# TRABAJO DE FIN DE MÁSTER

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# Biología y Tecnología Aplicada a la Reproducción Humana Asistida

**Extracellular vesicles: modulators of embryo maternal interaction in the bovine model**


Autor: **Daniel de la Fuente Toro**

Tutores: **Raquel Herrer Saura**

**Dimitrios Rizos**

**José María Sánchez Gómez**

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| | |
|---|---|
| **BOEC** | Bovine oviductal epithelial cells |
| **BSA** | Bovine serum albumin |
| **CL** | Corpus luteum |
| **CM** | Conditioned media |
| **COCs** | Cumulus-oocyte complexes |
| **DNA** | Deoxyribonucleic acid |
| **E2** | Estrogens |
| **EGA** | Genomic activation of the embryo |
| **EVs** | Extracellular vesicles |
| **FCS** | Fetal calf serum |
| **FSH** | Follicle stimulating hormone |
| **GV** | Germinal Vesicle |
| **ICSI** | Intracytoplasmic sperm injection |
| **IVC** | *In vitro* culture |
| **IVF** | *In vitro* fertilization |
| **IVM** | *In vitro* maturation |
| **IVP** | *In vitro* production |
| **LH** | Luteinizing hormone |
| **miRNA** | microRibonucleic acid |
| **MVs** | Microvesicles |
| **NTA** | Nanoparticle Tracking Analysis |
| **OF** | Oviductal fluid |
| **P4** | Progesterone |
| **PBS** | Phosphate buffered saline solution |
| **PGF2α** | Prostaglandin F2 alpha |
| **PGR** | Progesterone receptor |
| **RPMI** | Roswell Park Memorial Institute |
| **SOF** | Synthetic Oviductal Fluid |
| **UF** | Uterine fluid |
| **ZP** | Zona pellucida |

## RESUMEN

La comunicación materno-embrionaria desempeña un papel fundamental en el establecimiento y mantenimiento de la gestación en mamíferos. Dicha comunicación es clave para el correcto desarrollo temprano del embrión, y se encuentra mediada por diversas vías de señalización entre oviducto, útero y embrión. Una de las vías de señalización intercelular son las vesículas extracelulares (EVs), unas nanopartículas liberadas por las células de forma natural para establecer una comunicación entre ellas. Por ello, el objetivo de este estudio consistió en la validación de las condiciones óptimas para el establecimiento de un modelo *ex vivo* de comunicación embrio-materna (co-cultivo de embriones sobre explantes oviductales y endometriales) mediante la evaluación de la viabilidad de los explantes oviductales y endometriales y de embriones, así como su capacidad para excretar EVs al medio.

Para ello, los explantes de oviducto y endometrio fueron expuestos a diferentes condiciones de cultivo: 6 y 18 horas de incubación en medios Synthetic Oviductal Fluid (SOF) y Roswell Park Memorial Institute (RPMI). Paralelamente, embriones en estadio ≥8 células (día 2.5) y embriones en estadio de blastocisto (día 7) fueron cultivados en las mismas condiciones. En cuanto al cultivo de explantes, los resultados no mostraron diferencias respecto a la capacidad de liberación de EVs al medio a pesar de que su viabilidad se vio comprometida tras 18 horas de cultivo. Tampoco se encontraron diferencias en cuanto al desarrollo y calidad embrionaria. Finalmente, no pudimos detector la secreción de EVs mediante el uso de nuestra metodología.

Puede concluirse que el cultivo durante 6 y 18 horas en SOF y RPMI no afecta a la capacidad de producción de EVs por parte de los explantes ni a la supervivencia embrionaria. Sin embargo, es necesario optimizar tanto la metodología aislamiento de EVs embrionarias como del procesamiento de los explantes.



**Palabras clave:** Comunicación materno-embrionaria • Vesículas extracelulares • *In vivo* • *In vitro* • Explantes • Desarrollo embrionario

## SUMMARY

Embryo-maternal communication during preimplantation development plays a fundamental role in the establishment and maintenance of pregnancy in mammals. This communication is key in early development and is regulated by signaling pathways between the oviduct, uterus, and embryo. Extracellular vesicles (EVs) are a way of intercellular communication between the maternal reproductive tract and the embryo. EVs are small particles naturally released from cells to communicate with other cells. Therefore, the objective of this study was the validation of the optimal conditions for the establishment of an *ex vivo* model of embryo-maternal communication (co-culture of embryos on oviductal and endometrial explants) by evaluating the viability of oviductal and endometrial explants and embryos, as well as their ability to excrete EVs into the culture medium.

For this, oviductal and endometrial explants were exposed to different culture conditions: 6 and 18 hours of incubation in Synthetic Oviductal Fluid (SOF) and Roswell Park Memorial Institute (RPMI) media. In parallel, embryos at ≥8 cells stage (day 2.5) and at blastocyst stage (day 7) were cultured under the same conditions.

Regarding the culture of explants, the results did not show differences in the ability to secrete EVs to the medium regardless of the media and time of incubation. However, the viability of both oviductal and endometrial explants was compromised after 18 hours of culture. In addition, no differences in terms of development and embryonic quality were found. Finally, we could not detect EVs secreted by the embryos using our methodology. It can be concluded that the culture for 6 and 18 hours in SOF and RPMI does not affect the capability of the explants to secrete EVs nor embryo survival. However, it is necessary to optimize both the isolation methodology of embryonic EVs and the processing of the explants.



**Keywords:** Embryo-maternal communication • Extracellular vesicles • *In vivo* • *In vitro* • Explants • Embryo development

# INTRODUCTION

## 1. Background and current state of assisted reproductive technology

During the last 25 years, the number of assisted reproductive treatments has increased dramatically. Furthermore, given our economic and social evolution, this trend is expected to continue, due to the increasingly evident delay in the age of maternity. In Europe, more than half a million treatments are performed each year in different *in vitro* fertilization (IVF) laboratories, resulting in around 100,000 newborns. This figure corresponds up to 1.5% of all live newborns on this continent.

This growing social demand for access to the field of assisted reproduction has been accompanied by numerous scientific and technical advances that have facilitated the development of these techniques and patient access to them, adapting to their needs. However, despite best efforts, pregnancy rates per cycle have remained constant for the last years. This lack of improvement in the pregnancy rates achieved could be due to the population characteristics of the patients who undergo this type of treatment. One of the most distinctive characteristics of patients who access assisted reproduction treatments is their age since around 60% of them are between 35 and 43 years.

It is well known that one of the main indicators of success in achieving a live newborn is the age of the patient. There are several reasons why advanced maternal age (defined as ≥35 years) turns out to be a limitation during IVF treatments. The most evident of them is the increasing rate of aneuploidy that occurs in these embryos, which increases from a 30% baseline in women younger than 35 to > 90% in women older than 44 years old (1). Another cause is the low ovarian reserve of these patients and the consequent low response to and subsequent search and collection of oocytes (1). If we add to these two causes a lower rate of blastocyst formation, we find a greatly reduced fertility rate, which is aggravated by a miscarriage rate close to 40% in patients of 40 years and 80% in patients older than 44 years old.

Since there is no possibility of modifying the characteristics of the population that requires IVF treatments, the current objective should be aimed at improving the techniques used during IVF treatments to produce embryos of the highest possible quality, which would allow improving the potential implantation of these.

The improvement of the *in vitro* culture (IVC) conditions has been one of the keys to increasing the embryo rate. In order to mimic these *in vivo* conditions, most of the media

used for IVC have been designed based on the ionic composition and energetic substrates of the fluids in which the embryo is found during its first days of development (2).

Several studies have suggested that the composition of the media where embryos are cultured may have an impact on the quality of embryos generated in IVF / ICSI (Intracytoplasmic sperm injection) cycles thereby influencing implantation and pregnancy rates. For this reason, the way to optimize the *in vitro* production (IVP) process could be found in these culture media, therefore a medium that contains all the substrates present in the oviductal and uterine regions could increase the yield and embryo quality. However, a large part of the culture media currently used, in addition to the large varieties among them, only imitate this composition and bypasses the physiological environment in which the early embryo development occurs is highly dynamic and complex.

Recent research has shown that one of the keys to the correct early embryo development is their reciprocal interaction with the mother's reproductive tract, specifically between the developing embryo and the uterine endometrium (3). One of the main factors in this crosstalk, and therefore in reproductive success are the EVs. These EVs have been isolated and characterized in the different anatomical areas through which the embryo is transported from fertilization to implantation, which, as has been shown, actively participate in the signaling processes intercellular between them and the embryo in its first days of development. This crosstalk between mother and embryo has been mainly studied in the bovine model since its great similarities with human embryo development give great potential for clinical application. Cows, like humans, are mono-ovulatory mammals, with gestations of 9 months. Early bovine embryo development, although somewhat slower, follows a pattern very similar to the first days of human ones, producing blastocyst with a similar size and total cell count. In addition, the development of research in bovine reproduction has classically followed a path parallel to the human one, serving as a guide in several of the components used in the previously mentioned culture media. These are the reasons why this work has used the bovine model to study embryo-maternal communication, aiming to optimize the culture conditions and increase the IVF success rate by mimicking the conditions of development *in vivo*.

## 2. Reproductive physiology in the bovine model

The cow undergoes a series of changes in the reproductive system in periodic cycles called estrous cycles that are regulated by the hypothalamic-pituitary-gonadal axis. The hormonal changes that take place during the cycle are aimed at completing oocyte

maturation and preparing the female's oviductal and uterine environment for fertilization and the establishment and maintenance of pregnancy. The bovine estrous cycle lasts approximately 21 days and can be divided into 4 main phases:

**Estrus**: It lasts approximately 12-18 hours. It is considered the beginning of the estrous cycle (day 0). During this stage, there is a rapid increase in the levels of luteinizing hormone (LH), caused by high concentrations of estrogens (E2). The LH surge triggers ovulation of the dominant follicle. There is a slight peak in follicle stimulating hormone (FSH) that triggers the first wave of follicular recruitment that will not be able to recruit a dominant follicle. Therefore, the first follicular wave will not be able to trigger ovulation.

**Metaestrus**: lasts between 4 and 5 days. It is characterized by the formation of a corpus luteum (CL) precursor hemorrhagic body. The CL is a highly vascularized gland, whose function is to prepare the uterus and maintain a possible pregnancy, if the oocyte is fertilized, through the secretion of progesterone (P4). The moment of greatest vascularization of the CL is reached at the end of this stage, around day 4 of the estrous cycle.

