number. Adapted from the Istanbul Consensus, 2011 (Balaban et al., 2011)

Among all morphological characteristics considered, cell number can be regarded as the single most vital indicator for embryo quality and viability when evaluating cleavage-stage embryos. Normal mammalian embryos progress through a predictable timeline during their development from the 1-cell to 16-

cell stage. Thus, embryos cleaving too quickly and too slowly are linked with deranged embryonic development (Burruel et al., 2014). Studies have shown the direct correlation between cell number in day-3 human embryos and developmental potential, implantation rate, and live birth rate (Kong et al., 2016). Another important morphological parameter considered at this stage is embryo fragmentation. Fragments are anucleate and extracellular structures that originated from blastomeres (Keltz et al., 2006). When scoring embryos based on fragmentation, the percentage of the embryo volume occupied by the fragments is generally considered. Embryo fragmentation is negatively correlated with the implantation rate and developmental potential of embryos (Racowsky et al., 2003). However, a study by Gallardo et al. showed that the implantation of vitrified day 3 embryos after warming was not affected by embryo fragmentation (Fernandez Gallardo et al., 2016). Asymmetry of the blastomeres in terms of their sizes is also considered an abnormality in cleavage-stage embryos. It is reported that asymmetrical cleavage of blastomeres is detrimental to implantation rates and pregnancy (Hardarson et al., 2001). However, a relatively recent study did not show an association between embryo symmetry and implantation rate (Fernandez Gallardo et al., 2016). Multinucleation (more than one nucleus within a blastomere) is also considered anomalous. A study by Balakier et al. (2016) have shown that multinucleation frequency is higher in *in vitro* cultured human embryos and equally observed in both euploid and aneuploid embryos. However, they also observed that multinucleated embryos can self-correct during early cleavage divisions and further develop to euploid blastocysts giving rise to normal babies (Balakier et al., 2016). Therefore, considering all such factors when embryo scoring would better reflect the embryo quality.

When evaluating embryo morphology at day 4, the optimum embryo is expected to be compacting or compacted involving all the embryo volume and are in the 4^{th} round of the cell cycle. Table 7 illustrates the criteria for day 4 embryo grading developed by the Alpha Scientists in Reproductive Medicine and ESHRE Special interest Group of Embryology.

Table 7. Day 4 assessment (Morula stage)

Grade	Rating	Description
1	Good	Embryo entered into the 4^{th} round of cleavage. Embryo compaction involving almost all the embryo volume.
2	Fair	Embryo entered into the 4^{th} round of cleavage. Embryo compaction involving the majority of the Embryo volume
3	Poor	Embryo compaction is disproportionate and involves less 50% of embryo volume; 2–3 cells stay as discrete blastomeres

Adapted from the Istanbul Consensus, 2011 (Balaban et al., 2011)

When evaluating the embryo morphology at day 5, the development stage of the blastocysts and the quality of the inner cell mass and trophectoderm are evaluated. According to this system, the blastocysts were categorized into three grades based on their pattern of development and morphology. The most widely used scoring system for evaluating blastocysts was introduced by Gardner and Schoolcraft (Gardner & Schoolcraft, 1999) and Gardner et al. (Gardner et al., 2000). Here, the percentage of space occupied by the blastocoel within the whole embryo is considered, along with the cell number and the organization of the inner cell mass and trophectoderm. The blastocyst grading criteria illustrated in table 5 is adapted from the Istanbul consensus and is a numerical interpretation of the method described by Gardener and Schoolcraft (Gardner & Schoolcraft, 1999).

At the blastocyst stage, the embryo is expected to be ranging from fully expanded to hatched blastocyst with a prominent, easily discernible inner cell mass (ICM) consisting of many compacted and tightly adhered cells and a trophectoderm with many cells constituting a cohesive epithelium (Table 8) (Balaban et al., 2011). A recent study by Jihui et al. (2021) showed that, in the case of frozen-thawed single embryo transfers, higher expansion stage of the blastocyst, ICM grade, and TE grade are associated with better clinical pregnancy and live birth rates and lower miscarriage rates. Furthermore, ICM grade was the strongest predictor of live birth (Ai et al., 2021).

Table 8. Day 5 assessment (Blastocyst stage)

	Grade	**Rating**	**Description**
Blastocyst development stage	1		Early blastocyst
	2		Blastocyst
	3		Expanded blastocyst
	4		Hatched/hatching blastocyst
Inner cell mass (ICM)	1	Good	Prominent ICM; clearly noticeable; multiple cells which are compacted and tightly adhered to each other
	2	Fair	Easily noticeable ICM; many cells which are loosely grouped together
	3	Poor	Poorly discernable ICM; only few cells present
Trophectoderm (TE)	1	Good	A cohesive epithelium formed by many cells
	2	Fair	A loose epithelium formed by few cells
	3	Poor	Very few cells

Adapted from the Istanbul Consensus, 2011 (Balaban et al., 2011)

Even though a decade has passed since the Istanbul consensus for morphology-based embryo evaluation was established, these classification criteria are still used as the standard practice (Sayed et al., 2020). Furthermore, the embryo grading method developed by SART is also widely used (Hossain et al., 2016). In addition to these morphology-based embryo grading systems developed by professional societies/organisations, such systems have been designed by individual countries such as Spain (Cuadros et al., 2008) and UK (Cutting et al., 2008).

Since other factors such as endometrial receptivity are also involved in successful implantation, having a higher score in such grading systems does not guarantee a pregnancy (Saxtorph et al., 2020). The embryo development is a dynamic process, and morphology per se show only a snapshot of this continuous process. Even though morphology-based embryo evaluation is still the gold standard, other dimensions of embryo developments have to be explored to further improve the clinical outcome of ARTs. Embryo morphokinetics is another method of evaluating embryo development and is currently popular in the ART practice.

2.1.3.2. Morphokinetics based embryo evaluation – Time-lapse observations

The introduction of time-lapse microscopy (TLM) systems revolutionized embryo assessment and enabled continuous monitoring of embryos' morphology and kinetic parameters (Aparicio-Ruiz et al., 2018). Time-lapse incubator systems allow a digital camera to capture images of the embryo at set and frequent time intervals. These images can be played back as a continuous time-lapse sequence, thus facilitating the monitoring of embryo development, embryo morphology, and timing of various development events. Figure 2 illustrates the morphokinetic parameters of embryo development that are considered during time-lapse microscopy.

As the embryos are evaluated while still inside the time-lapse incubators, culture systems are not disturbed, which is the problem with the conventional embryo evaluation (Lundin & Park, 2020). However, one of the limitations of the TLM system is that it does not allow the rotation of the embryos, thus limiting the morphological assessment to some degree. This can be an issue when blastomeres overlap, and a high degree of fragmentation in blastomeres is present (Dolinko & Racowsky, 2019).

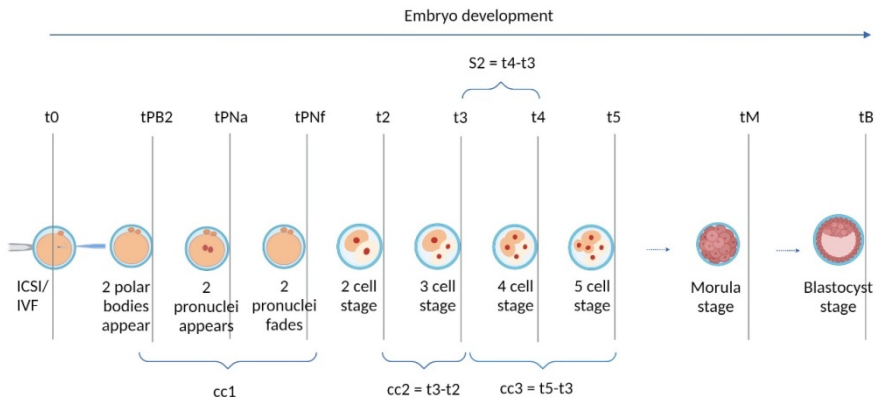

Figure 2. Morphokinetic parameters of embryo development used during time-lapse microscopy-based embryo evaluation. All the development stages are depicted. ICSI-intracytoplasmic sperm injection; tPB2-time from ICSI to the appearance of the second polar body, tPNA-time from ICSI to the appearance of pronuclei; tPNF- the time from ICSI to fading of pronuclei; t2-t5- the time from ICSI to corresponding division(2–5); tM and tB- the time from ICSI to embryo compacting into the morula stage and completing blastocyst formation respectively; cc1- first round of cleavage (t2-t0); cc2- second round of cleavage (t3-t2); S2- second synchronization parameter. Created with BioRender.com

Different algorithms have been created that can correlate the kinetic parameters of the development of early embryos with the blastocyst formation, chromosomal content, potential for implantation and the live birth rate (Aparicio-Ruiz et al., 2018). However, none of the existing algorithms is accepted universally. Adamson et al. (2016) demonstrated the benefit of combined TLM and morphology-based embryo selection in improving day 3 embryo transfers (Adamson et al., 2016). In the same year, Goodman et al. (2016) reported no added benefit of TLM for improving clinical reproductive outcomes based on a randomized controlled trial (RCT)(Goodman et al., 2016). Based on such previous findings, altogether, no solid and conclusive evidence suggests that Time-Lapse Technology improves the quality and the selection of embryos or the IVF success rates (Lundin & Park, 2020). However, a recent study using TLM and KIDScore™ algorithm, Boucret et al. (2021), showed that TLM systems could indicate certain anomalies of embryo development and predict the embryos with the highest implantation and pregnancy potential (Boucret et al., 2021). In another dimension, TLM may be useful in indicating the ploidy status of embryos. Desai et al. (2018) showed that evaluation of the morphokinetics can aid the selection of euploid embryos from blastocysts aged day 5/6 (Desai et al., 2018).

In most published studies, morphokinetic scoring is still dependant on subjective and intermittent annotations of embryo morphology and timings. Therefore, further improvements in the TLM based embryo evaluation would be dependant on new models and machine learning. In future, this would provide a

more precise and non-biased embryo selection (Blais et al., 2021; Q. Liao et al., 2021). While the subjectivity is a problem with morphology/ morpho-kinetics based embryo evaluation, non-morphological embryo assessments would be an alternative that can overcome such subjectivity.

2.1.3.3. Non-morphological evaluation of embryos

2.1.3.3.1. Preimplantation genetic testing (PGT)

Preimplantation genetic testing (PGT) is an invasive approach for evaluating embryos due to the performance of embryo biopsy to obtain nuclear material (Figure 3). Depending on the requirement, different modalities of PGTs are being described: for aneuploidy (PGT-A) (Donoso et al., 2007), for monogenic diseases (PGT-M) and for structural rearrangements of chromosomes (PGT-SR)(Harris et al., 2021). Despite different molecular techniques for genetic analysis of the biopsied materials, most clinics are increasingly using next-generation sequencing(NGS)-based screening for PGT.

An RCT by Rubio et al. showed that PGT-A in advanced maternal age dramatically decreases miscarriage rates and shortens the time to pregnancy (Rubio et al., 2017). Furthermore, a meta-analysis performed based on eight observational studies and three RCTs demonstrated significantly higher implantation rates with PGT-A (Dahdouh et al., 2015). Likewise, until recently, the available evidence supported PGT-A as a beneficial tool in increasing the live birth rates in patients with advanced maternal age. However, a recent randomized controlled trial has shown that PGT-A has no added benefit compared to morphology-based embryo grading alone (Munné et al., 2019). Furthermore, a systematic review, including 13 randomized controlled trials, concluded that the current evidence is insufficient to recommend PGT-A in routine clinical practice (Cornelisse et al., 2020).

Other main issues related to the use of PGT arise due to the possible mosaicism of embryos leading to false results, the invasiveness, and the involvement of expensive and time-consuming procedures. Chromosomal mosaicism is common during preimplantation embryonic development, especially at the cleavage stage. Therefore, this can affect the correctness of the results (Fragouli et al., 2019). Furthermore, as the embryo biopsy is an invasive procedure, the embryo biopsy could impact the subsequent embryo viability. According to a paired RCT, a relative reduction of 39% in the implantation rate was observed when a single blastomere biopsy was performed compared to the control group (Scott et al., 2013). Due to these reasons, PGT based screening of all embryos of all patients undergoing ARTs may not be justified.

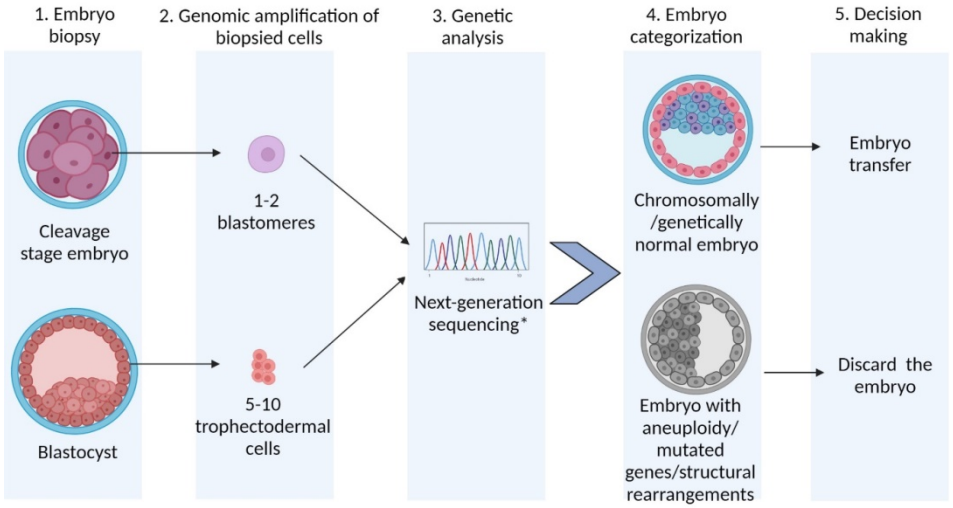

Figure 3: Schematic diagram to illustrate the steps of the PGT. Among the many molecular biological methods of genetic analysis, next-generation sequencing is commonly used. Created with BioRender.com

Due to the limitations of available embryo assessment methods, alternative embryo selection methods needs to be investigated. Evaluation of the embryo conditioned media is a broad spectrum of such alternative approaches currently under extensive research.

2.1.3.3.2. Non-invasive methods based on the analysis of embryo conditioned culture media/spent media

Embryo conditioned media is of no use at the end of embryo culture and is generally discarded. However, this media could reflect the embryo's function and the pathophysiological status during the embryo culture (Montskó et al., 2017). Furthermore, this method does not involve manipulating embryos and therefore is a non-invasive approach. Such methods would not affect posterior embryo development. Many groups have looked into the conditioned media within different areas of 'omics', including genomics (Huang et al., 2019), proteomics (Kosteria et al., 2017), metabolomics (Bracewell-Milnes et al., 2017) and small non-coding RNA (Russell et al., 2020).

A method that could replace PGT in the future has developed with the discovery of DNA within the embryo conditioned media and blastocoele fluid (BF). This relatively novel method is called non-invasive PGT (niPGT), or cell-free genetic testing of embryos (Leaver & Wells, 2020). While collecting blastocoel fluid is less invasive than embryo biopsy, collecting embryo conditioned media is entirely non-invasive. Therefore, this method would circumvent the limitations of the PGT, including the cost, invasiveness of the embryo biopsy, and the

associated training required (Brouillet et al., 2020). While some studies support the use of this method, another set of studies questions its validity (Table 9). Although some studies have shown specific results supporting its usefulness, wide variations observed in the proportion of samples that provides results and the variations in the concordance of niPGT results relative to PGT have become main concerns (Leaver & Wells, 2020). Moreover, the frequent detection of extra-embryonic DNA in embryo conditioned media (Brouillet et al., 2020; Hammond et al., 2017) also makes it difficult to arrive at conclusions on niPGT regarding its diagnostic efficiency and clinical efficacy.

Table 9. Recent studies that evaluated the cell-free DNA in the embryo conditioned media

Study	Species	Observations/comments
(Kuznyetsov et al., 2020)	human	High concordance with TE biopsy results and such concordance rates were not dependant on morphological embryo grade.
(Huang et al., 2019)	human	niPGT-A is less susceptible to errors arising due to embryo mosaicism. In that regard, reliable than TE-biopsy PGT-A.
(Rubio et al., 2019)	human	The concordance rate for ploidy with cell-free DNA was 78.7%
(Kuznyetsov et al., 2018)	human	A combination of blastocyst conditioned media and blastocoele fluid have embryonic DNA for total genomic amplification sufficient enough for accurate aneuploidy screening
(Feichtinger et al., 2017)	human	High concordance of culture media genetic analysis and polar body analysis. Maternal contamination is a problem
(Shamonki et al., 2016)	human	Cell-free DNA in the embryo conditioned media are consistent with trophectoderm biopsy
(Hammond et al., 2017)	human	DNA from culture media is of mixed origin and affect the reliability of its use for embryo genetic analysis.

Detection of small non-coding RNAs such as microRNA (miRNA) in the embryo conditioned media is another non-invasive embryo screening approach (Kropp et al., 2014). miRNAs are small (18–22 nucleotides), non-coding RNA molecules that are considered as negative gene expression regulators. miRNAs can destabilize or repress the translation of messenger RNA (mRNA) (O'Brien et al., 2018). The role and significance of miRNA during early embryogenesis and development are well studied in humans (Galliano & Pellicer, 2014) and other mammalian species, including cattle (Goossens et al., 2013; Rio & Madan, 2021). The global interest in developing miRNA as a disease biomarker would fuel the development of more sensitive miRNA detections. Hence, it

would invariably provide more evidence about the applicability of miRNA as biomarkers of embryo quality. Table 10 summarizes the recent studies that evaluated the miRNA in embryo conditioned media as a marker of embryo quality and transfer outcome. Further studies involving large patient cohorts or sample numbers and also in the form of RCTs are needed to confirm these findings. One of the main problems of using embryo conditioned media miRNA for embryo diagnostics is the possibility of having extra-embryonic miRNA that can be falsely labelled as of embryonic origin (Sánchez-Ribas et al., 2019). However, this problem is not limited to miRNA but is also applicable to other omics-based approaches.

Table 10. Studies that evaluated the RNAs of the embryo conditioned media

Study	Species	Observation in the culture media
(Hawke et al., 2021)	murine	Development stage based differences in the studied miRNA panel
(Rio & Madan, 2021)	bovine	miRNAs are detected in the embryo conditioned media along different embryo development stages.
(Kirkegaard et al., 2020)	human	No miRNAs were detected in the embryo conditioned media despite using state of the art detection methods
(Abu-Halima et al., 2020)	human	miR-19b-3p lower abundance in both embryo conditioned media and sperm samples was linked to positive pregnancy outcomes.
(S. J. Russell et al., 2020)	human	Significant differences in sncRNA between embryo conditioned media and control
(Borges et al., 2016)	human	miR-142-3p is a potential biomarker that indicates blastocyst implantation failure
(Rosenbluth et al., 2014)	human	Following correlations were observed: miR-645 (poor pregnancy outcomes); high miR-191 (with aneuploid embryos); high levels of miR-372 High miR-191 (with IVF failure)
(Kropp et al., 2014)	bovine and human	MiR-25, miR-302c, miR-196a2, and miR-181a abundance was higher in degenerating bovine embryos compared to blastocysts. These miRNAs were found in human embryo conditioned media too.

Proteomics based approaches have investigated the embryonic secretome in the culture media. Studies have shown that the secretome of the embryo change according to the embryo viability (Katz-Jaffe & Gardner, 2008). Many proteins have been investigated as potential biomarkers of embryo quality (Table 11). Though HLA-G entered multi-centre trials, they failed to prove its clinical benefit over morphological scoring (Rødgaard et al., 2015). Buttler et al. investigated the human chorionic gonadotropin (HCG), a signal for maternal recognition of pregnancy, in the secretome and found that the isoform HCGβ have the potential

to be used as a biomarker for embryo viability and transfer (Butler et al., 2013). Although much active research is underway to discover secretome biomarkers, the small sample volume and lower analyte concentration are the two main limitations. With the advancement of technology, highly multiplexed methods have been evolved, such as Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDIToF MS), to evaluate the secretome of the embryo conditioned media (Iles et al., 2019). Using such technologies, which are highly reproducible, further advancements in the proteomics-based embryo diagnostics would find better biomarkers of embryo quality and transfer success.

Table 11. Studies that evaluated the proteome of the embryo conditioned media

Study	**Species**	**Studied biomarker**	**Observation in the culture media**
(Lindgren et al., 2018)	human	EMMPRIN	High in embryos developing into blastocysts
		Caspase-3	Lower in high-quality embryos
(Kaihola et al., 2019)	human	Caspase-3	Biomarker of embryo quality Predict the outcome after transfer of day-2 cultured embryos
		HRG	Biomarker of embryo quality
(Iles et al., 2019)	human	A score based on characteristics within a specific mass range	This score can be used to predict embryos that can give rise to pregnancy
(Bouvier et al., 2017)	human	Soluble CD146	High CD146 levels in embryos that did not implant
(Pais et al., 2020)	human	Mass-spectral peak signatures –	Distinct peak signatures for euploid and aneuploid embryos
(Rodríguez Díaz et al., 2019)	human	Soluble HLA-G	Higher levels of sHLA-G secretion and pregnancy rate
(Freis et al., 2021)	human	PECAM-1 and TIMP4 and others	Highly expressed by implanted blastocysts
(M. S. Lee et al., 2020)	human	TNF-α and IL-1β	Embryos with similar morphology show different levels of IL-1β from day 3 to day 5–6
(Butler et al., 2013)	human	hCG	hCG isoforms have biomarker potential for embryo viability and transfer

The secreted metabolome is one of the intensively and widely studied groups of non-invasive biomarkers for embryo quality. Compared to genomics and proteomics, metabolomics encompasses a wide variety of smaller weight molecules, such as carbohydrates, amino acids, oxidation products and carboxylic acids (Zhang et al., 2020). The secretion or uptake of metabolites in the extra-

cellular environment can alter depending on cellular metabolic or environmental status. Therefore, metabolomes can be good indicators of cellular activity, hence may serve as promising biomarkers of embryo quality. Based on the used detection method, metabolomics gives a snapshot of all the metabolites in the sample. In the past, near-infrared (NIR) spectroscopy-based evaluation of embryo conditioned media had given promising results. However, a meta-analysis by Vergouw et al. did not further support those observations (Vergouw et al., 2014). Another meta-analysis published in 2017 indicates that evidence does not show that it can improve important reproductive parameters such as clinical pregnancy and live-birth rate (Bracewell-Milnes et al., 2017). More recent studies have used other different platforms to evaluate the metabolome and show promising findings. (Table 12). However, more studies in the form of randomized-controlled trials are needed for further evaluating these findings.

Table 12. Recent studies that evaluated the metabolome of the embryo conditioned media

Study	Species	Biomarker/variable	Observation in the culture media
(Inoue et al., 2021)	human	Organic acid metabolites based on GC-MS/MS	Five metabolites were higher, and three metabolites were lower in good quality embryo group compared to the low-quality embryo group
(Wiweko et al., 2020)	human	Spectral data from FTIR spectroscopy	FTIR spectroscopy can predict blastocyst formation from day-1 embryo conditioned media
(Liang et al., 2019)	human	Raman-based footprint profiling	Differences in the footprints of euploid and aneuploid embryos

GC-MS/MS-gas chromatography-mass spectrometer; FTIR – Fourier-transform infrared spectroscopy

Furthermore, the levels of specific metabolites or consumption of particular constituents of the culture media have been studied in association with embryo quality (Table 13). However, the number of studies based on such biomarkers are scarce. Hence further studies are needed to evaluate these potential bio-markers of embryo quality.

Table 13. Other variables studied in the embryo conditioned media

Study	Species	variable	Observation in the culture media/conclusion
(Han et al., 2020)	human	Oxygen consumption	embryo developmental potential and oxygen consumption are positively correlated.
(Huo et al., 2020)	human	Amino acids- ASP, SER, HIS, and ALA	peak height and peak area of amino acids in HPLC were distinct between pregnant and non-pregnant group
(Alegre et al., 2019)	human	Oxidative status (along with morphokinetics)	Combined morphokinetics and embryo conditioned media oxidative status may support embryo selection.
(D. K. Gardner et al., 2011)	human	Glucose consumption	Higher glucose consumption by embryos ended up in pregnancy than embryos that failed to develop following transfer.

Although a variety of non-invasive technologies for assessing human embryos have been developed, to date, none of such methods has been proven to be superior to the standard morphological evaluation of embryos. One of the issues is that most of these methods or potential biomarkers have not been studied in RCTs.

In terms of embryo evaluation using non-invasive methods, extracellular vesicles (EVs) in the embryo conditioned media also have the potential as a candidate biomarker, as reviewed in the subsequent chapters.

2.2. Extracellular vesicles (EVs)

2.2.1. Background

Extracellular vesicles (EVs) are cell-derived membranous biological nano-particles (NPs). These heterogenous particles are released by cells *in vivo* and *in vitro* under different pathophysiological conditions and play a crucial role in intercellular communication. EVs have been detected in almost all biological fluids, including but not limited to blood, urine, synovial fluid, respiratory secretions, and also in cell-conditioned culture media (Foster et al., 2016; Palviainen et al., 2019). Cells´ ability to release EVs has been conserved throughout the evolution from bacteria to plants and animals, including humans (Deatheragea & Cooksona, 2012; Robinson et al., 2016; Schorey et al., 2015).

The classification of extracellular vesicles is based on different factors such as their origin, size and molecular cargo. Considering the existing knowledge of their biogenesis, EVs can be broadly divided into three main types: exosomes,

microvesicles and apoptotic bodies (Figure 4). Of them, exosomes and microvesicles are the most interested and widely studied types of EVs. Exosomes (30–100 nm in diameter) are intraluminal vesicles (ILVs) that are generated by inward budding of the endosomal membrane during the maturation process of multivesicular endosomes (MVEs). Upon fusing MVEs with the plasma membrane, exosomes are released from the cells (Hessvik & Llorente, 2018). On the other hand, microvesicles (50 nm to 1,000 nm in diameter) are generated by the outward budding of the cell membrane and the subsequent release of vesicles into the extracellular space (Teng & Fussenegger, 2021). Their size differences make them comparable with other particles found in the biofluids (Figure 5). Due to the overlapping sizes of different subtypes of EVs and inefficient methods of their separation from each other, exosomes, microvesicles and apoptotic bodies are collectively studied together and referred to as EVs (Théry et al., 2018).

