



**İSTANBUL UNIVERSITY-CERRAHPAŞA  
INSTITUTE OF GRADUATE STUDIES**



**Ph.D. THESIS**

**EFFECTS of EXTRACELLULAR VESICLES DERIVED from SOEC on IN  
VITRO DEVELOPMENT and QUALITY of SHEEP EMBRYOS**

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**November, 2022**

## THESIS ACCEPTANCE AND APPROVAL

This thesis titled "**Effects of Extracellular Vesicles Derived from SOEC on in vitro Development and Quality of Sheep Embryos**", prepared by **Israa Faris MOHAMED FARIS** under the supervision of **Prof. Dr. Sema BİRLER**, was accepted as a **Ph.D. Thesis** after being **consensus** approved by our jury as a result of the exam held on **30/11/2022**.

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I dedicate my dissertation work to my daughters, Wahaj and Judy. Tremendous gratitude for my loving partner, Nail, and my parents, whose words of encouragement have supported me throughout the process. My sisters Safaa, Hanna and My brother Mohamed. I also dedicate this work to Abdullah Yaman, Prof.Dr. Ata Őenlikçi and Prof.Dr. Jacek Kuznicki supported me in obtaining this degree. I dedicate this work and give my special thanks to my best friend, Raba Zean and Züleyha Kılıç. I also dedicate this dissertation to Türkiye Bursları and TÜBİTAK.

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## LIST OF SYMBOLS AND ABBREVIATIONS

<b>Symbols</b>	<b>Description</b>
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<b>μ</b>	: Micron
<b>mL</b>	: Milliliter
<b>nm</b>	: Nanometer

<b>Abbreviations</b>	<b>Description</b>
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<b>SOEC</b>	: Sheep oviductal epithelial cells
<b>EV</b>	: Extracellular vesicles
<b>oEVs</b>	: Oviductal extracellular vesicles
<b>UC</b>	: Ultracentrifugation
<b>NTA</b>	: Nanoparticle tracking analysis
<b>FCS</b>	: Fetal Calf Serum
<b>TEM</b>	: Transmission electron microscopy
<b>PBS</b>	: Phosphate buffered saline
<b>DMEM</b>	: Dulbecco's modified eagle medium
<b>COCs</b>	: Cumulus-oocyte complexes
<b>IVM</b>	: In vitro maturation
<b>SOF</b>	: Synthetic oviductal fluid
<b>FCS/ FBS</b>	: Fetal calf serum/ Fetal bovine serum
<b>IVF</b>	: In vitro fertilization
<b>IVC</b>	: In vitro culture
<b>TE</b>	: Trophectoderm
<b>ICM</b>	: Inner cell mass
<b>miRNA</b>	: Micro messenger ribonucleic acid
<b>miRNA</b>	: Micro messenger ribonucleic acid
<b>FCM</b>	: Flow Cytometry
<b>CFSE</b>	: Superior fluorescent staining enable kit
<b>SSC</b>	: Side Scatter Signal

## GENİŞLETİLMİŞ ÖZET

### [DOKTORA TEZİ]

#### [OVIDUKT EPİTELYAL HÜCRE KÜLTÜRÜNDEN ELDE EDİLEN EKSTRASELÜLER VEZİKÜLLERİN KOYUN EMBRİYOLARIN GELİŞİMİ ve KALİTESİ ÜZERİNE ETKİLERİ ]

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[Ovidukt; gamet olgunlaşması, taşınması ve spermatozoanın oositlere doğru yönlendirilmesi için uygun mikro ortamı sağlayan, başarılı bir fertilizasyon ve erken embriyo gelişimi için elzem olan bir organdır. Embriyo ve annenin reproduktif kanalı arasındaki etkileşim (EV'ler gibi sinyallerin etkileşimi), başarılı embriyonik gelişim için çok önemlidir. Ovidukt, erken embriyonun maruz kaldığı ilk ortamdır ve bu yüzden embriyo ile anne arasındaki sinyallerin başlangıç noktası olduğu düşünülmektedir.

Oviduktal epitel hücrelerinden türetilen EV'lerin (oEV'ler) rolünün ne olduğu henüz tam olarak anlaşılmamıştır. Bu konuda son yıllarda gerçekleştirilen çalışmalarla EV'lerin reproduktif sistemde oynadıkları roller anlaşılmasına çalışılmaktadır. Oviduktal EV'lerle ilgili gerçekleştirilen en son çalışmalarda, EV'leri izole etmek için yoğunluk gradyanı, ultrasantrifüj/seri santrifüj, polimer bazlı presipitasyon ve ticari kitlerle immünoafinite gibi çeşitli yaklaşımlar kullanılmıştır. Mevcut literatüre bakıldığında, oviduktal EV'lere odaklanan çalışmaların sayısı, uterus EV'leri ve foliküler sıvı EV'leri gibi dişi reproduktif organlarından türetilen diğer EV'lerle karşılaştırıldığında daha azdır.

Çoğu çalışmada, ultrasantrifüj tekniği, oEV'leri izole etmek için tercih edilen bir teknik olmuştur. Çünkü bu çalışmaların çoğunda oEV'lerin gamet\embriyolar üzerindeki fonksiyonel etkilerini araştırmak amaçlanmıştır.

Sunulan çalışmada, yardımcı üreme tekniklerinde gebelik oranlarını iyileştirme konusunda esas olan in vitro embriyo üretimi üzerine ovidukt epitel kaynaklı EV'lerin etkilerine odaklanılmıştır.

Mevcut hipotezin amaçları, ilk olarak, koyun ovidukt epitel hücrelerinden (SOEC) koşullandırılmış (conditioned)-medyum toplamak için tek katmanlı SOEC hücre kültürünü elde etmektir. Daha sonra, ekstraselüler veziküller (EV), koşullandırılmış ortamdan sıralı ultrasantrifüj ile izole edildi ve nano-tracking analizi ile karakterize edilerek konsantrasyonu saptandı. SOEC-koşullandırılmış ortamdan oEV'lerin izolasyonundan sonra, SOEC'den türetilen EV'ler embriyo in vitro kültür ortamına eklenerek, EV'lerin embriyo gelişimi ve kalitesi üzerindeki etkileri araştırıldı.

Ovidukt epitel hücre kültürü, oEV'lerinin rolünü incelemek için temel bir işlemdir. Bu çalışmada, koyun ovidukt epitel hücrelerinin izolasyonunu gerçekleştirmek için iki yöntem kullanılmıştır. Üzerinde dominant folikül ve korpus luteum bulunmayan yumurtalıklar, uzunlamasına açıldı. Ovidukt iç yüzeyindeki hücreler steril bir lam kullanılarak (bir kez) kazındı, içinde doku kültürü medyumunu bulanan bir tüp içerisine toplandı ve sonra ekildi. Kullanılan diğer yöntem ise yıkama tekniğiydi. Bu teknikte öncelikle ovidukt boylamasına steril bir petri kutusunun içerisine sıklıdıktan sonra, ovidukt lümeni aynı petri kabı içerisine %10 FCS ilaveli 1 mL DMEM medyumunu ile yıkandı. Sağlıklı ve açık renkli hücre kümeleri toplandı ve kültür medyumunu (%10 FCS ilaveli DMEM) bulunan bir petri içerisine ekildi.

Kültür medyumunu, hücreler %90 konfluensiye ulaşana kadar her 48 saatte bir değiştirildi. Hücrelerin yayılması %90 civarına ulaştığında, medyum atıldı, yerine %10 oranında EV-depleted (EV'lerden arındırılmış) FCS ilave edilmiş DMEM eklendi ve hücreler 24 saat bu ortamda inkübe edildi. İnkübasyondan sonra, SOEC-EV'leri izole etmek için koşullandırılmış-medyum toplandı. Sıyırma yöntemi ile elde edilen 27 mL ve yıkama yöntemi ile elde edilen 10 mL olan koşullandırılmış-medyumuna sıralı ultrasantrifüj tekniği uygulandı. Bu yöntemde öncelikle, kalıntıları ve ölü hücreleri uzaklaştırmak için 300 g'de 10 dakika santrifüj yapıldı. Süpernatant alındı ve apoptotik cisimlerin uzaklaştırılması için 2000 g'de 20 dakika santrifüje edildi. Tekrar süpernatant alınarak büyük veziküllerin atılması için 10.000 g'de 30 dakika ve 20.000 g'de 40 dakika santrifüjlendi. Son olarak, süpernatant 100.000 g'de 60 dakika santrifüje

edildi. Son santrifüjden sonra süpernatant tamamen atıldı ve santrifüj tüpleri EV'leri çözmek için 1 mL fosfat tamponlu tuzlu su ile yıkandı.

Elde edilen EV'nin boyutlarını ve konsantrasyonunu belirlemek için Nano İzleme Analizi (NTA) gerçekleştirildi. Yıkama yöntemiyle elde edilen SOEC-EV'lerin konsantrasyonu ve tek bir EV'nin ortalama boyutu sırasıyla  $3.46 \times 10^9$  partikül/mL ve 200 nm olarak değerlendirildi. Kazıma yöntemiyle elde edilen SOEC-EV'lerin konsantrasyonu ve ortalama boyutu sırasıyla  $9.80 \times 10^9$  partikül/mL ve 184 nm olarak değerlendirildi.