**Diestrus**: It is the longest stage of the cycle, with a duration of 10-12 days. The CL completes its maturation by increasing in size and reaching its maximum production of P4 mid-cycle (day 9). If the oocyte is not fertilized or the early embryo fails to signal its arrival in the uterus through the secretion of Interferon tau, it will release the hormone prostaglandin F2 alpha (PGF2α) (days 16 to 20) that induces regression of the CL (luteolysis) and therefore the decrease in progesterone levels. The amount in the concentration of FSH allows a second follicular wave, with the subsequent follicular recruitment and selection of a dominant follicle.

**Proestro**: It lasts approximately 2-3 days. The regression of the CL, now dysfunctional (corpus albicans), causes the drop in P4 levels and again stimulates the secretion and the increase in the concentrations of FSH and LH. The development of the dominant follicle will trigger a further increase in the level of E2, causing a surge in LH and subsequent ovulation, thus completing the estrous cycle.

### 3. *In vivo* embryo development in the bovine model

Oocyte maturation begins at the first arrest of meiosis, during prophase I, and is maintained thanks to high levels of cyclic AMP. This oocyte is in a germinal vesicle (GV) state, and its re-entry into the meiotic cycle occurs during the transition from the preantral

follicle to the antral follicle, manifesting itself through the disappearance of GV. Oocyte maturation continues until a second meiotic arrest occurs in metaphase II, a stage in which it remains after ovulation and until its fusion with the male gamete. On the other hand, the sperm ejaculated in the cranial portion of the vagina is not viable for fertilization and requires biochemical changes that allow it to join the zona pellucida (ZP) of the oocyte. This process is called sperm capacitation and it occurs during the migration of sperm through the reproductive tract of the female to reach the place of fertilization in the oviduct in the transition zone between ampulla and isthmus. The embryo that results from fertilization is transported to the uterus through the oviduct. During this journey, the first mitotic divisions of the embryo occur. It is the process known as segmentation. The first division occurs in the oviductal isthmus 24-48 hours after ovulation. At the 8-16 cell stage, the genomic activation of the embryo (EGA) occurs (2), which consists of the activation of the embryo genome after the degradation of the maternal mRNA. At this stage, the embryo passes into the uterus through the uterotubal junction and forms a morula (day 5) composed of 16-32 cells, which begin to form intimate unions between them, with greater compaction in the outer cells of the embryo. The cells of the morula begin their differentiation into internal cells (inner cell mass) and external cells (trophectoderm), which allows the accumulation of fluid inside the embryo, forming a cavity called blastocele. Thus, between days 7 and 9 of development, the next stage called blastocyst is configured.

## 4. *In vitro* embryo development in the bovine model

Interestingly, IVP in cattle is of great relevance today with the number of *in vitro* produced embryos being much higher than the *in vivo* derived ones. To improve current methodologies, it allows the analysis of early embryo development throughout the preimplantation stage, also serving as a model to study embryo-maternal interactions in humans. The embryos selected for transfer to the recipient female are usually on blastocysts stage at day 7 of development. To minimize the differences with the *in vivo* process, the IVP process consists of 3 fundamental stages: the first of these is *in vitro* maturation (IVM), which, after the search and identification of the cumulus-oocyte complexes (COCs) in the follicular fluid aspirated from the ovaries, allows the development of the oocyte from the arrest of meiosis until the metaphase II stage, in which nuclear and cytoplasmic maturation occurs. Factors related to the origin of the

oocyte (such as follicle size), age, breed, and health status of the donors influence their competence.

The next stage is IVF, which aims at the fusion of the female and male gametes to achieve the fertilization of the oocyte.

The last is IVC, which is the longest stage of the IVP process. The blastocyst stage is not reached until days 7-8 post insemination (day 6-7 of culture). It should be noted that this stage is the one that constitutes a greater decrease in the development of the embryos so that only around 40% reach the blastocyst stage.

## 5. Embryo-maternal communication

Embryo-maternal interactions are critical to establishing a successful pregnancy. There is evidence of reciprocal interactions between the developing embryo and the uterine endometrium during early development. In fact, the process of elongation of the embryo after its hatching from the ZP is completely directed by the mother, which is why it does not occur *in vitro* or *in vivo* in the absence of uterine glands, whereas it normally occurs if blastocysts are transferred to synchronized receptors. In addition, P4 induces certain specific changes at the cellular level in the endometrial transcriptome, which are necessary to establish uterine receptivity. One of these changes is the downregulation of the nuclear progesterone receptor (PGR), both glandular and luminal epithelium. This regulation of the PGR allows the expression of certain genes necessary for the elongation of the embryo.

On the other hand, the role of the oviduct in embryo-maternal communication is less clear, with less knowledge about the cellular and molecular mechanisms through which the oviduct can influence embryo development and quality. However, the oviduct is considered the starting point for the search for any communication signal between the embryo and the maternal reproductive tract (4).

### 5.1 Communication between the oviduct and the embryo

Despite the clear evidence of bidirectional interaction between the uterus and the embryo in its early development (3), as previously indicated, the role of the oviduct in embryo-maternal communication is less known. The lack of knowledge of these mechanisms had traditionally led to the idea that the oviduct is nothing more than a simple passive conduit for gametes and embryos in their earliest stages, only performing a transport function to the uterine horn. However, just as the culture media are essential for the correct IVP, it

has been shown that the oviduct is an organ with a dynamic composition and a key function for the correct embryo development, providing a nutritious and conducive environment for its growth, in addition to transporting the embryo to the uterus through its muscular and ciliary activity. Today, it is proven that oviduct-embryo communication is a bidirectional process (3). This means that the oviduct can emit signals that the embryo receives and vice versa. The fact that EVs can be secreted and internalized by the bovine embryo and by the oviduct (4), supports the reciprocity of this communication. This crosstalk can occur through direct pathways, as reflected in the changes produced in the bovine oviductal epithelial cells (BOEC) transcriptome by contact with embryos during their *in vitro* culture; or indirect, through molecules known as embryotropins, traditionally characterized as biochemical messengers that include proteins, saccharides, lipids, DNA, miRNA, etc. This highlights the role of the EVs, which have gained great importance in recent years as intercellular mediators in the cell-to-cell communication between the oviduct and the embryo (4), exerting direct or indirect effects into the recipient cells (Figure 1).

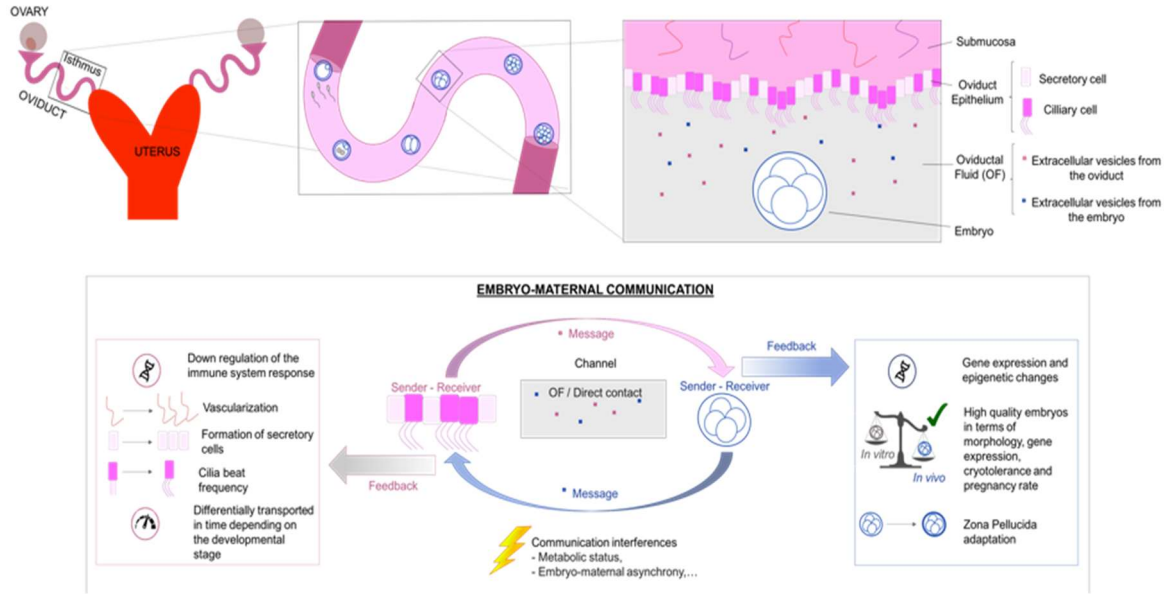



**Figure 1.** Representation of embryo-maternal communication in the oviduct (5).

### 5.1.1 Effect of the oviduct on the embryo

Understanding the effect of the oviduct on the embryo is essential given its influence on the quality of the embryo produced by IVF (4). It has been recently shown that the cultures which use different components related to the oviduct such as oviductal fluid (OF) or BOEC improve the quality of *in vitro*-produced embryos. Lopera-Vasquez *et al.* [6]

demonstrated that low concentrations of OF in bovine embryo culture media had a positive effect on the development and quality of the produced blastocysts. In addition, according to the results presented by Hamdi *et al.* [7], the use of concentrations of 1.25% of OF in embryo cultures in the absence of fetal calf serum (FCS) improves the quality of the embryo, concluding that OF provides better control of methylation of the embryo, improving its quality in terms of cryotolerance, cell count and gene expression. However, even though a significant number of studies demonstrate the positive effect that the oviduct exerts on the embryo, both the mechanisms and the exact components by which this improvement occurs are still unknown.

### 5.1.2 Effect of the embryo on the oviduct

In recent years, a limited number of studies have been published showing that the presence of the embryo has an effect on the oviduct (8). In general terms, the main identified mechanism by which the embryo acts on the oviduct is the modulation of the immune system. *In vivo*, the oviduct has to deal with the presence of pathogens while allowing the presence of an embryo that immunologically can be considered as semi allogenic. The embryo's ability to regulate the expression of genes related to the immune system indicates that it is a very important factor in preventing its own rejection (8).