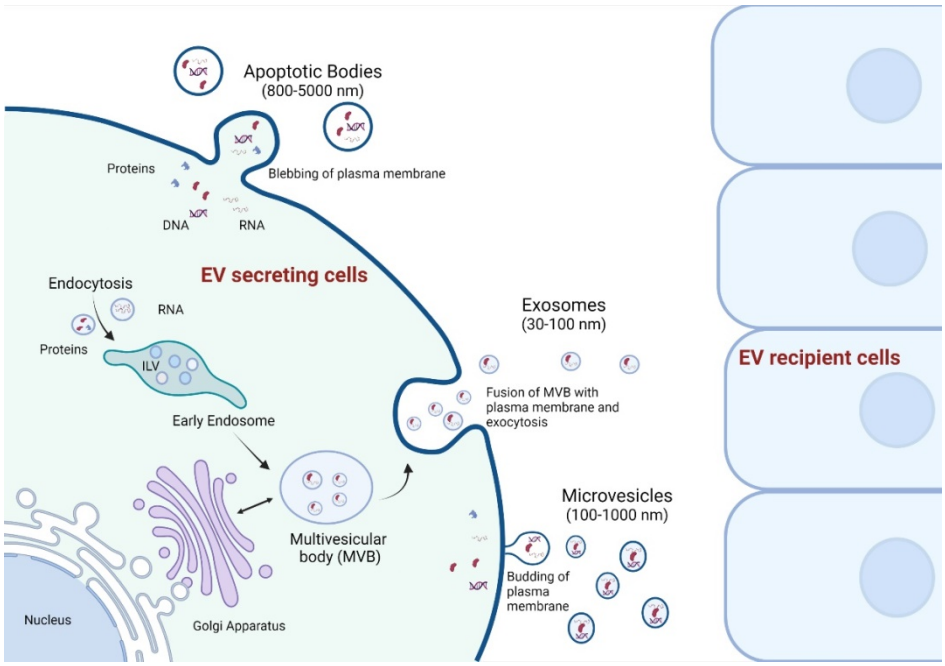

Figure 4. Biogenesis of extracellular vesicles (EVs). Types of EVs differ based on their biogenesis, size and molecular cargo. Created with BioRender.com

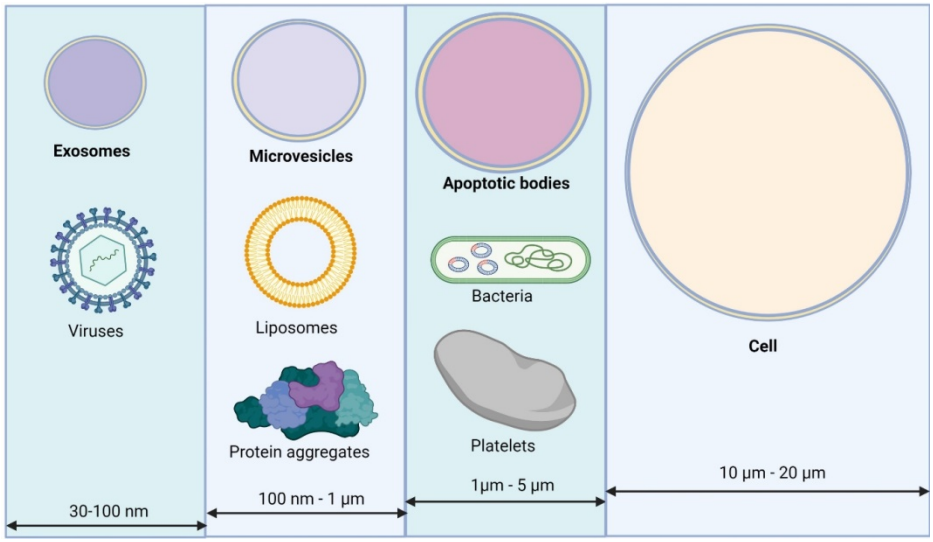

Figure 5. Size comparison of different types of EVs with similarly sized particles and a eukaryotic cell. Created with BioRender.com

The cargo of EVs consist of proteins, nucleic acids such as mRNA and miRNA and lipids (Chen et al., 2019; O'Brien et al., 2020; Uzbekova et al., 2020). Having compartmentalized inside EVs, these labile biomolecules are protected from inactivation and destruction in the hostile extracellular environment (figure 6) (Kalra et al., 2016). EVs released by cells carry the molecular cargo, which subsequently is internalized by the target cells. This cell to cell transport of EVs can occur in a microenvironment or remote site by entering biological fluids. By such transferring of biomolecules, EVs have the ability to change the cellular activity and phenotype of the target cells (Boyer et al., 2020). Furthermore, it is known that EVs can also induce (epi)genetic modifications in cells (Abeysinghe et al., 2020).

In the beginning, the EV secretion was considered as a method of cellular excretion, in other words, a mode of removing unwanted cellular compounds (Baixauli et al., 2014; Hessvik et al., 2016; Johnstone et al., 1987). However, more and more research during the last two decades proved that EVs are more than waste carriers, as previously thought. Therefore, in the past decade, EV related research increased dramatically, especially related to disease diagnosis and therapeutics (Srivastava et al., 2020). Furthermore, by being able to convey a multitude of biomolecules such as miRNA, cytokines and mediators of inflammation, now it is apparent they are involved in many physiological and pathological events such as maintenance of homeostasis, angiogenesis, cancer metastasis, promotion of repair, among others (Hanayama, 2021). Most interestingly, EVs are gaining a wide interest as potential biomarkers (Lane et al.,

2018) and therapeutic drug delivery systems (Bunggulawa et al., 2018), which has boosted EV related research in the recent past.

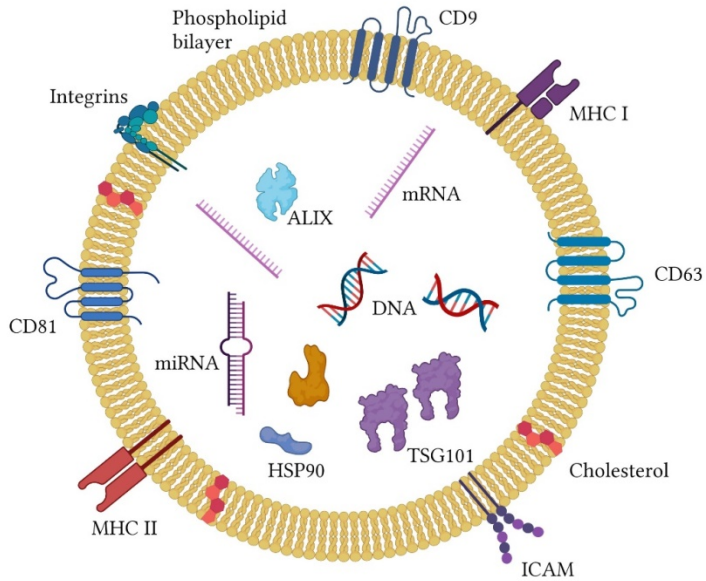

Figure 6. Schematic diagram to illustrate the structure of exosomes, a types of EVs. Exosomes are enriched in tetraspanins such as CD9, CD63 and CD81. Created with BioRender.com

2.2.2. Diagnostic and therapeutic applications of EVs

EVs are considered potential biomarkers for the early detection and diagnosis of certain diseases such as cancer (Yee et al., 2020). In the last two decades, circulating EVs have been broadly explored as biomarkers in liquid biopsy. The fundamental reason for the exponential rise in the scientific community's interest in EV research is the high stability of EVs and their abundance in all body fluids. Noninvasive biopsy of blood has drawn significant interest and attention mainly in terms of tumour diagnosis and the assessment of treatment response. Tumor-derived EVs were found to be associated with tumour progression, tumour metastasis, and immune evasion (Raimondo et al., 2020). EVs have been detected in tumour tissues and body fluids such as malignant effusions (Luo et al., 2020) and in the urine of patients with cancer (Rikkert et al., 2020). Since EVs are released in large numbers from each cell of origin, their quantification in biological fluids provides advantages compared to the scarcity of circulating tumour cells and circulating cell-free DNA (Krebs et al., 2010; Lee et al., 2018).

In patients with cancer, the quantity of EVs is raised, and the composition of EV proteins, mRNAs, and miRNAs were varied depending on the disease status (Ohno et al., 2013). For example, glypican-1 (GPC1), a cell surface proteoglycan, was found to be expressed explicitly by exosomes isolated from the serum of pancreatic cancer patients (Melo et al., 2015). Moreover, the levels of GPC1+ exosomes are reported to be correlated with the tumour burden and survival. In addition to primary tumours, the use of EVs in the early detection of cancer metastasis have been investigated. For example, MDA-9 and GRP78 proteins have a significantly higher expression in exosomes isolated from the serum of patients with metastatic melanoma, indicating the potential of these vesicle-associated proteins as biomarkers for early detection of metastasis (Guan et al., 2015).

In addition to cancer, the potential applicability of EVs as a diagnostic tool has been evaluated and discussed in many non-cancer disease conditions in the nervous (Andjus et al., 2020; Beard et al., 2020), cardiovascular (Dickhout & Koenen, 2018), reproductive (Simon et al., 2018), respiratory (Ibrahim et al., 2021) and genitourinary (Zhang et al., 2016) systems.

Regardless of the open challenges linked to the use of EVs, mainly related to the standardizing the EV isolation and characterization, most of the studies indicate that EVs could serve as a promising source for early diagnosis and prognosis of many pathological and physiological conditions.

From the therapeutic point of view, EVs are under extensive research considering their ability to transfer their molecular cargo to target cells and the ability to pass through biological barriers such as the blood-brain barrier (Hernandez-Oller et al., 2020). EVs can support tissue regeneration (Wiklander et al., 2019), immune modulation (Seo et al., 2019), and are potential alternatives to stem cell therapy (Ong & Wu, 2015). Using bioengineered exosome-mimetics or intrinsic EVs, it is possible to deliver therapeutic agents to diverse target cells. Recently, more attention has been drawn to using EVs in the development of vaccines. EVs can be bioengineered in such a way that they can display viral antigens. Hence, these antigen-presenting EVs can induce high and specific CD8(+) T cell and B cell reactions making EVs a novel virus-free vaccine strategy. Currently, EV-based vaccines against viral infections, such as Covid-19, are developing (Sabanovic et al., 2021). The used methods involve displaying SARS-CoV-2 Spike protein on the surface of exosomes or the EV based delivery of mRNAs of viral proteins.

2.2.3. Isolation of EVs

Successful isolation of EVs from biological fluids and cell culture media is crucial for the successful downstream applications of EVs. Many different techniques have been evolved over the years based on various properties of EVs such as size, charge and affinity. The most popular techniques nowadays are ultracentrifugation (UC), size-exclusion chromatography (SEC), density gradient ultracentrifugation, ultrafiltration (UF), immunoaffinity capture and polymer-based precipitation (Table 14).

EV research is a relatively new field; hence novel EV isolation methods are constantly being developed. Many novel methods, such as microfluidics-based approaches (Contreras-Naranjo et al., 2017; Meng et al., 2021) and asymmetric flow field-flow fractionation, which fractionate EV subpopulations (Zhang & Lyden, 2019), are receiving more focus.

During microfluidics-based EV isolation, EV capture is based on immuno-affinity (Sharma et al., 2018) and physical or mechanical characteristics of EVs such as size, density and compressibility (Guo et al., 2018). High-throughput and the precision of the technique, the necessity of only a low sample volume and low cost per sample are the main advantages of these methods. This list goes on every year, and more and more technologies are being developed and added to it. Emerging methods of EV isolation have similar positive characteristics with regards to shorter procedural duration, easy to use, and isolate EVs with high purity and integrity. However, they need further optimization for different types of samples.

The combination of multiple methods of EV isolation is considered superior to single EV isolation methods, as the former has produced EVs with high purity and specificity (Stam et al., 2021). UC method followed by density gradient centrifugation, SEC or AF4 is such a combined method (Tauro et al., 2012; Théry et al., 2006; Vaswani et al., 2017). Similarly, ultrafiltration and subsequent SEC have produced a significantly high yield of EVs compared to UC alone and preserved EVs' biophysical properties (Nordin et al., 2015).

There is no gold-standard method of EV isolation that fits all types of samples. The isolation method needs to be selected and optimised based on the sample type and the downstream applications (Théry et al., 2018).

Table 14. Common methods of isolating EVs from biological fluids/culture media samples

Method	Principle	Advantage	Limitations	Used in the thesis
Ultracentri-fugation (UC) (Coughlan et al., 2020)	EV separation based on buoyant density	Isolate EVs from a large volume of samples; No additional chemical require-ments	Long procedural duration; Lower reproducibility; Higher impurities; Damage to EVs	-
Size exclusion chromatography (SEC) (Dissanayake et al., 2021)	Size based separation of EVs when eluting through a porous polymer matrix	Scalable, robust and simple; Short procedural duration; Fewer impurities and preserved EV functionality	Limited sample volume; Single used columns; Co-isolation on large protein aggregates; Processing a single sample in each run	+
Density gradient centrifugation (Pavani et al., 2019)	EV separation based on density differences	High purity and less contamination; No additional chemicals	Complexity; Sample loss; Viral contamination (when Sucrose is used)	-
Ultrafiltration (Kim et al., 2021)	Molecular weight or size-based separation when passing through filtration membranes.	Scalable, effective and a time-efficient method Require mild pressure	Separation dependent on filter membrane quality and the homogeneity of membrane pore size distribution; can affect EV integrity; EVs can be bound to filter and lost	+
Polymer-based precipitation, e.g. polyethylene glycol (PEG) (Karttunen et al., 2019)	EVs are precipitated using polymers	High yield; Good purity; Straightforward workflow; Preservation of EV integrity; Simultaneous pro-cessing of a large number of samples	High protein contamination and aggregation; Co-precipitation of large non-EV particles; Aggregation of vesicles; Poor reproducibility;	-
Immunoaffinity-based EV isolation e.g. using antibodies (Foroni et al., 2020)	specific antibody-antigen interactions that target specific EV populations	Good purity; Acquisition of a target-specific EV population	Expensive; Possible nonspecific binding	-

2.2.4. Characterization of EVs

It is necessary to characterize the isolated EVs using multiple and complementary methods. This is needed to quantify the EV yield and verify that all the subsequent functional analysis and downstream applications are mediated by EVs per se and not by other co-isolated nanoparticles. The international society of extracellular vesicles has issued guidelines about the minimum information requirements when reporting EV studies (MISEV2018)(Théry et al., 2018). EVs can be characterized by physical and biochemical methods. As shown in the recent studies, the most commonly used physical characterization methods of EVs are: transmission electron microscopy (TEM) to visualize the EV structure and nanoparticle tracking analysis (NTA) to quantify the concentration and size profiles of EVs. Table 15 summarizes popular EV physical characterization methods.

Table 15. Common methods of physical characterization of EVs

Method	Measured features	Advantages	Limitations	Used in the thesis
Nanoparticle Tracking Analysis (NTA) (Dissanayake et al., 2021)	Estimate of the concentration and size distribution and zeta potential of EVs; In the fluoroscent mode (fl-NTA), antibody based EV detection	Accurate measures even in polydisperse samples; Fast assessment; Fl-NTA distinguish EVs from others.	Scattered light may misrepresent the EV concentration and size distribution in the complex biofluids (due to light scattering from other sources like protein aggregates	+
Dynamic light scattering (DLS) (Lyu et al., 2021)	Determine the size distribution and concentration	Simplicity; Fast assessment	Better for analysing monodispersed samples; Limited use with minimally-processed samples.	-
Tunable Resistive Pulse Sensing (tRPS) (Phan et al., 2021)	Determine the size, concentration and surface charge of EVs.	Higher sensitivity and accuracy	Difficulty in analyzing minimally-processed biological samples due to obstruction of the pore by larger EVs.	-
Transmission electron microscopy (TEM) (Comfort et al., 2021)	Visualize size and morphology of individual EVs	High-resolution images	Artefacts such as cup-shaped EVs; Structure may deform; Need specialized training; Time consuming	+

Method	Measured features	Advantages	Limitations	Used in the thesis
Cryo-electron microscopy (Rizzo et al., 2021)	Visualize size and morphology of individual EVs	Higher quality; EV spherical shape preservation	Expensive; Limited accuracy for estimating EV concentration; Need specilized training	-
Scanning electron microscopy (SEM) (Cavallaro et al., 2021)	Visualizes the EV surface topography	Direct visualization of EV surface	Requires fixation and drying of the sample; Altered native morphology	+
Atomic force microscopy (AFM) (Sharma et al., 2020)	Imaging the topology of surfaces with nanometer resolution; estimating their size and structure	Performed witout any sample labelling; Data about EV stiffness and elasticity	Low throughput; requirement of specific skills;	-

There are multiple methods to characterize EVs from a biochemical standpoint. The most common and straightforward approach is by proving the presence of EV proteins (Kowal et al., 2016). In this regard, desired methods target the quantification and identification of specific EV proteins. On the other hand, mass-spectrometry based proteomics approaches try to identify all proteins in the EV samples (Bandu et al., 2019). EV specific markers would invariably support the development of EVs as a diagnostic and prognostic tool (Arbelaiz et al., 2017). The two broad categories of commonly used biochemical EV characterization methods are conventional protein analysis such as immuno-blotting and capturing specific EVs by techniques such as immunosorbent EV assays (Table 16).

Table 16: Methods of biochemical EV characterization

Method	Measured features	Advantages	Limitations	Used in the thesis
Immunoblotting				
Westernblot (Bonsergent et al., 2021)	Demonstrate the presence of EV-associated proteins such as CD9, CD63, Tsg101)	Quick and simple detection of the EV protein content	Semi-quantitative analysis; No information on the protein content of individual EVs or their hetero-geneity; Requires a large sample volume; Extensive sample processing	-
Immunosorbent EV Assays				
Flow Cytometry (Maia et al., 2020)	Measures scattering or fluorescence intensity of particles illuminated by a laser	Identification of specific EV subpopulations ability to analyse biomarkers on individual vesicles down to 100 nm in size	Low detection sensitivity; Inability to detect the common small vesicles individually; Need high sensitive detectors for less abundant and specific EVs; require highly specialized equipment	-
EV array (Jørgensen et al., 2021)	Capturing and detection of EVs using a selected array of antibodies	Multiplexing; Need low sample volume; Sensitive detection	Not widely available	+

2.3. Models used to study the early human embryo development and embryo-maternal communication

2.3.1. Animal models of preimplantation embryo development

Even though human embryos produced during ART are donated for research, the use of such embryos in research is less common due to various ethical and legal constraints and the limited availability of such embryos. This has

necessitated the use of animal and other models for embryological research. *In vivo* and *in vitro* produced animal embryos as surrogates of human embryos is a viable option. However, even animal embryos can raise many ethical and legal limitations (Hyun, 2019).

Embryos of mice, zebrafish, cattle and pigs are among the animal models of human embryos. However, when considering using an animal model for human embryos, the first requirement is to define the objective of using the model. It is quite challenging to select a specific model for human embryos as there are multiple differences across species, including reproductive physiology. However, over the years, mouse (Mittnenzweig et al., 2021) and bovine models (Simmet et al., 2018) have been used to study human IVF and early human embryogenesis that mimic different aspects or stages of early human embryogenesis (Table 17). While bovine embryos are considered suitable for studying early human embryogenesis (Simmet et al., 2018), mouse embryos are better for learning the implantation process (Zhang et al., 2019). In general, mouse embryos have facilitated the understanding of the physiological and biochemical regulation of mammalian embryos. Moreover, mouse embryos (one-cell mouse embryo assay- MEA) are widely used as a 'pharmacotoxicological tool' to assess the suitability and the quality of the materials and methods used in human IVF (Gilbert et al., 2016).

Table 17: Comparison of mouse and bovine as a model for with human embryo studies

	Mouse	**Cattle**	**Human**
2-cell stage	24 hours	24 hours	24 hours
Morula stage	72–80 hours	144 hours	72–80 hours
Blastocyst stage	4–5 days	6–8 days	5–6 days
Implantation	4–5 days	16–18 days	8–13 days
Zygote volume	5–8 times < human	Similar to human	-
Lipids in the cytoplasm	+	+++	++
Activation of embryo genome	2-cell stage	8–16 cell stage	4–8 cell stage
The maternal and paternal regulatory process	Dissimilar from human	Similar to human	-
Amino acid metabolism	Dissimilar from human	Dissimilar from human	-
Communications between the embryo and the corpus luteum	Similar to human	Dissimilar from human	-
Length of gestation	19–21 days	283 days	280 days
Type of ovulation	Polyovulatory	Monoovulatory	Monoovulatory

Despite the presence of specific important differences between cattle and humans, cattle are frequently being used as a model for early human preimplantation embryogenesis(Ramos-Ibeas et al., 2020). Also, it is worth mentioning that much of the early human ARTs were dependant on findings based on cattle ARTs (Sirard, 2018). A study by Jiang et al. (2015) investigated the expression profiles of 26 imprinted genes in bovine *in vivo* produced oocytes and embryos using RNA-seq, and compared them with human, mouse and pig counterparts. The transcriptional abundance of MEST and PLAGL1 genes decreased during the preimplantation embryo development in the bovine from oocyte to blastocyst stage (Jiang et al., 2015). Transcription levels of these genes decreased early (at 2–4 cell stage) in mouse and pig embryos, whereas cattle and human embryos had their decrease at the 8-cell stage. This study indicates the closeness of human embryos to bovine embryos compared to mouse embryos.

Furthermore, a study by Simmet et al. has added evidence to use bovine embryos as an animal model for early human embryogenesis. In their research, OCT-4 knocked out somatic cell nuclear transfer (SCNT) bovine blastocysts failed to express NANOG in the inner cell mass. This finding was in line with the OCT4 deficient human blastocysts but in contrast with the OCT4-null mouse blastocysts where NANOG expression in the ICM is unaffected (Simmet et al., 2018).

When using animal models for embryo research, using domestic mammals such as cows is also advantageous for animal welfare (Sjunnesson, 2020). Due to the meat industry, cattle and pig ovaries hence oocytes are abundantly available from the discarded materials in the abattoir. Using oocytes from these materials to produce embryos *in vitro* minimizes the need to use animals in research. However, cell culture-based embryo surrogates have been developed and would revolutionize and fasten the research on early embryo development.

2.3.2. Cell culture-based models for embryo-maternal communication and implantation

In vivo, the human embryo meets the endometrium about five days after the fertilization, when it is developed to the blastocyst stage, and the ZP is hatched. The aim of this meeting is the implantation. Implantation is a very complex event with strict regulation of intercellular communication and gene expression. This involves three steps: (a) apposition, (b) attachment, and (iii) invasion through the basement membrane to the underlying stroma (Figure 7). During these steps, embryo endometrial interactions are under tight regulation that involves both intercellular and cell–extracellular matrix interactions. However, the physiological and molecular mechanisms underlying the successful embryo implantation is not fully elucidated.

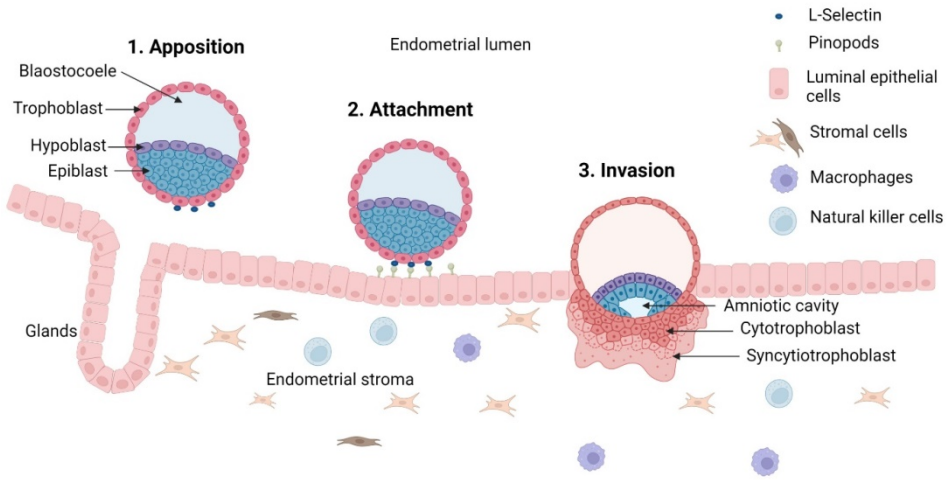

Figure 7. Main steps of human embryo implantation. During the apposition stage, the embryo expresses L-selectins. These L-selectins are needed when blastocyst interact with the L-selectin ligands on the pinopodes of luminal epithelium. This happens only during the window of implantation. During the invasion stage, the trophoblastic cells penetrate the endometrial epithelial cells and grow towards the endometrial stroma. Immune cells including natural killer cells and macrophages are vital during subsequent decidualization process and make the environment suitable for implantation. Created with BioRender.com

Embryo implantation is the least understood and most studied key rate-limiting step in the process of reproduction (Kim & Kim, 2017). Decoding the mechanisms underlying the embryo-maternal communications at the endometrium and process of implantation is crucial to improving the outcomes of ART. However, studying human embryo-maternal communication and embryo implantation, as *in vivo* studies using human resources, are restricted by ethical and legal constraints and technical limitations (Peng et al., 2020). This has directed researchers to use animals for this purpose. Even though *in vivo* mouse models could mimic human embryo implantation, they may not translate well to humans and have certain limitations (Carter, 2020). Furthermore, this also has raised certain ethical concerns and limitations. Table 18 illustrate the potential advantages and limitations of using animal models when studying embryo implantation.

Representative functional models are needed to understand the complex embryo–endometrial interactions that are taking place during implantation. These models should represent both embryo and endometrium. *In vitro* models have been developed as alternatives to *in vivo* animal models that mimic the early and late stages of human embryo implantation. Based on the contemporary molecular biological techniques, these models are providing strong evidence to fill the knowledge gap in the human implantation process.

Table 18: Advantages and limitations of using *in vivo* and *in vitro* models for studying embryo implantation

Advantages	Limitations
***In vivo* models**	
The use of a complete organism facilitates to study of the complex interaction between two entities	Ethical consideration of animal experiments
Reduce the risk of artefacts	Experiments consume longer times
Defined laboratory animals available	Translational relevance can be low
Techniques are already established	Evaluation of the implantation is complex and difficult
***In vitro* models**	
Convenient to follow up the experiments	Initial setting up can be challenging
It can be studied using human materials	All relevant tissues may not be available
Certain individual signalling pathways can be studied	Systemic effects cannot be studied
Manipulation is easy	Surrogates may have less translational value
The need for animals for research is minimized	Using certain human material may raise ethical issues
Standardized protocols with less variability	
Better reproducibility	

Adapted from (Ban et al., 2020)

The endometrial counterpart of *in vitro* models is different endometrial cell lines and primary endometrial cells. Cell lines are preferred as surrogates of the endometrial epithelium. RL95-2 (Es-Haghi et al., 2019), ECC-1(Kinnear et al., 2019), Ishikawa cells (Rodriguez-Caro et al., 2019), and HEC-1A (Vergaro et al., 2019) are such preferred cell lines. Even though they are deriving from uterine adenocarcinoma cells, most have retained typical characteristics of endometrial cells, such as expression of integrins and response to steroid hormones. However, in terms of implantation potential, these cell types show differences. RL-95-2, Ishikawa and HEC-1A are considered highly receptive, moderately receptive and non-receptive, respectively, in terms of their adhesiveness (Vergaro et al., 2019). The main advantages of using cell lines are easy accessibility, easy handling and cost-effectiveness.