SOEC'den türetilen EV'lerin embriyo gelişimi ve kalitesi üzerindeki etkilerinin incelenmesi amacıyla, izole EV'lerin in vitro embriyo kültür ortamına eklendi ve embriyoların yarıklanma ve blastosist oranları, ayrıca embriyo kalitesi değerlendirildi. Embriyo kültür medyumuna (SOFaa) içerisine, düşük EV ( $9.8 \times 10^6$  EV/50 $\mu$ L), orta EV ( $49 \times 10^6$  EV/50 $\mu$ L) ve yüksek EV ( $98 \times 10^6$  EV/50 $\mu$ L) konsantrasyonlarında SOEC'den türetilen EV'ler katıldı ve hiç EV katılmayan kontrol grubu ile karşılaştırıldı. Yarıklanma (2. Gün) ve blastosist gelişimi (8. Gün) değerlendirildi. Tüm grupların (Kontrol, Düşük EV, Orta EV ve Yüksek EV) yarıklanma ve blastosist oranları sırasıyla %68.40, %64.66, %66.00, %59.53; ve %23.39, %33.54, %29.09 ve %26.80'dir. Gruplar arasında dikkate değer farklılıklar olmasına rağmen, istatistiksel olarak sonuçlar arasındaki farklar anlamlı olmadı ( $p > 0.05$ ).

Kontrol, Düşük EV, Orta EV ve Yüksek EV gruplarındaki blastosistlerde toplam hücre sayısı ( $\pm$ SEM) sırasıyla  $142.75 \pm 11.93$ ,  $298.25 \pm 36.11$ ,  $225.33 \pm 15.38$  ve  $279.38 \pm 15.32$  olarak saptandı. EV eklenen gruplarda blastosistlerin daha kaliteli oldukları saptandı ( $p < 0.05$ ).

Sunulan çalışma, koyun ovidukt epitel hücrelerinden elde edilen EV'lerin ilk kez tanımlandığı bir çalışmadır. Ayrıca elde edilen sonuçlar, embriyo-ovidukt kanalı ilişkilerinin modülatörleri olarak kabul edilen EV'ler gibi, SOEC'den üretilen EV'lerin embriyo gelişimini ve kalitesini iyileştirdiğini göstermektedir. ]

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**Anahtar kelimeler:** [SOEC-kaynaklı-Ekstraselüler Veziküller, Koyun, IVF, Embriyo Gelişimi ]

## **ABSTRACT**

**[Ph.D. THESIS]**

**[EFFECTS of EXTRACELLULAR VESICLES DERIVED from SOEC on IN VITRO DEVELOPMENT and QUALITY of SHEEP EMBRYOS ]**

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[The oviduct provides an appropriate micro-environment for gamete maturation and transport, and the direction of spermatozoa toward the oocytes conducting successful fertilization and sustaining early embryo development. The interaction of signals such as EVs between the embryo and the mother environment is crucial to successful embryo development. The oviduct is the first environment in which the zygote is exposed and is assumed to be the starting point of signals between the embryo and the mother.

The role of oviductal epithelial cells-derived EVs (oEVs) needs to be more adequately understood; several experiments have attempted to achieve further knowledge. In most recent studies of oEVs, various approaches were used to isolate the EVs such as polymer-based precipitation, density gradient, ultracentrifugation\serial centrifugations, and immunoaffinity. Regarding the existing publications, studies focused on the oEVs is few compared with other EVs (uterine and follicular fluid EVs).

Ultracentrifugation was the preferred technique to isolate the oEVs because most aimed to investigate the effects of oEVs on gametes and/or embryos.

This study focused on oviductal epithelial-derived-EVs' effects on in vitro embryo production, which can be essential in improving pregnancy rates in mammals ART.

The aims of the current hypothesis were, firstly, to obtain a monolayer cell culture of sheep oviductal epithelial cells (SOEC) to collect conditioned media. Then, extracellular vesicles were isolated from the conditioned oviductal cell culture media by sequential ultracentrifugation, quantified, and characterized by the nano-tracking analysis. After the isolation of oEVs from SOEC-conditioned media, the SOEC-derived-EVs were supplemented into the embryo culture medium and investigated their effects on the development of embryos and quality.

Oviductal epithelial cell culture is a fundamental step for studying the role of the oviducts oEVs. In this study two methods were used to perform the isolation of sheep oviductal epithelial cells for culturing. Ovaries that demonstrated follicle growth and absence of the corpus luteum were picked as the follicular phase oviducts were cut longitudinally. Cells were scraped by a glass slide and seeded. The other method was the flushing technique. After squeezing the oviduct longitudinally, the oviductal lumen was flushed with 1 mL of DMEM supplemented with FCS (10%) in a petri dish. Healthy and light-colored clumps of cells were collected and seeded.

The medium was substituted every 48 hours until the cells reached 90% confluency. Therefore, the medium was discarded, replaced by DMEM supplemented with EV-depleted FCS (10%), and incubated for 24 hours. After the incubation, the conditioned media were collected. Ultracentrifugation technique was applied to conditioned media which were 27 mL of scraping method and 10 mL of flushing method in order to isolate the SOEC-EVs. Centrifugation was started at 300 g for 10 minutes to remove the debris and dead cells. The supernatant was centrifuged at 2000 g for 20 minutes to pellet apoptotic bodies. The volume was centrifuged at 10.000 g for 30 minutes and 20.000 g for 40 minutes to remove the large vesicles; the last centrifuge was achieved at 100.000 g for 60 minutes. After the last centrifugation, the supernatant discarded, and the tubes were washed with 1 mL PBS to dissolve the EVs.

Nano Tracking Analysis (NTA) was used to characterize and quantify the size of a single EV and the concentration. The SOEC-EVs concentration and mean size of a single EV obtained by the flushing method were assessed as  $3.46 \times 10^9$  particles/mL and 200 nm, respectively. The SOEC-EVs concentration and mean size of a single EV obtained by scraping method were assessed as  $9.80 \times 10^9$  particles/mL and 184 nm respectively.

The examination of SOEC-derived EVs' effects on the development of embryos and quality was performed by supplementing the isolated EVs into embryo culture media. Presumptive zygotes were cultured in SOFaa supplemented with SOEC-derived EVs in different concentrations of low EV ( $9.8 \times 10^6$  EVs/50 $\mu$ L), medium EV ( $49 \times 10^6$  EVs/50 $\mu$ L), and high EV ( $98 \times 10^6$  EVs/50 $\mu$ L). The cleavage rates and the blastocyst development were evaluated at Day 2 and Day 8, respectively. Cleavage and blastocyst rates of all groups (Control, Low EV, Medium EV, and High EV) were 68.40%, 64.66%, 66.00%, 59.53%; and 23.39%, 33.54%, 29.09% and 26.80%, respectively. Although there were remarkable differences between groups, yet were not important statistically ( $p > 0.05$ ).

The total cell number of embryos ( $\pm$ SEM) in Control, Low EV, Medium EV and High EV groups were counted at Day 8 blastocysts as  $142.75 \pm 11.93$ ,  $298.25 \pm 36.11$ ,  $225.33 \pm 15.38$  and  $279.38 \pm 15.32$ , respectively. The qualities of blastocysts were more reasonable in EV-added groups than in the control ( $p < 0.05$ ).

Our research provides the first definition of sheep oviductal epithelial cells derived-EVs, demonstrating the impact of SOEC-derived EVs as modulators of the embryo–oviduct relations. Moreover, our results indicate SOEC-derived EVs as means to improve embryo development and quality especially total cell numbers under *in vitro* conditions. ]

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**Keywords:** [SOEC-derived-Extracellular Vesicles, Sheep, IVF, Embryo Development]

## **1. INTRODUCTION**

### **1.1. EXTRACELLULAR VESICLES**

Extracellular Vesicles (EVs) are tiny spherical packages released by cells into the extracellular environment. EVs are isolated from several cells and a lot of biological fluids such as follicular fluid, milk, blood plasma, urine, and saliva. They influence recipient cells literally via the transfer of bioactive cargo (protein, lipids, mRNA, etc.) or directly by impacting the cellular epigenome [3,18,36,50]. Through endocytosis and cell membrane fusion, EVs transfer their molecular cargo to recipient cells. Standardization of isolation, quantification and storage methods for EVs will significantly improve the future promises for diagnostic and therapeutic applications of EVs [92].

#### **1.1.1. Nomenclature**

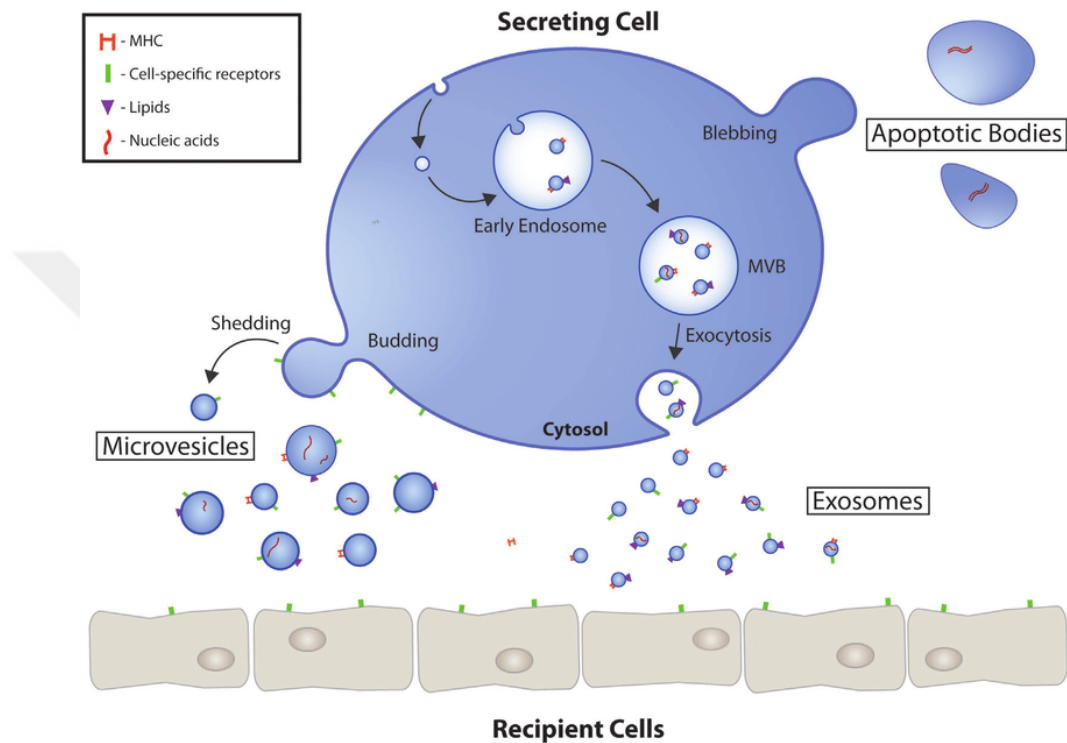
The recent incapability to acquire an agreement on various aspects of the nomenclature of vesicles reflects natural contrasts of thought about the value of scientific pattern, the comparative values of functional versus biogenetic techniques for naming vesicles, and scientific conflict about the accuracy of current standards of vesicle biogenesis [32].