## 5.2 Communication between the uterus and the embryo

The achievement of pregnancy, both in humans and bovine, depends directly on the implantation of the embryo in the maternal uterus. The implantation of the embryo occurs only during a period denominated as the window of implantation. In humans, the receptive phase occurs during the mid-luteal phase; implantation outside this window is associated with spontaneous miscarriages. The three main tissue of the uterus (luminal and glandular endometrial epithelium, stroma, and myometrium) can regulate pregnancy, being the luminal epithelium perceived as the crucial site for uterine receptivity transmitting signals to other compartments, due to the blastocyst adhesion occurs in it (9). In the same way that the use of OF in the culture media on days 1 to 4 of development favors the production of higher quality blastocysts, a similar influence was observed by the uterine fluid (UF) on days 4 to 9 (7). Furthermore, a recent study showed that the specific pattern of the endometrial transcriptome is altered by the presence of a day 7 embryo, with more notable change effects in the endometrial tissue corresponding to the region where the embryo was deposited (8). This fact indicates that intercellular signaling

between the uterus and the embryo does not occur in a single direction but is a bidirectional dialogue in which both parts can modulate each other.

## 6. Extracellular vesicles

EVs are bilipid nanoparticles secreted by cells to the extracellular space that can be classified according to their size, biological content, biogenesis, or method of release from the cell. Regarding size, EVs are classified in a range from 30 to 1000 nm that are subdivided into exosomes (30-90mm) and microvesicles (MVs) (above 100nm). EVs have been shown to contain mRNAs and miRNAs that can be transferred to other cells where they can be translated into functional proteins.

The view on EVs changed when their immune effects were tested. These evidences opened the possibility that EVs could play an important role in intercellular communication, generating interest, and discovering different types and in different tissues *in vivo*. Since then, EVs have been described in biological fluids such as plasma, urine, amniotic fluid, and follicular fluid (10).

### 6.1 Extracellular vesicles and embryo-maternal communication

The first evidence of the role of EVs in reproduction was reported by da Silveira *et al.* [10], who isolated EVs and exosomes from equine follicular fluid and characterized their cargo (lipids, proteins, and miRNAs). In addition, this group reported a high degree of commonality between the EVs and exosomes isolated in the ovarian follicular fluid and those isolated in granulosa cells (10), suggesting that EVs may play an important role in ovarian cell communication. Regarding bovine follicular fluid, cellular communication mediated by exosomes and miRNA was demonstrated shortly thereafter (10). It has been shown that the different EVs isolated from the follicular fluid promote changes in the gene expression of the cells of the granulosa and provides support for the expansion of cumulus-oocyte complexes (COCs) *in vitro*. How EVs influence these processes depends on the stage of development of the follicle. Its potential role in reproduction has increased significantly in recent years, in which its presence has been discovered in seminal plasma, follicular fluid (10), OF (6), and UF (11). As mentioned above, this crosstalk can also occur between the oviduct and the embryo through direct pathways, as reflected in the changes in the BOEC transcriptome by contact with embryos during *in vitro* culture; or indirect, through molecules such as EVs. It has been also shown that bovine blastocysts

secrete EVs in culture media and that the concentration of EVs secreted from day 7 to day 9 varies according to the competence and the origin of the embryo.

In human reproduction, only a few studies have addressed the secretion of EVs from embryos and explored their potential as biomarkers for its own selection (12). Two main interests have driven the most recent studies about EVs in IVF embryo conditioned media (CM). On the one hand, the potential role of EVs is mediating the embryo-endometrial crosstalk with the endometrium for proper implantation. On the other hand, the profile of secreted EVs as an indicator of embryo fitness, being a promising tool for the assessment of embryo quality and competence (12).

### 6.2 Oviductal Extracellular vesicles

Oviduct-derived EVs, also known as oviductosomes, have attracted the recent attention of numerous studies since they might act as natural nanoshuttles that bring key components from the oviduct into gametes / embryos, developing a main role in their capacitation and development (4,6).

Hormones derived from the hypothalamic-pituitary axis induce significant changes in the oviductal transcriptome and the OF composition throughout the estrous cycle. Due to EVs are part of the OF composition, it's right to infer that the main contents of these EVs may also vary along the cycle, being regulated by these hormones (13). These changes are essential to provide an optimal environment for gamete capacitation and transport, fertilization, and early embryo development. However, the impact that this dynamic regulatory environment may have on the secretion and composition of the oviductal EVs is still not fully understood. Despite this, thanks to the sequencing of its RNA content, different functions have been revealed, such as the expression of cilia in the cell epithelium, embryo development, and transcripts that encode ribosomal proteins, in addition to the expression of genes involved in the modification of embryo chromatin. Recent studies have observed that EVs originating from BOEC monolayer cultures can be isolated and implemented in embryo IVC, improving the quality of the blastocysts produced (Reviewed by Rodriguez-Alonso *et al.* [8]).

Moreover, the implementation of EVs from OF and UF at concentrations less than 5% in the culture media in the absence of FCS increased embryo quality. It was observed that the embryos produced in these media increased their antioxidant activity, in addition to their quality, which was measured by survival rate to the vitrification and devitrification processes, total cell count, and gene expression.

### 6.3 Uterine Extracellular vesicles

Although most of the studies on the role of EVs in embryo-maternal communication have focused on EVs derived from OF, it has recently been shown that the protein content of uterine EVs released by human endometrial epithelial cells during the IVC is altered by the implementation of E2 and P4. This could confirm that the hormonal changes derived from the female reproductive cycle can vary the content and function of EVs in order to adapt to the needs of each stage.

Regarding the function of these EVs derived from the UF, it is accepted that the successful implantation of the embryo depends on the coordination between it and the endometrium, this being a process in which the uterine EVs actively participate (7). Therefore, it has been suggested that the endometrial epithelium releases EVs that are involved in the transfer of signaling miRNAs and adhesion molecules to the blastocyst or adjacent endometrium into the uterine cavity (13). The EVs originating from the UF are also capable of release their specific RNA, miRNA, and protein content. Other main evidence of this fact, beyond the reciprocal modulation between both, is found in the origin of these EVs, which emanate from both the embryo trophectoderm and the uterine epithelium, supporting their important biological role for the establishment and maintenance of pregnancy (11).

It can therefore be concluded that the study of EVs seems a fundamental requirement to understand their role in the interactions between embryo-oviduct / uterus and their implications in reproductive success, turning these nanoparticles into valuable tools with potential applications to human reproductive medicine.

## 7. *Ex vivo* bovine model

*Ex vivo* models of co-culture with endometrial explants represent an alternative to those mentioned above. The main advantage of these models is that they provide a cellular architecture similar to the uterus *in vivo* (14). Furthermore, studies that do not use the explant model find difficult to analyze and detect the signals induced by embryos in the endometrium after collecting these tissues *in vivo*, since they have a local effect. The use of endometrial explants has made it possible to overcome this problem by co-culturing them with blastocysts in the Roswell Park Memorial Institute (RPMI) medium (14), detecting changes in the gene expression of the endometrium in response to embryos. Thanks to this, various studies have reported changes in the endometrial transcriptome

depending on the stage of development of the embryo (14), as well as differences from the endometrium to day 14 embryo depending on whether the endometrial explants were obtained from the uterine horn ipsilateral or contralateral to the CL. However, the main limitations of this system are the requirement of a minimum number of 5 embryos to generate an appreciable response (14) and the limited time of co-culture, since the explants begin to show signs of necrosis and apoptosis after 48 hours of culture (15). These limitations reinforce the need to develop a co-culture model that establishes optimal conditions for both the embryos and the explants, also allowing the detection of EVs signals. In addition, *ex vivo* model studies open the possibility to develop new co-culture models similar to those described with the endometrium that implements the use of oviductal explants.

## 8. Hypothesis and Objectives

The reciprocal interaction between the embryo and the mother's reproductive tract is key for an adequate development of the embryo. Developing an *in vitro* embryo co-culture system that allows the study of these interactions is essential, due to mimicking the *in vivo* conditions, and a great strategy to produce embryos of the highest possible quality, improving their potential of implantation. The results presented by the *ex vivo* models (14,15) reflect the need to establish ideal co-cultivation conditions for the use of this model. These conditions must favor embryonic development and preserve the integrity of the explants, optimizing, in turn, the detection of signals (EVs) secreted by both the embryo and the explants. It should be noted that the use of oviductal explants had not yet been carried out for the study of their EVs. That is the reason why this work aims to study the viability of embryos, endometrial and oviductal explants under different culture conditions, as well as the concentration of their secreted EVs. Therefore, the explants and embryos were cultured in a medium previously developed for the culture of explants (RPMI) (14) and in a medium designed for the culture of embryos ((Synthetical Oviductal Fluid) SOF) (2). In order to preserve the functionality of the explants while giving the embryos enough time to secrete a concentration of detectable EVs, the culture times were established at 6 and 18 hours. The embryos were cultured in two critical phases of their development: embryos ≥8 cells (52 hours) and blastocysts (day 7), while the explants were selected from both the ipsilateral oviduct to the ovary containing the CL of heifers in stage 1 (days 1-4 of the estrous cycle) and from the ipsilateral uterine horn to the CL of heifers in stage 2 (days 5-10 of the estrous cycle).

The outcome from the above models will be used in future studies where embryos ≥8 cells will be co-cultured with oviductal explants and blastocysts on day 7 will be co-cultured with endometrial explants, thus mimicking the contact conditions *in vivo*. Consequently, the specific objectives of our study are:

- Evaluating the viability of endometrial and oviductal explants, as well as the survival and development of embryos after being cultured in SOF and RPMI media for 6 and 18 hours.
- Determining the capacity of the endometrial and oviductal explants as well as the embryos to secrete detectable concentrations of EVs after being cultured in SOF and RPMI media for 6 and 18 hours.

# MATERIALS AND METHODS

## 1. Experimental Design

The objective of this work was to establish an *ex vivo* model for the study of embryo-maternal communication in cattle. Specifically, embryonic survival, oviductal / endometrial explant viability, as well as their ability to secrete EVs, were analyzed in different culture conditions. 54 oviductal explants obtained from the oviduct ipsilateral to the CL of 6 females (9 explants per female) in the phase of the estrous cycle corresponding to the EGA in the 8-cell stage (Stage 1) and 54 endometrial explants obtained from the uterine horn ipsilateral to the CL of 6 females (9 explants per female) in the phase of the estrous cycle corresponding to the stage of blastocyst development (Stage 2), were processed. (Figure 2a). Both groups of explants were cultured in RPMI and SOF medium supplemented with Bovine serum albumin (BSA) for 6 and 18 hours.