Moreover, there are reasonable surrogates for endometrial stromal cells. KD02-44D and T HESCs have been used as cell lines analogue of the endometrial stroma. However, for studying the endometrial stromal cells, researchers have relied mostly on primary cells. The establishment of the improved culture of biopsied endometrial cells, their cryopreservation and ability to passage multiple times have made it advantageous to use them (Turco et al., 2017). The main limitation of these endometrial epithelial or stromal cell-based models is that they do not mimic the complete endometrium. For example, uterine glands

(or their surrogates) that interact with implantation crypts have not been considered in such models (Table 17).

The lack of structural analogy of the previously mentioned models to the endometrium has been addressed by developing organoids. Organoids are entities deriving from tissue, embryonic stem cells or induced pluripotent stem cells (iPSC). They can self-organize as 3D cultures due to their capacity to self-renew and differentiate (Wei et al., 2021). They can mimic specific organs and tissues and have revolutionized biomedical research during the past decade. Different organoids for reproductive research have been developed, such as blastocyst organoids, trophoblast organoids and endometrial organoids. Such models are useful when investigating the mechanisms of embryo implantation (Rawlings et al., 2021). The advances in developing organoids have created hormone-responsive organoids with a secretory element resembling uterine glands. Organoids may not fully represent *in vivo* counterparts, and the random and uncontrolled nature of their growth can be a limitation. However, organoids based models would be more representative than other models in Reproductive Biology research (Heidari-Khoei et al., 2020).

Embryonic counterparts of *in vitro* models could be real *in vitro* produced embryos (Christodoulou et al., 2019). However, considering the limited availability of such embryos and ethical and practical constraints, embryo surrogates have been employed for this purpose. The most popular surrogates are the trophoblast spheroids (cell clusters that form spheres) derived from cell lines. These models are more attractive as they are readily available and easy to manipulate. The most widely used trophoblast cell line for spheroid generation is the JAr cell line (Es-Haghi et al., 2019). JAr cell lines are easy to handle and express human chorionic gonadotrophin (HCG) and progesterone. Apart from the JAr cell line, BeWo, Jeg-3, and Sw.71 have been used to generate trophoblast spheroids (Rothbauer et al., 2017). Despite being originated from carcinoma cells, these cell lines have gained popularity as an analogue of embryos due to their ability to perform certain functions of trophoblasts such as secretion of hormones, barrier function and transport of glucose. However, being deriving from the first-trimester placenta, these cell lines better mimic advanced embryonic stages than the implantation stage. JAr, BeWo and Jeg-3 were found to have different attachment rates to RL-95-2 cell lines, one of the analogues of the endometrial epithelium. Furthermore, these embryo surrogates represent extra-villous cell lineage that is ideal for representing invasion rather than apposition and adhesion stages of implantation (Ban et al., 2020).

Recent studies led to the development of *in vitro* embryo analogues such as embryoid bodies, consisting of a collection of embryonic stem cells (Spangler et al., 2018), and trophoblast organoids (Sheridan et al., 2020). These artificial analogues would become more popular than multicellular spheroids made up of trophoblastoid cells such as JAr, as they can better imitate embryonic architecture and early embryogenesis. Even though such models are not yet widely used in reproductive biology research, such methods would speed up the progress of

research on embryo implantation, enabling the identification of the mechanisms behind implantation failure.

2.4. Embryo derived-EVs and their role during the embryo-maternal communication

2.4.1. Pre-implantation embryo-derived EVs

Several studies have detected EVs released by preimplantation mammalian embryos such as bovine (Mellisho et al., 2017), porcine (Saadeldin et al., 2015), murine (Pallinger et al., 2018) and humans (Giacomini et al., 2017) when cultured *in vitro*. A study by Giacomini et al. showed that EVs released by human pre-implantation embryos are uptaken by primary human endometrial epithelial cells and stromal cells. Another study showed that bovine embryo-derived EVs could be internalized by the embryos (Pavani et al., 2019). Such studies support and add evidence to the contribution of EVs during embryo-maternal and inter-embryo communication, respectively.

A previous study has shown that individually cultured bovine blastocysts, produced by IVF and parthenogenetic activation (PA), release EVs to the culture media during day 7 to 9 of *in vitro* culture. They further showed that the concentration of vesicles was higher in the media of IVF blastocysts with arrested development compared to competent PA blastocysts during day 7–9 of culture (Mellisho et al., 2017). The same researchers further showed the release of EVs by single bovine embryos during the blastulation stage (Mellisho et al., 2019). However, these studies have investigated the release of EVs from the bovine embryos of the advanced stage of preimplantation embryo development. Therefore, the release of EVs by pre-implantation embryos at earlier embryonic development stages is yet to be investigated.

A recent study investigated the release of EVs by human preimplantation embryos at different developmental stages (Vyas et al., 2019). Based on electron microscopy, they detected CD9 positive vesicles on the plasma membrane surface, in the perivitelline space and along the zona pellucida. However, they did not observe such vesicles in metaphase II oocytes. This may indicate that EV release commences only after the fertilization of the oocytes.

As the EVs tend to reflect the pathophysiological status of their cell of origin, it is possible that single pre-implantation embryo-derived EVs be different depending on the quality and developmental stage of embryos. These differences could be apparent in EVs' physical and biochemical characteristics, such as the concentration or the size profile of EVs or molecular cargo, including proteins and nucleic acids. If such differences exist, EVs can be used as biomarkers of embryo quality and future development potential.

2.4.2. Embryo-maternal communication at the oviduct

Following the fertilization, the developing embryo passes through the oviduct while undergoing a series of developmental changes (Figure 8). The oviduct is not merely a conduit for the passage of gametes and embryos. Having lined by simple columnar epithelium with ciliated and secretory cells that secrete the oviductal fluid, it supports many early reproductive events. Several studies have demonstrated the contribution of the oviductal fluid during sperm capacitation (Zapata-Carmona et al., 2020), fertilization and early embryo development (Li & Winuthayanon, 2017). Furthermore**,** oviductal fluid derived EVs were found to play an important biological role during fertilization of oocytes (Al-Dossary et al., 2013) and early embryonic development (Almiñana & Bauersachs, 2019; Lopera-Vasquez et al., 2016).

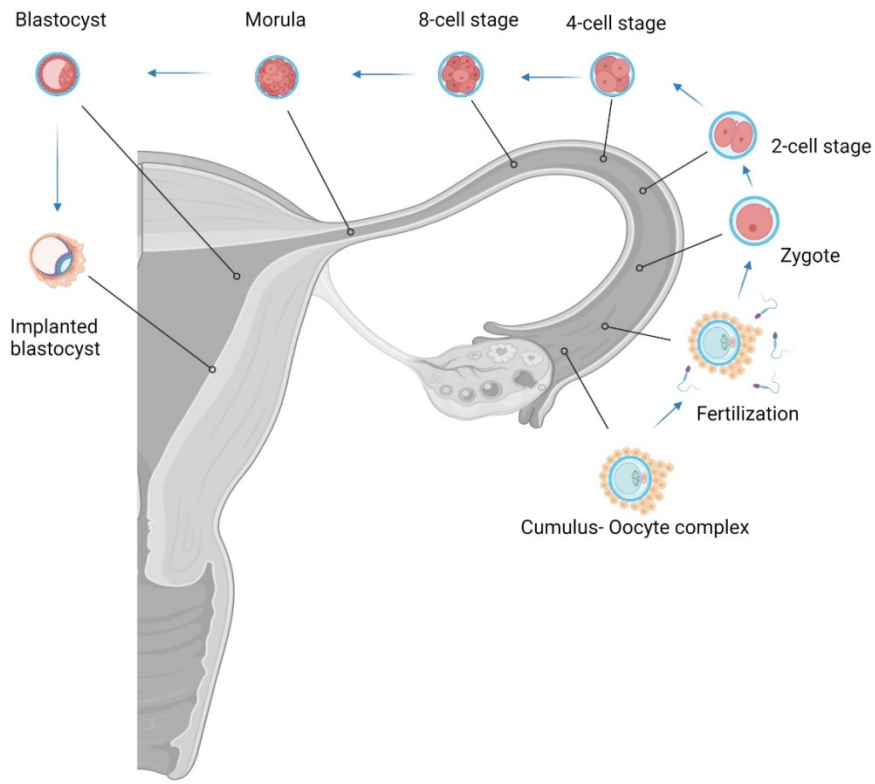

Figure 8. The path of the pre-implantation embryo along the maternal tract. Created with BioRender.com

Embryo-maternal communication at the oviduct is vital for the subsequent embryonic development, and errors in this dialogue would be detrimental for the prospective implantation. Furthermore, the mother can influence the epigenetic programming of the embryo during these communications, thus, influencing the health and development of an individual (Wu & Sirard, 2020). Uncovering the effects that the embryo and mother can have on each other, mechanisms of such effects and identifying the mediators involved would answer questions related to reproductive medicine and biology, such as recurrent implantation failure and ectopic pregnancy.

Up to date, different studies have been conducted using *in vivo* and *in vitro* models to understand the embryo-maternal communication at the oviduct (Kölle et al., 2020). It has been shown that when preimplantation bovine embryos were co-cultured with bovine oviductal epithelial cell (BOECs) monolayer cultures, the early embryo development and quality, as assessed by expression of specific genes, were improved (Cordova et al., 2014). Furthermore, they showed a significant improvement in the blastocyst rates when the first four days of embryo culture was a co-culture with BOECs. This reflects the positive paracrine influences from the maternal tract to embryonic development. Moving forward along this research line, the supplementation of the BOECs derived EVs was found to improve the quality of *in vitro* produced bovine embryos as indicated by the increased number of cells in the trophectoderm and total cells and increased cryosurvival after vitrification (Lopera-Vasquez et al., 2016)**.** The same researchers subsequently showed that EVs secreted from isthmus oviductal fluid, not the EVs from ampullary oviductal fluid, could increase the survival rate, development and quality of the blastocysts produced *in vitro* (Lopera-Vasquez et al., 2017), indicating the regional effects of oviductal EVs on embryos. Such effects could be mediated following the uptake of EVs by the embryos, as shown by Carmen Almiñana et al. They showed that EVs deriving from bovine oviductal fluid could be uptaken by *in vitro* produced bovine embryos. Moreover, those EVs were able to increase the blastocyst rate and improve the embryo survival and quality (Almiñana et al., 2017). Proving the supporting effects of EVs on embryos, the supplementation of donor oviductal fluid derived EVs to the embryo transfer medium was found to improve the live birth rate in mice significantly. This suggests that donor oviductal fluid EVs improve the efficiency of embryo transfer (Almiñana & Bauersachs, 2019). The same study showed that EV supplementation lowered the expression of Bax (an apoptosis activator gene) and enhanced the expression of Bcl2 (an apoptosis blocking gene) and Oct4 (a gene essential for pluripotency). These studies illustrate the influence that the oviduct have on embryos via EVs or other mediators.

On the other hand, embryos also influence the oviduct mediated by different paracrine signalling pathways. Schmaltz-Panneau et al. and García et al. both showed that bovine embryos could alter the transcriptome of BOECs, when co-cultured (García et al., 2017; Schmaltz-Panneau et al., 2014). Moreover, García et al. (2017) investigated the involvement of the bone morphogenetic protein

(BMP) signalling pathway during the early embryo-maternal crosstalk (García et al., 2017). They found out that when embryos were co-cultured with BOECs, specific genes involved in the BMP signalling pathway were downregulated in the oviductal cells. These findings have been further strengthened by an *in vivo* study by Maillo et al. (2015), who investigated the effects of the presence of single or multiple embryos on the gene expression of the bovine oviductal epithelium. Although the presence of a single embryo did not make a detectable transcriptomic change in the bovine oviductal epithelium, following the transfer of 50 embryos *in vivo*, 123 and 155 genes were up and downregulated, respectively, in the oviductal epithelium (Maillo et al., 2015). Different soluble factors such as proteins could mediate such dialogues using different mechanisms. However, precise mechanisms are yet to be discovered.

Thus, the positive effects of oviductal secretions and, specifically, oviductal EVs on the embryo-maternal communications and early embryonic development and successful pregnancy are apparent. Also, the overall effects of embryo-derived signalling in the oviduct have been investigated. However, embryonic EV mediated such effects on the transcriptome of the oviduct have not been investigated. As preimplantation embryos may use EVs as a mediator to notify the future mother about their presence or quality, studying such EV mediated embryonic effects on the oviduct would be an exciting area of research in Reproductive Biology.

2.4.3. Embryo-maternal communication at the endometrium

Understanding the mechanisms underlying embryo-maternal communications would be supportive in developing therapeutic interventions for recurrent implantation failures. It is reported that suboptimal endometrial receptivity is a cause of implantation failure in two-thirds of the cases, whereas the other third is due to embryo quality (Idelevich & Vilella, 2020). Many studies have evaluated pre-implantation embryo-maternal communication at the embryo-endometrial interface (Das & Kale, 2020; Evans et al., 2019; Sponchiado et al., 2017)**.** Such dialogues during the "window of implantation" helps the implantation process by making certain structural and biochemical modifications in the uterine epithelium and stroma (Salamonsen et al., 2016; Sharma et al., 2016).

Embryo implantation makes a long term physical contact with the maternal tract tissues and is the most crucial step in the establishment of pregnancy. At first, the embryo loosely adheres to the endometrial epithelium and then invades the uterine stroma (Kim & Kim, 2017). Simultaneously, the endometrial epithelium and the underlying stroma undergo major alterations that facilitate the implantation process. However, interestingly, most of the anatomical, physiological, biochemical changes are extremely confined to the implantation site. This led to the rise of the hypothesis that the embryo itself guides and directs the necessary changes that need to be accomplished for the implantation to take place in the endometrium (Massimiani et al., 2020; Schumacher & Zenclussen, 2019).

The failure to successfully carry out this intercellular dialogue would be the reason for implantation failure during natural and assisted reproduction. It is reported that nearly 5% of women are experiencing two consecutive losses of pregnancy, and of them, 75% cases are caused by implantation failure (Bashiri et al., 2018). Therefore, understanding the molecular mechanisms behind embryo-maternal communication would help to explain pathological conditions such as recurrent implantation failure (Mrozikiewicz et al., 2021).

Different signalling methods have been investigated as involved in mediating embryo-maternal communication at the endometrium. For example, hormones and cytokines are known to regulate endometrial tissue during the window of implantation (Piccinni et al., 2021). However, it is not possible to explain the embryo-maternal cross-talk phenomenon solely based on biochemical means. As is the case in the oviduct during the embryo-oviductal communication, EVs may be involved in setting up this communication at the embryo-endometrial interface (Figure 9). A study by Gregory W Burns et al. illustrated that fluorescently labelled EVs deriving from pregnant ewes' uterine fluid were taken by embryonic trophectoderm and endometrial epithelial cells when infused into the uterine cavity of pregnant ewes (Burns et al., 2016). A study by Keigo Nakamura et al. showed that EVs isolated from the uterine fluid of pregnant ewes, compared to their cyclic counterpart, could upregulate the interferon-stimulated genes (ISGs) and attributes this to the interferon-tau (IFN-τ) containing exosomes released by conceptus (Nakamura et al., 2016). A recent study by Liu et al. demonstrated that EVs isolated from the endometrial cell cultures of patients with recurrent implantation failure hampered the growth and invasion of murine embryos (Liu et al., 2020). These studies show the role played by EVs in mediating the embryo-maternal communication during pre-conception period.

Considering the immune tolerance that needs to be developed in the endometrium during periconception, it is rational to consider regulatory RNA-mediated signalling as a possible means to prevent the rejection of semi-allogenic embryo. This regulatory RNA mediated intercellular communication has been observed in many physiological and pathological conditions where immune-tolerance plays a role, for instance, during host-parasite interactions (Shahid et al., 2018; Wang et al., 2018) and cancer metastasis (Probert et al., 2019; J. P. Wang et al., 2018), respectively. Most of these investigations emphasise non-coding RNA such as microRNA (miRNA), small single-stranded non-coding RNA molecules (containing 18–22 nucleotides) that play crucial roles in regulating gene expression. It is well established that miRNA, once released by one cell type, is transported to their target cells and affects those cells' transcriptome by degrading mature mRNA (Van Deun et al., 2017). A previous study by Es Hagi et al. has observed that other non-coding RNA such as long intergenic non-coding RNA (lincRNA) and even coding RNA molecules are transferred from trophoblast cells to endometrial cells, and alter their gene expression (Es-Haghi et al., 2019). Hence, regulatory RNA could be involved in mediating embryo-maternal communication. Regulatory RNA,

especially miRNA, are packaged and protected in extracellular vesicles (EV) to increase the probability of survival in the RNAse rich extracellular space (Boon & Vickers, 2013).

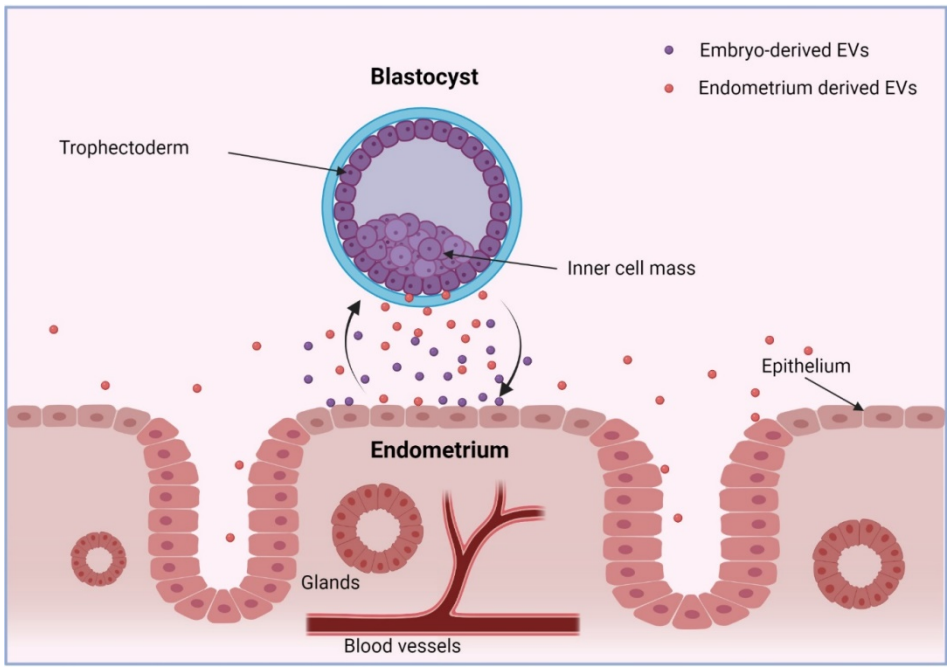

Figure 9. **Embryo-maternal communications at the endometrial interface**. EVs may play a crucial role in negotiations between the two entities when preparing for embryonic implantation. Created with BioRender.com

In addition to RNAs, EVs are known to carry other biomolecules such as proteins, lipids and DNA. These EV cargo molecules also would attribute to EV mediated embryo-maternal communication (Diaz-Garrido et al., 2021).

Current data on EV physiology, their diversity, cellular uptake and cargo delivery is not sufficient to provide a precise mechanistic explanation about EV mediated effects on target cells. As a result, despite evidence for EV-mediated embryo-maternal communication, little is known about EVs' functional specificity in mediating embryo-maternal cross-talk. Therefore, further research is warranted to disclose the functional specificity of EVs in the maternal tract.

2.5. Summary of the literature

Infertility is a disorder in the reproductive system, and according to the World Health Organization (WHO), infertility will be the 3rd most prevalent disease in the 21st century. Assisted reproduction technology plays a vital role during the management of infertility and includes IVF and ICSI. Unfortunately, the success of ART is not up to the expectations. One of the ways to improve the success is to identify better or complementary methods to assess embryo viability and predict embryo transfer success. The current gold standard, morphology-based evaluation of embryo quality, is not without limitations. Even though many different methods, including time-lapse imaging, pre-implantation genetic testing, and non-invasive assessment of embryo conditioned media have been investigated, no solid data support these methods' superiority over traditional morphology-based embryo assessment. Therefore, a dire need exists to find alternative or supplementary ways to indicate embryo quality and transfer success.

Due to the ethical and practical limitations and legal restrictions of using human embryos in research, animal models have been used in research. Studies have shown that bovine embryos are more suitable for studying early human embryogenesis. Furthermore, embryo and endometrial epithelial cell mimics have been developed using immortalized cell lines and stem cells. These models can be used as models to study the implantation and embryo-maternal cross-talk.

Extracellular vesicles are a relatively newly recognized domain of cell-cell communication and have drawn wider attention from the scientific community. Cells use these nanoparticles to convey messages to nearby or distant target cells by attaching to the cell surface membrane or delivering their molecular cargo to the target cells. This molecular cargo of EVs could indicate the pathophysiological status of the cells and thereby have the potential as a biomarker. EVs as biomarkers has been studied extensively in many disease conditions, as cancer. However, research investigating embryo-derived EVs in reproductive biology has been limited mainly due to the technical limitations of isolating and characterizing EVs from culture media. This has mainly limited studying the EVs released from individually cultured embryos. Therefore, the available EV isolation and characterization technologies should be further improved to deal with limited quantities of EVs. Furthermore, although EV release from single embryo cultures has been shown, none of the studies has investigated embryo-derived EVs from a biomarker standpoint involving all the pre-implantation developmental stages.

Studies have shown the positive effect that maternal tract EVs have on embryo growth and development. However, the role of embryonic EVs during embryo-maternal is less studied. To the best of my knowledge, embryonic EVs effects on the transcriptome of the maternal tract and functional specificity of such EVs have not been studied. Understanding the EV-mediated embryo-maternal communication at the embryo-oviductal and embryo-endometrial interface can be crucial to elucidate the mechanisms underlying successful implantation and recurrent implantation failure.

3. AIMS OF THE STUDY

The general aim was to investigate the potential of the embryo-derived extracellular vesicles (EVs) in indicating the embryo quality and the role of embryonic EVs and their specificity during embryo-maternal communication, using bovine embryo and human cell culture models.

The specific aims of this study were,

1. To isolate and characterize individually cultured preimplantation bovine embryo-derived EVs and assess their relationship with embryo quality and developmental stage (sub-study I).
2. To investigate the effects of supplementation of EVs released by individually cultured good quality and degenerating pre-implantation bovine embryos on the gene expression profile of primary bovine oviductal epithelial cells cultured *in vitro* (sub-study II).
3. To investigate the effects and specificity of JAr cell spheroid-derived EVs, compared to HEK293 cell spheroids-derived EVs, on differential expression of genes in RL-95-2 cells, and to investigate JAr EV RNA cargo compared to the HEK293 EV RNA cargo (sub-study III).

4. MATERIALS AND METHODS

4.1. Overall study design

The whole project aimed to investigate the potential of the bovine embryo-derived EVs in indicating the quality of the *in vitro* produced preimplantation embryos, and the role of such embryos or embryo surrogate JAr cell spheroid derived EVs during embryo-maternal communication. All the experiments were performed using bovine embryos (sub-study I and II) and a human cell culture model (JAr/RL-95-2 cells) (sub-study III). Figure 10 illustrates the overall study design of the project.

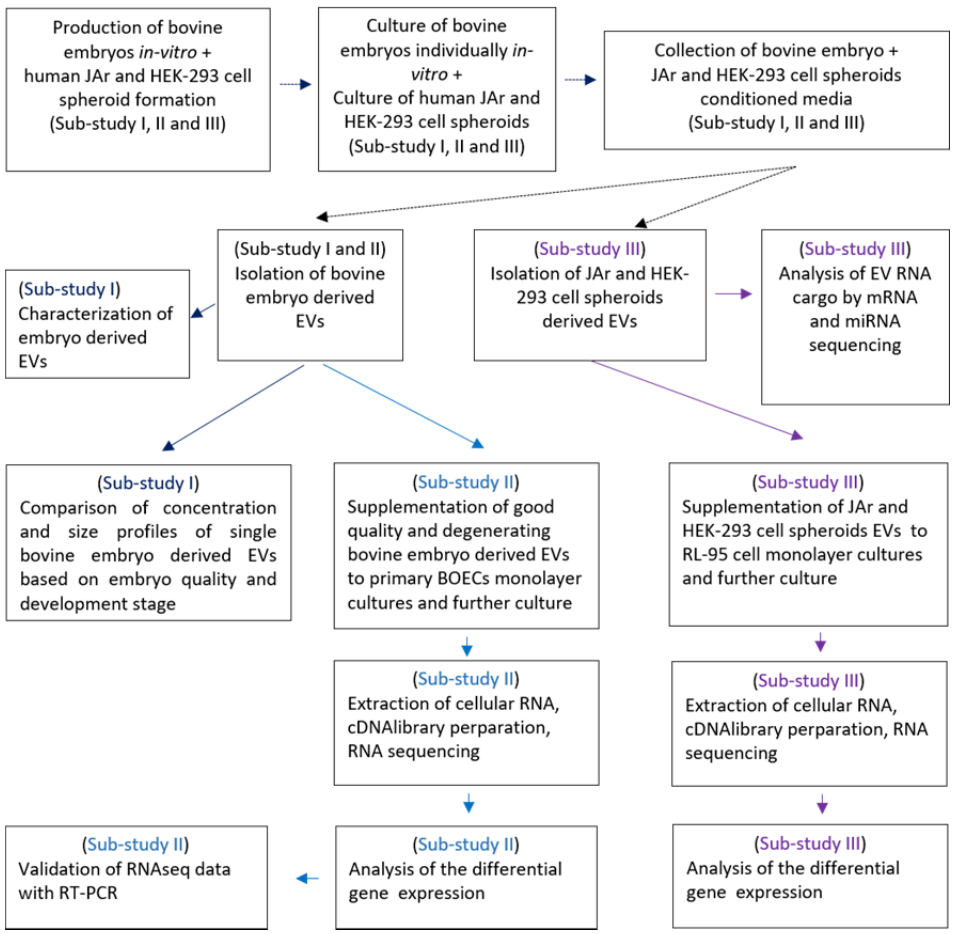

Figure 10. Study design of the whole project. The objectives of the study were investigated by 3 sub-studies

4.2. Ethics statement

In vitro production of bovine embryos was based on the oocytes obtained from slaughterhouse derived cattle ovaries and commercial semen samples. Therefore, no animals were sacrificed or used for these experiments. *In vitro* produced such embryos were used for 1st and 2nd sub-studies, whereas the 3rd sub-study was based on human immortalized cell lines. Therefore, ethical approval was not sought for the conduct of these experiments.