Exosome and microvesicle terms have historically been burdened by varied, contradictory definitions and false expectations of unique biogenesis [85]. The research community expresses the EVs' nomenclature [34,90] which is associated with their origin, biogenesis, and size.

According to this nomenclature, EVs are named according to physical characteristics, such as size their size; small EVs are called exosomes (30-150 nm), 100-1000 nm are microvesicles, and apoptotic bodies their size of up 5  $\mu\text{m}$  (Figure 1.1). The nomenclature also includes terminology related to the characterizations of circumstances or the origin of cells (large oncosomes, apoptotic bodies, podocyte EVs and hypoxic EVs). For example, EVs named isolated from uterine luminal fluid as uterosomes, from oviduct fluid as oviductosomes, from the epididymal fluid as epididymosomes, and from the prostate as prostasomes [18,22,80,88].

### 1.1.2. Biogenesis

The term described by the biogenesis of EVs classified as exosomes are released by combining multivesicular with the plasma membrane, microvesicles are shed from the outer plasma membrane by their outward budding, and apoptotic bodies are originated at the same time apoptosis occurs and contain degraded cell fragments from programmed cell death [17,22,81].



**Figure 1.1:** The schematic illustration of the significant pathways for biogenesis of EVs. The early endosomes are secreted and combine the inward budding of multivesicular bodies with the plasma membrane to form exosomes. The direct external budding of the plasma membrane produced microvesicles. Apoptotic bodies are created by membrane blebbing after programmed cell death [34].

### 1.2. ISOLATION METHODS OF EXTRACELLULAR VESICLES

The suitable method of EVs' isolation depends first on starting materials (e.g., milk, plasma, urine, cell culture, etc.), then the scientific questions, a critical downstream analysis, the preferred end product (e.g., total EVs or CD63+), and equipment available in the laboratory [8,51,79,89,93].

Collected samples must be maintained on ice, and concern should be provided to remove cells, bacteria, and sustenance debris before the isolation of EVs which can be done by low-speed centrifugation.

### 1.2.1. Differential Ultracentrifugation

Differential centrifugation is the most widely applied (81%) direct isolation technique across all applications, even increasing to 85% for the isolation of EVs from conditioned media.

The isolation of EV populations was published by They et al. [84]. The exact speed and duration may vary in each step, but this is the general procedure of multiple centrifugations from 300 g up to 100,000 g. After removing the supernatant, the tube must be kept at an angle so the pellet is covered with supernatant (half a centimeter of liquid is still covering the pellet) [84].

Depending on starting material, a conditioned media or body fluids can centrifuge at 300 g to pellet removing cell debris; this step is crucial; when a high centrifuge is performed, the cells can burst, and the cell's intercellular vesicles can contaminate the samples. The supernatant used for the next step will centrifuge at approximately 2000 g, pelleting the apoptotic bodies. For pelleting microvesicles, the G-force must be between 10.000 and 20.000, then the supernatant centrifuges at 100,000 g for isolation of the smallest EVs and the exosomes (Figure 1.2) [93,84].

Ultracentrifugation seems to be the most reasonable method for the subsequent use of EVs for biological studies, proteomics, flow cytometry, lipidomics, and RNA studies. Furthermore, the use of ultracentrifugation maintained the biological activity and integrity of isolated EVs.

Additionally, current studies have suggested that maintaining the same protocol with differential centrifugation is an essential step when comparative analyses of oEVs are conducted since the various kinds of rotors and particular tubes utilized in rotors can influence the K-factor as well as the period of centrifugation, creating 2-3-fold contrasts in pellet result [4,5,6,7,33].

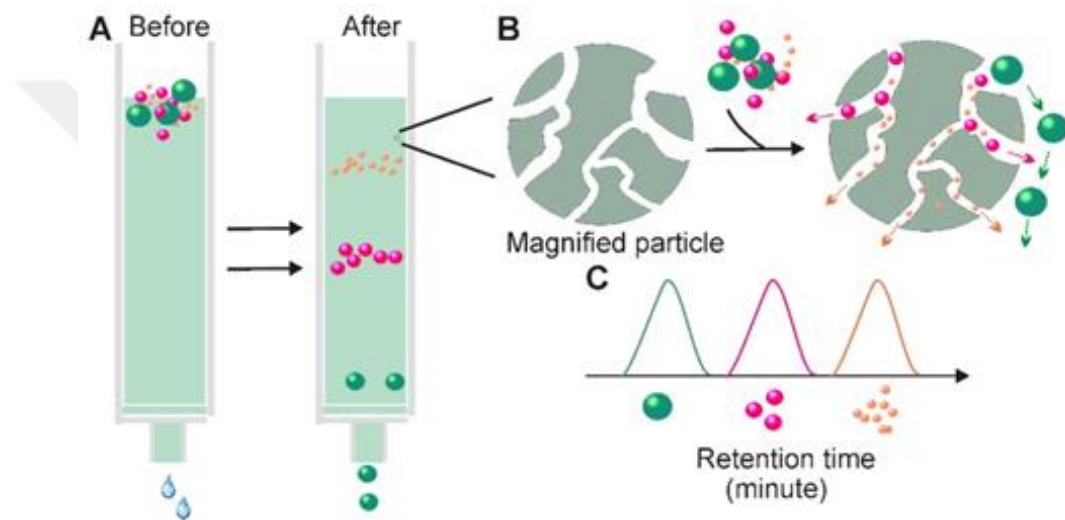


**Figure 1.2:** The chart illustrates the steps of EV isolation by sequential ultracentrifugation from body fluid and/or conditioned medium.

### 1.2.2. Size Exclusion Chromatography (SEC)

SEC is a size-based separation method. For separation in SEC, the resin is contained a permeable matrix of spherical molecules that lack reactivity. The commonly used resin is Sepharose 2B, and the diameter of the pores is about 70 nm [94].

The isolation of EVs by SEC is commonly used with centrifugation after removing the cell debris and the large-sized vesicles, then running the sample to concentrate the EVs sample [10,20,40,41]. If the sample is viscous, the dilution can be considered with normal saline or PBS before SEC [43,48]. There is an optimal ratio between the applied EV-containing sample volume and the column volume [50,51].



**Figure 1.3:** The direction of exosome isolation by size exclusion chromatography. When the sample passes via a stationary phase porous resin particles, EVs can be isolated based on size (A). While particles with radii smaller than the stationary phase penetrate the pores, (B) larger particles cannot enter the pores and push straight around the resin (B) [94].

In the process, there are two phases: a mobile phase is represented by the sample (biofluid), and the stationary phase is a porous gel filtration polymer. The character of the stationary phase permits differential elution: larger particles pass first, followed by smaller vesicles, and last is non-membrane-bound proteins (Figure 1.3).

SEC is the most practical technique that retrieves isolated exosomes' morphology and function integrity and purifies it from protein. SEC has been applied as a thriving method to isolate and purify exosomes from various body fluids, including serum, plasma, synovial fluid, milk, urine, saliva, tears, cerebrospinal fluid, nasal lavage, adipose tissue, and seminal fluid [13,14,15].

### **1.2.3. Immunoprecipitation (immuno-affinity capture)**

The immuno-affinity capture technique isolates specific exosomes based on the expression of their surface proteins. Generally, using antibodies against characteristic surface markers of exosomes, the tetraspanins: CD9, CD63, and CD81 [8,40].

Exosome isolation applying immuno-affinity capture can be conducted by setting the sample with magnetic beads or gold-loaded ferric oxide nanocubes, which are covered with antibodies against the external proteins. The immuno-affinity EV isolation technique utilizes antibodies to target surface markers on EVs to perform higher isolation particularity and purity than other methods. However, releasing the seized EVs is challenging because substantial binding of antibody-antigen or the antibody-substrate is commonly indestructible [52].

## **1.3. CHARACTERISTICS OF EXTRACELLULAR VESICLES**

Cells yield and release a heterogeneous EV content varying in size, chemical and physical characteristics.

### **1.3.1. Chemical characterization**

Biochemical characterization is required to deliver information on EV's composition. EVs have specific markers that distinguish between exosomes and microvesicles.

#### ***1.3.1.1. Membrane proteins***

EV's identity is assessed by detecting the existence of anchored proteins located at the outer membrane such as tetraspanin transmembrane (i.e., CD9, CD63, and CD81), heat shock proteins (HSPs; i.e., HSPA1A, HSPA8, and HSP70) and tumor susceptibility gene 101 (TSG101). These are known as exosomal markers. Microvesicle markers comprise arrestin-domain-containing-1 (ARRDC1), beta-1 integrin, TSG101, vesicle-associated membrane protein3 (VAMP3), and MT1MMP (membrane type1-matrix metalloproteinase) [8,38,40].