As a control group to evaluate the viability of the explants, one from each tissue and each animal was processed immediately (time 0). These explants were divided into two, one of the halves was used to examine their histology while the other half was frozen in liquid nitrogen and stored at -80ºC for the analysis of gene expression in later studies. The histological analysis made it possible to study the viability of the different groups of explants thanks to the assessment of the maintenance of the tissue structure. Furthermore, negative control for each culture medium at each time was generated to evaluate the capacity to secrete EVs. These groups consisted of the medium without any explant being cultured.

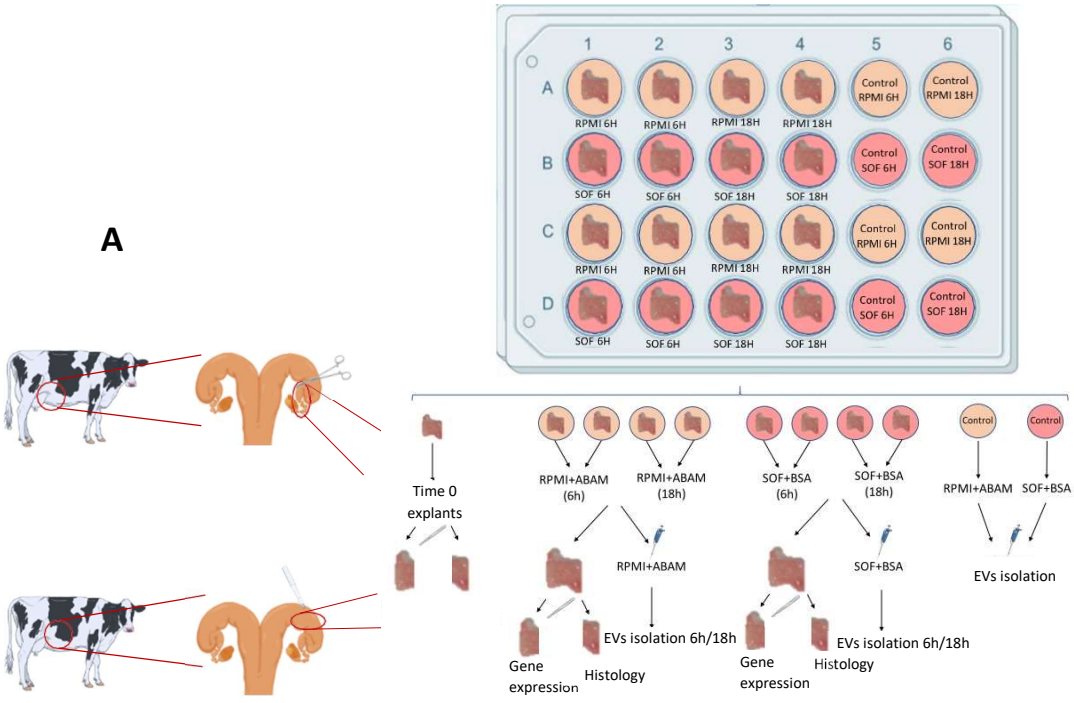
As a result, the following experimental groups were established (Figure 2a):

- Oviductal / endometrial explants for 6 hours in RPMI medium
- Oviductal / endometrial explants for 18 hours in RPMI medium
- Oviductal / endometrial explants for 6 hours in SOF medium
- Oviductal / endometrial explants for 18 hours in SOF medium
- Oviductal / endometrial explant for 0 hours (control)
- 1 well of RPMI medium for 6 hours (negative control)
- 1 well of RPMI medium for 18 hours (negative control)
- 1 well of SOF medium for 6 hours (negative control)
- 1 well of SOF medium for 18 hours (negative control)

On the other hand, IVP was performed as shown in Figure 2b to obtain ≥8 cell-stage embryos (day 2.5) and blastocysts (day 7). Between 52-54 hours after fertilization,

the embryo cleavage rate at the ≥8 cell stage was assessed by direct observation under a stereomicroscope. Selected ≥8 cells embryos were cultured in RPMI and SOF media supplemented with BSA for 6 (112 embryos in SOF and 82 embryos in RPMI) and 18 hours (112 embryos in SOF and 82 embryos in RPMI). After this incubation, every group of embryos was transferred to a fresh culture media of 500 µL well of SOF + BSA to continue their development. On day 7, the percentage of embryos at the blastocyst stage was recorded and they were subjected to the same treatments described for embryos ≥ 8 cells (16 blastocysts in both SOF groups and 11 blastocysts in both RPMI groups). The development capacity of the blastocysts after day 7 (days 7 and 8), including their expansion and hatching from the ZP, was studied as a marker of embryo quality. As a result, 8 experimental groups were established based on the culture medium and time for the 2 embryonic stages:

- Embryos / Blastocysts in SOF medium for 6 hours.
- Embryos / Blastocysts in RPMI medium for 6 hours.
- Embryos / Blastocysts in SOF medium for 18 hours.
- Embryos / Blastocysts in RPMI medium for 18 hours.
- 1 well of SOF medium for 6 hours. (negative control)
- 1 well of RPMI medium for 6 hours. (negative control)
- 1 well of SOF medium for 18 hours. (negative control)
- 1 well of RPMI medium for 18 hours. (negative control)



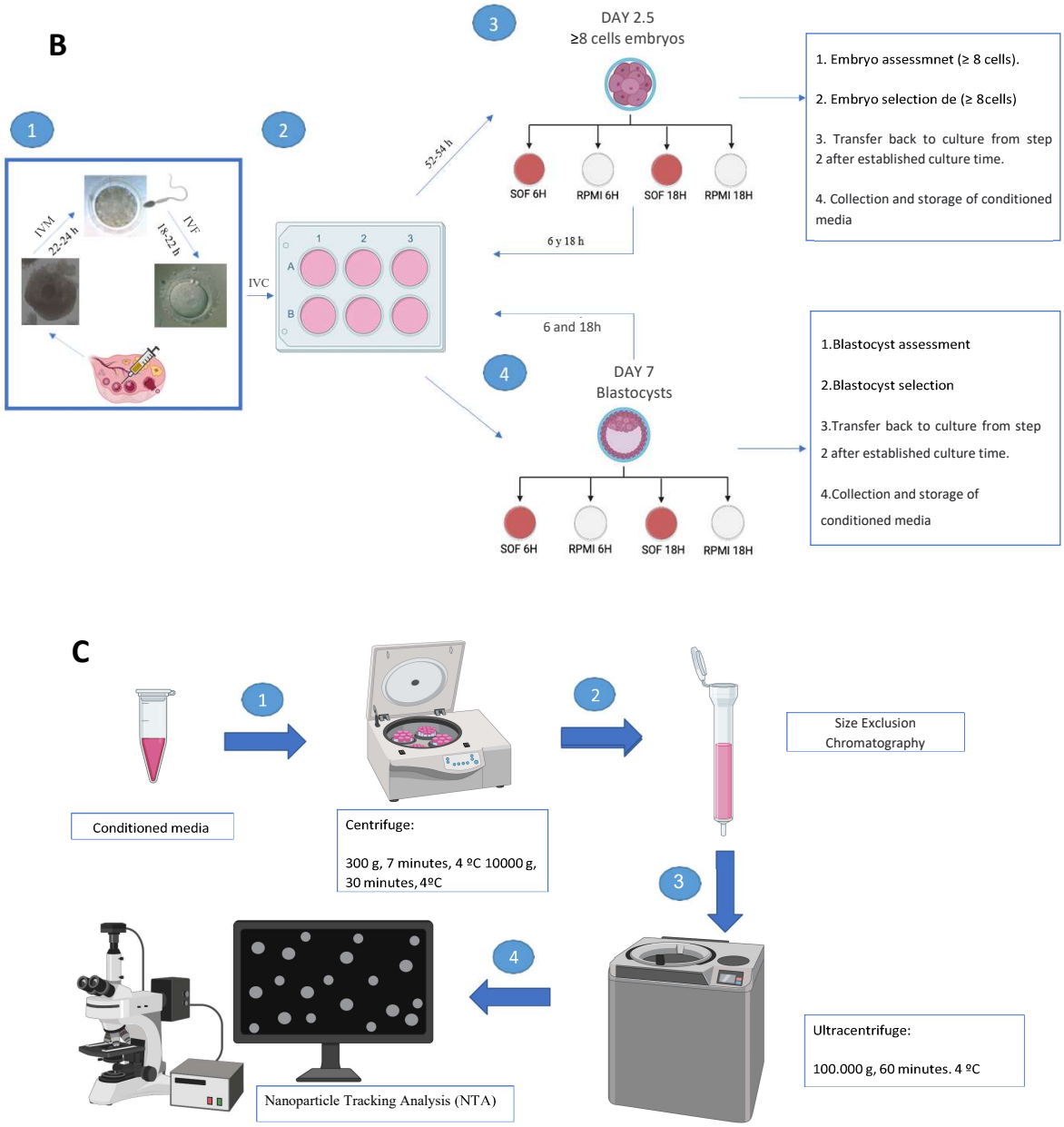



**Figure 2.** ***Experimental design:*** *(a) Development of embryos of ≥8 cells selected at 52 hpi (day 2) and of blastocysts (day 7) cultured for 6 or 18 hours in SOF medium (optimal for embryo culture) and RPMI (optimal for culture of explants). (b) Oviductal and endometrial explants cultured for 6 or 18 hours in SOF medium (optimal for embryo culture) and RPMI (optimal for culture of explants). (c)* Isolation, concentration, and determination of EVs from the conditioned media from each group.

Finally, and concerning all the experimental groups described above, CM was collected from both the embryos and the explants to analyze the concentration of EVs using Nanoparticle Tracking Analysis (NTA) (Figure 2c).

## 2. Collection, evaluation, and processing of the reproductive tracts

The reproductive tracts were recovered postmortem at a local slaughterhouse. To obtain oviductal explants, only oviducts from females around day 3.5 (stage 1) of the estrous

cycle were used, while to obtain endometrial explants, uterus from animals that were around day 7 (stage 2) were recovered. Of note, the selected reproductive tracts were free of any pathology. The stage of the estrous cycle was estimated based on the morphology of the ovarian structures, especially the CL. Briefly, in stage 1 (days 1 to 4) the CL is small and red, and the epithelium has not yet covered the point of follicular rupture. In stage 2 (days 5 to 10) the CL is somewhat bigger, vascularization is observed around it, the apex is reddish in color and the epithelium has already covered the rupture point. Each tract was independently deposited in a plastic bag and transported to the laboratory on ice in a polystyrene box 2 hours after collection. The tracts were washed with cold water to remove blood traces and were placed on trays on ice where they were dissected to separate the oviducts or uterine horns from all adjacent tissues. Subsequently, they were washed with cold 1% D-PBS (Dulbecco's phosphate-buffered saline solution; Gibco, ThermoFisher Scientific) supplemented with 1% ABAM (antibiotic / antifungal; Gibco, ThermoFisher Scientific).