4.3. *In vitro* production of embryos (IVP) (sub-study I and II)

The production of bovine embryos was based on the method described by Nõmm et al. (Nõmm et al., 2019). Cattle ovaries obtained from the slaughterhouse, irrespective of the breed, age or stage of the oestrus cycle, were used to obtain oocytes. Cumulus-oocyte complexes (COCs) were aspirated, and quality code 1 oocytes (Bó & Mapletoft, 2013) were washed and matured in *in vitro* maturation (IVM) medium at 38.5°C with 5% CO_2 for 22–24 h. *In vitro* matured COCs were fertilized using frozen-thawed semen obtained from Estonian Holstein Breed (EHF). The COCs and sperms were co-incubated in 4-well plates in groups in Fert-TALP media at 38.5°C with 5% CO_2 for 18–20 h.

After co-incubation, cumulus cells were removed mechanically from the presumptive zygotes by vortexing. Subsequently, denuded embryos were added to 60 µl droplets (one embryo per droplet) of *in vitro* culture (IVC) media under mineral oil (Nõmm et al., 2019) for single embryo culture. IVC media was prepared by supplementing 0.8% BSA to modified Synthetic Oviductal Fluid with amino acids and myo-inositol. Such individual embryo culture was performed to sample conditioned media at different time points of embryo culture. Presumptive zygotes were cultured for 8 days in an incubator with 5% CO_2 and 90% N_2 at 38.5°C. Embryos were evaluated, based on morphological criteria, at day 2, 5 and 8 post-fertilization, and the developmental stages and embryo quality were recorded (Bó & Mapletoft, 2013). Development stages that were morphologically evaluated were cleavage (day 2), morula (day 5) and blastocyst (day 8) stages. As the control, embryo culture media droplets were incubated in parallel to embryo cultures but without embryos. IVP was done by Monika Nomm and Yosra Ressaisi

In sub-study 1, embryo conditioned media samples (50 µl) were obtained from the droplet cultures at day 2, day 5, and day 8 post-fertilization from different batches of individual embryo cultures. Subsequent to the collection of embryo conditioned media at day 2 or 5, the embryos were cultured further in the residual culture media droplet until day 8 post-fertilization. The collected media samples were refrigerated at -80°C till used for downstream analysis.

The collected conditioned media samples were classified according to the morphology-based evaluation of the embryos at three-time points following the fertilization as follows.

At day 2: Day 2 good quality embryo media; media conditioned by embryos cleaved by day 2 and subsequently developed to blastocysts by day 8, and Day 2 bad quality embryo media; media conditioned by embryos that cleaved by day 2 but after that degenerated.

At day 5: Day 5 good quality embryo media; media conditioned by embryos that developed to morula by day 5 and subsequently developed to blastocysts by day 8, and Day 5 bad quality embryo media; media conditioned by embryos developed to morula by day 5 but after that degenerated

At day 8: Day 8 good quality embryo media; media conditioned by embryos that developed to blastocysts by day 8, and Day 8 bad quality embryo media; media conditioned by embryos developed to morula by day 5 but after that degenerated.

As the control samples, the same embryo culture media samples were incubated in droplets (without embryos) for 2, 5 or 8 days, and denoted as "Day 2 control", "Day 5 control" and "Day 8 control", respectively.

In sub-study 2, embryo conditioned media samples (50 µl) were collected at day 5 after fertilisation from the bovine embryos cultured individually. After the conditioned media collection, embryos were cultured further in the residual media until the 8th-day post-fertilization. The collected embryo conditioned and control media samples were stored at -80°C until the isolation of EVs. The collected conditioned media samples were labelled retrospectively, depending on the morphological evaluation. Two categories of embryo conditioned media samples allocated for sub-study 2 were: Day 5 good quality embryo media" (culture media conditioned by embryos that developed to morula by day 5 and later became blastocysts by day 8) and "Day 5 degenerating embryo media" (culture media conditioned by embryos that cleaved by day 2 and after that degenerated). As the control samples, culture media samples were incubated similar to embryo cultures but without embryos, and were collected at day 5.

4.4. Cell culture

Primary bovine oviductal epithelial cell (BOEC) culture (sub-study II)

Bovine oviducts were acquired from the abattoir. An oviduct from a cow in the post-ovulatory stage (Day 0–3 post-ovulation) of the oestrous cycle was selected guided by the ovulation site of the ipsilateral ovary (Ireland et al., 1980). The chosen oviduct was cleaned properly by washing with wash solution I (DPBS solution + 1% Penicillin/Streptomycin + 1% Amphotericin B), and the connective tissues were dissected out. The isthmus and the ampulla of the oviduct were identified and separated from the ampullary-isthmic junction. Oviductal mucosa was cautiously expelled by gently squeezing the oviduct with the aid of a sterile glass slide. The cells were collected and moved into a tube

containing washing medium II (DPBS solution + 5% FBS +1% Penicillin/ Streptomycin + 1% Amphotericin B). Harvested cells were washed two times in washing media II by centrifuging at 180 g for 2 minutes at 4°C. Following the washing steps, the cell pellet was dissolved in 5 ml of BOEC culture medium (DMEM/F12 medium+ 10% FBS + 1% Penicillin/Streptomycin + 1% Amphotericin B). The cell suspension was added to a 100 mm Petri dish and topped up to a 10 ml total media volume, and cultured at media at 38.8°C in a 5% CO_2 incubator. Initially, cells were cultures for 72 h uninterrupted before the cells were evaluated. Subsequently, the culture medium was replaced every 48 h. Once the cells became 80% confluency, the cells were split once and cultured to increase the cell population. When the cell subculture became 80% confluent, they were detached by adding trypsin and subsequently cryopreserved in separate aliquots.

Prior to the EV supplementation experiments, one of the cryopreserved vials of BOECs (ampullary region) was thawed at a time and cultured in BOEC culture medium as previously described. Following 48 h of culture, the BOEC monolayer was washed, trypsinized, and then sub-cultured in 4-well plates (Nunc, Denmark) by adding ~50,000 cells per well.

JAr and HEK293 cell spheroid formation (sub-study III)

As an analogue of human trophoblasts, a human choriocarcinoma cell line (JAr) deriving from the first trimester trophoblasts (ATCC® HTB-144™, Teddington, UK) was used. A vial of frozen JAr cells was thawed and cultured in RPMI 1640 media (Gibco, Scotland) supplemented with 10% FBS, 1% Penicillin/ Streptomycin and 1% L-glutamine in a 5% CO_2 incubator at 37°C. When the JAr cells approached the confluency, they were washed with Dulbecco's phosphate-buffered saline (DPBS, Verviers, Belgium), then detached using trypsin-EDTA (Gibco® Trypsin) and finally pelleted by centrifuging at 250 g for 5 min at RT. Consequently, JAr cells were counted, and one million cells were added per 60 mm Petri dish in 5 ml of media and incubated in a 5% CO_2 incubator at 37°C. During the culture, the Petri dishes were placed on a gyratory shaker (Biosan PSU-2T, Riga, Latvia) set at 295 rotations per min (rpm) inside the incubator and incubated for 18 h. The multicellular spheroids formed at the end of the culture were used as analogue of trophoblast cells *in vitro*.

As an analogue of human non-trophoblast cells (control), human embryo kidney cell line (HEK-293, ATCC® CRL-3216™) was used. A HEK293 cell vial was thawed and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS (Gibco) and 1% L-glutamine (Sigma) in T75 flasks at 37°C in a 5% CO_2 incubator. Until the cells reached the confluency, the media were changed every second day. After detaching the cells using trypsin-EDTA, one million cells were counted and added per 60 mm Petri dish and cultured overnight on a gyratory shaker to form multicellular spheroids as described above.

EV depleted FBS was prepared using the ultrafiltration method previously described (Kornilov et al., 2018). When preparing EV depleted complete media

for each cell type, such EV depleted FBS was supplemented at 10% of the total volume of the respective media. Following the formation of spheroids, they were moved into 60 mm dishes containing EV depleted cell culture media (5 ml per dish). Approximately 5,000 spheroids were added to each dish. Then, the spheroids were incubated while kept on a slow rotating gyratory shaker for 24 h. Following the incubation, conditioned media samples (approximately 100 ml) were collected and used for isolating EVs. Cell culture was carried out by Kasun Godakumara and Keerthie Dissanayake.

RL-95-2 cell culture (sub-study III)
RL95-2 cells (ATCC® CRL-1671) were used as an analogue of the human receptive endometrium. They were cultured in DMEM medium supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% L-glutamine and 5 µg/ml Insulin at 37°C in a 5% CO_2 incubator (Godakumara et al., 2021). The media was changed every other day till the cells reached confluency. Once they got 80% of the confluency, cells were washed, trypsinized and sub-cultured in 12 well plates till they reached 80% confluency.

4.5. Isolation of EVs (sub-study I, II and III)

In sub-study 1, initially, individual embryo conditioned media samples (~50 µl) were thawed on ice. Once thawed, 45 µl of each media sample was diluted in sterile-filtered DPBS to a final volume of 150 µl. These diluted media samples were then passed through two centrifugation steps. Firstly, the samples were centrifuged at 400 g for 10 min at 4°C for removing dead cells and cell debris. Subsequently, 145 µl of the supernatant was moved to another tube and centrifuged at 2,000 g for 10 min to get rid of apoptotic bodies. From the supernatant, 140 µl were collected to a tube and kept on ice before EV isolation. EVs from individually cultured embryo conditioned media were isolated using qEVsingle size exclusion chromatography columns (Izon Science Ltd. UK). In brief, the columns were positioned vertically in a column holder. The lower and upper lids of the SEC column were detached, and the preservative fluid in the column was allowed to drain. Subsequently, the SEC columns were equilibrated by letting 10 ml of freshly filtered DPBS pass through the column. Then, the 140 µl of pre-processed embryo media sample was placed on top of the SEC column, and the collection of fractions (200 µl each) was commenced straightaway. The column was topped up with DPBS to make sure the flow continued without pause during the period of fraction collection. The first five fractions were the void volume, and the subsequent four fractions (fraction number 6–9) were expected to be the fractions containing embryo derived EVs. Those four fractions were pooled before subsequent processing.

In sub-study 2, samples belonging to “Day 5 good quality embryo media” (n = 40), “Day 5 degenerating embryo media” (n = 40), and “Day 5 control media” (n = 40) were identified and thawed on ice and then pooled according to

their category. Pooled conditioned media (~2 ml) and control media (~2 ml) samples were subjected to double centrifugation steps as described previously. Supernatants were moved to new tubes and concentrated to 150 µl by centrifugation using 10K Amicon ultra-2 centrifugal filter units. The concentrated media samples were subjected to EV isolation, similar to study 1. Following the isolation of EVs, fractions 6–9 from each sample type were concentrated separately to ~220 µl. Twenty microlitres of the concentrated EV samples were used for the quantification by NTA. The remaining 200 µl sample from each group was aliquoted into 50 µl fractions and refrigerated at -80°C till EV supplementation experiments were performed.

In sub-study 3, EVs were isolated from media conditioned by JAr and HEK293 spheroids. These media samples were sequentially centrifuged at 400 g for 10 min, 4,000 g for 10 min and finally at 20,000 g for 15 min to get rid of cells, cell debris and apoptotic bodies, respectively. After each centrifugation step, the supernatants were transferred to new 50ml tubes for the next step of the sequential centrifugation. Subsequently, the conditioned media were concentrated to 500 µl using 10K Amicon® Ultra-15 centrifugal filter devices. Degradation of EV RNA during the isolation procedure was inhibited by adding RNase inhibitor (1u/µl) to each conditioned media sample. EVs were purified based on SEC method using Econo-Pac® Chromatography columns (10 cm, Bio-Rad, 7321010)) packed with cross-linked 4% agarose beads of 90 µm size (Sepharose 4 fast flow™, GE HealthCare Bio-Sciences AB, Uppsala, Sweden). Fraction numbers 7–10 (fraction size 1 ml) were retrieved as the fractions containing EVs, and these fractions were pooled and subsequently concentrated as previously described. Trophoblast (JAr) and non-trophoblast (HEK293) spheroid derived EVs were isolated by Kasun Godakumara.

4.6. Characterization of bovine embryo-derived EVs (sub-study I)

Nanoparticle Tracking Analysis (NTA)

The concentration and size profile of NPs isolated from embryo conditioned media and control samples were measured using the nanoparticle tracking analyzer-Zetaview®. Zetaview® was auto-aligned using 100 nm-sized standards (Applied Microspheres BV, Netherlands. Catalogue no. 10100). Samples were measured (concentration and size profile) in the scatter mode. Following settings were used for sample measurements in the scatter mode; sensitivity: 75, shutter: 100, frame rate: 30 fps and number of cycles: 3. Each of the samples was measured three times. After measuring each sample, the measurement chamber ("cell") of the instrument was properly cleaned using Milli-Q® water and DPBS before the injection of the next sample.

EV Array

EV array technology was used to characterize EVs, based on the EV marker proteins (Jørgensen et al., 2013). Conditioned media samples obtained at day 5 post-fertilization from embryos that developed to morula stage by day 5 (n = 40) and culture media samples incubated *in vitro* till day 5 without embryos as controls (n=40) were used. Each pooled media sample was subjected to two-step sequential centrifugation, as described earlier, to remove potential larger particles. For direct testing of the media (before purification), 100 µl fractions were moved to two separate fresh tubes and labelled as media before EV isolation (sample and control). The rest of the embryo conditioned media sample and the control samples were used for isolating EVs using qEVsingle columns. EV isolation was performed as previously described and concentrated to a final volume of ~100ul. All four samples (conditioned media and control samples before and after purification) were refrigerated at -80^{0}C till performing EV array.

Using a sciFLEXARRAYER S12 (Scienion AG, DE), microarray slides used for the EV array were produced. In brief, antibodies were printed on epoxy-coated slides (SCHOTT Nexterion, Germany) with a coated PDC4 size 60 (Scienion AG). As the positive and negative controls, respectively, biotinylated human IgG (100 mg/mL) and PBS with 5% glycerol were used. EV array was produced using twenty-two antibodies, including one anti-bovine antibody (CD63, clone CC25, BioRad, CA, USA) and 21 anti-human antibodies (Dissanayake et al., 2020). Finally, antibodies were diluted in PBS (with 5% glycerol) and printed in triplicates at 200 mg/mL concentration.

Based on the method described by Jørgensen et al. (2013), EV array was performed with some alterations. In brief, initially, the microarray slides were blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0), and subsequently, 40 µL sample diluted in wash-buffer (PBS/0.05% Tween®20) was added and incubated. The incubation was carried out in Multi-Well Hybridization Cassettes (ArrayIt Corporation) at RT for two hours followed by overnight incubation at 4°C. To detect retained EVs, a biotinylated anti-bovine antibody against CD9 (Antibodies-Online.com, DE, dilution 1:1500), and Cy5-labelled streptavidin (Life Technologies, MA, USA, dilution 1:1500) were used. Scanning of fluorescence and the detection of spots were carried out as previously described (Bæk & Jørgensen, 2017). EV array was performed Malene Møller Jørgensen and Rikke Bæk at the Department of Clinical Immunology, Aalborg University Hospital, Denmark.

Transmission electron microscopy (TEM)

For the TEM imaging, EVs isolated from pooled day 5 single embryo conditioned media samples (n = 60) were used. Isolated EVs were concentrated as described previously and refrigerated at -80^{0}C till shipped for electron microscopy facility. From each of the purified sample, a drop was placed on formvar/carbon-coated 200 mesh grids (Agar Scientific, Stansted, UK) and allowed to adsorb for 20 min.

Subsequently, samples were fixed using Karnovsky fixative (2% paraformaldehyde and 1% glutaraldehyde; Sigma-Aldrich, Germany; Polysciences, USA, respectively). Fixed samples were contrasted in uranyl oxalate. In the next step, samples were embedded in a mixture of methylcellulose (Sigma-Aldrich, Schnelldorf, Germany) and uranyl acetate (Polysciences, Warrington, USA). For imaging of the samples, a JEM 1400 transmission electron microscope (JEOL Ltd. Tokyo, Japan) was used at 80 kV. Using a numeric camera (Morada TEM CCD camera, Olympus, Germany), digital images were obtained. TEM imaging were done by Aneta Andronowska at the Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Poland

Scanning electron microscopy (SEM)

For the SEM imaging, EVs isolated from a pooled day 5 single embryo conditioned media samples (n = 30) were used. The concentrated EV sample was refrigerated in a mechanical freezer at -80°C before being shipped to a SEM facility. At the SEM facility, the sample was thawed, and a drop from the EV sample was placed on an aluminium foil and kept overnight for drying. The following day, the dried sample was sputter-coated with gold and imaged in a Hitachi S-4300 SEM microscope. SEM imaging were done by Sourav Bhattacharjee at the School of Veterinary Medicine, University College Dublin (UCD), Belfield, Dublin 4, Ireland

4.7. Supplementation of embryo and cell spheroid derived EVs to epithelial cell cultures (sub-study II and III)

In sub-study II, when the BOEC monolayer approached 80% confluency, the media was discarded and washed once with 0.8% BSA supplemented SOF medium. Subsequently, 0.8% BSA supplemented SOF media (450 µl) with added 50 µl of either EVs (from embryo conditioned media) or NPs (from control media) were added to the corresponding BOEC monolayer culture. With reference to the amount of EVs supplemented per well, each well with BOEC monolayer culture received either EVs isolated from 10 single bovine embryo culture samples or NPs isolated from 10 droplets of control media samples. Following the supplementation of EVs/NPs, BOECs were further cultured for another 8 hours at 38.5°C with 5% CO_2 and 90% N_2. This experimentation was performed four times using BOECs cultured on four separate days.

In sub-study III, once the 80% confluency was reached, conditioned culture media were discarded and replaced with EV depleted culture media supplemented with ~1×10^8 EVs (per well) from JAr cell spheroids (trophoblast analogue) and HEK293 cell spheroids (non-trophoblast). As controls, RL95-2 cells cultured in EV-depleted media were used without supplementation of EVs. Cells were further cultured for 24 h. After the culture, the media were discarded, and the cells were used for total RNA extraction. The study was carried out three times on three separate days to obtain materials for three replicates.

4.8. Extraction of cellular and EV RNA (sub-study II and III)

Extraction of the total RNA from BOECs, RL-95-2 cells, and JAr and HEK293 EVs were performed using QIAzol lysis reagent (Qiagen® Sciences, USA). Briefly, 300 µl of QIAzol lysis reagent was added to each cell monolayer and incubated at RT for 10 min. In the case of JAr and HEK293 EVs, ~ 1×10^{12} EVs from each sample were used. The suspensions were mixed thoroughly by pipetting and moved to sterile microcentrifuge tubes separately. Following the addition of 150 µl of chloroform and vortexing for 15 sec, the mixtures were incubated at RT for 3 min. Following the incubation, the samples were centrifuged at 12,000 g for 15 min at 4°C. At the end of centrifugation, 3 distinct layers were formed. Of the 3 layers, the aqueous phase was collected to a new tube. A similar quantity of isopropyl alcohol was added. RNA yield was increased by adding 20 µg of glycogen (UltraPure™ Glycogen). Subsequently, the sample was incubated at RT for 10 min, and then centrifuged at 12,000 g for 30 min at 4°C. The RNA pellet was precipitated and washed three times using 500 µl of 70% ethanol each time and centrifuging at 12,000 g for 5 min at 4°C. Following the washing step, the RNA pellet was air-dried and diluted in 20 µl of nuclease-free water by incubating at 70°C for 10 min. The quantification of RNA was carried out using Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific, USA). The quality of RNA was determined by Bioanalyzer Automated Electrophoresis instrument (Agilent Technologies, USA) using Agilent RNA 6000 nano Kit (Agilent Technologies, USA). RNA extraction procedures were performed by Keerthie Dissanayake and Kasun Godakumara.

When isolating miRNAs from JAr and HEK293 spheroid derived EVs, 100 µl of RLT buffer (Qiagen) was mixed with EVs (1×10^{8}) isolated from 10 ml of conditioned medium of each type of spheroids. Then the mixture of EV/RLT buffer was mixed with 2 µl of pellet paint (Merck Millipore) and briefly vortexed. Then, 19 µl of 3M Sodium Acetate and 300 µl of 100% ethanol were added to it and briefly vortexed. The mixture was incubated overnight at 4°C. The following day, the mixture was centrifuged at 16,000 g for 15 min at 4°C, and the supernatant was removed cautiously. The pellet was washed carefully two times using 1 ml of fresh 80% ethanol and air-dried. Finally, the RNA pellet was dissolved in RNase free water (10 µl) and refrigerated at -80°C.

4.9. Preparation of the sequencing libraries and RNA sequencing (sub-study II and III)

The preparation of RNA sequencing (RNAseq) libraries from RNA isolated from BOECs, RL-95-2 cells and JAr and HEK293 EVs were performed using the Smart-seq2 methodology (Picelli et al., 2014) with slight modifications. The modifications made in the protocol were the use of 20 ng of total RNA for cDNA synthesis instead of using single cells, the performance of 10 cycles of PCR for pre-amplification and the replacement of KAPA HiFi DNA

polymerase with Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Using the Illumina Nextera XT DNA Sample Preparation Kit (FC-131-1024), 2 µL of diluted cDNA was applied to dual-index library preparation. For all cleanup steps and the size selection of 200-700 bp fragments of the final libraries, AMPure XP beads (Beckman Coulter) were used. RNA sequencing libraries were prepared by Kersti Jääger and Agne Velthut-Meikas. All the samples were pooled into single library mix by equal concentration. RNA sequencing was done on an Illumina NextSeq500 desktop sequencer using High Output Flow Cell v2.5 (single-end, 75 bp) at the Institute of Genomics Core Facility, University of Tartu, Estonia.

The preparation of small RNA libraries was carried out from miRNAs extracted from JAr and HEK293 cell-derived EVs, using the protocol described by Faridani et al. (Faridani et al., 2016; Hagemann-Jensen et al., 2018). The purification of the amplified libraries was carried out using AMPure XP beads mixed 1:1 with the sample and collected in 10 µl of RNase free water. The quantification of DNA was done using Qubit HS DNA assay (Thermo Scientific), and the QC of the DNA library was based on the Bioanalyzer 2100 station (Agilent). Finally, DNA from each library (5 ng) was mixed and sequenced 1x100 bp using Illumina NovaSeq platform (National Genomics Infrastructure, SciLifeLab, Sweden). Nageswara Rao Boggavarapu and Omid R. Faridani optimized miRNA sequencing system and performed miRNA-seq.

4.10. Processing, alignment, and quantification of RNA sequencing (RNAseq) reads of BOEC and RL-95-2 cell samples (sub-study II and III)

The quality of the raw reads obtained following the RNAseq was evaluated by FASTQC v0.11.8 (Brown et al., 2017). Reads trimming and the removal of adaptor sequences was performed by Trimmomatic v0.39 (Bolger et al., 2014).

In the case of BOECs RNAseq data, the remaining reads were aligned to ARS-UCD1.2 *B. taurus* genome assembly using HISAT2 alignment programme (Kim et al., 2019). Splice site annotations acquired from the corresponding genome assembly annotation file (version 1.2.97) was also included (Kim et al., 2019). Using featureCounts programme, reads were counted at the gene level considering only uniquely mapping reads (mapping quality score ≤ 8) (Liao et al., 2014). Genes with at least 10 counts for three out of the four replicates in at least one of the experimental groups were used for differential expression analysis.

In the case of RL95-2 cell, JAr and HEK-293 EV RNAseq data, reads were aligned to the hg38 human reference genome using HISAT2 alignment programme (Kim et al., 2019). The featureCounts programme was used to obtain Gene-level read counts (Liao et al., 2014). Ensembl H. sapiens annotation file (GRCh38.97) was used for genomic feature annotations. Subsequent differential

expression testing was based on the genes that had a minimum of 10 counts for all the replicates in at least one of the experimental groups. Processing, alignment, and quantification of RNA sequencing (RNAseq) reads were carried out by Freddy Lättekivi.

In the case of JAr and HEK-293 EV miRNA data analysis, Partek Flow bioinformatics software (Partek Inc, USA) was used. Reads were aligned to human genome Hg38 using Bowtie 2 aligner and quantified to Hg38 miRBase mature miRNAs database. Based on the prepared miRNAseq libraries from JAr and HEK293 EV samples, miRNA alignment counts were examined to identify putative JAr EV-specific miRNAs. The number of miRNAs detected over 1, 3, 5, and 10 raw count thresholds in at least 2 of the 3 libraries of either of JAr or HEK293 EVs were counted. Thus, the cut off set for miRNA to be JAr EVs specific was having a minimum of five counts in 2 of the 3 JAr EV libraries, whereas not detecting it at all in any of the HEK293 EV libraries. A list of predicted target transcripts was acquired from miRDB, and they were filtered to keep high-confidence targets (target score of ≥90). REFSEQ transcript IDs were transformed to ENSEMBL gene IDs using the R package AnnotationDbi to obtain the list of predicted miR targets at the gene level. Thus, putative miRNA targets were identified in the RL95-2 gene expression data based on the ENSEMBL IDs. Subsequently, the number of putative targets that were differentially and non-differentially expressed within the RL95-2 gene expression data for each miRNA were counted.

4.11. Quantitative real-time PCR (RT-qPCR) validation of BOECs RNAseq data (sub-study II)

For the validation of results obtained from BOECs RNAseq data, RT-PCR was performed based on the same RNA samples utilized for RNAseq. . Designing the primers was carried out using NCBI primer blast (Ye et al., 2012). Gene exon-exon junctions were included during primer design. Primer quality was verified using Integrated Genome Technologies-IDT™ (Idt, 2014). Designed primers were obtained from Microsynth AG (Wolfurt, Austria).

RNA samples were reverse transcribed using FIREScript RT cDNA Synthesis mix™ with Oligo (dT) and random primers (Solis BioDyne, Estonia). RT-qPCR was performed using HOT FIREPol® EvaGreen® qPCR Supermix (Solis BioDyne, Estonia) on a QuantStudio 12K Flex™ real-time PCR system (ThermoFisher Scientific). The instrument settings used during the RT-qPCR were: enzyme activation at 95°C for 15 min; denaturation at 95°C for 20 s, 40 cycles; annealing at 57°C for 20 s, 40 cycles; and extension at 72°C for 20 s, 40 cycles.