### **1.3.2. Physical characterization**

The evaluation of morphology, concentration and size distribution of vesicles can be obtained by direct imaging methods to evaluate the precise size of EVs, such as SEM (scanning electron microscopy), TEM (transmission electron microscopy), and cryo-electron microscopy. Furthermore, Nano Tracking Analysis (NTA) or flow cytometry (FC) methods can be utilized to measure the concentration and a single EV size of EVs in a solution.

### **1.3.2.1. Electron microscopy**

#### **Scanning Electron Microscopy (SEM)**

The electrons from SEM scatter on the sample surface, which creates the image of the sample surface. It has not been used very extensively in EV research.

SEM has various techniques:

1. Conventional SEM uses to illustrate the EVs.
2. Three Dimensional SEM includes two procedures, the slice and view procedure and FIM/B.

#### **Transmission Electron Microscopy (TEM)**

TEM is one of the many accepted methods for visualizing extracellular vesicles, particularly exosomes. Transmission electron microscopes' extensive range differs in the type of lens' system, electron gun, and detector device. The electron sources in TEM are thermionic and field emission guns [67]. TEM creates EVs image that can function in three various methods. The most common technique is to place EVs onto a grid and image them by applying negative staining. This technique is the most straightforward and the most widespread one.

EVs are variable in morphology. Besides, they are recognized as round-shaped, elongated, oval, and complex-shaped particles (Figure 1.4). Nevertheless, TEM, when applying negative staining, most EV images illustrate the deflated balls, which is called the cup-shaped morphology. This morphology is characteristic and highly illustrated; considered occur due to the dehydration and adsorption of the EVs onto rigid surfaces. Operating atomic force microscopy sometimes also scans cup-shaped EVs [62,73].

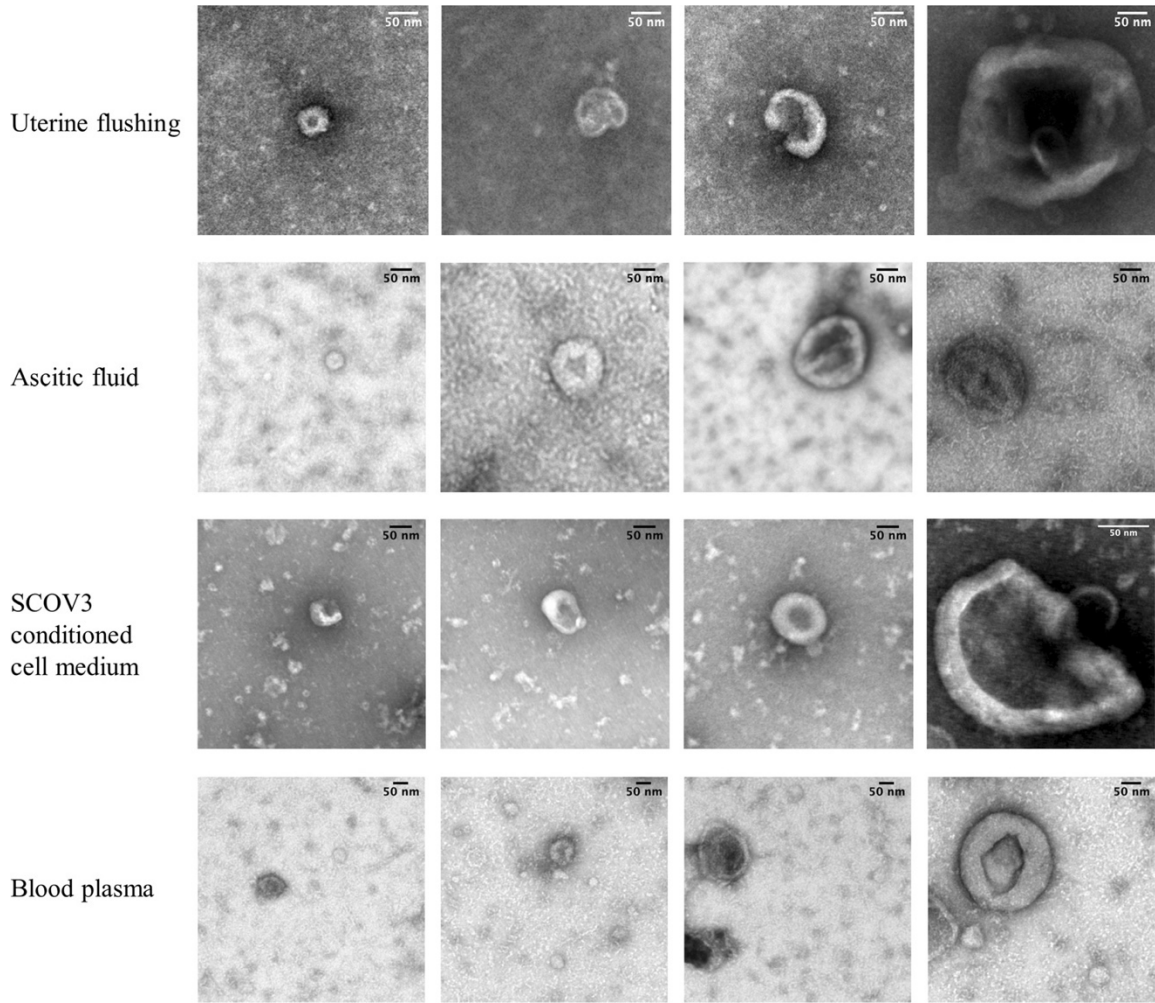
TEM has been operated to detect and characterize a single EV morphology and precise size and is one of many accepted methods for visualizing extracellular vesicles, particularly exosomes. Many different methods can achieve imaging EVs by TEM:

#### **Negative Staining Technique**

The fastest and most popular technique is perpetrated in a vacuum environment. Confirm EV presence quickly; samples require fixation and dehydration. EVs mainly illustrate deflated ball morphology which is called cup-shaped morphology. This morphology is assumed to occur due to the adsorption and staining of the EVs onto the grid surface.

#### **Cryo-Transmission EM**

Cryo-TEM is the most practical approach, and samples are directly spread onto an EM grid and vitrified in amorphous ice then visualized. Thus qualifying for the characterization of EVs close to their native shape, without applying staining, chemical fixative, and a high-pressure frozen thin section. Their size and morphology are well preserved.



**Figure 1.4:** Images illustrate individual cup-shaped EVs of various sources such as, uterine fluids, SCOV3 conditioned cell medium, ascitic fluids, and plasma. Frames were shortened to  $700 \times 700$  pixels [62].

The advantages of cryo-TEM are that the lipid bilayer structure is confined, which allows distinguishing EVs from other types of particles, and image EVs in pure body fluids, so sample processing such as ultracentrifugation is not required, which may cause artifacts. Cryo-TEM equipment is regarded as the most practical approach, whereas it is more costly and uncommon than traditional TEM. All the techniques described here can be formulated by immunogold labeling.

#### **Immunogold labeling-TEM**

This technique detected EV surface protein expression using immunogold labeled antibodies and analyzed with cryo-TEM. It shows the vesicle morphology and proteins can be detected inside and on surface of EVs. There are various immunolabeling-TEM techniques such as immuno-negative stain, immune of plastic cryo-section, and Tokuyasu cryo-section. TEM has been used to detect and characterize a single EV morphology and the precise size. Furthermore,

utilizing immunogold labeling in TEM could provide data about the biochemical properties of EV surface proteins.

#### **1.3.2.2. Nano tracking analysis**

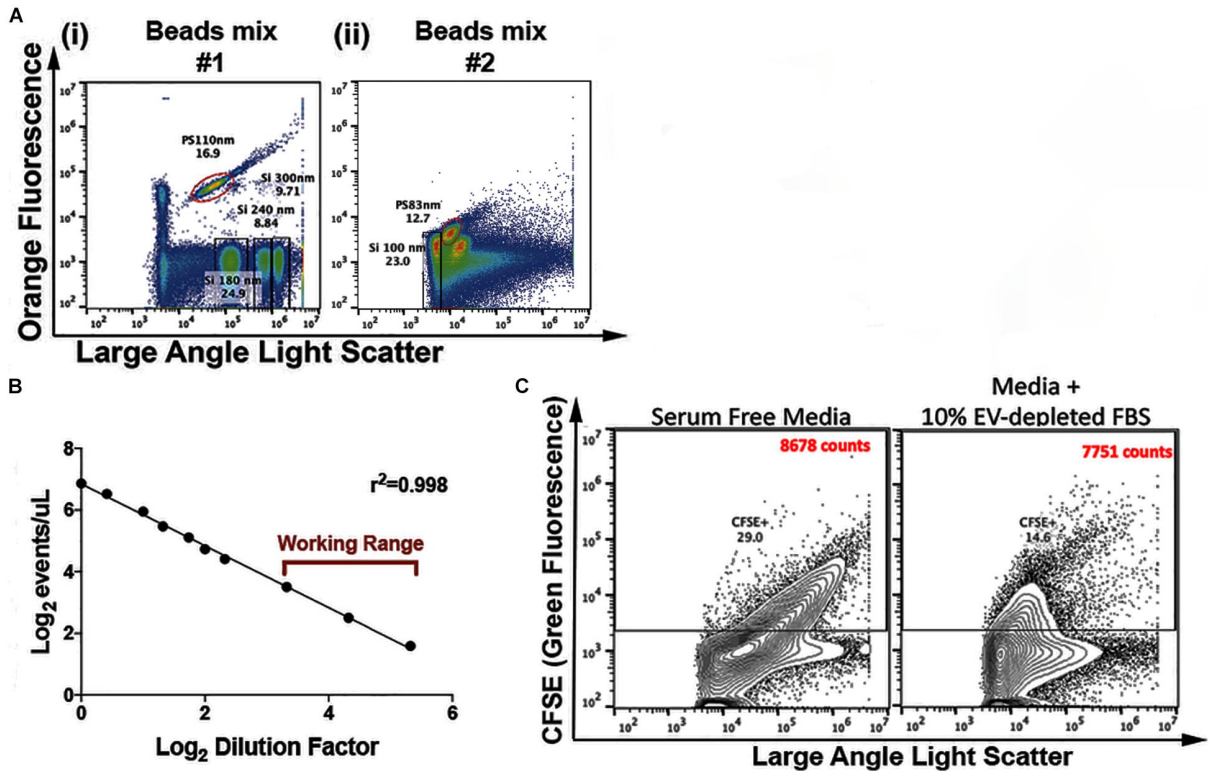
Nano-tracking analysis (NTA) based on the principles of light distribution qualifies the tracking of the Brownian signal of each particle within the EV sample. NTA is essential for assessing the precise concentration (particles per milliliter), and size distribution of a single EV (10 nm to 2  $\mu\text{m}$ ) [24,68].