### 3. Obtention of the oviductal and endometrial explants

The oviducts ipsilateral to the CL were divided at the junction between the ampulla and isthmus to separate these anatomical regions. Then, the isthmus was cut into 1 cm sections and each of these sections was opened longitudinally with sterile scissors. Subsequently, each section was cut in half, obtaining two explants of 0.5cm. Overall, 9 explants were obtained from each female. These explants were washed with HBSS (Hank's balanced salt solution, Gibco, ThermoFisher Scientific) supplemented with 1% ABAM. The uterine explants were processed as described by Borges *et al*. [15]. Briefly, the uterine horn ipsilateral to the CL was opened longitudinally on the antimesometrial side with sterile scissors to expose the endometrium. Tissue was then harvested from the intercaruncular areas of the upper third of the uterine horn with a sterile 8 mm diameter biopsy punch (Henry Schein). The myometrium was dissected with a scalpel to obtain the endometrial explants. Again, 9 endometrial explants were obtained from each female, which was washed in HBSS supplemented with 1% ABAM.

### 4. Culture conditions

The explants were individually placed with the epithelial surface up in a 24-well CELLSTAR® plate (Greiner Bio-One,) with 1 ml of SOF or RPMI media and incubated in a Lab-Line Model 316 incubator (American Laboratory Trading) at 5% CO2 at 38.5ºC

for 2 hours. Then, the media was renewed and the explants were cultured for 6 or 18 hours. After culturing, like the t0 explants, they were divided into two halves for histological and gene expression analysis.

### 5. Histological analysis

Half of each explant was individually fixed in 1 ml of paraformaldehyde for 24-48 hours. After this time, the explants were washed twice with PBS and stored in 1 ml of 70% ethanol at 4 °C for a maximum of one week. Subsequently, the explants were dehydrated in a gradual series of ethanol (80%, 90%, and 100%), then, introduced in toluene and, finally, included in paraffin for their fixation. Once paraffinized, histological sections of 6µm from the periphery and the central area of the explant (since the central area has less access to the culture medium) were obtained using a Leica RM2125 RTS rotary microtome (Leica Biosystems) to check the integrity of the tissue. Finally, these sections were stained with hematoxylin and eosin and were observed under the Olympus DP72 microscope (Galileo Equipment) for histological analysis.

### 6. *In vitro* embryo production

#### 6.1 Oocyte retrieval

Ovaries from heifers or cows were collected in the slaughterhouse and transported to the laboratory in saline solution (0.9% NaCl; Sigma) supplemented with 0.1% gentamicin (Sigma G1272) at 35-37 ºC.

Once in the laboratory, the ovaries were washed first with warm water (35-37 ºC) and then with saline solution at the same temperature to eliminate the remains of blood. The ovaries were kept in saline solution in a water bath to maintain the temperature between 35-37 ºC during the aspiration process. Follicular fluid was aspirated from 2 to 8 mm ovarian follicles using a 5 ml syringe connected to an 18g needle and deposited in a sterile 50 ml Falcon tube that was also in the water bath. The aspirated follicular fluid was allowed to settle for 10 min. After this time, the supernatant was removed and the pellet containing the COCs was resuspended with PBS. This suspension was poured into a 100mm culture plate and search under a stereomicroscope. Only grade I and II COCs were selected, which are characterized by greater compaction between the cumulus cells and homogeneous ooplasm.

### 6.2 *In vitro* maturation

Selected grade I and II COCs were washed three times in PBS and two times in maturation medium (TCM-199 supplemented with 10% FCS and 10 ng / ml EGF). Pools of 50 COCs were then placed in 4-well plates (Thermo Scientific) with 500 µl of maturation medium in each of the wells and cultured for 22-24 hours at 38.5 °C, 5% $CO_2$, and maximum humidity.

### 6.3 Sperm selection and *in vitro* fertilization

Frozen semen of a previously tested bull was thawed in a water bath at 37 ºC for 30 seconds. It was then centrifuged at 280 g for 10 min in a gradient of 1 ml of Bovipure 40% and 1 ml of Bovipure 80% according to the manufacturer's specifications (Nidacon AB Laboratories, Gothenburg, Sweden). The sperm pellet was isolated and washed in 3 ml of Boviwash (Nidacon) by centrifugation at 280g for 5 min. Once centrifuged, the supernatant was removed, and the pellet was resuspended in the remaining 300 µl of Boviwash. Sperm concentration was determined by counting these cells in a Thoma chamber and was adjusted to reach a final concentration of $1x10^6$ sperm/ml for IVF. The male and female gametes were then co-incubated for 18-22 hours in 500 µl of fertilization medium (Tyrode medium, Calbiochem) in a 4-well plate at 38.5 °C, 5% CO2, and maximum humidity.

### 6.4 *In vitro* culture of embryos

Approximately 20 hours after IVF, the presumptive zygotes were transferred to a 15 ml Falcon tube containing 2 ml of PBS and vortexed for 3 minutes to remove cluster cells and sperm. The tube washing was repeated with 1 ml of PBS. The presumptive zygotes were then placed in a Petri dish and subsequently washed three and two times in PBS and SOF, respectively. Finally, they were transferred in groups of approximately 50 to 4-well plates with 500 µl of culture medium and were incubated at 38.5 ° C under an atmosphere of 5% CO2, 5% O2, 90% N2, and maximum humidity.

## 7. Obtention of conditioned medium and Extracellular vesicles isolation

The CM from each group were recovered and transferred to an Eppendorf tube (Thermo Fisher Scientific; Massachusetts, United States). The media were centrifuged at 300 g for 7 min and the supernatant was recovered, which was subjected to an additional 10,000 g

centrifugation for 30 min to remove cell debris and apoptotic bodies. The supernatant was transferred to a new Eppendorf Protein LoBind tube (Thermo Fisher Scientific) and stored in a -80°C freezer. To determine the presence of EVs secreted by explants and embryos in the media, size exclusion chromatography columns (HansaBioMed Life Sciences) were used to separate them from the rest of the particles. First, the column was washed with 30 ml of PBS, and then the CM was passed through. Once the entire volume of the sample was filtered, 11 ml of PBS were added. In each filtration, 23 of 500 µl fractions of the sample can be isolated. Fractions 1-6 (3 ml), containing small particles, and fractions 14-23, containing proteins, were discarded. The EVs were concentrated in fractions 7-11 in a volume of 2.5 ml that was recovered and frozen at -80°C. To concentrate the EVs, the filtered samples were thawed and ultracentrifuged at 100,000 g for 1 hour at 4°C. After the samples were ultracentrifuged, the supernatant was discarded, and the EVs pellets were resuspended in 250 µl of PBS.

### 8. Determination of the concentration and size of EVs in conditioned media

The concentration of EVs and their size were determined by applying a Nanoparticle Tracking Analysis (NTA) with the Nanosight LM10 equipment integrated with the NTA 2.3 software (Nanosight; Wiltshire). The NTA uses scattering properties and Brownian motion to obtain measurements of concentration and size distribution of particles in a liquid suspension after passing a laser over the sample. The light scattered by the moving nanoparticles can be visualized thanks to a 20x microscope coupled to a camera, capturing 30 fields/second.

### 9. Statistical analysis

Data obtained had a normal distribution and a homogeneous variance, for which they were statistically analyzed using the SigmaStat software (Systat Software Inc.) with a one-way ANOVA, followed by a post-hoc test for comparisons between treatments.

These data were obtained and processed in compliance with the social commitment required by the Code of Good Research Practices of the European University.

The correct treatment of data was evaluated and approved by the Research Ethics Committee of the European University (Annex 1).

## RESULTS

### 1. Determination of the concentration and size of EVs in explant conditioned media

The number of particles per field in samples from a single oviductal explant cultured in SOF for 6 hours was 1.1 ± 0.3 (Average ± sem). On the other hand, the data obtained from an endometrial explant cultured under the same conditions revealed several 4.7 ± 0.9 particles per field (Table 1). Although the software provided data on the size and concentration of EVs, a minimum of 20 particles/field is required for an adequate analysis of those parameters (16). Thus, we decided to repeat the analysis using pools of 4 explants.

**Table 1.** Representative data of analysis by NTA of isolated EVs corresponding to a single oviduct and endometrial explant cultured in SOF for 6 hours versus 4 explants cultured under the same conditions. Isolated particles per field are reported.

| | | 1 EXPLANT | 4 EXPLANTS |
|---|---|---|---|
| | | OVIDUCT | OVIDUCT |
| | | PARTICLES/FIELD (Average ± sem) | PARTICLES/FIELD (Average ± sem) |
| SOF | 6H | 1,1 ± 0,3 | 68,2 ± 3,4 |
| | | ENDOMETRIUM | ENDOMETRIUM |
| | | PARTICLES/FIELD (Average ± sem) | PARTICLES/FIELD (Average ± sem) |
| SOF | 6H | 4,7 ± 0,9 | 117,6 ± 26,1 |

**Sem:** standard error mean

As it is shown in Table 1, using a pool of 4 oviductal or endometrial explants cultured in SOF for 6 hours, we observed 68.2 ± 3.4 and 117.6 ± 26.1 (Average ± sem) particles/field, respectively. Therefore, the remaning analysis of the concentration and size of EVs were carried out in a total of 4 pooled samples to obtain conclusive and homogeneous results. As described in Figure 3, pooled samples showed much greater homogeneity compared to individually cultured explants. The latter presented heterogeneous results with disparate values, which caused the analysis to be neither representative nor significant.