4.12. Statistical data analysis

In the sub-study, I, embryo's survival distributions in EV depleted and regular IVC media culture conditions were evaluated using the Log-rank test, with the correction of the p-values for multiple testing based on Bonferroni adjustment. The statistical significance in the differences in the NP concentration and their average size in the three experimental groups and developmental stages were tested using linear mixed models (LMM) fitted by residual maximum likelihood (REML) approach. The resulting estimated marginal means (EMMs) were subjected to t-test to obtain the p-values, and were adjusted with Tukey's multiple comparison test.

In the sub-study II, the differential expression of genes in BOECs in response to the supplementation of embryo derived EVs were analyzed with a generalized linear model followed by likelihood ratio tests. The differential expression of genes was considered statistically significant if the false discovery rate (FDR) ≤ 0.05. Gene set enrichment analysis (GSEA) was conducted using the clusterProfiler package (Yu et al., 2012) and pathway annotations from KEGG Pathway database (Kanehisa et al., 2017). Results were regarded as statistically significant if FDR ≤ 0.05. Gene expression data obtained from qPCR test was compared using Mann-Whitney U Test. Benjamini-Hochberg method was used to adjust the p-values for multiple testing. Gene expression values were standardized (z-score) within genes for the comparison of qPCR and RNAseq results.

In sub-study III, analysis of the differentially expressed genes in RL-95-2 cells in response to EV supplementation compared to control was similar to sub-study 2. ReactomePA package and Reactome Pathway database annotations were used for GSEA and pathway over-representation analysis.

For the statistical data analysis in sub-study I, R programme (R, 2017) was used along with its lme4 (Bates et al., 2015) and emmeans (Russell et al., 2018) packages. EdgeR (version 3.26.8) (Robinson et al., 2009), was used to analyze differentially expressed genes in BOECs and RL-95-2 cell in response to the supplementation of respective EVs (in sub-study II and III). Using the prcomp function of the R package, principal components were calculated. Heatmaps were prepared using the pheatmap package (Kolde, 2019). All the figures were generated by ggplot2 package (Wickham, 2016). For the statistical data analysis of sub-studies Freddy Lättekivi, James Ord and Kasun Godakumara and Keerthie Dissanayake contributed.

5. RESULTS

5.1. Sub-study I: Isolation and characterization of individually cultured bovine embryos, and evaluating the association of concentration and size of EVs with embryo quality and development stage

5.1.1. Study design

In sub-study I, two experiments were performed. 1) investigating the impact of depleting EVs from culture media before embryo culture for subsequent bovine embryonic development: 2) isolating and characterizing EVs deriving from individually cultured bovine embryos in regular embryo culture media and comparing their concentration and size differences based on embryo quality and development stage (Figure 11).

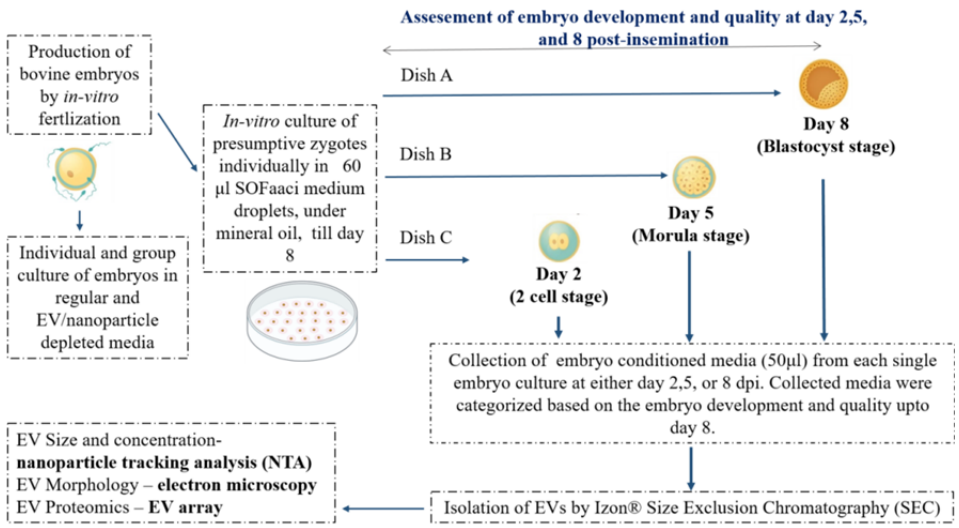

Figure 11. Sub-study I experimental design.

5.1.2. Effects of EV/nanoparticle depletion from culture media for the individual and grouped embryo development *in vitro*

Under EV/nanoparticle depleted culture conditions, while 54.5% of individually cultured embryos reached the morula stage, only 3% of the embryos were developed to the blastocyst stage (Table 19). On the other hand, 39.3% of the individually cultured presumptive zygotes in regular IVC media were developed to the blastocyst stage. Therefore, the overall survival was poor when pre-implantation bovine embryos were cultured individually in media depleted of EVs (p=0.013) compared to culturing them in regular IVC media (without EV depletion). However, the overall survival of preimplantation bovine embryos was better when they were cultured as groups in the media depleted of EVs (p=0.014) compared to culturing them individually in media depleted of EVs. Thus, the blastocyst rate when presumptive embryos were cultured in groups in EV-depleted medium was similar to the blastocyst rate when embryos were cultured individually in regular IVC media.

Table 19: Preimplantation embryo culture- individually and as groups in EV-depleted and regular IVC media.

	Individual embryos cultured in regular IVC media	**Individual embryos cultured in EV-depleted IVC media**	**Group culture of embryos in EV-depleted IVC media**
	n (%)	n (%)	n (%)
Oocytes	33 (100)	33 (100)	63 (100)
Zygotes	31(93.9)	27 (81.8)	53 (84.1)
Morula	20 (60.6)	18 (54.5)	37(58.7)
Blastocysts	13 (39.3)	1 (3.0)	25 (39.6)
p-value	0.013*		0.014**

Data are represented as numbers (n) or percentages (%).* p-value is obtained from the Log-rank test performed for the overall survival of bovine embryos when cultured individually EV-depleted media in comparison to regular media. ** p-value was obtained from Log-rank test performed for overall survival of bovine embryos cultures in groups in EV-depleted media in comparison to bovine embryos cultured individually. Adapted from (Dissanayake et al., 2020).

This experiment showed that filtration of BSA solution loses certain factors such as EVs/NPs or biomolecules that are needed for the growth and development of individually cultures embryos to the blastocyst stage. Therefore, individual embryo culture for the subsequent experiments was performed in regular IVC media.

5.1.3. Individual culture of bovine embryos and morphological assessment of their development

Individual presumptive zygotes were *in vitro* cultured in regular IVC media (60 µl droplets) under mineral oil until day 8 post-fertilization. Following the collection of a fraction of embryo conditioned media (50 µl) from each media droplet at day 2 or day 5, those embryos were further cultured in the remaining media droplet till day 8. A sum of 105 presumptive zygotes were *in vitro* cultured (35 zygotes for each of the three replicates) for each time point of media collection. Embryo quality was evaluated using morphology-based criteria at day 2, day 5 and day 8 post-fertilization.

In the day 2 group (conditioned media collection at day 2), approximately 25% of the presumptive zygotes became blastocysts by day 8. In the same group, ~27% presumptive zygotes were cleaved by day 2 and later degenerated (Table 20). In the day 5 group (conditioned media collection at day 5), ~28% of the presumptive zygotes were developed to the blastocysts by day 8. In this group, ~17% presumptive zygotes that developed to morula by day 5 later degenerated by day 8. Finally, in the day 8 group (conditioned media collection at day 8), while 23% of the presumptive zygotes became blastocyst, 21% of the presumptive zygotes degenerated after becoming morula by day 5.

Table 20: Morphological assessment of embryo development

	Timepoint of media collection		
Embryo development	**Day 2**	**Day 5**	**Day 8**
Presumptive zygotes (n)	35	35	35
Not cleaved by day 2 (%)	26.66 ±2.05	23.80±3.10	24.76±3.11
Cleaved by day 2, but subsequently degenerated (%)	26.66 ±4.11	31.42±3.56	31.42±4.85
Developed to morula by day 5, but subsequently degenerated (%)	21.90 ±1.55	17.14±1.34	20.95±3.39
Developed to blastocysts by day 8 (%)	24.76± 2.05	27.61±2.80	22.85±4.85

Data are shown as counts (n) or mean ± SEM of percentage. Corresponding to each time point, embryo conditioned media samples were obtained from 105 samples. There were three replicates with 35 samples in each replicate. Adapted from (Dissanayake et al., 2020).

Overall, the collection of media before the end of embryo culture is unlikely to have had a negative impact on the embryo development as similar blastocyst rates were observed in 3 groups. EVs isolated from the conditioned media of these sets of embryos were used for the EV characterization and comparison of the EV size profile and concentrations.

5.1.4. Biochemical and physical characterization of the individually cultured bovine embryo-derived EVs

Characterization of the pre-implantation bovine embryo-derived EVs was carried out using biochemical means; EV array and physical means; nanoparticle tracking analysis (NTA), scanning (SEM) and transmission electron microscopy (TEM).

For biochemical characterization of EVs, isolated nanoparticles were phenotyped to prove their EV identity based on the EV array. Here, twenty-two different antibodies were used to capture EVs onto a microarray (EV Array). An anti-bovine CD9 antibody was used for the detection of the captured EVs on the microarray. The EV Array illustrated that NPs isolated from culture media conditioned by bovine embryos show strong positivity for tetraspanin proteins CD9 and CD81. Furthermore, CD63, CD82, p53, Alix and HLA G were weakly positive in the NPs isolated by media conditioned by bovine embryos cultured individually (figures 12A and 12B). In comparison, NPs isolated from control culture media samples were only very weakly positive for CD9, CD81, CD82, HLA G, Alix and p53. Therefore, EVs were considered to be absent among the NPs isolated from control media. Furthermore, EVs were enriched following their isolation using SEC as evidenced by enhanced signal intensity for EV markers in sample I against sample II. Likewise, a very weak increase in the fluorescent signal intensity was detected in the control media samples (sample III vs sample IV).

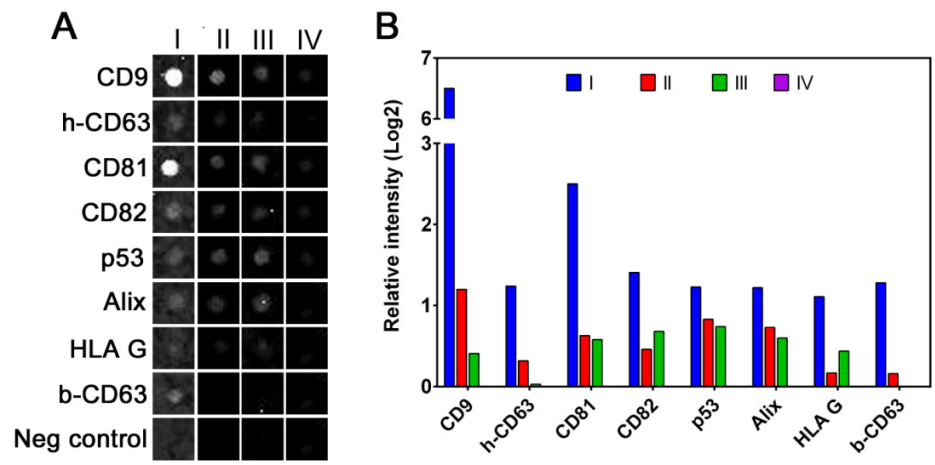

Figure 12. Biochemical characterization of embryo-derived EVs using EV array. Sample I: Day 5 embryo conditioned media-derived NP sample, Sample II: Day 5 embryo conditioned media (before SEC), Sample III: Day 5 control media derived NPs, and Sample IV: Day 5 control media (before SEC). (A) Microarray spots generated by detecting fluorescent signals with anti-bovine CD9 antibody. Positive signals were only seen in the spots visualized. (B) The histogram visualizes thelog2 transformed fluorescence intensities compared to negative control spots. Adapted from (Dissanayake et al., 2020).

According to the EV array findings, embryo conditioned media derived NPs were referred to as EVs, whereas NPs derived from control media were referred to as NPs themselves. This is due to the positive proteomic proof for particles isolated from embryo conditioned media, whereas particles isolated from the control media sample did not have positive proteomic evidence.

Figure 13 depicts the average size distributions of EVs isolated from embryo conditioned media derived EVs, and the control media derived NPs of day 2, 5, and 8 samples. Most of the particles were observed to be sized between 30–330 nm. This, in general, is the average size distribution of EVs where exosomes are known to be 30–100 nm and microvesicles ranging from 100–1000 nm.

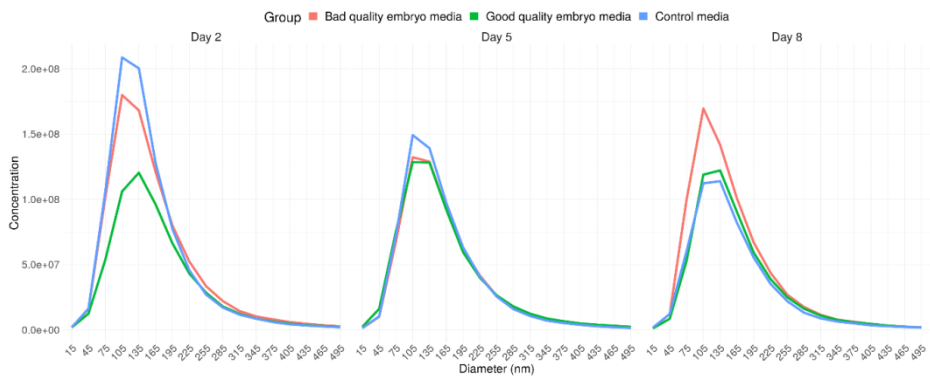

Figure 13. NTA based physical characterization of EVs. “Day 2 good quality embryo media” (n = 23); “Day 2 bad quality embryo media” (n = 22); “Day 2 control” (n = 15); “Day 5 good quality embryo media” (n = 25); “Day 5 bad quality embryo media” (n = 19); “Day 5 control” (n = 15); “Day 8 good quality embryo media” (n = 24); “Day 8 bad quality embryo media” (n = 20); “Day 8 control” (n = 15). Each of these groups are defined in the methods section. Concentrations are presented as particles/ml. Adapted from (Dissanayake et al., 2020)

For the TEM based EV characterization, EVs isolated from day 5 good quality embryo media were used (n = 60) (figure 14A). TEM images illustrated EVs, and most of these particles were sized between 50–150 nm (supplementary material 2 of (Dissanayake et al., 2020)). Further characterization was performed using SEM. EVs isolated from day 5 good quality embryo media were used (n= 30). The imaging visualized the spherical shape of the EVs (figure 14B). Therefore, direct visualization of the samples based on 2 modes of electron microscopy confirmed the existence of EVs in the embryo conditioned media.

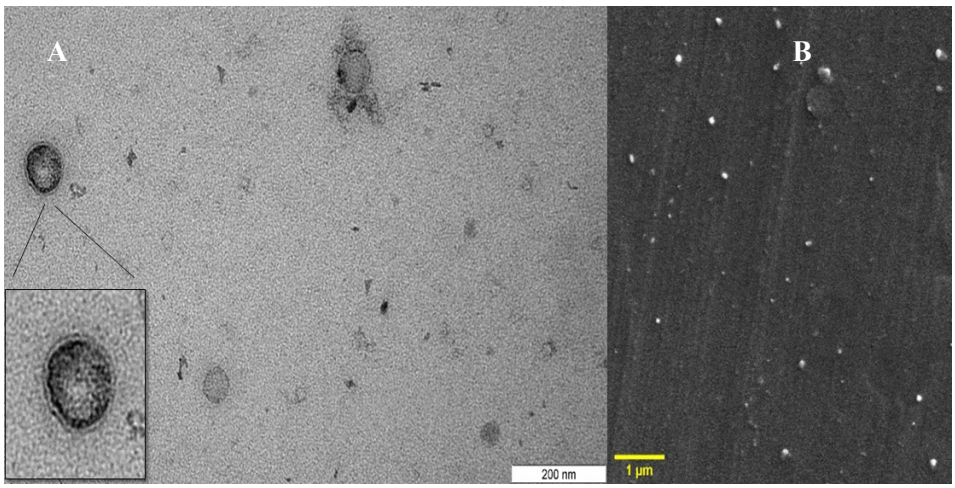

Figure 14. Physical characterization of EVs based on electron microscopy (A) Transmission electron microscopy (TEM) of EVs from day 5 embryo conditioned media (n = 60). Most of the EVs were sized between 50–150 nm. The magnified section of the image is a single EV demonstrating a cup-like shape characteristic of EVs. The scale bar is 200 nm. (B) Scanning electron microscopy (SEM) of EVs from day 5 embryo conditioned media (n = 60). White dots appearing in the grey background are EVs. They are heterogeneous in terms of their size. The scale bar is 1 µm.

Overall, physical and biochemical characterization steps confirm EVs' presence in individually cultured bovine embryo-conditioned media samples. However, the nano-particles isolated from control media samples do not have the characteristics of EVs.

Having confirmed the presence of EVs in the embryo conditioned media, in the next step, we evaluated the association of the mean concentration and size of EVs, as measured by NTA, with embryo quality and development stage.

5.1.5. Association of the mean concentration and size profile of EVs with embryo quality

At day 2, the mean concentration of bad quality embryo media derived EVs (8.25×10^8/ml) were 1.41 times higher than good quality embryo media derived EVs (5.86×10^8/ml, $p<0.05$) (figure 15A and 15B). Moreover, at day 2, the mean of the control media derived NPs (8.65×10^8/ml) were as high as the mean of the bad quality embryo media derived EVs.

In contrast, at day 5, the mean concentration of good quality and bad quality embryo media derived EVs were similar. In comparison to day 2 and 5, at day 8, the average concentration of bad quality embryo media derived EVs (7.17×10^8/ml) was 1.26 times higher in comparison to that of good quality embryo media derived EVs (5.68×10^8/ml, $p<0.05$) (figure 15A and 15C). Furthermore, the mean concentration of the control media sample derived NPs were gradually

dropped with the extension of the media incubation duration (8.65×10^8/ml, 6.47×10^8/ml, and 5.37×10^8/ml at day 2, day 5 and day 8, respectively).

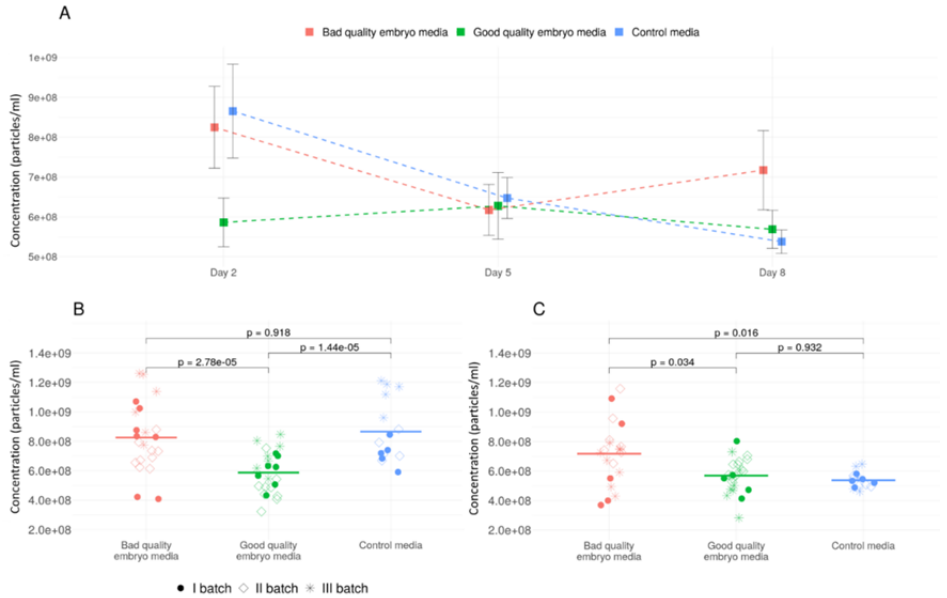

Figure 15. Concentrations of EVs (per ml) deriving from single bovine embryos cultures and the NPs (per ml) deriving from corresponding control media. (A) Mean concentrations of individually cultured embryo media derived EVs and corresponding control media derived NPs at day 2, 5 and 8. (B) Statistical comparison of the concentrations of day 2 good quality embryo media and day 2 bad quality embryo media derived EVs and day 2 control media derived NPs. (C). Statistical comparison of the concentrations of day 8 good quality embryo media and day 8 bad quality embryo media derived EVs and day 8 control media derived NPs. Different shapes in B and C illustrate the samples measurements from 3 replicates. Each such shape represents the average value of the three technical measurements of each biological sample. Differences between groups were statistically significant if $p<0.05$. Error bars represent 95% confidence intervals. The figure was adapted from (Dissanayake et al., 2020).

Further analysis of the EV concentration data was carried out to investigate differences between groups based on specific EV size ranges (figure 16A). Size ranges used for this comparison were 61–90 nm (figure 16B), 91–120 nm (figure 16C), and 121–150 nm (figure 16D) as NTA results are more reliable in these size ranges and exosomes are sized within this range. At day 2, good quality embryo media and control media were different with reference to 61–90 nm and 91–120 nm size ranges (figure 15B and 15C). Furthermore, at day 2, good quality embryo media and bad quality embryo media were different with reference 91–120 nm size range (figure 12C). Furthermore, at day 8, good quality embryo media and bad quality embryo media samples were different

with reference to 61–90 nm and 91–120 nm size ranges. Conversely, at day 5, three study groups were not different with respect to any of the size ranges considered.

Overall, the concentration of EVs isolated from embryo conditioned media differs based on embryo quality in two of the three development stages studied.

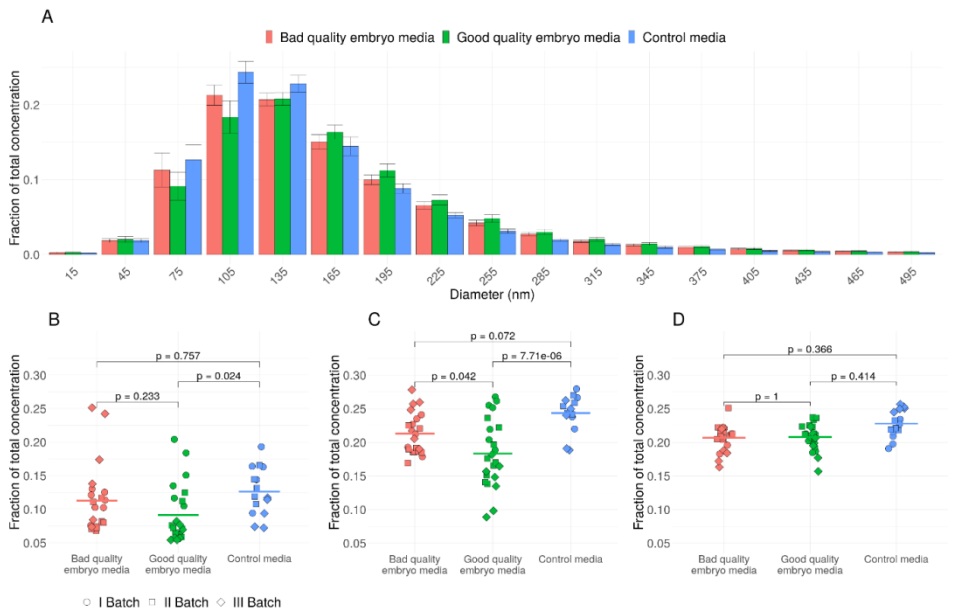

Figure 16. Size range based comparison of the concentrations (per ml) of EV from day 2 good (n = 23) and bad quality (n = 22) embryo media, and corresponding concentration of NPs from day 2 control media (n = 15). (A) The overall distribution of the particles isolated from 3 groups. Data are given as fractions of the total concentration. B, C, D visualize the comparison of the EV/NP concentrations of the three media types, based on size ranges: B; 61–90 nm, C; 91–120 nm, D; 121–150 nm. Different shapes in B, C, and D, denotes samples measured in three replicates. Each such shape indicates the average value of three technical measurements done for each biological sample. Differences between groups were statistically significant if $p < 0.05$. Error bars indicate 95% confidence intervals. Adapted from (Dissanayake et al., 2020).

Further comparison of the EVs were performed by comparing the size differences. The mean diameters of embryo-derived EVs and control media derived NPs had differences at day 2 and day 8 (figure 7 of Dissanayake et al. (2020)). The mean (±CI) diameter of day 2 good quality embryo media and day 2 bad quality embryo-derived EVs were 166±9 nm and 159±8.8 nm, respectively. These average EV sizes were larger than day 2 control media derived NPs, which was 150±9 nm ($p<0.05$). However, at day 5, none of the groups was

different based on the mean diameter of EVs or NPs. Finally, at day 8, the mean (±CI) diameter of good quality embryo media derived EVs(163±7 nm) was larger than bad quality embryo media derived EVs(154±11 nm) ($p<0.05$). As the incubation duration extended, the mean diameters of the NPs isolated from control media samples were increased from day 2 to day 8.

Intermediate summary: Sub-study I

- The depletion of EVs from BSA by filtering leads to loss of certain factors that are needed for the development of individual cultured bovine embryos to the blastocyst stage.
- Individually cultured bovine embryos release EVs to the culture media as confirmed by EV array, NTA, SEM and TEM. However, the EV array based biochemical analyisis did not confirm the presence of EVs in contrl media samples.
- NTA based EV comparison indicates that the concentration and average size of EVs isolated from embryo conditioned media differs based on embryo quality and their development stage.

Following the confirmation of the release of EVs by individually cultured bovine embryos and showing the embryo quality dependant differences in the concentration and size profiles of EVs, in the next step (sub-study II), we were opted to investigate the role of embryo-derived EVs in embryo-oviductal communication.

5.2. Sub-study II: Role of bovine embryo-derived EVs during embryo-oviductal communication

5.2.1. Study design:

In sub-study II, how the bovine embryo-derived EVs affected the primary bovine oviductal epithelial cells' transcriptome was evaluated. EVs/NPs isolated from individually cultured and subsequently pooled Day 5 good quality embryo media, Day 5 degenerating embryo media and Day 5 control media samples were supplemented to primary BOECs monolayer cultures, and their effects on differential expression of genes were evaluated by RNAseq and later validated with RT-PCR (Figure 17).