#### **1.3.2.3. High-resolution flow cytometry (FCM)**

Protein expression analysis of the EVs' surface can define protein quantity and type. It can be accomplished by labeling them with fluorescent reporters and specific antibodies (Figure 1.5).

FCM is a precise and generally used technique in clinics with high results and the prospect of simultaneous observance of various external antigens; FCM accurate EV detection has many challenges [53]. For example, EVs' size distribution is mainly heterogeneous, with the majority diameter smaller than 100 nm; the surface area is antigen/ proteins expression of these small EVs is minimal. Therefore, labeling EV surface proteins with fluorescent markers/ antibodies yields fluorescent with weak signals. The conventional FCM is generally inadequate for detecting and examining small EVs' fluorescence weaknesses. FCMT characterizes the size and counting of the EVs by light distribution and fluorescence. The EVs' stream moves over a laser beam; the interchange of the laser beam and EVs result in the light distribution in all movements. FCM is generally combined to assemble the scattered light in two directions, forming a forward scatter (FSC) signal. In contrast, the scattered light is assembled in the vertical direction and manifested as a side scatter (SSC) signal.

Flow cytometry (IFCM)-based single EV analysis method using CD63-enhanced green fluorescent protein (eGFP)-tagged EVs as biological reference material. Recently, IFCM has been demonstrated as a sensitive and robust method for examining fluorescently labeled small EVs (50-150 nm) in human plasma samples and cell culture samples. Also, different parameters were evaluated to optimize this procedure and demonstrated that small EV subpopulations stained with distinct fluorescent antibodies could be analyzed in a multiparametric mode. However, different parameters influencing EV staining procedures demand further evaluation, optimization, and standardization. Tertel et al. [83] have presented a straightforward method to estimate the influence of the incubation temperature. This approach can be an example of application parameters in evaluating EV by flow cytometry.



**Figure 1.5:** EVs' flow cytometry parameter. (A) Flow cytometry analysis of a mix of polystyrene (PS) and silica Beads (Si), (B) Events/ $\mu$ L concerning the dilution factor of sample, (C) Representative properties of serum-free CFSE-labeled and EV-depleted FBS (10%) medium; the shown totals (CFSE+) conform to the possibilities within the CFSE+ gate [53].

### 1.3.3. Molecular characterization

Most cells release EVs which harbor miscellaneous cargoes such as lipids, RNAs, DNAs and proteins. That can be means of intercellular communication at paracrine and systemic levels transported and exchanged between cells [92].

#### 1.3.3.1. Lipidomics

Lipidomics is the characterization and quantitation of lipid species in EVs samples. Approximately 2000 kinds of lipids were determined from secreted EVs by various cell origins [35,38].

EVs can transmit lipids that regulate multiple pathologic functions such as atherogenesis, inflammation and tumor development. Furthermore, EV lipidomic biomarkers may be utilized for disease diagnostics, prognosis, and therapy [63].

#### 1.3.3.2. Proteomics

EVs from various sources comprise protein content analyzed by SDS-PAGE, marked by immunoblotting, protein staining, and/or proteomic analysis [69]. Proteomics has determined roughly 3500 categories of proteins obtained from EVs released by separated cells [19]. EV

proteins are derived from endosomes, plasma membranes, and cytosol depending on biogenesis and types of cells [8,22]. Certain proteins can differ between EV categories and subtypes and mediate cellular contact [40, 54].

Mass spectrometry and liquid chromatography techniques can routinely determine thousands of proteins in hours [79]. The PTM (post-translational modifications) and proteomic profiling can be accomplished by recent techniques such as data searching algorithms, high-resolution liquid chromatography, and mass spectrometry [9,20,29,38].

### **1.3.3.3. Sequencing**

The RNAs named microRNAs (miRNAs), which are EV-associated small non-coding regulatory and other non-coding RNAs, have function as intercellular messengers. miRNAs can be categorized inside the vesicle or released outside the cell, as reported in the studies about the mesenchymal stromal cells in equine and human experimentations [17,77].

Exosomes are a steady source of miRNA in biofluids. Despite there are many techniques for the extraction of exosomes and miRNA analyzes, their usefulness for diagnostics in clinical applications is debatable. Moreover, exosomal miRNAs can modify the behavior of the tumor and stromal cells to boost oncogenesis, which shows their important influence on cancer cell communication [86].

## **1.4. APPLICATIONS OF EXTRACELLULAR VESICLES ON DIAGNOSIS AND THERAPY**

### **1.4.1. EV-Based Laboratory Developed Test (LDT)**

Development of an EV-based LDT is manufactured, and only used in CLIA-approved laboratory (Clinical Laboratory Improvement Amendments). EVs have provided a tremendous possibility to discover disease-specific markers which can be used for disease diagnosis, observing of disease progression, and predicting the prognosis.

Accelerating the translation of EV-based biomarkers will demand researchers to use vital methodologies to overcome the limitations, thus establishing a gold standard for the isolation and analysis of EVs and identifying clinically applicable EV-based markers for disease diagnosing and monitoring [95].

The therapeutic usage of EVs (exosomes, microvesicles) is a growing interest. Since there need to be high standards for therapeutics, mainly in stem cell-related approaches; the evolution of EV-based techniques has emphasized standardization at the preclinical and clinical levels [70].

EVs have crucial information cargoes in physiological and pathological circumstances [80,90]. The physiological status of spreading EVs in the whole body (serum, tissues, or other biological fluids) varies regarding the animal model, duration of the disease, and the EV analysis methods. This information could assist in identifying the potential therapeutic dose [22,88].

In the genital tract, EV-mediated transmission between cells and embryo-maternal communication is vital in pathologic and physiologic events [55,56]. The molecular cargoes of EVs create changes in the pathological cases. And these situations influence the conservation and prognosis of obstetrics-related and reproductive pathologies (Table 1.1).

Recently, many studies were carried out about the physiological functions of exosomes exponentially, while the relationship between exosomes and reproduction has yet to be understood entirely.

Recent reproductive studies pointed out that exosomes play an essential role in reproductive mechanisms, especially in oocyte development [44]. Table 1.1 demonstrates the studies that utilize EVs' molecular cargoes as biomarkers in reproduction which are categorized by various pathological circumstances illustrating the origin and technique of EV isolation and the principal of obtained effects [7,31,39,55,56,57].

According to the present research, the function of the EVs' cargoes (RNA and proteins) have been confirmed in embryo implantation. However, the effects of lipids have been disregarded in the endometrial EVs. In sheep, studies have pointed out that lipids in EVs provide the conceptus elongation in vivo [64].

In the liver, cargoes of EVs (hepatocyte-derived) contain hormones, albumin, various enzymes, xenobiotics and nutrients. The hepatocytes are also produced the EVs containing a many kinds of RNAs that can manage the other hepatic cells' functions, as well as hepatic SCs. EVs released from hepatic SCs are seized by neighbor Kupffer cells and LSECs, generating glycolysis [46].

The TIMPless fibroblast cells secrete exosomes that carry the protease ADAM10, improving cancer cell motility and inducing the transformation of stromal fibroblasts into more invasive CAFs cells [21].

**Table 1.1:** EVs role in different physiological and pathological conditions.

EVs	Sample type	Species	Isolation Method	Main Results	Ref.
OC-EVs	OEC conditioned media	Bovine	Ultracentrifugation	Functions in fertilization and early embryonic phase	[1]
Breast cancer-derived	Conditioned media	Human	Ultracentrifugation	Contribute to cancer progression	[63]
OC-EVs	Oviductal cell-derived	Canine	Centrifugation + Exosome Isolation Reagent	Role on oocyte maturation	[45]
Embryonic EVs	Media of embryo cultures	Bovine	Centrifugation + SEC	Gamete maturation and embryo implantation	[3]
Hepatocyte-derived EVs	Liver tissue culture Serum	-	Ultracentrifugation	Regulate functions in other hepatic cells, including HSCs	[46]
Uterine luminal fluid derived EVs	Endometrial epithelia	Sheep	Ultracentrifugation	Elongate conceptus during early pregnancy.	[64]
Human prostasomes	Seminal plasma	Human male	Ultracentrifugation	Modulate sperm capacitation	[18]
oEVs	Oviductal fluid and oviductal culture media	Bovine	Sequential centrifugation	Quality of the embryos	[49]
Endothelial cell (EC)-derived EVs	Serum	Human	Commercial kits	Support in improving of different cardiovascular diseases	[34]
Epididimosome	Normozoospermic seminal fluid	Human	-	Enhancing motility and triggering the capacitation of sperm	[28]

### 1.4.2. Extracellular Vesicles in Animal Reproduction

Cell communication is essential for many molecular pathways. These pathways allow cells to exchange information with each other in the whole organism [59,61].

Maternal relationships between gametes and embryos are assumed as the foundation of reproductive success. The signals of interaction between maternal organs and gametes which are also affecting the destiny of embryos, may be divided into two types: long-range and short-range signals. Long-range signals contain sex hormones, and they have function in the gamete maturation, transport, fertilization, and early embryonic growth. However, short-range signals are located in the surroundings of gametes and embryos within female reproductive organs [27].