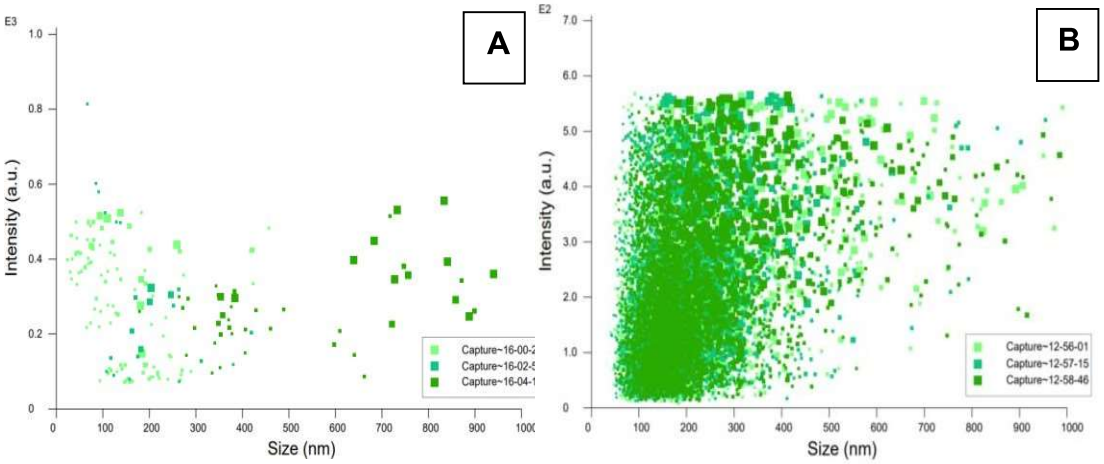



**Figure 3.** Comparison of the analysis of NTA of EVs isolated from CM. The light scattering intensity is plotted against the size of the isolated EVs. (A) 1 explant of oviduct cultured in SOF for 6 hours. (B) Pools of 4 oviductal explants cultured in SOF for 6 hours.

We did not observe differences in the size and concentration of the EVs secreted by the oviductal explants cultured in RPMI or SOF after 6 or 18 hours (Table 2, Figure 4). Similarly, no differences in the size and concentration of the EVs secreted by the endometrial explant were found when they were cultured in different conditions (RPMI *vs* SOF and 6h *vs* 18h) (Table 2; Figure 5).

**Table 2.** Results of the analysis of ATN of isolated EVs. The size mode and concentration / mL of EVs isolated from the CM of 4 oviduct and 4 endometrial explants cultured for 6 and 18 hours in RPMI and SOF media are reported.

| | | **OVIDUCTAL EVs** | |
|---|---|---|---|
| | | **MODE SIZE (nm)** (Average ± sem) | **CONCENTRATION/ml** (Average ± sem) |
| **RPMI** | **6H** | 148,6 ± 22,8 | 4,94e+008 ± 2,81e+008 |
| | **18H** | 171,7 ± 10,7 | 1,28e+009 ± 5,79e+008 |
| **SOF** | **6H** | 163,2 ± 7,4 | 7,27e+008 ± 3,09e+008 |
| | **18H** | 174,4 ± 13,6 | 6,31e+008 ± 6,49e+007 |
| | | **ENDOMETRIUM EVs** | |
| | | **MODE SIZE (nm)** (Average ± sem) | **CONCENTRATION/ml** (Average ± sem) |
| **RPMI** | **6H** | 157,0 ± 14,0 | 1,80e+010 ± 1,42e+010 |
| | **18H** | 163,7 ± 20,3 | 6,64e+010 ± 4,48e+010 |
| **SOF** | **6H** | 160,4 ± 4,8 | 3,34e+009 ± 1,54e+009 |
| | **18H** | 220,7 ± 72,8 | 2,27e+010 ± 1,13e+0010 |

**Sem:** standard error mean

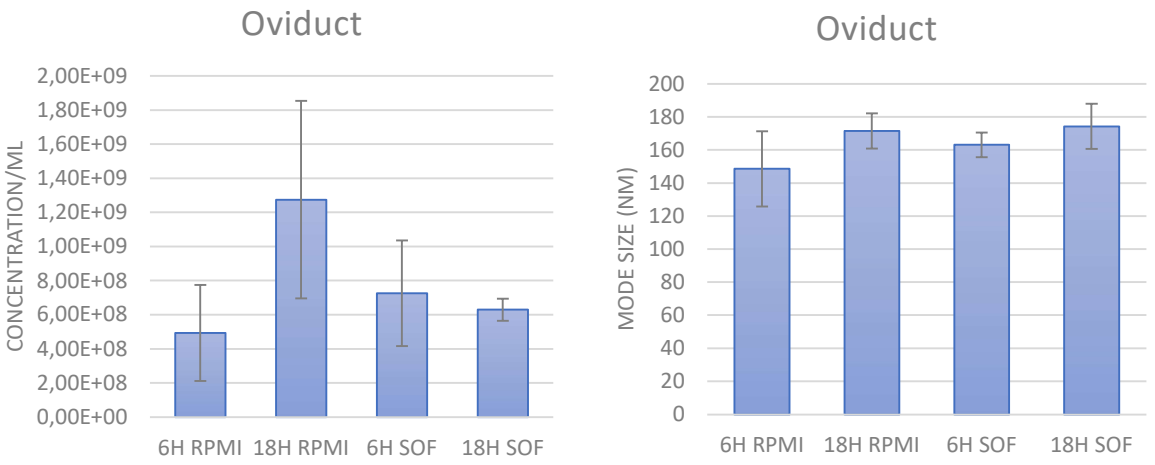



**Figure 4.** Graphic representation of the results obtained in the NTA of the conditioned media of the oviductal explants in the different culture conditions (A) Concentration / mL of EVs. (B) Mode size (nm) of the EVs.

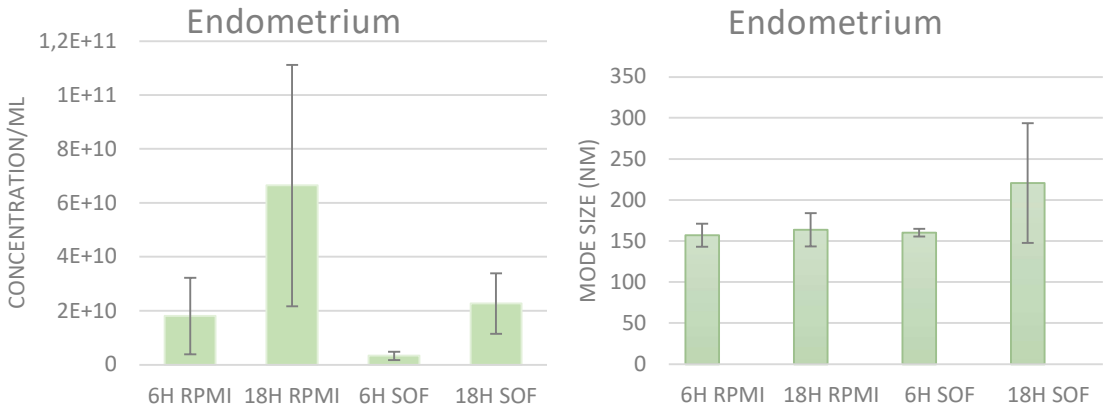



**Figure 5.** Graphic representation of the results obtained in the NTA of the conditioned media of the endometrial explants in the different culture conditions. (A) Concentration / mL of EVs. (B) Mode size (nm) of the EVs.

## 2. Histological evaluation of the oviductal and endometrial explants

The viability of the control explants (time 0) and after culture for 18 hours was evaluated. Representative images of explants of all groups are presented in Figure 6. t0 Endometrial explants (Figure 6A) showed normal tissue architecture: they preserved the lining epithelia (columnar pseudostratified epithelium) and the glandular epithelia. In contrast, the histological analysis of endometrial explants cultured for 18 hours (Figure 6 B-C), it was observed that they had lost both the lining epithelia and the glandular epithelia. A vacuolar degeneration was observed in the connective tissue cells, being less in the explants cultured in SOF medium. Regarding the t0 oviductal explants (Figure 6D), a small detachment of the lining epithelium was observed, while the rest of the tissue maintained a normal architecture. Oviductal explants cultured for 18 hours (Figure 6 E-F) did not show degeneration, while they conserved both muscular and serosa layers. Lining epithelia, like in t0 oviductal explants, also suffered some loss in determined areas.