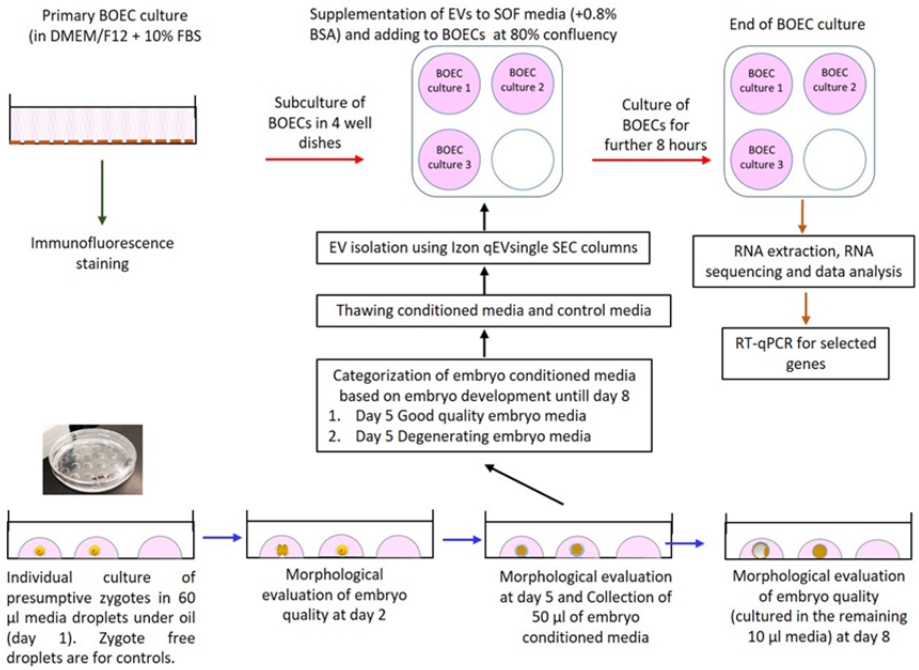

Figure 17. Experimental design of sub-study II experimental design. Adapted from (Dissanayake, Nõmm, et al., 2021)

5.2.2. Effects of bovine embryo-derived EVs in altering the gene expression of primary BOECs

Differential expression of genes was analyzed by comparing 1. good quality embryo-derived EV supplemented BOECs against the Control BOECs; 2. degenerating embryo-derived EV supplemented BOECs vs control BOECs and 3. good quality embryo-derived EV supplemented BOECs vs degenerating embryo-derived EV supplemented BOECs.

In the overall gene expression profile of the samples of oviductal monolayer cultures, a considerable degree of inter-group and intra-group variation was observed (Figure 18).

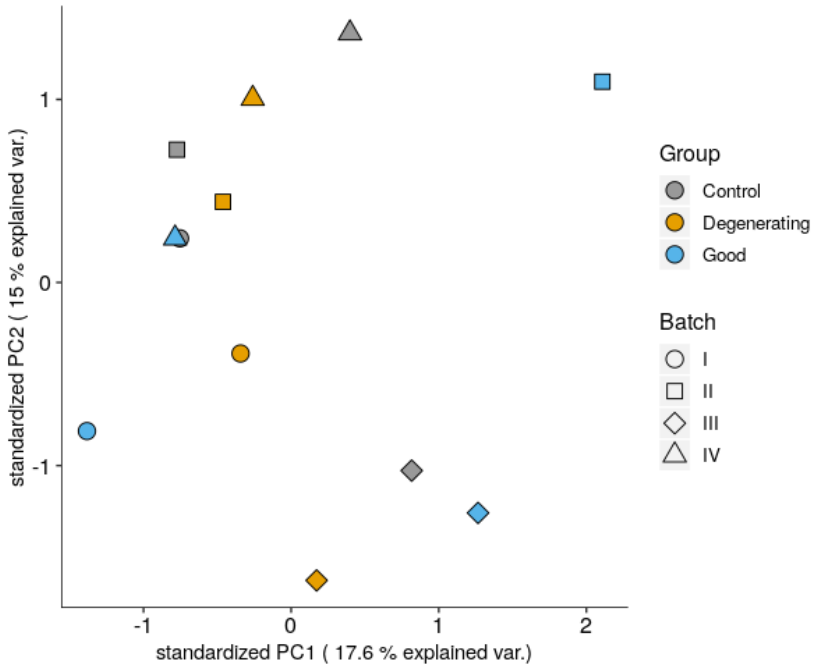

Figure 18. Gene expression profile of the good and degenerating embryo-derived EV-supplemented and control BOECs samples. Two leading principal components of standardised (z-score) counts per million (CPM) values of the expressed genes in BOECs. Three experimental groups were: Good: good quality embryo-derived EV supplemented BOECs; Degenerating: degenerating embryo-derived EV supplemented BOECs; Control: Control media derived NP supplemented BOECs. BOECs- bovine oviductal epithelial cells, EVs- extracellular vesicles, NPs- nanoparticles. Adapted from (Dissanayake et al., 2021)

The comparison between the gene expression of BOECs supplemented with good quality embryo-derived EVs and the control group BOECs showed 7 up-regulated genes and 18 downregulated genes. Interestingly, among the 7 upregulated genes, 4 were found to be interferon-induced genes (ISG-15, MX1, OAS1Y, and LOC100139670) (Table 3). However, between the gene expression of BOECs supplemented with degenerating embryo-derived EV and the control group BOECs yielded only a single, uncharacterized gene that was differentially expressed (ENSBTAG00000051364, log2FC = 0.83, FDR = 0.046). Furthermore, the gene expression of good quality embryo-derived EV-supplemented BOECs and degenerating embryo-derived EV-supplemented BOECs were compared, which resulted in 4 upregulated genes and 11 down-regulated genes. Similar to comparing good quality embryo-derived EV-supplemented group and the control group BOECs, the upregulated genes included interferon-induced ISG-15, MX1, OAS1Y, and LOC100139670 (Table 21 and Figure 19).

Table 21. Differentially expressed gene lists when EVs from day 5 good quality embryo EV supplemented BOECs were compared with the control BOECs and day 5 degenerating embryo EV supplemented BOECs

	Good embryo EVs vs Control			**Good embryo EVs vs Degenerating embryo EVs**			
Gene name	**Log2FC**	**LogCPM**	**FDR**	**Log2FC**	**LogCPM**	**FDR**	**Putative Function**
OAS1Y	1.83	3.66	4.35e-13	1.61	3.66	1.37e-09	Immune response, anti-viral
MX1	1.40	2.65	0.0004	1.19	2.65	0.0115	Antiviral, apoptosis
LOC100139670	1.33	1.73	0.0404	1.73	1.73	0.0039	Unknown
ISG15	1.23	3.71	3.93e-06	0.95	3.71	0.0039	Protein modification
*ENSBTAG00000051364**	1.07	4.67	2.28e-06	-	-	-	Unknown
*ENSBTAG00000053545**	0.88	4.32	0.0012	-	-	-	Unknown
CYP1A1	0.46	7.57	0.0067	-	-	-	Metabolism of endogenous substrates
ALKBH4	-1.29	2.49	0.0118	-	-	-	Transcription regulation
MADD	-1.28	2.37	0.0456	-1.45	2.37	0.0081	Cell proliferation, survival and death
HIP1R	-1.25	4.07	1.12e-06	-0.88	4.07	0.0119	Support early stages of endocytosis
C28H1orf198	-0.98	4.60	3.11e-05	-	-	-	Unknown
HID1	-0.97	4.46	4.38e-05	-0.82	4.46	0.0058	Unknown
CDC42EP1	-0.93	4.60	8.69e-05	-0.80	4.60	0.0061	Organization of the actin cytoskeleton
UNC13D	-0.85	5.56	2.78e-06	-0.79	5.56	8.87e-05	Innate immune response
AGPAT1				-0.79	4.44	0.0061	Cell metabolism
ALDH16A1	-0.80	3.86	0.0393	-		-	Oxidoreductase activity
CAPN1	-0.78	4.38	0.0118	-0.87	4.38	0.0039	Cytoskeletal remodelling and signal transduction
BAK1	-	-	-	-0.66	4.80	0.0287	Role in the mitochondrial apoptosis

	Good embryo EVs vs Control			Good embryo EVs vs Degenerating embryo EVs			
Gene name	**Log2FC**	**LogCPM**	**FDR**	**Log2FC**	**LogCPM**	**FDR**	**Putative Function**
PXDN	-0.74	5.58	9.08e-05	-0.62	5.58	0.0081	Extracellular matrix formation
*ENSBTAG00000043565**	-0.70	4.65	0.0114	-	-	-	Unknown
CPSF1	-0.68	4.48	0.0337	-	-	-	3-prime processing of pre-mRNAs
HGH1	-0.65	4.56	0.0473	-	-	-	Unknown
ARHGEF2	-0.63	4.89	0.0309	-	-	-	Cell cycle regulation and innate immune response
LAMB3	-0.58	6.25	0.0015	-	-	-	Cell signalling
FSTL3	-0.57	5.53	0.0162	-	-	-	Transcriptional regulation
RHBDF2	-0.49	6.39	0.0162	-	-	-	Cell survival, proliferation, migration and inflammation
SLC7A8	-	-	-	-0.47	7.97	0.0039	Molecular transport
MYC	-0.45	6.95	0.0212	-		-	Transcription factor
TGM2		3.66		-0.43	7.63	0.0212	Protein modification

BOECs – bovine oviductal epithelial cells, Log2FC- log2 fold change, LogCPM – Log counts per million, FDR – false discovery rate, *Genes without a particular gene name are mentioned with Ensembl symbol. FDR < 0.05 are considered significant. Adapted from (Dissanayake, et al., 2021)

However, no significantly enriched pathway was detected according to the GSEA with KEGG pathway annotations performed using the results of differential gene expression tests.

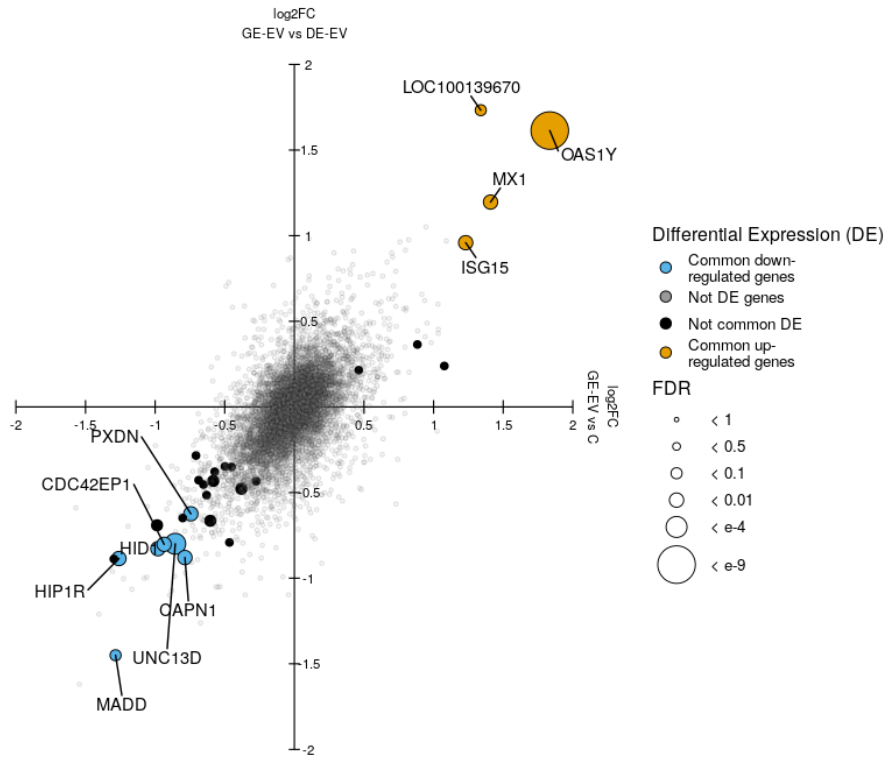

Figure 19. The similarity of genes expressed differentially when differential gene expression was tested between (1) good quality embryo EV supplemented BOECs(GE-EV) compared to the control (C); and (2) GE-EV group compared to the degenerating embryo EV supplemented BOECs (DE-EV). The mean FDR of the two comparisons is presented in the figure. BOECs – bovine oviductal epithelial cells, FDR- false discovery rate. Adapted from (Dissanayake et al., 2021)

RT-qPCR based validation was conducted with the three genes (OAS1Y, MX1, and LOC100139670) that implied the most relevance in the context of this system, based on previously published studies in this field. The expression levels of the three genes were quantified in BOECs that were supplemented with good quality embryo-derived EVs and in the control group BOECs. These three genes displayed a similar trend of upregulation as observed based on the RNAseq data (figure 21 A-C), thus adding more confidence to these genes being upregulated in BOECs in our experimental system as the result of supplementation with good quality embryo-derived EVs. Two of the genes – OAS1Y

and MX1 – were detected to be significantly upregulated (FDR ≤ 0.05, Mann-Whitney U test, Benjamini-Hochberg Procedure correction) based on the RT-qPCR data.

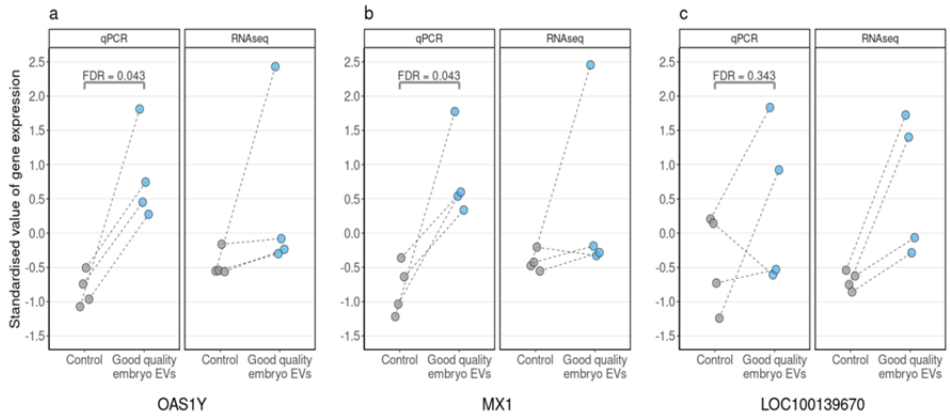

Figure 20. **Validation of the selected differentially expressed genes from RNAseq data using RT-qPCR**. Standardized (z-score) -ΔΔqC and counts per million (CPM) values for the three upregulated genes belonging to the interferon-τ pathway: (a) OAS1Y, (b) MX1, and (c) LOC100139670. Relative quantification of mRNA expression in day 5 good quality embryo EV supplemented BOECs was carried out using RT-qPCR. Same four replicates used for mRNA sequencing experiments were used for RT-qPCR. Dashed lines connect the replicates from the same experimental batch. Regarding RT-qPCR data, the group comparison was performed using Mann-Whitney U test with Benjamini-Hochberg Procedure to correct for multiple testing. Adapted from (Dissanayake et al., 2021)

Intermediate summary: Sub-study II

- Preimplantation bovine embryo derived EVs can alter the gene expression in the primary bovine oviductal epithelial cells.
- Oviductal response to the embryo-derived EVs is dependant on the embryo quality.
- Good quality embryo derived EVs could induce the genes belonging to the interferon-τ pathway indcating embryo derived EVs carry interferon τ.

After observing that bovine embryo-derived EVs can alter the maternal tract's gene expression, specifically in oviducts, we were intrigued to see if the maternal tract response is specific to the embryonic EVs. Therefore, in sub-study 3, we investigated this specificity using a cell-culture-based human embryo-maternal communication model.

5.3. Sub-study III: Role of trophoblast cell-derived EVs during embryo-endometrial communication

5.3.1. Study design

In sub-study III, two experiments (Figure 21) were performed to achieve the objectives. In the first experiment, the RNA cargo of JAr (analogue for trophoblasts of the pre-implantation embryo) and HEK293 (non- trophoblasts) cellular spheroid-derived EVs were examined using mRNA and miRNA sequencing. The second experiment studied how the JAr and HEK293 spheroid-derived EVs alter the RL95-2 cell (analogue for receptive endometrium) gene expression.

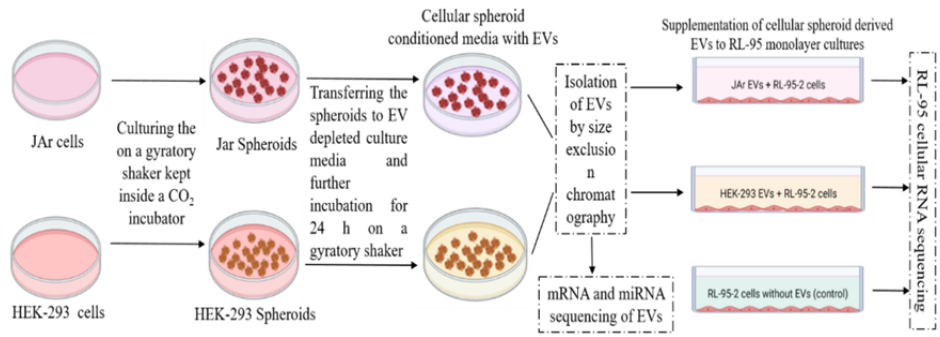

Figure 21. Experimental design of sub-study III

5.3.2. Effects of JAr cell spheroid and HEK293 cell spheroid derived EVs on differential gene expression in RL95-2 cells

Differential expression of genes in the JAr EV and HEK293 EV supplemented RL-95-2 cell culture groups were analyzed in comparison to the untreated RL-95-2 cell culture group. It was observed that the gene expression profile of RL95-2 cells supplemented with JAr spheroid derived EVs (RJ) was apparently different from both the untreated RL95-2 cells (R) and the RL95-2 cells treated with HEK293 spheroid-derived EVs (RH) **(**figure 22A**)**. One of the RH samples had to be excluded from the further analysis as it was found to be an outlier during the analysis. Nonetheless, it was observed that EV untreated RL95-2 cells and HEK293 EVs treated RL95-2 cells were clustered closer to each other, indicating no considerable effect of HEK293 derived EVs on RL95-2 cells.

Differential expression (DE) analysis showed that in the RJ group (JAr EV treated), in comparison to the R group (untreated), 1166 genes were upregulated while 588 genes were downregulated. However, comparison of the gene expression in RH group (HEK293 EV treated) compared to R group (untreated) did not yield any significant differential expression of genes. According to the

expression levels of previously mentioned DEGs, the similarity between R and RH groups is apparent (figure 22B).

Therefore, the analysis of the differential expression of genes indicates that while trophoblast cell analogue JAr spheroid derived EVs could alter the receptive endometrial cell analogue RL95-2 transcriptome significantly, non-trophoblast cell analogue HEK293 cell spheroid derived EVs do not have such a capacity.

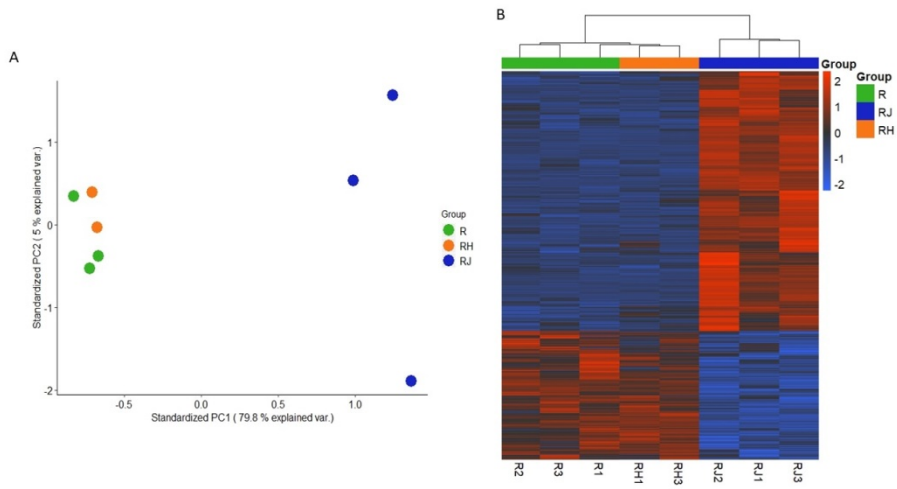

Figure 22. RL95-2 cells' gene expression profile in response to JAr EVs and HEK293 EVs supplementation. Three experimental groups were: JAr EV supplemented RL-95-2 cells (RJ); HEK293 EV supplemented RL-95-2 cells (RH); and RL-95-2 cells without EV supplementation (R). (A) PCA of all the genes considered as expressed in any of the three experimental groups i.e. RJ, RH and R. Figure shows the first two principal components. (B) Heatmap and the euclidean distance-based unsupervised hierarchical clustering of the 1,754 differentially expressed genes in the RJ in comparison to R. Adapted from (Godakumara et al., 2021)

Such observed differences in RL-95-2 cell transcriptome indicates the functional specificity of EVs during embryo-maternal communication. Subsequently, the functional enrichment analysis was performed to see any significance of these differentially expressed genes to the cellular function of endometrial epithelium during the pre-implantation period.

5.3.3. Gene set enrichment analysis (GSEA) of differentially expressed genes in RL95-2 cells in response to JAr EVs

Table 22 illustrates the most significantly enriched pathways in RL95-2 cells when supplemented with JAr spheroid derived EVs. "Signalling by G-protein coupled receptor (GPCR)" and "extracellular matrix (ECM) organization" are the two major pathways induced in the RL-95-2 cells in response to the supplementation of JAr spheroid derived EVs. Other significantly enriched pathways were events belonging to the previously mentioned two major pathways. For example, GPCR downstream signalling pathway is among the two first-level events of the "signalling by GPCR pathway". Furthermore, "collagen formation pathway" is an event of the ECM organization pathway. A net positive Normalized Enrichment Score (NES) value for these pathways shows that the genes affiliated to these pathways were mostly upregulated in RL95-2 cells.

Table 22. Gene Set Enrichment Analysis (GSEA) results based on DE analysis of JAr EV supplemented RL-95-2 in comparison to the control group.

Reactome ID	Description	NES	FDR
R-HSA-372790	Signalling by G-protein coupled receptor (GPCR)	1.267	0.010
R-HSA-388396	GPCR downstream signalling	1.289	0.010
R-HSA-1474228	Degradation of the extracellular matrix (ECM)	1.479	0.010
R-HSA-1474244	Extracellular matrix (ECM) organization	1.448	0.010
R-HSA-1474290	Collagen formation	1.490	0.010
R-HSA-216083	Integrin cell surface interactions	1.549	0.010
R-HSA-3000171	Non-integrin membrane-ECM interactions	1.456	0.011
R-HSA-3000157	Laminin interactions	1.568	0.019

NES- Normalized Enrichment Score, FDR- False Discovery Rate (FDR). Adapted from (Godakumara et al., 2021)

Interestingly, most of these enriched pathways have certain roles in the preparation of the endometrium for implantation. Hence, JAr cell spheroid-derived EVs seem to specifically alter the RL-95-2 cells, which could mimic the endometrium's preparation for the implantation by embryo-derived EV signalling.

5.3.4. JAr cell spheroid derived EV specific mRNA cargo in comparison to HEK293 cell spheroid derived EV mRNA.

The calculation of the enrichment of mRNA in EVs was performed by contrasting the JAr EV mRNA abundance against HEK293 EV mRNA abundance. Considering the population of fragments of mRNA aligning to known genes, it was observed that JAr EV mRNA and HEK293 EV mRNA are significantly different (figure 3A of Godakumara et al. (2021)). Differential enrichment analysis showed that 400 mRNA were enriched considerably, and

501 mRNA were significantly depleted in JAr EVs compared to HEK293 EVs (figure 3B of Godakumara et al. (2021)). Therefore, these data suggest that the EV mRNA cargo is significantly reliant on the cellular source of EVs. However, the effect of this differential enrichment of mRNA could not explain the differential expression of genes in RL-95-2 cells.

5.3.5. JAr cell spheroid derived EV specific miRNA cargo in comparison to HEK293 cell spheroid derived EV miRNA

Similar to the mRNA, miRNA content was also different between JAr and HEK293 cell spheroid derived EVs. It was observed that miRNA filtering criteria used for the analysis affected both the total number of miRNAs detected in either of the two EV types studied (figure 23A), and the number of miRNAs that were exclusively found either in JAr or HEK293 EVs (figure 23B).

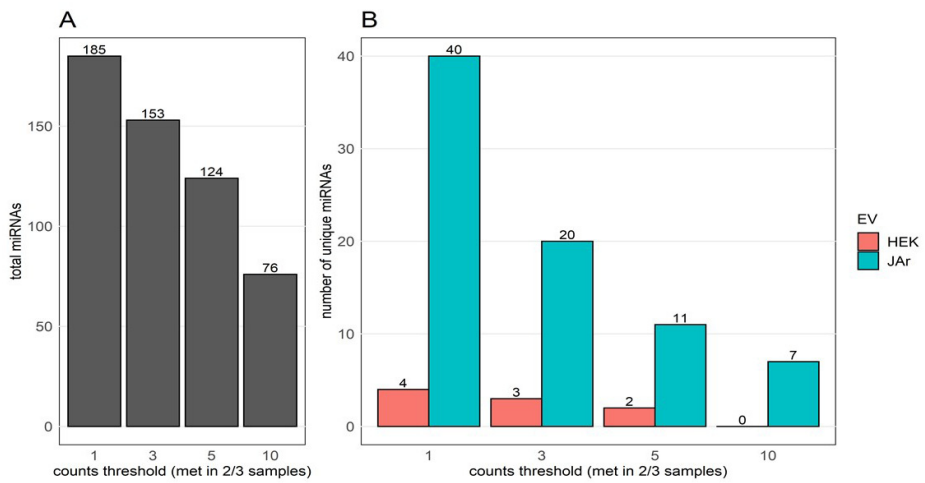

Figure 23. Results of the miRNA content analysis of JAr EVs and HEK293 EVs. (A). Number of miRNAs identified in at least two of the three libraries in either of JAr EVs or HEK293 EVs at four different raw count thresholds (1,3,5,10). (B) Four bars represents the miRNA numbers unique to JAr EVs or HEK293 EVs following getting through the respective raw count threshold. Adapted from (Godakumara et al., 2021).

However, it is apparent that the effect of miRNA filtering criteria has on the total miRNAs and unique miRNAs detected in each type of EVs. When a raw read count threshold of five was applied and only the miRNAs detected in at least 2 of the 3 libraries within a given group (JAr or HEK293) were considered, 11 JAr EVs unique microRNAs and 2 HEK293 EVs unique miRNAs were detected. These JAr EV specific 11 microRNAs were further analysed with respect to their target genes in RL-95-2 cells.