Extracellular vesicles play an essential role in reproductive processes and mediate cross-talk interaction in female reproductive organs with gametes and embryos. In the female reproductive tract, EVs are released by the oviductal, follicular, embryonal, placental and endometrial cells. Germ cells (oocytes) and follicular cells (cumulus, granulosa, and theca) release the follicular fluid EVs (ffEVs), which govern oogenesis, follicular growth, and oocyte maturation. ffEVs were first observed and illustrated in the human, horse and bovine investigations [18,29,77].

In male reproduction, EVs play an essential role in gamete function. Epididymosomes are vital for the maturation of spermatozoa. However, the function of the other EVs such as prostasomes are unclear in the acrosome reaction [28]. In mice, cattle, and cats, capacitation and sperm motility are regulated by oviductal EV secretion (oEV) [2].

In avian species, uterine fluid EVs play a vital function in conserving sperm function [73]. The presence of EVs in the uterine fluid was shown in the uterine fluid in sheep. Furthermore, the fetus can interact via secreting EVs with endothelial cells [4,6,39].

Placental EVs characterize maternal-fetal relations during pregnancy. During pregnancy, placental-originated extracellular vesicles [exosomes, large EVs (microvesicles and apoptotic bodies)] are distributed to the maternal and fetal circulation in humans. Placental EVs interact with maternal tissues during pregnancy and regulate many functions of the mother such as immune response and vascular reactivity, as well as passing into the fetal circulation and affecting the development of the fetus [60].

The placental EVs maintain proteins affected in immunomodulation, which is critical for a successful pregnancy [23,43,87].

### 1.5. SHEEP OVIDUCT EPITHELIAL CELLS (SOEC)

The physiological environment of the oviduct has been under investigation for many decades. The oviduct provides an optimal environment regarding nutrients, osmotic pressure, pH, oxygen, and temperature to newly fertilized zygotes and early-developing embryos. The oviduct is also presumed to be a source of signals, molecules, and extracellular vesicles to regulate and communicate with gametes, and zygotes throughout ovulation, fertilization, and early embryo development [31,39].

The impacts of the oviduct on embryo growth were *in vitro* investigated by using co-culture of oviductal epithelial cells which positively impacted embryo development. Many studies were established to understand the oviduct epithelial cells' secretion and communication with gametes and embryos [49].

The co-culture studies of the oviduct epithelial cells showed the function was not at the same level of development in the *in vivo* environment. For example, it has long been realized that *in vitro* produced embryos of domestic species take longer to grow to blastocysts and contain rarer cells than their *in vivo* embryos [37,47].

One of the oviductal fluid's elements is extracellular vesicles, which have been suggested as crucial modulators of remarkable cross-talk. Few studies identified their content and their function in oviduct-embryo communication. In many species (cats, dogs, pigs, cows and mice), oviduct-derived EVs are integrated with the oocytes, sperm, and embryos [49,75].

Sheep oviductal epithelial cells (SOEC) secrete various elements to maintain reproductive procedures [23]. Oviduct epithelial-derived EVs have been displayed to enhance fertilization and embryo growth and similarly reduce the occurrence of polyspermy. OEC-EVs carry molecular cargoes (lipids, proteins, miRNAs, mRNAs), which may be transferred to recipient cells.

The oviduct-derived extracellular vesicles have been investigated in different species (humans, rodents, reptiles, domestic and farm animals). However, many breakdowns on characterization of oEVs have been conducted in pigs and cows.

Since the current knowledge on the effects of oviduct epithelial cell secretion *in vivo* and *in vitro* (OEC) is slight; we aimed to investigate the impact of sheep oviduct epithelial cell (SOEC) on embryo development and its quality.

SOEC was isolated from sheep oviduct epithelial cells and cultured *in vitro* until it reached the 90% confluence; then, the conditioned medium was discarded, and the cell culture medium was supplemented with 10% FCS depleted for EVs to ensure that SOECs secreted the

EVs. Proposed the SOEC- derived EVs transmit molecules and components that may regulate embryo growth.



## **2. CONCEPTUAL FRAMEWORK**

The conceptual framework of this thesis project is built on emerging EVs' literature and provides new ways to use EVs in the reproductive area.

To achieve these goals, this project aimed to (1) isolate and characterize EVs from a culture medium conditioned by the sheep oviductal epithelial cells and (2) verify if these EVs are functionally active.

The specific scientific purposes of the recent thesis can be explained as follows:

1. To obtain a monolayer cell culture of sheep oviductal epithelial cells (SOEC) in order to collect conditioned media.
2. To isolate extracellular vesicles from the conditioned media by sequential ultracentrifugation technique.
3. To quantify and characterize the extracellular vesicles isolated from conditioned media.
4. To supplement the SOEC-derived-EVs into the embryo culture medium and investigate their effects on embryo development and quality in vitro.

### 3. METHOD

#### 3.1. MATERIALS

##### 3.1.1. Biological

- Slaughterhouse materials: Whole reproductive tract including ovaries, oviducts, and uterine horns.
- Ram semen samples

##### 3.1.2. Consumables and equipments

###### 3.1.2.1. *Chemical consumables*

- NaCl
- KCl
- NaH<sub>2</sub>PO<sub>4</sub>
- HEPES Sodium
- HEPES
- NaHCO<sub>3</sub>
- D-Glucose
- Sodium Pyruvate
- L-Glutamine
- CaCl<sub>2</sub>.2H<sub>2</sub>O
- MgCl<sub>2</sub>.6H<sub>2</sub>O
- Penicillin-G
- Streptomycin Sulfate
- Kanamycin Sulfate
- Phenol Red
- BSA Fraction V
- Sodium Lactate
- Hyaluronidase
- M-199
- FBS

- ETS (Embryo Tested Water)

### **3.1.2.2. *Plastic and Glass Consumables and Equipments***

- Petri dishes
- Flasks
- 35 cm, 60 cm Petri dishes for cell culture
- Four well dishes
- Centrifuge tubes 15, 50 mL
- Ultracentrifuge tubes
- Automatic pipettes& tips
- Graduated cylinder
- Sterile syringes
- Small animal surgical sets
- Slides and coverslips
- Scalpels
- Surgical gloves
- Sterile gauges
- 0.22  $\mu\text{m}$  pore size hydrophilic membranes
- Magnetic Sensor Analytical Balance
- Thermometer
- Beakers
- Ultra-micro lab balance
- Converted microscope
- Stereomicroscope
- Phase-contrast microscope
- Fluorescent microscope
- CO<sub>2</sub> incubators
- Bench-top centrifuge
- Ultracentrifuge
- Laminar flow hood
- Autoclave
- PH meter
- Osmometer

- Refrigerator
- Freezer -20°C
- Freezer -80°C
- Washing Machine
- Nano Tracking Analysis Device

### 3.1.2.3. *Media and Reagents*

Most media and reagents were prepared in the laboratory before the day of tissue culture. All stocks of tissue culture media and chemicals utilized were dissolved in distilled water, stirred, and adjusted their pH between 7.2 to 7.4. Osmolarity was 270-275 mOsm afterward, sterilized by membrane filtration with a diameter of 0.22  $\mu\text{m}$ , and stored at 4°C in the dark to suppress the growth of opportunistic microorganisms. Before utilizing the media were warmed to a suitable temperature for cell maturation.

#### **Tissue culture medium (DMEM)**

<b>Chemical Substances</b>	<b>Amount</b>
DMEM	95 mg
NaHCO <sub>3</sub>	22 mg
Sodium pyruvate	1 mg
BSA	150 mg
Penicillin -G	1000 IU
Stereptomycin sulphate	100 $\mu\text{g}$
MQ water	10 mL

10 mL of millipore water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled to the final volume, then filtered by membrane filtration with a diameter of 0.22  $\mu\text{m}$  and adjusted the pH 7.3-7.4 and 270-275 mOsm, and kept at 4°C. Antibiotics represent 1% of the total volume. Stored at 4°C.

#### **Physiological saline (Sodium chloride)**

<b>Chemical Substances</b>	<b>Amount</b>
Sodium chloride	9 g
Millipore water	1 L
Penicillin	100 $\mu\text{g}\text{mL}$
Streptomycin	100 $\mu\text{g}\text{mL}$

800mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final, filtered and adjusted the pH 7.4 and 270-275 mOsm, and kept at 4°C.

**Red blood cells lysis solution**

Ammonium chloride lyse (10X concentration)

Chemical Substances	Amount
NH <sub>4</sub> Cl	8.02 g
EDTA (disodium)	0.37 g
NaHCO <sub>3</sub>	0.84 g
Millipore water	100 mL

80 mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final 100 mL volume, filtered and adjusted book and mOsm, and kept at 4°C.

**Working solution**

Dilute 10 mL 10X concentrate with 90 mL Millipore water stored at 4°C until use.

**Phosphate-Buffered Saline (PBS)**

Chemical Substances	Amount
Na <sub>2</sub> HPO <sub>4</sub>	2.16 g
KCl	200 mg
KH <sub>2</sub> PO <sub>4</sub>	200 mg
NaCl	8 g
Penicillin	300 mg
Streptomycin	250 mg
Neomycin	250 mg
Distilled water	1 L

1000 mL of distilled water was prepared in a suitable beaker; first, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, filtered and adjusted the pH and Osm, and kept at 4°C.