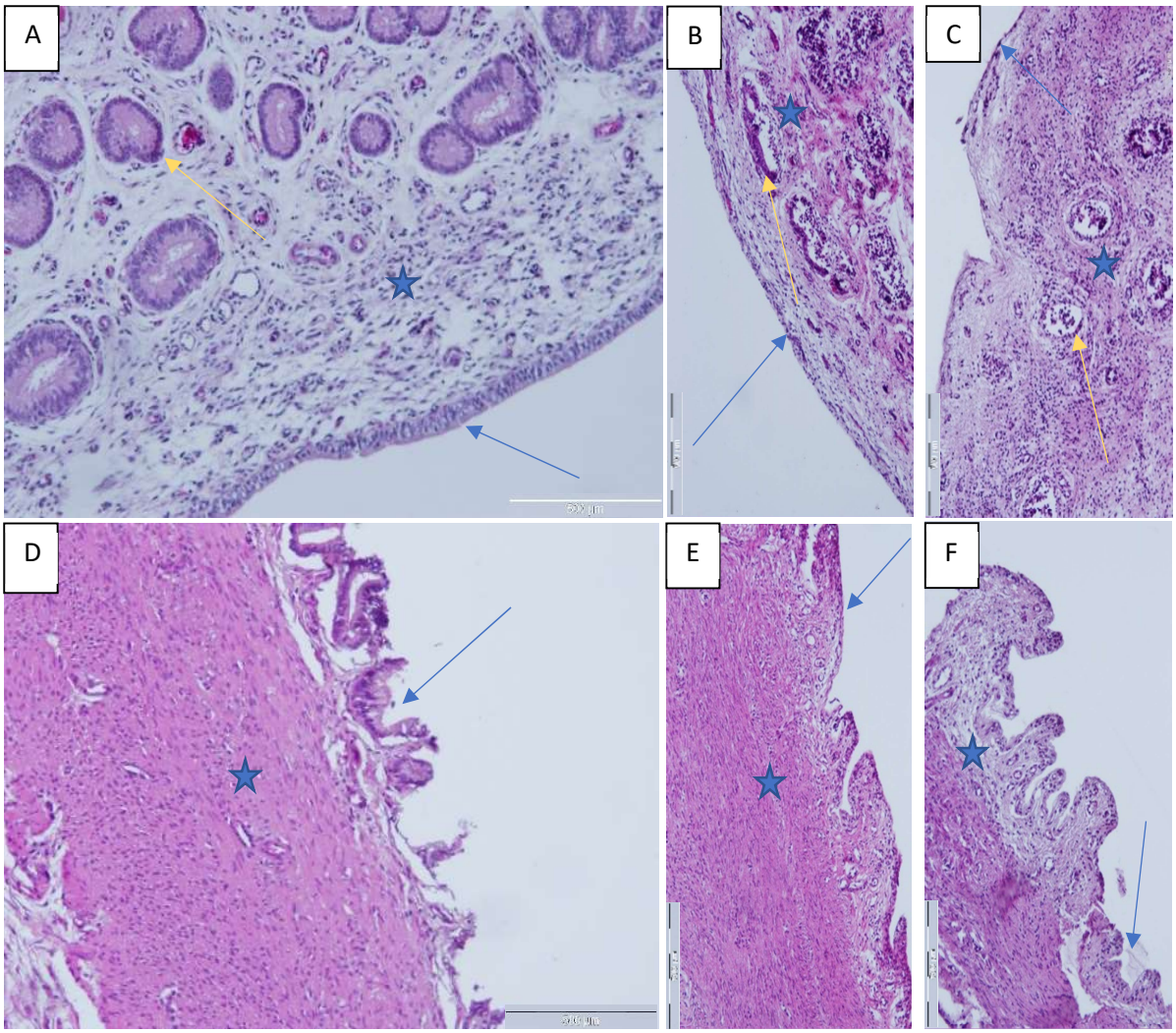



**Figure 6.** Representative images of the histological analysis of the following groups of explants: (a) Endometrium t0. (b) Endometrium SOF t18h. (c) Endometrium RPMI t18h. (d) Oviduct time 0. (e) Oviduct SOF t18h. (f) Oviduct RPMI t18h. Blue arrows indicate lining epithelia. Blue stars indicate representative areas of tissue architecture. Green arrows indicate glandular epithelia (500µm).

### 3. Effect of media and culture time on development and embryo quality

The number of embryos ≥8 cell stage at 52 hpi and the production of blastocysts on day 7 after IVP are shown in Table 3. The proporsion of embryos ≥8 cell stage at 52 hpi was ~41% (400 ≥8 cells embryos out of 991 presumptive zygotes). In the same way, no differences in blastocyst rate were observed when >8 cells embryos were exposed to different culture conditions (SOF vs RPMI and 6h vs 18 h; Table 3). Embryos cultured during 6h in SOF and RPMI presented 30,21 ± 12,46% and 39,59 ± 10,42% blastocyst rate, while the groups cultured during 18 hours in the same media also showed a similar blastocyst rate (32,71 ± 2,29% and 21,88 ± 4,25% respectively)

**Table 3.** Effect of culture media and time on embryonic development

| **Total presumptive zygotes (Day 1)** | **Embryos ≥8 cells 52 hpi (Day 2)** | | | **Blastocyst production (Day7)** |
|---|---|---|---|---|
| | ≥8 cells embryos | Experimental groups (≥8 cells embryos) | | |
| 991 | N (%±sem) | Treatment | n | Blastocysts (%±sem) |
| | 400 (41,35 ± 3,62) | SOF 6H | 112 | 28 (30,21 ± 12,46) |
| | | RPMI 6H | 82 | 31 (39,59 ± 10,42) |
| | | SOF 18H | 112 | 36 (32,71 ± 2,29) |
| | | RPMI 18H | 82 | 17 (21,88 ± 4,25) |

**N** = Number of embryos ≥ 8 cells; **n** = Number of embryos ≥ 8 cells assigned in the experimental groups
**Sem** = Standard error of the mean

In terms of embryo quality, we did not observe diferences in the proportion of blastocysts, expanded blastocysts and hatched blastocysts on Day 8 when Day 7 blastocysts were exposed to different culture conditions. The resulting blastocyst profile is found in Table 4.

**Table 4**. Effect of the medium and culture time on embryo quality at day 8.

| **Experimental Groups (Day 7)** | | **Blastocysts morphological quality (Day 8)** | | |
|---|---|---|---|---|
| Treatment | N | Blastocysts (% ±sem) | Expanded Blastocysts (% ±sem) | Hatched Blastocysts (%±sem) |
| SOF 6H | 16 | 3 (13,64 ± 13,64) | 11 (77,27 ± 22,73) | 2 (9,09 ± 9,09) |
| RPMI 6H | 16 | 3 (18,33 ± 1,67) | 8 (53,33 ± 13,33) | 5 (36,67 ± 3,33) |
| SOF 18H | 16 | 3 (16,67 ± 16,67) | 12 (74,60 ± 3,18) | 1 (7,14 ± 7,14) |
| RPMI 18H | 11 | 3 (28,33 ± 11,67) | 6 (53,33 ± 13,33) | 2 (18,33 ± 1,67) |

**N**= number of blastocysts evaluated at day 8; **Sem**: Standard error mean

## 4. Determination of the concentration and size of EVs in the embryo conditioned media

To determine the presence of EVs secreted by embryos, we analyze the CM of groups of embryos with a different number of ≥8 cells embryos (ranging from 10 to 50) or Day 7 blastocyst (ranging from 4 to 25) cultured in SOF media for 6 or 18h (Table 5). Of note, the number of particles per field detected in each group was below the limit required by the NTA to reliably carry out particle size and concentration analysis (Table 5). Therefore, RPMI samples were not further analyzed. Diagrams were obtained in which the light scattering intensity of the particles was represented according to their size. (Figure 7).

**Table 5**. Concentration (particles / ml), mode size (nm) and particles / field of EVs analyzed by NTA and isolated of SOF media conditioned by different samples of ≥ 8 cells embryos and blastocysts for 6 and 18 hours of culture

| SAMPLES | PARTICLES/ml | MODE SIZE (mm) | PARTICLES / FIELD |
|---|---|---|---|
| SOF control | 529.000 | 55,3 | 0 |
| **≥ 8 cells embryos (N)** | | | |
| SOF 6H (10) | 25.500.000 | 72,5 | 1,3 |
| SOF 6H (30) | 152.000.000 | 143,7 | 7,7 |
| SOF 18H (10) | 207.000.000 | 129,7 | 10,5 |
| SOF 18H (30) | 289.000.000 | 194,8 | 14,7 |
| SOF 18H (50) | 22.600.000 | 216,7 | 1,1 |
| **Blastoscysts (N)** | | | |
| SOF 6H (5) | 253.000.000 | 114,9 | 12,8 |
| SOF 6H (12) | 330.000.000 | 142,5 | 16,7 |
| SOF 18H (4) | 183.000.000 | 200,7 | 9,3 |
| SOF 18H (5) | 181.000.000 | 192,6 | 9,2 |
| SOF 18H (25) | 2.160.000 | 178,1 | 0,1 |

**N**= number of embryos evaluated to analyze EVs secretion; **Sem**: Standard error mean

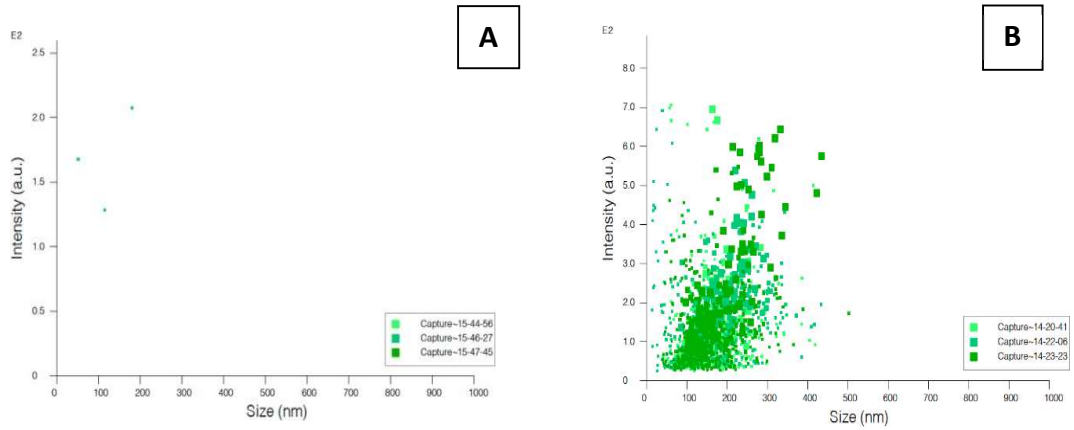



**Figure 7.** Comparison of light scattering intensity diagrams versus particle size obtained by NTA. (a) SOF medium without embryos. (b) SOF medium with 10 embryos ≥ 8 cells grown for 18 hours

## DISCUSSION

The oviduct and uterus are the anatomical locations of the reproductive tract where both fertilization and early embryonic development take place. The physiological environment in which this embryonic development occurs has a great influence on the short and long-term growth of the embryos (2). In fact, a great percentage of the embryos produced *in vitro* do not reach the blastocyst stage.

The establishment of adequate maternal-embryonic communication during the preimplantation stage is one of the essential requirements for the correct development of the embryos. (8). However, *in vivo* studies are not only expensive and difficult to design, but they also do not allow us to differentiate early spontaneous miscarriages. For this reason, *ex vivo* studies that mimic conditions in the early embryonic environment are essential to understand the mechanisms of maternal-embryonic interactions. Even though the co-culture of embryos with explants has represented a new method of studying maternal-embryonic communication that more precisely replicates the physiological conditions *in vivo*, there are still some limitations. Among them, the explant culture time (15) and the complications in the detection of communication signals stand out (12,14).

This study, based on the importance of these interactions during the first days of embryo development and the environment in which it is found, aims to establish an *ex vivo* co-culture model between embryos and explants from the oviduct and uterus. For this, the viability and the ability to secrete EVs by the two elements that make up this *ex vivo* co-culture model (embryo and tissue) were studied in SOF and RPMI media at different time points of incubation (6 and 18 hours).

Our study revealed that the endometrial explants did not maintain their viability after 18 hours of culture, while the oviductal explants, despite presenting some epithelial detachment, maintained the 2 layers of tissue and their architecture. The secretion of EVs by these tissues did not show differences between all groups. Embryos cultured under the same conditions did not show differences in development or quality, in addition to the fact that the concentration of secreted EVs was not enough to be detected in our analysis.

Regarding the explants, a first collection of the CM was made from the culture of a single oviduct and endometrial explant during the established 6 and 18 hours. However, the NTA analysis revealed that the EVs extracted were at a concentration lower than the 20-100 particles per field established to detect a representative signal (16). To increase the

concentration of EVs and therefore the signal detected by NTA, it was decided to analyze the CM of pools of 4 oviductal explants and 4 endometrial explants in all culture conditions. Since the detected signal determined an adequate particle per field concentration (20-100 particles per field), this 4-sample system was fixed in the following runs.