5.3.6. The correlation of JAr spheroid derived EVs miRNA abundance and the extent of downregulation of target genes in RL95 cells

It was observed that the 11 JAr-specific miRNAs had 1,188 high-confidence putative gene targets in the MicroRNA Target Prediction Database (miRDB). Further analysis showed that, of these gene targets, 744 genes were detected within the RL95 gene expression dataset. However, of them, only a small proportion was differentially expressed i.e. 53 genes were downregulated and 68 genes were upregulated.

Moreover, out of the eleven JAr EV specific miRNAs, six had a greater number of downregulated targets than the upregulated ones, whereas four miRNAs had a greater number of upregulated ones than the downregulated targets (figure 24A). JAr EV specific hsa-miR-524-5p was found to have the highest number of putative targets in our gene expression dataset.

The mean log2FC of downregulated target genes had a moderate negative correlation with the abundance of a given JAr EV miRNA (weighted Pearson's correlation, r = -0.65, p = 0.041; figure 24B). Among the JAr specific 11 miRNAs found, has-miR-1323 was the most abundant. However, hsa-miR-1323 had the second-lowest log2FC for the downregulated targets, and the lowest was hsa-miR-526b-5p. The negative correlation was affected by the used target score cut-off (90), and the FDR cut-off as more relaxed criteria did not yield such correlation (supplementary figure 2 of (Godakumara et al., 2021).

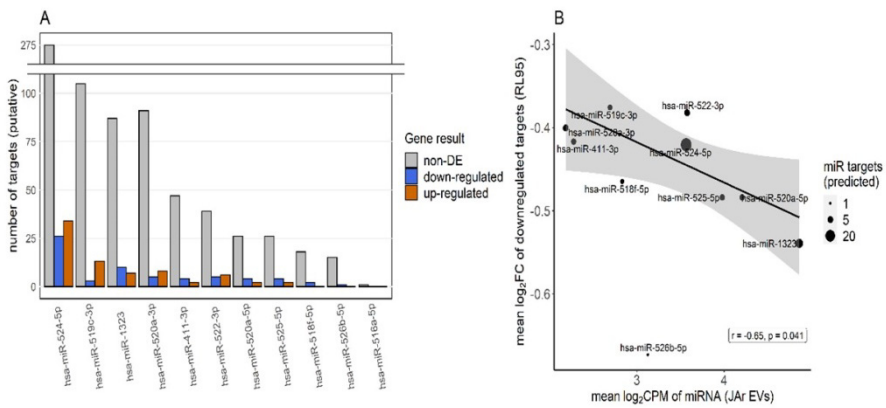

Figure 24. Target prediction of JAr EV specific miRNA (A) JAr EV specific 11 miRNAs and the numbers of putative high-confidence gene targets for each of the miRNA. Numbers of non-differentially expressed, downregulated and upregulated target genes are indicated by grey, blue and orange bars, respectively. (B) Association of the abundance of JAr EV specific miRNAs (expressed as mean log2cpm) and the extent of down regulation (expressed as mean log2FC) of putative high-confidence targets in RL95 cells (FDR ≤ 0.05). The size of the each point represents the down-regulated putative target number for each miRNA. A weighted regression line with 95% confidence intervals of the mean is presented. Adapted from (Godakumara et al., 2021).

Target prediction analysis was not conducted for the gene expression data set of RH group (RL-95-2 cells treated with HEK293 EVs) as no differentially expressed genes were observed there. Altogether, these data support the role of JAr EV specific miRNA in altering the transcriptome of RL-95-2 cells and the effect of the abundance of such miRNAs on the down-regulation of gene targets.

Intermediate summary: Sub-study III

- While supplementation of JAr cell spheroid derived EVs could alter the transcriptome of RL-95-2 cells, HEK293 cell spheroid derived EVs could not alter the RL-95-2 cell transcriptome. This indicates the functional specificity of EVs in mediating embryo-maternal communication
- The RNA cargo of JAr and HEK293 cell spheroids are different based on the results of mRNA and miRNA sequencing.
- A small proportion of the differential expression of genes in RL-95-2 cells in response to the supplementation of JAr EVs could be explained by JAr EV miRNA cargo suggesting that most of the altered gene expression could be induced by other biomolecules such as EV proteins.

6. DISCUSSION

Overall, this study broadly aimed 1) to isolate and characterize individually cultured embryo-derived EVs and to assess their association with embryo quality and developmental stage and 2) to study the role of embryonic in mediating embryo-maternal communication. This was accomplished by using *in vitro* produced bovine embryos and embryo-surrogate JAr spheroids as models. We managed to isolate EVs secreted by individually cultured bovine embryos and show that EVs' concentration and size profile vary depending on embryo quality and prospective embryonic development. Furthermore, primary oviductal epithelial cells reacted only to good quality bovine embryo-derived EVs but not to the degenerating embryo-derived EVs proposing the differences in the EVs, as a whole, depending on the embryo quality. Finally, we demonstrated that endometrial epithelial cell analogue RL-95-2 cells reacted only to JAr spheroid derived EVs but not to the control HEK293 derived EVs, suggesting that the maternal tract uniquely responds to embryonic signalling. Hence, all three studies collectively demonstrated the embryonic EV's physical differences, depending on the embryo quality and functional specificity.

Detecting differences in embryo-derived EVs based on embryo quality and further development and differential response of the maternal tract to EVs depending on the embryo quality are "proof of concept" research that would be translatable to human ART practice when embryo selection before uterine transfer. However, further studies recruiting human materials are warranted. Furthermore, all these findings add new knowledge to reproductive biology hence facilitating its progress.

6.1. Single bovine embryo-derived EVs, their isolation and characterization

The process of isolating individually cultured preimplantation bovine embryo-derived EVs is challenging. Such a challenge is due to 1) the small sample volume where embryos are cultured, 2) the limited quantity of EVs released by preimplantation embryos during the culture period, and 3) technical limitations in the current EV isolation and detection technologies for materials with limited EVs. However, we endeavoured to explore EVs deriving from individually cultured bovine embryos, considering their potential translational value in human ART practice. We successfully demonstrated EVs released from individually cultured bovine embryos as confirmed by NTA, TEM, SEM and EV array.

Even though a previous study reported EVs deriving from individually cultured bovine blastocysts, embryos in that study had been cultured initially in groups up to day 7 to select the blastocysts for subsequent individual culture from day 7–9 (Mellisho et al., 2017). Another study investigated the release of

human embryo-derived EVs using CD9 as an EV marker. They demonstrated the release of EVs by embryos of all development stages, thus supporting the findings of our study (Vyas et al., 2019). However, they had not compared the physical characteristics of EVs in terms of concentration and size.

The standard practice is to deplete EVs from the culture media before using them in cell culture-based EV research (Théry et al., 2018). This is because culture media are usually supplemented with FBS or its derivatives that can introduce EVs to the complete media, thus contaminating EVs released by studied cells. In the case of bovine embryo culture, the media is supplemented with BSA, whereas in human embryo culture, the media are supplemented with human serum albumin. These serum derivatives can be an external source of EVs to the culture media. Therefore, in this experiment, initially, the embryos were cultured *in vitro*, both individually and in groups, in culture media depleted of EV to observe how the embryos develop in EV-depleted conditions. We observed that blastocyst rate was significantly low when embryos were cultured individually in EV-depleted media (3%) compared to their culture in regular media (39.3%). This initial experiment provided evidence that once the culture media used for embryo culture was depleted of EVs, if there were any, by ultrafiltration, some biomolecules/factors have been removed from the media that are crucial for the embryo development individually. As such a drop was not seen when embryos were cultured in groups, we deduced that co-cultured embryos mutually compensated for the missing factor. Indeed, the improved embryo development when cultured in groups is an established observation (Swain, 2021). Due to the drop in embryo development in EV depleted conditions, subsequent individual embryos cultures were performed in regular embryo culture media.

Biochemical characterization of EVs using protein markers is crucial as it provides definitive evidence for the presence of EVs. Hence, all studies on embryo-derived EVs have relied on different methods when characterizing EVs, such as western blot (Giacomini et al., 2017), immunofluorescence (Saadeldin et al., 2014) and flow cytometry (Mellisho et al., 2017). However, as the current study investigated individually cultured embryo-derived EVs, the lower abundance of EVs in our samples was challenging. Therefore, we opted to depend on a sensitive detection method for EV proteins as techniques such as western blot require a high quantity of EV proteins to obtain results. Thus, EV array technology was used to detect the EVs in this study. (Jørgensen et al., 2021). This antibody-based EV detection method utilizes antibodies against EV-, surface- or surface-associated markers. As the NPs isolated from day 5 embryo conditioned media were strongly positive for EV markers such as CD9 and CD81, we concluded that individually cultured bovine embryos release EVs as early as day 5. However, the NPs isolated from day 5 control media samples were insignificant for the EV array test. Although the control media samples had a high quantity of NPs, as measured by NTA, these particles had no biochemical characteristics suggestive of EVs. This observation corroborate with previous studies that show NPs derivng from BSA used to supplement

embryo culture media either do not contain EVs (Pavani et al., 2019) or are distinct from EVs (Stolk & Seifert, 2015). Therefore, it is acceptable to refer to embryo conditioned media derived NPs as 'EVs'. As such supportive evidence was not obtained for the control media sample derived NPs, they were referred to as 'NPs' itself. Moreover, the EV array test of the current study demonstrated that EVs are enriched following their isolation using the SEC method, compared to the control embryo conditioned media sample, highlighting the validity of the technique used for EV isolation in the current study.

Physical characterization of EVs was performed using NTA, TEM and SEM. According to the NTA based characterization, EVs from embryo conditioned media were between 30-300 nm. However, the control media samples also had NPs most abundantly in the same size range. Previous observation according to the EV array that control media samples does not carry EVs, and NTA based similarity in size ranges of particles isolated from two sample types indicates that the isolation of EVs using SEC cannot separate EVs from other non-EV nanoparticles the same size ranges. However, SEC can still be considered a better method of isolating EVs than most of the other available popular methods of EV isolation (Sidhom et al., 2020). Furthermore, in the scatter mode, NTA tracks all the nanoparticles in the sample irrespective of whether they are EVs or not. As a result, NTA instruments such as ZetaView® cannot specifically detect and distinguish EVs from other nanoparticles in the sample. These are some of the limitations of current technology in EV research. However, the advances in NTA technology, such as fluorescent NTA (fl-NTA), can detect immunolabeled EVs overcoming such limitations, at least partially (Thane et al., 2019). TEM imaging demonstrated EVs in size range of 50–150 nm. However, the number of EVs in a given field was not high enough in these images. This could be due to the scarcity of EVs in the embryo conditioned media samples or their degradation during sample processing. SEM also demonstrated almost circular-shaped NPs suggestive of EVs. Hence, the used physical characterization methods confirmed the presence of EVs in the embryo-conditioned media.

Limitations of the currently available technology led us to pool embryo-conditioned media samples for EV characterization by EV-array and electron microscopy. This is due to the less abundance of EVs released by a given single embryo and the limited sensitivity of the current technologies. Therefore, more advances in the current technology are needed to characterize EVs released from single embryos without pooling the embryo conditioned media. With the confirmation of EVs in the embryo-conditioned media, subsequently, we compared the association of EV concentrations and size with the embryo quality and developmental stage.

6.2. Size profile and the concentration of bovine embryo-derived EVs vary depending on the embryo quality and their development

As mentioned earlier, the release of EVs by cells may vary under different physiological and pathological situations of those cells, and hence could serve as biomarkers in many health and disease conditions (Pang et al., 2020; Simeone et al., 2020). In this respect, it is paramount to study the differences of EVs deriving from single embryos as it would indicate the physiological or pathological status of the embryos. Thus, in this study, we explored that if there are differences in the EVs deriving from individually cultured preimplantation embryos depending on the embryo quality and development stage. However, we had to rely only on NTA based particle concentration and size profiles as the tool of comparison.

The mean EV concentration of day 2 good quality embryo media was less than day 2 bad quality embryo media. However, on morphological parameters, at day 2, embryos of both the categories were similar though their subsequent development upto day 8 differed. Hence, the concentration of EVs isolated from day 2 conditioned media could have predicted the prospective embryonic development, as those individual embryos that release less quantity of EVs have a better chance of developing to the blastocyst stage. It is possible that the consumption and secretion of the same nutrients, such as glucose, by embryos may mask the correct picture of the nutrient flux (Herrick et al., 2020). Similarly, the true release of EVs by embryos could be masked by EV uptake by the same embryo (Melo-Báez et al., 2021). Such dynamic exchange of EVs and non-EV NPs between the embryo and its surrounding microenvironment could be the reason for a net reduction in the concentration of total nanoparticles in day 2 good quality embryo conditioned media compared to control samples. However, at day 5, no differences were observed in embryo-derived EVs.

The mean EV concentration of day 8 bad quality embryo media was higher than that of day 8 good quality embryo media. Based on this observation, we can assume that degenerating embryos release more EVs to the culture media. A recent study is in line with this observation where it is shown that the mean concentration of EVs from non-viable early blastocysts was higher than EVs from viable early blastocysts (Mellisho et al., 2019). Most of the EVs released by degenerating cells could be apoptotic bodies of larger diameters than other EV types. However, with the advancing technology, it has been shown that, in addition to regular apoptotic bodies, degenerating cells release small-sized vesicles such as apoptotic microvesicles (ApoMVs) or exosome-like ApoEVs (Li et al., 2020). This could be the reason for detecting more EVs in the degenerating embryo conditioned media at day 8. Such smaller vesicles could carry biomolecules, including specific proteins, indicating the embryonic status. The comparison of particle concentrations depending on specific size ranges

also showed differences between good quality and bad quality embryo media samples (at day 2 and day 8 but not at day 5). This would add further evidence to prove the differences in EVs based on embryo quality.

Most of the embryo conditioned media derived EVs' were sized between 30–300 nm, hence corroborating with previous studies (Mellisho et al., 2017; Pavani et al., 2019). At day 2, the mean size (diameter) of the EVs isolated from good quality and bad quality embryo media derived EVs were larger than control media derived NPs. Furthermore, the average diameter of day 8 good quality embryo media derived EVs was larger than day 8 bad quality embryo media derived EVs. On the contrary, no difference was observed between the mean diameters of competent and non-competent blastocysts-derived EVs (Mellisho et al., 2017). Such differences between the studies could be a result of using different EV isolation and NTA methods. However, the embryo quality dependant differences in average EV size observed in our study may indicate that subtype of EVs released by EVs is dependant on embryo quality.

The concentration and average size of day 2 and day 8 embryos derived EVs show differences based on embryo quality. Further research using human embryo conditioned media is needed to investigate these findings' consistency across species. In the presence of such differences even in human embryo conditioned media, alone or in combination with morphological parameters, NTA-based EV assessments could be developed as an embryo scoring system. Such combined methods would augment the embryo grading and selection process compared to simple morphology-based embryo grading as EVs may indicate the functionality of the developing embryos. This would facilitate embryologists when deciding the best embryos for uterine transfer. A recent study developed such a model that enables to classify viable embryos based on embryo morphokinetics and EV characteristics (Mellisho et al., 2019). This type of combined non-invasive method of embryo scoring has the potential to advance fertility treatment outcomes.

The observed differences in EVs even manifest at the EV molecular cargo level, such as proteins or RNA. Many studies have evaluated the embryo conditioned media targetting different aspects of OMICs to identify biomarkers of embryo quality (Hernández-Vargas et al., 2020). These studies have looked at the whole embryo conditioned media without focusing on the EVs. From a translational point of view, studying the whole conditioned media may be more convenient than studying the OMICs of the embryo-derived EVs. However, to the best of our knowledge, such methods are yet to change the ART practice. Therefore, further studies are warranted to investigate the OMICs of embryo-derived EVs. As the current study is a proof of concept research, further experiments are needed to evaluate the translational possibility of embryo-derived EVs as a biomarker in human ART practice.

6.3. Only the good quality bovine embryo derived-EVs could alter the transcriptome of the BOECs

Having observed embryo quality dependant differences in the physical characteristics of EVs, we determined to study the functionality of such EVs. EV functionality was investigated in terms of EV's role during embryo-maternal communication. Even though previous studies had demonstrated the embryo-induced transcriptional changes in oviductal epithelial cells (Hamdi et al., 2019; Maillo et al., 2015; Schmaltz-Panneau et al., 2014), none of those studies had focused on EVs during this dialogue. Therefore, we specifically aimed to investigate if EVs deriving from preimplantation embryos can alter the oviductal transcriptome and if such oviductal responses are dependant on the embryo quality. The obtained results showed that good quality embryo-derived EV supplementation could induce particular transcriptional changes in the BOECs, which were not observed when supplemented by degenerating embryo-derived EVs. Supplemented EVs are the ones released by early bovine embryos during their development up to day 5. On average, *in vivo*, bovine embryos spend the first four days in the oviduct before entering the uterus. Therefore, the current experiment delivers a robust *in vitro* model to investigate the oviductal epithelial cell response to the pool of early embryo-derived EVs.

Among the upregulated genes in response to good quality embryo-derived EV supplementation, *ISG-15*, *MX1*, *OAS1Y*, and *LOC100139670* have particular significance. These genes are collectively referred to as interferon-stimulated genes (ISGs) and ISGs are memberts of the interferon tau (IFN-τ) pathway. Previous studies have shown that ISGs are upregulated in the epithelium in response to interferon-**τ**, a type 1 interferon, secreted by the trophoblastic cells in days 13–21 of bovine pregnancy. Like HCGβ is considered the pregnancy recognition signal in human pregnancy, IFN-τ is its counterpart in ruminants. It inhibits the expression of oxytocin receptors and PGF2α synthesis, prevents the breakdown of the corpus luteum and sustains the pregnancy (Forde & Lonergan, 2017). However, Talukder et al. showed that 16-cell stage bovine embryos (day 4) release IFN- τ, when co-cultured with BOECs (Talukder et al., 2018). The evidence from that study and current study suggest that pre-implantation bovine embryo-derived EVs convey biomolecules such as IFN-τ to maternal tract recipient cells hence altering their transcriptome. As we supplemented embryo free-culture media derived NPs to the control BOECs, it is apparent that embryo-derived EVs induced the observed transcriptomic changes. Furthermore, we validated these findings by performing RT-qPCR for three of the upregulated genes identified by RNAseq. The RT-qPCR results backed the upregulation of MX1 and OAS1 in BOECs but *LOC100139670.*

Two independent studies have previously reported that these ISGs are also upregulated in the oviductal epithelial cells when co-cultured with embryos or in the oviduct in the presence of embryos *in vivo* (Schmaltz-Panneau et al., 2014; Smits et al., 2016). Schmaltz-Panneau B et al. witnessed that *ISG15*, *MX1*, and *OAS1* were upregulated in BOECs when co-cultured for 7 days with

bovine embryos. In the study by Smits et al., the upregulation of *ISG15*, *MX1*, *OAS2*, and *OASL* in the equine oviductal epithelium with the presence of *in vivo* embryos 4 days after the ovulation was observed (Smits et al., 2016). Excitingly, the current study also reports the upregulation of some ISGs, i.e. *ISG-15*, *MX1*, and *OAS1* in BOECs mediated by the good quality embryo-derived EVs; however, in the absence of embryos. Therefore, the current study suggests that pre-implantation embryo-derived EVs can alter the gene expression in the oviduct *in vivo*, and such effects depend on embryo quality.

Among the genes down-regulated in BOECs following the supplementation of EVs deriving from good quality embryos, *UNC13D* and *ARHGEF2* are involved in the immune response. Innate immune response and neutrophil degranulation are the roles of the protein-coding gene *UNC13D* alias *Munc13-4* (Galgano et al., 2020). Similarly, innate immune response, epithelial barrier permeability, antigen presentation, cytokinesis, and cell cycle regulation, are some of the roles of *ARHGEF2*, which encodes GEF-H1 (Guanine nucleotide exchange factor H1) (Birkenfeld et al., 2008; Zhao et al., 2012). Embryos, being foreign entities, should avoid immune rejection by the mother if successful implantation occurs (Schumacher & Zenclussen, 2019). Therefore, regulating the immune response in the maternal tract is crucial during the pre and post-implantation phases of embryonic development. Almiñana et al. showed that the expression of several genes related to the immune reaction of the maternal tract could be down-regulated by *in vivo* pig embryos (Almiñana et al., 2012), hence overcoming the immune rejection. Therefore, the embryonic influences on the maternal tract immune tolerance for the implanting embryos are apparent.

There is a substantial difference in the cellular activities in good quality embryos and degenerating embryos (Betts & King, 2001; Graf et al., 2014). Furthermore, it has shown that even the embryonic global transcriptome could reflect their future development competence (Salilew-Wondim et al., 2021). Therefore, such differences in cellular activity, including gene expression, could be mirrored in the embryo-derived EV cargo, which also could reflect the embryos' developmental competency. This was partly evidence in sub-study 1, where embryo-derived EVs' physical characteristics differed based on the embryo quality. Hence, embryonic EVs with different molecular cargo could be distinguished by the maternal tract during embryo-maternal communication.

Several extrinsic factors may have influenced the observed gene expression profiles of BOECs, including, but not limited to, the quantity of EVs supplemented to BOECs, duration of incubation following EV supplementation, phenotypical characteristics of the cows from which oviductal epithelial cells were harvested, such as age, nutritional status, and certain disease conditions. Future experiments warrant investigating if and how the changes in supplemented EV quantity and incubation duration impact the gene expression of BOECs. Such studies would demonstrate the temporal nature of the observations of the current study. As we had to rely on oviducts from the abattoir, we were unaware of the phenotypes of the cows from which the oviducts and subsequently oviductal epithelial cells were recovered.

This study suggests that pre-implantation bovine embryo-derived EVs display molecular signatures indicative of embryo quality and developmental potential. Furthermore, such distinct biomolecules displayed or carried in EVs could signal the maternal tract distinctly. Thus, following the findings of this 'proof of concept' research, it would be possible to develop the oviductal epithelium or epithelial cells as an embryo sorting tool to identify the embryo quality. This tool may potentially complement the current morphology-based embryo quality evaluation, facilitating embryologists to pick the best embryo when performing elective single embryo transfer during ARTs.

6.4. JAr cell spheroid derived EVs, having a distinct RNA cargo, can uniquely alter the transcriptome of receptive endometrium analogue RL-95-2 cells

Having observed the differential expression of genes in the oviductal epithelial cells in response to the supplementation of embryo-derived EVs in a bovine model, we determined to study the functional specificity of the embryonic EVs when altering the gene expression of maternal tract epithelium. Hence, the functional specificity of embryonic EVs during embryo-maternal communication was investigated using a human cell culture model. It was observed that JAr cell spheroid (analogue of the pre-implantation embryo) derived EVs induced significant alterations in the transcriptome of RL95-2 cells (analogue of receptive endometrium). However, interestingly, EVs deriving from HEK293 cell spheroids (non-embryonic control) could not cause similar alterations in the transcriptome of the RL95-2 cells. Therefore, the current study demonstrated the functional specificity of the JAr cell spheroid derived EVs during intercellular communication. Furthermore, compared to HEK293 spheroid derived EVs, JAr EVs posed a unique mRNA and miRNA cargo. Such differences in the molecular cargo could explain, at least partly, the observed functional specificity to EVs during embryo-maternal communication though the underlying mechanisms are not clear.

EV uptake or internalization specificity has not been universally accepted. While some studies claim that EVs can be uptaken by any cells without specificity, other studies have demonstrated that EV uptake is a highly specific process in which both the EVs and the cells should pose the correct type of ligands and receptors for the coordinated interactions and internalization (Kwok et al., 2021). On the other hand, the functional specificity of EVs has been demonstrated, especially in cancer metastasis. In those studies, cancer cell-derived EVs regulate the target cell reactions during metastasis (Bae et al., 2018; Wu et al., 2017). To the best of our knowledge, this study and the previous study (Dissanayake et al., 2021) are the first studies that illustrated the functional specificity of EVs during embryo-maternal communication. However, the mechanisms underlying such differences in the effects induced by EVs are not

apparent. This warrants further research to elucidate the underlying mechanisms of EV mediated embryo-maternal communication.

GSEA analysis indicated that biological pathways enriched in the RL-95-2 cells, in response to JAr cell spheroid derived EVs, highlighting the functional importance of the effects that JAr EVs have on RL-95-2 cells. Among the enriched pathways, the extracellular matrix (ECM) organization pathway is fundamental as remodelling of the ECM of the endometrium is a critical morphological and biochemical modification needed for establishing a successful pregnancy (O'Connor et al., 2020). In addition, GPCRs pathway-based signalling (R-HSA-372790) was also found to be significantly enriched. Regarding the endometrial microenvironment, GPCRs are used as receptors by many ligands, such as hCG, prostaglandin E2, cytokines and progesterone, to execute their effect in altering their effect the endometrial microenvironment during the window of implantation (Su & Fazleabas, 2015). The enriched GPCR downstream signalling pathways (R-HSA-388396) are secondary messengers that are able to modify the morphology of the endometrium hence facilitating the implantation. Moreover, phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway, which regulates cell proliferation, metabolism and survival, is known to be involved in endometrial cellular migration, a crucial step in embryo attachment and invasion (Makker et al., 2018). Altogether, it is apparent that JAr spheroid EV induced changes in the RL-95-2 transcriptome are geared to endometrial modifications for impending implantations and priming and modifying the epithelial cell receptors for better receipt of embryonic signals. As we used JAr/RL-95-2 cell culture system to mimic the uterine microenvironment, we deduce that JAr EVs effects on RL-95-2 cells are not merely random but represent the effects of embryonic EVs during embryo-maternal communication at the uterine interface.

Molecular cargo of the EVs would play a critical role when mediating the intercellular communication, as the transfer of those cargo molecules from one cell to the other via EVs is pivotal in executing phenotypic changes in the recipient cells (Es-Haghi et al., 2019). Here, we explored the RNA cargo of EVs isolated from JAr cell spheroids and HEK293 cell spheroids for subsequent analysis and interpretation of the mechanisms behind EV induced transcriptomic changes in RL-95-2 cells. The unlimited capacity to obtain EVs from cell-culture based embryo surrogates is advantageous compared to real embryo derived-EVs when studying the EV molecular cargo. When JAr EVs were contrasted to that of HEK293 derived EVs, statistically significant differences in both the mRNA and miRNA cargo between the 2 sample types were observed. Even though we were unable to find an association between the altered transcriptome of the RL-95-2 cells and the mRNA cargo of the supplemented JAr EVs, the unique miRNA in JAr EVs could partly explain, at least partly, the differential expression of genes in RL-95-2 cells.