**Oocyte washing media (OWM)**

Chemical Substances	Amount
TCM199	4950 mg
NaHCO <sub>3</sub>	168 mg
Heparin	5 mg
HEPES	1260 mg
Sodium HEPES	1365 mg
Kanamycin	38 mg
Penicillin	37.5 mg
Streptomycin	25 mg
BSA fraction V	1500 mg
Millipore water	500 mL

400mL of distilled water was prepared in a suitable beaker; first, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then

fulfilled the volume to the final volume, filtered 0.22  $\mu\text{m}$  and adjusted the pH to 7.2-7.4 and 273-290 mOsm/kg, and kept at 4°C.

#### Oocyte maturation media (OMM)

Chemical Substances	Amount
TCM-199 stok	9 mL
FBS	1 mL
Na piruvat	1,1 mg
L-glutamine	3.2 mg
Östradiol	10 $\mu\text{g}$
FSH	1 IU
LH	1 IU

Was filtered 0.22  $\mu\text{m}$  and adjusted the pH to 7.2-7.4 and 273-290 mOsm/kg, and kept at 4°C.

#### In vitro fertilization media (BSOF)

Chemical Substances	Amount
NaCl	314.7 mg
KCl	26.70 mg
NaH <sub>2</sub> PO <sub>4</sub>	2.7 mg
NaHCO <sub>3</sub>	105 mg
CaCl <sub>2</sub> .2H <sub>2</sub> O	500 $\mu\text{l}$
MgCl <sub>2</sub> .6H <sub>2</sub> O	500 $\mu\text{L}$
Na lactate	70.5 $\mu\text{L}$
Na pyruvate	5.5 mL
L-glutamine	7.3 mg
Penicillin	3.75 mg
Streptomycin	2.5 mg
Phenol red	0,1 mg
ETS water	50 mL

40 mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, 0.22  $\mu\text{m}$  filtered, and adjusted the pH 7.2-7.4 and 273-290 mOsm/kg, and kept at 4°C.

#### Synthetic Oviductal Fluid (SOF)

Chemical Substances	Amount
NaCl	314.7 mg
KCl	26.70 mg
NaH <sub>2</sub> PO <sub>4</sub>	2.70 mg
NaHCO <sub>3</sub>	105 mg
CaCl <sub>2</sub> .2H <sub>2</sub> O	500 $\mu\text{L}$
MgCl <sub>2</sub> .6H <sub>2</sub> O	500 $\mu\text{l}$
Na lactate	23.50 $\mu\text{L}$
Na pyruvate	5 mg
Glutamine	5 mg

Glucose	12.96 mg
Penicillin	3.75 mg
Streptomycin	2.5 mg
BSA fatty acid free	400 mg
MEM nonessential aa	500 $\mu$ L
MEM Amino Acid	1 $\mu$ L
Millipore water	50 mL

40 mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, filtered and adjusted the pH and Osm, and kept at 4°C.

### HSOF

Chemical Substances	Amount
NaCl	629.4 mg
KCl	53.4 mg
NaHCO <sub>3</sub>	33.6 mg
NaH <sub>2</sub> PO <sub>4</sub>	5.4 mg
Na HEPES	274 mg
HEPES	252 mg
CaCl <sub>2</sub> .2H <sub>2</sub> O	40.32 mg
MgCl <sub>2</sub> .6H <sub>2</sub> O	10 mg
Na Lactate	47 $\mu$ L
Glucose	43.2 mg
Na pyruvate	11 mg
L-glutamine	14.6 mg
Penicillin	7.5 mg
Streptomycin	5 mg
BSA fraction V	400 mg
Phenol red	0.1 mg
MEM non esensiyal	1 mL %1
ETS	100 mL

### CaCl<sub>2</sub>.2H<sub>2</sub>O Stock

Chemical Substances	Amount
Distilled water	50 mL
CaCl <sub>2</sub> .2H <sub>2</sub> O	1260 mg

40mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, filtered and adjusted the pH and Osm, and kept at 4°C.

**MgCl<sub>2</sub>.6H<sub>2</sub>O Stock**

Chemical Substances	Amount
Distilled water	50 mL
MgCl <sub>2</sub> .6H <sub>2</sub> O	500 mg

40 mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, filtered and adjusted the pH and Osm, and kept at 4°C.

**Phenol Red Stock**

Chemical Substances	Amount
Distilled water	50 mL
Phenol red	10 mg

40 mL of distilled water was prepared in a suitable beaker; First, powdered substances were added, then the stock solutions. Stirred the mixture well until components had gone, then fulfilled the volume to the final volume, filtered and adjusted the pH and Osm, and kept at 4°C.

**Percoll-SOF**

Chemical Substances	Amount
Percoll	10 mL
Na Hepes	27.4mg
Hepes	25.2 mg
NaCl	62.94 mg
KCl	5.34 mg
NaH <sub>2</sub> PO <sub>4</sub>	0.54 mg
CaCl <sub>2</sub> .2H <sub>2</sub> O	2.52 mg (100 µL stock)
MgCl <sub>2</sub> .6H <sub>2</sub> O	1 mg (100 µL stock)

Final medium must not be filtered.

**Extracellular vesicles-depleted FCS**

FCS is always used to supplement culture medium in cell biology experimentations, contended to supply some necessary factors. Significantly, FCS despite being filtered contains many EVs, exosomes. In this study, for depletion, FCS (F7524; Sigma Aldrich) was subjected to centrifugation (Beckman Coulter; 18 h) at 100.000 g at 4°C. Supernatant was then collected and stored at -20°C until used for culture media supplements [77].

## 3.2. OBTAINING SHEEP OVIDUCTAL EPITHELIAL CELL CULTURE

### 3.2.1. Sample collection and preparation

The oviducts with the ovaries attached were collected from the local slaughterhouse (Hadımköy Slaughterhouse-İstanbul) and transferred to the laboratory at a thermos containing warmed physiological saline (0.9% sterile sodium chloride solution at 30°C). The ovaries that demonstrated dominant or Graafian follicle and the absence of an active corpus luteum were picked as the follicular phase. Firstly, the organs were washed three times in prewarmed sodium chloride physiologic saline solution, then moved to a beaker and soaked in warmed saline. The next step trimmed all the tissues and ligaments around the oviduct (Figure 3.1).



**Table 3.1:** Trimming all the tissues and ligaments around oviducts.

The oviductal epithelial cells were perpetrated from the oviduct by two techniques.

#### 3.2.1.1. *Scraping technique*

After being washed the whole organs in sodium chloride buffer solution three times, the organs were moved to a beaker, soaked in warmed saline, and freed from the oviduct from the connective tissue thoroughly. Subsequently, oviducts were dissected longitudinally. The oviductal inner faces were scraped using a clean glass slide, and the cells were collected from the scraped materials with a tissue culture medium in a tube. The cell suspensions were centrifuged (300 g for 5 minutes), and the supernatant was discarded. Then 1mL of a red blood cell lysis buffer was added to the pellet for a minute to lyse the red blood cells. Afterward, the

pellet was resuspended with a culture medium, and centrifuged at 300 g for 10 min. After washing the epithelial cells three times with a fresh culture medium, epithelial cells were distributed into suitable (65 mm) tissue culture dishes in 5 ml of culture medium (DMEM). The plates were incubated at 38.5°C and 5% CO<sub>2</sub> in an atmosphere. The culture medium was replaced every 48 hours until the confluency reached 90%. After confluence, the media were discarded, replaced by 10% depleted FCS, and incubated for 24 hours more. After the incubation, the conditioned media were collected [71].

#### **3.2.1.2. Flushing technique**

The oviductal lumen was flushed with 1 mL of DMEM supplemented with FCS (10%); healthy clumps of cells were collected and seeded in a culture medium supplemented with FCS (10%). The plates were incubated at 38.5°C and 5% CO<sub>2</sub> in an atmosphere. Every 48 hours, the medium was replaced until the cells reached 90% confluency. After confluence, the media were discarded, replaced by 10% depleted FCS, and incubated for 24 hours more. After the incubation, the conditioned media were collected [71].

### **3.3. ISOLATION OF SOEC-DERIVED EXTRACELLULAR VESICLES**

The collected conditioned media were centrifuged at 300 g for 10 minutes to remove the debris and dead cells. Then the supernatant was centrifuged three times for 2000 g/20 minutes; 10.000 g/30 minutes, and 20.000 g/40 minutes by discarding the pellet at the end of each step. Then the last centrifuge was carried out at 100.000 g for 60 minutes, the supernatant was discarded and the tube was washed with 1 mL phosphate buffer saline [58,78,91]. All the centrifugation steps were conducted at 4°C.

### **3.4. CHARACTERIZATION OF SOEC-DERIVED EXTRACELLULAR VESICLES**

#### **3.4.1. Nano tracking analysis**

Nano tracking analysis (NTA Version: NTA 3.4 Build 3.4.4) was performed to characterize the EV's size and evaluate EV particle concentration per milliliter [5,24,68].

NTA (NS500 model, Nanosight Technology, Malvern, UK) was used to determine the concentration and size distribution of EVs. The configuration of the camera level and detection threshold were 14 and 8 respectively. The suspensions were diluted ten times to obtain  $120.5 \pm 2.4$  particles/frame. During the analyses, the video records were kept on for 30 sec and replicated three times for each sample. The records were analyzed by NTA 3.1 software.

### **3.5. IN VITRO EMBRYO PRODUCTION**

#### **3.5.1. Oocyte Collection and Maturation**

Sheep ovaries were collected within two hours after the sacrifice from the local slaughterhouse (Hadimkoy slaughterhouse) and transferred to the laboratory in 0.9% sterile NaCl solution (30°C). The ovaries were washed twice in warm physiological saline.

The follicles with a diameter of 2-6 mm were sliced to collect the cumulus-oocyte complexes (COCs). Optimal growth of the cumulus mass is crucial for cytoplasmic maturation. The process of cumulus expansion co-occurs with modifications of gap junctions that occur; there is an absolute requirement for a soluble oocyte-secreted factor [1]. Only the oocytes with uniformly granulated cytoplasm and surrounded by compact cumulus cell layers (at least three layers) [26,30,71] were selected and cultured in groups of 40-50 COCs in 500 µL OMM. They were incubated in 5% CO<sub>2</sub> in the air for 24 h at 38.5°C [11,12, 25,42,65,66]. After maturation, oocytes are presumed to have already reached metaphase II (MII) [96].