The results obtained by the NTA analysis of the CM from the pools of 4 oviductal explants reflected a mean size of the EVS between 148.6 nm and 174.4 nm, while those from the endometrial explants had a size between 157.0 and 220.7 nm. These results are similar to those obtained previously in the OF and UF, in which it was observed that the isolated EVs had a size between 150-200 nm (6). Regarding the concentrations of EVs, it was observed that the EVs extracted from the CM of the oviductal explant pools ranged between $4.94x10^8$ and $1.28x10^9$ particles/ml, while the EVs isolated from the CM of the endometrial explant pools were found in concentrations between $3.34x10^9$ and $6.64x10^{10}$ particles /ml. In comparison with the results presented by Lopera-Vasquez *et al.* [6], which describe a concentration of $9.28 \times 10^8$ particles/ml in the OF, they are very similar to those observed in the oviductal explant pools. For its part, the EVs concentration found in endometrial explant pool cultures is also similar to the $4x10^9$ particles/ml described by Hamdi *et al.* [13] in the UF. In addition, no differences were observed in terms of the size and concentrations of the EVs in the different media and incubation times of our oviductal or uterine explants.

Thus, our results demonstrate that both oviductal and endometrial explants are able to secrete EVs at different culture conditions.

Regarding the viability of the explants of both tissues, we only performed the histological analysis of the controls tissues and the explants cultured for 18 hours in both SOF and RPMI media. This response is to the basis of our hypothesis that if the explants were viable at 18 hours of culture, they would also be viable at 6 hours. For the control groups, the endometrial explants showed correct preservation of the luminal and glandular lining epithelia, in addition to a conserved tissue structure. Similar results were observed in the oviductal explants, although in this case detachment of the lining epithelium was observed, which could be due to the processing of the samples of this tissue, whose manipulation is more complex. Furthermore, it should be noted that up to now there are no studies available evaluating the viability of the oviductal explants under different culture conditions. On the other hand, endometrial tissues cultured for 18 hours showed

degenerative changes, suffering a loss of the luminal and glandular lining epithelia with necrosis foci.

Oviductal tissues cultured for 18 hours did not show degenerative changes in their architecture, conserving both muscular and serosa layers. While, lining epithelia suffered some loss in determined areas.

Even though the RPMI medium is the one designed for the culture of explants our results showed that the tissue better conserved its structure after being cultured in the SOF medium, observing less ballooning degeneration and cell necrosis. The differences found in comparison to the study by Borges *et al.* [15], whose explants did maintain viability after different culture times (6, 24, and 48 hours), could be due to the different compositions of the medium. While Borges *et al*. [15] used RPMI supplemented with 10% FCS, we did not use FCS. Our culture media were not supplemented with serum, which contains a high concentration of EVs that would have interfered in the isolation of EVs derived from our explants, either through false positives or burying the signal emitted by the tissue in noise. background. The absence of supplementation of our RPMI medium could have negatively influenced the viability of the explants since FCS is a source of proteins and growth factors of great importance during tissues and cell cultures. In addition, the time since the animal is slaughtered until the tissue is processed in the laboratory may be one of the reasons for the dissimilarities with the results reported by Borges *et al.* [15] as we already observed detachment of the lining epithelium in some time 0 oviductal explants (control). Taking it all together, these variables must be considered in future studies to improve the viability of the explants.

Regarding embryonic development, we hypothesized that differences in the composition between RPMI (without essential nutrients for embryo development and high concentrations of glucose) and SOF media would result in a poorer developmental rate in those embryos cultured in RPMI. However, our results did not show differences in embryonic development and quality when ≥8 cells embryos and Day 7 blastocysts were exposed to different culture media for 6 or 18 hours. The blastocyst rate was similar to the results obtained by Rizos *et al.* [17], who performed the culture in SOF medium supplemented with BSA and BSA + FCS. In our work, only BSA was used, since the presence of EVs in the serum could interfere with the objective of detecting EVs secreted by embryos as occurred in the culture of explants.

Blastocyst rate on day 7 from ≥8 cells embryos cultured during 6 and 18 hours in SOF supplemented with BSA presented 30.21 ± 12.46% and 32.71 ± 2.29% blastocyst rate

respectively, while the day 7 blastocyst rate showed by Rizos *et al.* [17] were 28.00 ± 9.00% for SOF supplemented with BSA groups and 32.00 ± 1.00% for those embryos cultured in SOF supplemented with BSA + FCS. Embryo quality results based on morphological criteria on day 8 did not show differences between the different groups. Therefore, the results obtained in this work ensure the viability of the embryos when cultured in RPMI or SOF media for 6 and 18 hours. These results, together with those reflected in the culture of explants, validate the use of both media for the co-culture of explants and embryos. Table 5 shows the concentration and size distribution of EVs secreted by samples of different numbers of embryos in the SOF medium at 6 and 18 hours and in the control (SOF medium without embryos). Despite carrying out different approaches such as the ultracentrifugation of the CM to increase the concentration of EVs and increasing the number of embryos in the culture medium, we did not detect the minimum of 20-100 particles/field required to perform an adequate analysis of the size and concentration of embryonic EVs by NTA (16). In the work of Pavani *et al*. [18], EVs could be correctly detected by NTA, however, pools of 500 embryos were necessary. Although increasing the number of embryos could amplify the signal they emit, it should be remembered that our work is focused on mono-ovulatory animals, therefore, physiologically, only one embryo is found in the oviduct and uterus. Since the objective of our work is to evaluate the ideal conditions for embryo culture with endometrial and oviductal explants, the use of a high number of embryos could alter the interactions between the two and not reflect the physiological conditions that occur in *in vivo* development. Therefore, the number of cultured embryos must be optimized to detect a signal while not distorting the results compared to what happened *in vivo*.

In addition, it should be considered that in the same work by Pavani *et al*. [18], a different isolation methodology (density gradient centrifugation) was used than that used in our study (differential centrifugation + size exclusion chromatography). Pavani *et al*. [18], demonstrated that density gradient centrifugation is a more efficient method for the separation and purification of EVs compared to the techniques used in our work since this technique allows efficiently separating EVs from ribonucleoprotein complexes, lipoproteins, and other particles. Therefore, it is worth highlighting the importance of the isolation and concentration methods of EVs used since various methods can generate large variations in the results obtained. On the other hand, the study by Bridi *et al*. [19] demonstrated the secretion of embryonic EVs using groups of 10 blastocysts cultured in SOF medium for 48 hours. These results could indicate that the lack of detection of EVs

in our study could be due to the shorter time of culture (6 or 18 hours). Although a logical solution could be to increase the hours of culture, the ultimate objective of this experiment was to establish the ideal conditions to detect EVs in a coculture of embryos and explants. Considering this objective, the modification of the protocol used to detect EVs in this experiment represents a more successful proposal, since increasing the culture period would cause the degeneration of explants (15), highlighting the need for future experiments with an adequate methodology for the measurement of EVs. In addition, this study aims to focus on the maternal-embryonic interactions at specific moments of development (day 2.5 and day 7), so increasing the culture time would not make sense. If embryonic development were studied for a longer period of time, the results obtained would not represent these development stages.

It should be noted that previous studies demonstrated the variation of the transcriptomic profile of the bovine endometrium in the presence of an embryo (14), as well as the ability of embryos to vary the gene expression and metabolic profile of BOECs (20). Therefore, despite not having detected a sufficient signal for the analysis by NTA, the final objective of this study was to establish a co-culture model, so the signal that will be detected in the CM co-culture between embryos and explants will be indirectly amplified. These mechanisms will allow the presence of EVs to be assessed not only by their concentration but also by their effect on the tissues in which they are cultured.

## CONCLUSIONS

- Oviductal and endometrial explants are capable of secreting EVs into culture media without differences in concentration between SOF and RPMI media and incubation times of 6 and 18 hours.
- *In vitro* culture of bovine embryos (≥ 8 cells embryos and day 7 blastocysts) in SOF and RPMI media for 6 or 18 hours does not affect development or embryo quality.
- Our experimental model did not allow the detection of an adequate amount of EVs secreted by the embryos, highlighting the need to explore new methodologies for the processing and analysis of embryonic CM.

**AGRADECIMIENTOS**

*En primer lugar, me gustaría mostrar mi agradecimiento a dos de mis tutores de TFM, los Dres. Dimitrios Rizos y José María Sánchez. Gracias por confiar en mí desde el primer momento y permitirme formar parte del gran grupo del INIA. Pero, sobre todo, gracias por enseñarme y aconsejarme día a día.*
*Especial agradecimiento a mi también tutora de TFM la Dra. Raquel Herrer de IVI Zaragoza, quien, además, ha guiado y tutelado mis primeros pasos en un laboratorio de embriología humana.*

*Gracias también a mis compañeros de laboratorio: Beatriz, Sandra, Paula, Iván, Andrea, Celia, Vasilis… Habéis formado parte de mi día a día y habéis hecho que todo el esfuerzo de este año fuese un poco menos.*

*A mi familia y amigos, por demostrarme que las ganas y el esfuerzo me llevarán a lograr aquellos que me proponga. Gracias por creer incondicionalmente en mí y servirme de constante apoyo.*

*A mis profesores y compañeros del máster, a quienes, dadas las circunstancias, no he podido llegar a conocer todo lo que hubiera deseado. A pesar de todo hemos formado un gran equipo.*

*En definitiva, gracias a todas las personas que han sido parte de mi vida durante este tiempo y han permitido que este trabajo sea una realidad.*

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Universidad Europea

# ANNEX 1

*Comité de Ética de la Investigación de*
*la Universidad Europea*

**Daniel de la Fuente Toro**

**Universidad Europea de Madrid**

Villaviciosa de Odón, 16 de julio de 2021.

Estimado investigador:

En relación al Proyecto de Investigación titulado:

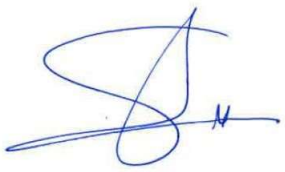
**"Vesículas extracelulares: moduladores de la interacción materno-embrionaria y de la fertilidad en el modelo bovino"**

este Comité de Ética de la Investigación ha procedido a la revisión del mismo y ha acordado que está en situación de:

**APROBADO**

Al proyecto se le ha asignado el código interno **CIPI/213006.24**

Atentamente,



Fdo.: Lola Pujol

Secretaria del Comité de Ética de la Investigación