As a regulator of gene expression, miRNAs play pivotal roles in intercellular communication, including embryo-maternal (Paul et al., 2019). It is well known that miRNAs use different mechanisms to inhibit, cleave and destabilize the

transcripts when regulating gene expression. Several studies have shown different miRNA profiles in EVs depending on their cellular source of origin (Liu et al., 2019; Temoche-Diaz et al., 2019). Such differences in the miRNA profiles, as observed between JAr and HEK293 cell spheroid derived EVs, enabled, at least partly, the interpretation of the observed EV induced effects on RL95-2 cellular transcriptome. As only a limited number of RNA copies are carried in EVs, the altered expression of most of the DEGs may not be attributed to either mRNA or miRNA carried to the recipient cells via EVs (Albanese et al., 2020; Chevillet et al., 2014). Therefore, we can speculate that other types of biomolecules carried in the EVs, such as transcription regulation enzymes or cytokines and other non-coding RNAs, could play a role in driving these observed changes in the endometrial transcriptome.

There are many other investigations that can be carried out to understand the functional specificity of EVs further. These include, but are not limited to, studying EV uptake specificity by RL95-2 cells for JAr and HEK293 spheroid derived EVs and investigating the differences in the surface and cargo proteome between the EVs from two sources. Furthermore, studying the target specificity of trophoblast spheroid derived EVs directed towards target cells of endometrial and non-endometrial/non-reproductive origin and exploring the mechanisms used by target cells to transduce the signals delivered by EVs would shed light on the functional specificity of embryonic EVs during embryo-maternal.

6.5. Future perspectives

The current project investigated the potential of EVs as biomarkers of embryo quality and their involvement in mediating embryo-maternal communication. The findings of these studies would open avenues for future research that would have a higher translational value in changing the current practice of ARTs.

In the 1st study, EVs from cleavage and blastocyst developmental stage embryos were studied, and the EV concentration and the size profile differed based on embryo quality. Most importantly, early developmental stage embryo-derived EVs would provide valuable information that could facilitate the decision making during fresh embryo transfers compared to studying EVs deriving from the blastocyst stage. However, blastocyst-stage embryo transfer is more common than cleavage-stage embryo transfers in the current human ARTs. Therefore, frozen-thawed embryo transfers may have to be considered if a decision is to be taken based on blastocyst stage embryo-derived EVs. While the study findings would directly relate to bovine ARTs, as a continuation, it is vital to study the same hypothesis using human embryo culture media in future research. It would demonstrate the release of EVs from individually cultured human embryos to the culture media and how comparable the findings of this study are with human embryo-derived EVs. However, we used bovine embryos as a model species for early preimplantation embryonic development in humans. The current study tested only the physical properties of EVs, such as particle size and concentration,

to evaluate embryo quality. However, future studies need to look into the molecular cargo (proteins and nucleic acids) of embryo-derived EVs in individual embryo culture settings. Analyzing EV molecular cargo of single preimplantation embryo-derived EVs would enable omics-based EV biomarker discoveries. However, this would need further development of current platforms, especially their sensitivity for detecting low abundant biomolecules.

To date, many omics-based studies have been carried out using embryo conditioned media to understand their relationship with embryo quality. For instance, many proteins, including human leucocyte antigen-G (HLA-G), platelet-activating factor, agrocranin, leptin, and ubiquitin have been investigated as embryo quality markers. Furthermore, human chorionic gonadotropin (HCG), a signal for maternal recognition of pregnancy, have been studied in this regard, and authors report that its isoform β-HCG have the biomarker potential. However, these studies have not considered whether such biomolecules were in the embryo conditioned media as free form or are part of EVs' molecular cargo. Therefore, investigating the molecular cargo of embryo-derived EVs would give a different perspective than detecting such biomolecules in the whole culture media.

The 2nd and 3rd sub-studies included in the current thesis investigated the effects of the embryo and trophoblast spheroid-derived EVs on the gene expression of oviductal and endometrial epithelial cells, respectively. They add more evidence to the biomarker potential of embryonic EVs by showing that their effects are dependant on the embryo quality and specific to the embryonic signalling. Furthermore, it shows the biosensor potential of maternal tract epithelium. Currently, we are investigating the maternal tract epithelial cell response as a tool for embryo quality assessment in human ARTs. This method tests the functionality of the embryo-derived EVs along with their cargo. However, it would be vital to determine the specific biomolecule/s responsible for inducing the said changes in the maternal tract. In this regard, more focus could be given to EV proteins than nucleic acids such as micro-RNA, as the third sub-study showed that the contribution of the embryonic EV micro-RNA for differential gene expression in endometrial epithelial cells is relatively smaller.

Therefore, future investigations must be directed towards studying the potential contribution of the embryonic EV cargo proteins for elucidating the mechanisms behind differential gene expression responses of the endometrial epithelial cells. Direct quantification of such EV cargo biomolecules would have a better translational value in human ARTs. In the second sub-study, where the effects of the bovine embryo-derived EV on the transcriptome of oviductal epithelial cells were studied, interferon-stimulated genes or genes belonging to the interferon-τ pathway were among the upregulated genes. This finding suggests that interferon-τ is a potential inducer of differential gene expression of bovine oviductal epithelial cells, and interferon-τ may be carried in EVs. A previous study has shown that interferon-τ are part of the molecular cargo of EVs (Nakamura et al., 2016). Therefore, discovering and detecting similar EV cargo biomolecules, which can notify the maternal tract about the embryo quality, would increase the translational value of the EV based embryo quality biomarker research.

7. CONCLUSIONS

The following conclusions are drawn based on the findings of the three sub-studies presented in this thesis.

1. Preimplantation bovine embryos cultured individually secrete EVs to the culture media as proved by the physical and biochemical methods. The concentrations and the average size of EVs in the media conditioned by individually cultured embryos are dependent on the quality and the development stage of embryos. Hence EVs may have the potential to be used as a non-invasive embryo quality marker. However, further advancements in the current technologies are warranted to facilitate the profiling of EVs deriving from embryos with a very limited number of cells, such as zygotes and 2 cell staged embryos. This would offer more useful data about the quality of early preimplantation embryos and lead to identifying novel EV based-biomarkers that would be pivotal in selecting the best embryo for uterine transfer.
2. The results of this study suggest that bovine embryo-derived EVs are capable of altering the gene expression of primary BOECs. However, this effect was seen only when good quality embryo EVs, not degenerating embryo EVs, were supplemented. Thus, embryonic signalling to the maternal tract seems to be different based on the embryo quality and as a result maternal tract sense the quality of the embryos. Such sensing of the embryo quality may help the mother to decide if it is needed to invest resources in pregnancy or not. Furthermore, this observed effect of embryo-derived EVs on BOECs could serve as a non-invasive method of evaluating embryo quality.
3. EVs deriving from trophoblast analogue JAr cells could induce alterations in the transcriptome of the receptive endometrium analogue RL-95-2 cells. Such effects were unique to JAr EVs as HEK293 EVs could not have any such effects on the RL-95-2 transcriptome. This observation indicates the functional specificity of JAr EVs in mediating embryo-maternal communication. Some of the observed changes in the transcriptome, such as those linked to extracellular matrix remodelling and GPCR mediated signalling, would indicate functional components of the embryo-maternal communication during the peri-implantation period. These unique effects mediated by JAr cell-derived EVs, compared to HEK293 cell-derived EVs, could be attributed, at least partly, to the distinct RNA cargo of the EVs found in the two cellular sources. However, as the JAr EV RNA cargo could be putatively associated with only a small proportion of changes in the RL-95-2 transcriptome, more studies in the future should investigate the role of different biomolecules and molecular pathways underlying EV-mediated transcriptomic changes.

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SUMMARY IN ESTONIAN

Varastest embrüotest pärit ekstratsellulaarsed vesiikulid: potentsiaal embrüokvaliteedi markeritena ja roll embrüo-emaka suhtluses[1]

Sisukirjeldus

Viljatus on globaalne probleem, mis mõjutab hinnanguliselt umbes 48.5 miljonit paari. Abistav reproduktiivtehnoloogia (ART), sealhulgas *in vitro* viljastamine (IVF), on oluline viljatuse raviviis. Kuigi esimesest IVF lapse sünnist on möödunud neli aastakümmet ja embrüokultuurisüsteemides on tehtud märkimisväärseid edusamme, ei ole ART-järgne rasedus ja sündimus endiselt ootuspärane. Üheks kitsaskohaks on kvaliteetsete embrüote tuvastamine, mis suurendaks implantatsiooni tõenäosust. Klassifitseerimiseks embrüoid kasutatakse kliinilises praktikas näiteks morfoloogia-põhiseid ja teisigi meetodeid, nagu morfokineetika ja embrüobiopsia. Lisaks uuritakse embrüo kvaliteedi mitteinvasiivse hindamise ja siirdamisedukuse ennustamise meetodeid. Paraku on neil kõikidel puudujääke. Seetõttu vajame uusi või täiendavaid diagnostilisi meetodeid, et hinnata embrüo elujõulisust ja siirdamise eduvõimalust.

Rakuvälised ehk ekstratsellulaarsed vesiikulid (EVd) on kahekihilise lipiidmembraaniga nanoosakesed, mida toodavad peaaegu kõik rakutüübid erinevates patofüsioloogilistes konditsioonides. Vesiikulid sisaldavad erinevaid aineid nagu proteiinid, nukleiinhapped (sealhulgas mRNA ja mikro-RNA) ja lipiidid. EVd vahendavad rakuvahelist suhtlust erinevate mehhanismide kaudu ja võivad mõjutada retsipientrakkude fenotüüpi. Kõige olulisem on see, et EVd peegeldavad päritolurakkude patofüsioloogilist seisundit. Seetõttu võivad bioloogiliselt erinevate omadustega EVd sobida biomarkeriteks.

Uuringud on näidanud, et *in vitro* kasvatatud implantatsiooni-eelse perioodi embrüotest vabanevad EVd kultuurisöötmesse. Siiski pole varem uuritud embrüonaalsete EVde ja embrüo kvaliteedi seost individuaalsetes rakukultuurisüsteemides, mis hõlmaks embrüo arengu kõiki järke. Varem on pakutud, et endomeetrium suudab ise hinnata embrüo kvaliteeti, kuid pole teada kas seda teeb ka munajuha. Embrüo elujõulisuse tuvastamine suguteedes *in vivo* oleks evolutsiooniliselt kasulik, et kulutada ressursse vaid nendele embrüotele, mis panevad aluse tiinusele/rasedusele. Selleks peaks emakas hindama embrüo elujõulisust sõltuvalt embrüo enese saadetud signaalidest. On teada, et EVd aitavad vahendada embrüo ja emaka suhtlust, kuid teadmata on embrüotest vabanevate EVde mõju ja nende spetsiifilisus emaka geeniekspressioonile.

Selles doktoritöös uuriti implatatsiooni-eelse perioodi embrüote EVsid kui embrüokvaliteedi indikaatoreid ning nende rolli embrüo ja emaka suhtluse vahendajana. Veise embrüot peetakse heaks mudeliks uurimaks inimese varase

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embrüo arengut. Seetõttu kasutati uuringutes *in vitro* kultiveeritud veise-embrüoid. Individuaalselt kultiveeritud embrüote EVd isoleeriti kasvusöötmest ja need tuvastati füüsiliste ning biokeemiliste meetoditega. Seejärel võrreldi ühest embrüosöötmest isoleeritud EVsid embrüo arengustaadiumi ja kvaliteediga. Embrüonaalset päritolu EVde funktsionaalsuse hindamiseks lisati neid munajuha epiteelrakkude kultuuri ja analüüsiti geenide diferentsiaalset ekspressiooni. Lõpuks hinnati rakukultuuri mudelsüsteemi abil EVde vahendatud embrüo ja emaka suhtluse spetsiifilisust embrüo-endomeetriumi tasemel.

Doktoritöö eesmärgid

Töö üldine eesmärk oli uurida embrüo EVde sobivust embrüokvaliteedi markeritena ning nende spetsiifilisust embrüo ja emaka suhtluses.

Täpsed eesmärgid:

1. Eraldada ja iseloomustada EVsid, mida saadakse individuaalselt *in vitro* kasvatatud veise-embrüote söötmest, lähtudes nende kvaliteedist, arengujärgust ja loodetavast arengust (uuring 1)
2. Uurida individuaalselt kultiveeritud kvaliteetsete ja hääbuva implatatsioonieelse staadiumi embrüote EVde mõju *in vitro* kasvatatud veiste munajuha primaarsete epiteelirakkude geenikspressioonile (uuring 2)
3. Uurida embrüo EVde mõju endomeetriumi transkriptoomi muutustele, transkriptoomi muutuse spetsiifilisust sõltuvalt EVde päritolust ja nende muutuste seost EVdes sisalduva RNAga (uuring 3)

Materjalid ja meetodid

Esimeses uuringus loodi *in vitro* viljastamisega veise embrüod. Selleks hangiti tapamajast koduveise (*Bos taurus*) munasarjad ja neis olevatest folliikulitest aspireeriti munaraku ja follikulaarepiteelirakkude kompleksid. Järgmiseks etapiks valiti välja 1. kvaliteediklassi munarakud, mida kasvatati *in vitro* ning siis viljastati külmutatud ja sulatatud spermaga. Arvatavaid sügoote kultiveeriti individuaalselt 60 µl söötmetilkades, kust koguti 50 µl söötmetilgaproovi 2., 5. või 8. päeval pärast viljastamist. Vaatamata söötmeproovi kogumisele jätkati embrüokultuuride kasvatamisega allesjäänud söötmetilgakoguses kuni 8. päevani. Embrüoarengut hinnati 2. päeval (lõigustumisel), 5. päeval (moorula-staadiumis) ja 8. päeval (blastotsüsti-staadiumis) pärast viljastamist. Kogutud söötmetilgaproovid liigitati embrüote morfoloogiapõhise hindamise põhjal 2., 5. ja 8. päeval pärast viljastamist. Kogutud proovidest isoleeriti EVd suuruseralduskromatograafia meetodil qEVsingle® kolonniga. EVde olemasolule individuaalselt kasvatatud embrüote söötmetilgaproovist anti kinnitus iseloomustades vesiikuleid järgmistel meetoditel: nanoosakeste jälgimise analüüs (*nanoparticle tracking analysis* – NTA), EV-kiibi analüüs (*EV-array*), transmissiooni elektronmikroskoopia ja skaneeriv elektronmikroskoopia. Lisaks võrreldi individuaalselt kasvatatud embrüokultuuride EV kontsentratsiooni- ja suurusprofiili, et hinnata võimalikku EVde erinevust sõltuvalt embrüokvaliteedist ja arengustaadiumist.

Sarnaselt esimese uuringuga kasvatati teises uuringus arvatavaid sügoote individuaalselt *in vitro* söötmetilkades kuni 8. päevani. Embrüonaalset arengut hinnati 2., 5. ja 8. päeval. Söötmetilgaproov koguti 5. päeval pärast viljastamist ja pandi kokku vastavalt embrüoarengu staadiumile (kvaliteetse embrüo sööde *vs.* hääbuva embrüo sööde). Kogutud proovist isoleeriti EVd suuruseralduskromatograafia meetodiga. Tapamajast saadud veise munajuhadest kasvatati veise munajuha epiteelrakud (*bovine oviductal epithelial cells* – BOECs). Kvaliteetse ja hääbuva embrüo EVd lisati esmastele BOEC ühekihilistele rakukultuuridele. Pärast 8-tunnist EVdega kultiveerimist eraldati rakukultuurist RNA ja see järjendati. Diferentsiaalse geenieksprессiooni analüüsi tulemuste põhjal kontrolliti valitud hulka erinevalt avaldunud geene veel reaalaja kvantitatiivse polümeraasi ahelreaktsiooni (*real time quantitative polymerase chain reaction* – RT-qPCR) meetodil.

Kolmandas uuringus kasvatati trofoblastide analoog JAr-rakud (inimese koriokartsionoomi rakuliin) ja moodustati neist sferoidid jäljendamaks embrüoid. HEK293 rakkudest moodustati kontrollgrupi sferoidid. Valminud sferoide kasvatati veel 24 tundi söötmes, kust vesiikulid olid eemaldatud. Järgmisena eraldati sferoididest vabanenud EVd suuruseralduskromotograafia teel söötmest. Trofoblastide analoogi (JAr-rakkude) sferoididest eraldatud EVd lisati endomeetriumi analoogi (RL 95-2) rakukultuurisüsteemi. HEK293 sferodidest eraldatud EVd lisati RL-95-2 rakkudele ja kontrolliks kasutati RL-95-2 rakukultuuri, kuid seda vesiikulitega ei rikastatud. RL-95-2 rakuliine kasvatati veel 24 tundi, kust eraldati RNA ja see jäljendati, uurimaks transkriptoomimuutusi. Lisaks uuriti EVde sisu jäljendades mRNA ja mikro-RNA.

Tulemused

Esimeses uuringus leiti kasutatud meetodite toel kinnitust EVde olemasolu individuaalselt kasvatatud veise embrüo söötmes. EV-kiibi analüüsi tulemused näitasid, et embrüosöötme nanoosakesed olid tugevalt positiivsed EV-markerite CD9 ja CD81 ning nõrgalt positiivsed CD63 ja Alixi suhtes. Transmissiooni elektronmikroskoobi fotodel oli näha, et EVdel on tüüpiline kausjas kuju, ja skaneeriv elektronmikroskoop näitas EVdel sfäärilist kuju. Selle alusel järeldati, et söötmetilgast eraldatud nanoosakesed on EVd. Kontrollsöötmest eraldatud nanoosakesed olid rakuväliste vesiikulite markerite suhtes negatiivsed. Teisel päeval pärast viljastamist isoleeritud embrüosöötme EVdel tehtud nanoosakeste jälgimise analüüs näitas, et hääbuva embrüo EVde kontsentratsioon ($8{,}25 \times 10^8$/ml) on kõrgem võrreldes embrüote kontsentratsiooniga, mis arenesid blastotsüstiks ($5{,}86 \times 10^8$/ml, $p < 0{,}05$). Samamoodi 8. päeval isoleeritud hääbuva embrüo EVde kontsentratsioon ($7{,}17 \times 10^8$/ml) oli kõrgem võrreldes blastotsüstis leitud kontsentratsiooniga ($5{,}68 \times 10^8$/ml, $p < 0{,}05$). Lisaks oli 8. päeval isoleeritud keskmine hääbuva embrüo EVde diameeter (153,7 nm) väiksem kui blastotsüsti EVde keskmise diameeter (163,5 nm, $p < 0{,}05$).

Teises uuringus tuvastati 7 suurenenud ja 18 allasurutud geeni avaldumine veise munajuha rakukultuuris, millele oli lisatud kvaliteetse embrüo EVsid.

Suurenenud oli interferoon τ-indutseeritud geenide nagu OAS1Y, MX1 ja ISG15 avaldumine. Erinevate uuringute põhjal toimub see munajuha epiteelrakkudes, embrüo olemasolul. Suurenenud ekspressiooniga geenidest kinnitati RT-qPCR abil OAS1Y ja MX1. Vaid üks suurenenud ekspressiooniga geen tuvastati veise munajuha rakukultuuris, kui sellele oli lisatud hääbuva embrüo EVd. See näitab, et munajuhavastus EVdele sõltub embrüo kvaliteedist.

Kolmandas uuringus põhjustasid trofoblasti sferoidide EVd suuri transkriptoomi muutusi endomeetriumi rakkudes, kuid sama ei põhjustanud teiste rakutüüpide EVd. See näitab, et EVde päritolu on oluline kutsumaks esile funktsionaalseid muutusi. Geenikomplekti rikastus analüüsi (*gene set enrichment* analysis – GSEA) põhjal leiti, et endomeetriumi vastus EVdele keskendus rakuvälise maatriksi ümberkujundamisele ja G-valgu retseptoritega seotud signaalidele. Mõlemal sündmusel on funktsionaalne tähtsus endomeetriumi retseptiivsusele. Ligikaudu 9% endomeetriumi rakkudes allasurutud geenidest olid suure usaldusväärsusega prognoositud mikro-RNAde sihtmärgid, mida tuvastati eranditult trofoblastide analoogi EVdest. See viitab, et ainult väike osa endomeetriumi rakkudes vähenenud geeniekspressioonist võib tingitud olla EVdes leiduva mikro-RNAst.

Arutelu

Antud uurimistöö eesmärk oli uurida veise embrüo päritolu EVde sobivust embrüokvaliteedi markerina ning hinnata EVde rolli embrüo ja emaka suhtluses. Esimese uuringuga kinnitati, et implantatsiooni-eelsel perioodil vabastab arenev embrüo EVsid, mis võimaldas võrrelda EVsid sõltuvalt embrüo kvaliteedist. Veise embrüo EVde suurus ja kontsentratsioon erines sõltuvalt embrüo kvaliteedist 2. ja 8. arengutsükli päeval. Lähtudes neist tulemustest võib eeldada, et veise embrüo päritolu EVd on potentsiaalsed embrüokvaliteedi markerid. Vajadus on täiendavate uuringute järele inimestelt pärinevate proovidega selleks, et hinnata EVde rolli tõlgendavust erinevate liikide embrüokvaliteedi markerina. Antud teadmised tooksid kasu inimese ART rakendamises. Paraku hetkel olemasolevad tehnoloogilised vahendid ei võimalda täpselt iseloomustada ühe embrüo EVde omadusi. Võimalik oli vaid võrrelda EVde NTA mõõtmistulemusi. Edasised uuringud võrdlemaks EVdes leiduvaid biomolekule nagu proteiinid ja RNA aitaksid paremini mõista võimalike biomarkerite rolle ja liikide vahelisi sarnasusi. Sellised uuringud võivad avastada unikaalseid biomolekule, mis aitavad paremini hinnata embrüo kvaliteeti.

Teine uuring antud uurimistöös näitas erinevuste mõju EVde sisus, kus rikastati embrüo päritolu EVdega veise munajuha epiteelrakkude kultuuri, mille tagajärjel toimus rakkudes transkriptoomi muutused sõltuvalt embrüo kvaliteedist. Selleks, et mõista muutuse põhjuseid on vaja tulevikus uurida EVde molekulaarsisu. Embrüotest pärinevate EVde funktsionaalne spetsiifilisus embrüo ja emaka suhtluses oli seni teadmata. Seda uuriti viimases uuringus, kus JAr-rakkude trofoblastide sferoidide EVd muutsid RL-95-2 rakukultuuri geeniekspressiooni, mida HEK293 rakkude sferoidide EVd ei teinud. See oli esimene uuring,

mis näitas embrüo päritolu EVde funktsionaalset spetsiifilisust. Kindla RNA olemasolu JAr EV sisus võiks osaliselt selgitada mõju RL-95-2 rakukultuurile. Paraku on EVdes piiratud kogus RNA koopiaid, siis võivad eelnevalt kirjeldatud muutuseid mõjutada ka teised EVde biomolekulid nagu proteiinid. Seetõttu EVde proteoomi uurimine JAr ja HEK293 päritolu EVdest võimaldaks täpselt seletada JAr sferoidide päritolu EVde põhjustatud RL-95-2 rakukultuuri transkriptoomi muutuseid.

Kokkuvõte

Kokkuvõtteks võib öelda, et individuaalselt kasvatatud implatatsiooni-eelse perioodi veise-embrüod eraldavad EVsid kultuurisöötmesse. Embrüo kvaliteet mõjutab vesiikulite kontsentratsiooni ja suurust. Need võivad näidata embrüote arengupotentsiaali. Lisaks sai kinnitust hüpotees, et embrüost pärinevad EVd võtavad osa embrüo ja emaka suhtlusest munajuhas ning munajuha vastus sõltub embrüo kvaliteedist. Lõpuks tõestati rakukultuuril põhinevate katsetega, et trofoblasti EVd muudavad endomeetriumi geeniekspressiooni, millel võib olla funktsionaalne tähtsus embrüo ja emaka suhtluses implantatsiooni-perioodil.

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List of publications:

1. Dissanayake K, Nõmm M, Lättekivi F, Ressaissi Y, Godakumara K, Lavrits A, et al. Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers. Theriogenology 2020;149:104–16. doi:10.1016/j.theriogenology.2020.03.008
2. Dissanayake K, Nõmm M, Lättekivi F, Ord J, Ressaissi Y, Godakumara K, et al. Oviduct as a sensor of embryo quality: deciphering the extracellular vesicle (EV)-mediated embryo-maternal dialogue. J Mol Med 2021; 99:685–97. doi:10.1007/s00109-021-02042-w

3. Godakumara K, Ord J, Lättekivi F, Dissanayake K, Viil J, Boggavarapu NR, et al. Trophoblast derived extracellular vesicles specifically alter the transcriptome of endometrial cells and may constitute a critical component of embryo-maternal communication. Reprod Biol Endocrinol 2021;19. doi:10. 1186/s12958-021-00801-5.
4. Dissanayake K., Midekessa G., Lättekivi F., Fazeli A. (2021) Measurement of the Size and Concentration and Zeta Potential of Extracellular Vesicles Using Nanoparticle Tracking Analyzer. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_15
5. Dissanayake K., Godakumara K., Fazeli A. (2021) Isolation of Extracellular Vesicles (EVs) Using Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC). In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_13
6. Reshi Q.U.A., Hasan M.M., Dissanayake K., Fazeli A. (2021) Isolation of Extracellular Vesicles (EVs) Using Benchtop Size Exclusion Chromatography (SEC) Columns. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_14
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8. Midekessa, G., Godakumara, K., Ord, J., Viil, J., Lättekivi, F., Dissanayake, K., Kopanchuk, S., Rinken, A., Andronowska, A., Bhattacharjee, S., Rinken, T., & Fazeli, A. (2020). Zeta Potential of Extracellular Vesicles: Toward Understanding the Attributes that Determine Colloidal Stability. ACS Omega, 5(27), 16701–16710. https://doi.org/10.1021/ acsomega.0c01582
9. Dissanayake, K. P., Dissanayake, K., Etulgama, S. B., & Hettiarachchi, K. (2021). Flexible Fibreoptic Bronchoscopy Performed Through a Laryngeal Mask Airway (FFB-LMA) in a Paediatric Population: Experience of a Tertiary Care Children's Hospital in Sri Lanka. Sri Lankan Journal of Anaesthesiology, 29(2), 88. https://doi.org/10.4038/SLJA.V29I2.8693
10. Midekessa, G., Godakumara, K., Dissanayake, K., Hasan, M. M., Reshi, Q. U. A., Rinken, T., & Fazeli, A. (2021). Characterization of Extracellular Vesicles Labelled with a Lipophilic Dye Using Fluorescence Nanoparticle Tracking Analysis. Membranes, 11(10), 779. https://doi.org/10.3390/membranes11100779

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1. Dissanayake K, Nõmm M, Lättekivi F, Ressaissi Y, Godakumara K, Lavrits A, et al. Individually cultured bovine embryos produce extracellular vesicles that have the potential to be used as non-invasive embryo quality markers. Theriogenology 2020;149:104–16. doi:10.1016/j.theriogenology.2020.03.008
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