#### **3.5.2. *In vitro* Fertilization**

Semen samples were freshly collected from two healthy rams, pooled and the motility was evaluated. The Percoll gradient technique was used to select healthy and motile spermatozoa. 200 mL of semen sample was put onto the top of the Percoll layers (on the top, 45 and 90%, respectively), diluted with BSOF, and centrifuged at 600 g for 5 minutes. The sperm pellet was then washed in an IVF-BSOF medium. The final concentration of spermatozoa was 1x10<sup>6</sup> motile sperm per 1 mL of BSOF. Gametes were incubated for 18–20 h in 500 mL fertilization media in 5% CO<sub>2</sub> in the air at 38.5°C [11,12,14,30,65,66,76,87].

#### **3.5.3. *In vitro* Culture of Embryos**

Presumptive zygotes were cultured in SOFaa media supplemented with oEVs at different concentrations of 9.8x10<sup>6</sup>EVs\50µL, 49x10<sup>6</sup>EVs\50µL, and 98x10<sup>6</sup>EVs\50µL. The embryos were incubated in the droplets under mineral oil at 38.5°C in a humidified 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> gas mixture. The cleavage (Day 2) and the blastocyst development (Day 8) rates were evaluated.

### **3.5.4. Assessment of embryo quality**

#### **3.5.4.1. *Differential Staining***

Blastocysts on Day 8 were used to evaluate the embryo quality by the differential cell count of intercell mass and trophectoderm cells. The inner cell mass (red) cells were stained with propidium iodide, and trophectoderm (TE) cells with Hoechst (blue).

Blastocysts obtained from four different groups on the 8th Day of in vitro culture, a control, low EV, medium EV, and high EV groups, were incubated at room temperature for 7 s in SOF-aa containing Triton X-100 (1%) and propidium iodide (100 mg/mL), then transferred in pure ethanol with Hoechst 33258 (25 mg/mL), overnight at 4°C. The embryos were mounted with glycerol on slides and observed under a fluorescent microscope by using G2A and UV filters. For each blastocyst, the cell number was counted by using an ImageJ Mac OS X software program.

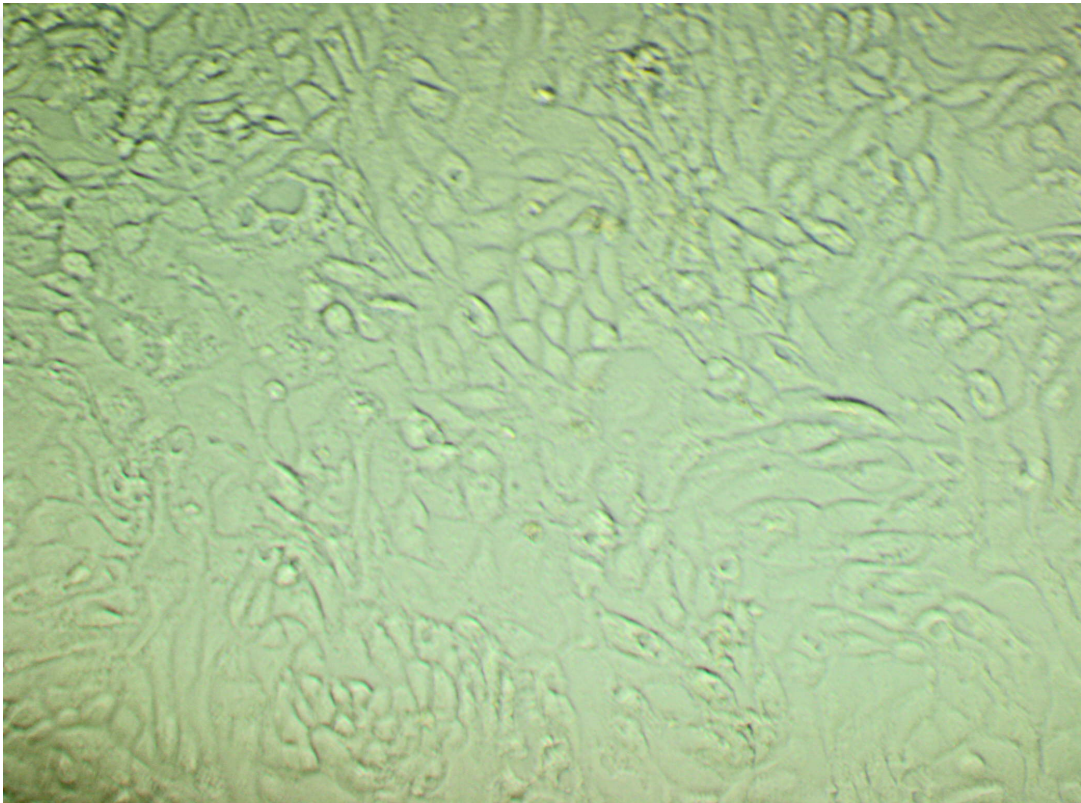
### **3.6. STATISTICAL ANALYSIS**

Statistical analysis for embryo development rates (cleavage rate and blastocyst rate) were performed by chi-square analysis using IBM SPSS Statistics 21. The embryo total cell numbers were evaluated by one-way ANOVA technique with post-hoc analysis (Duncon test). The embryo total cell numbers were presented as means  $\pm$  SEM.  $P < 0.05$  was considered statistically important.

## 4. RESULTS

### 4.1. OVIDUCTAL EPITHELIAL CELL CULTURE

SOECs scraping technique reached confluence faster than the SOECs flushing technique; the average time to get 90–100% confluence (Figure 4.1) was 4 days and 7 days, respectively. EVs were obtained after serial centrifugations for two techniques each and kept at -20°C before characterization.



**Figure 4.1:** SOEC culture confluency (90%).

### 4.2. CHARACTERIZATION OF SOEC-DERIVED-EVS

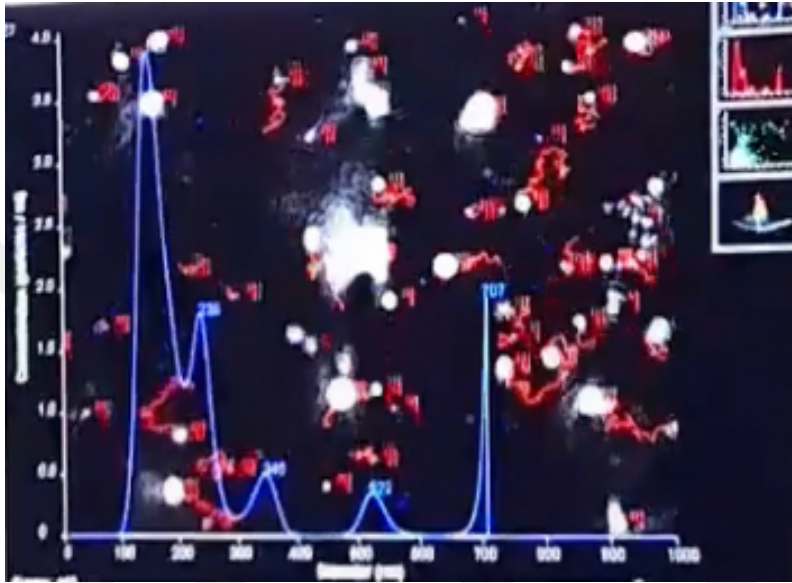
#### 4.2.1. Nano Tracking Analysis

Nano tracking analysis (NTA Version: NTA 3.4 Build 3.4.4) was performed to characterize the EVs' size and evaluate EV particle concentration per milliliter.

The Brownian motion of each nanoparticle and the scattered light were documented while analyzing the sheep oviductal epithelial-derived EVs (Figure 4.2).

The mean size of a single diameter and concentration of SOEC-EVs (obtained by flushing method) measured as 200.0 nm,  $3.46 \times 10^9$  particles/mL (Figure 4.3).

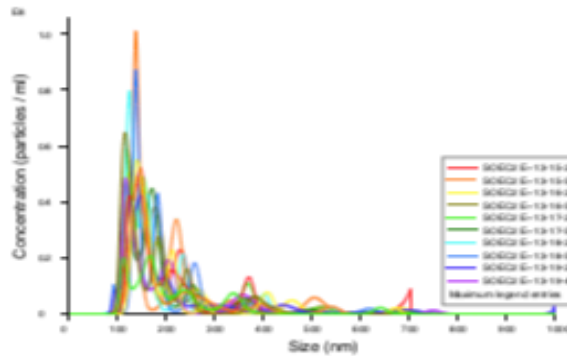
Similarly, in SOEC-EVs (obtained by scraping method), the concentration was measured as  $9.8 \times 10^9$  particles/mL, and the mean size of a single diameter was 184.0 nm (Figure 4.4).



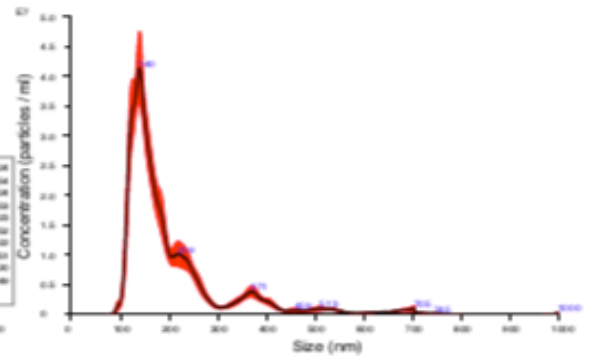
**Figure 4.2:** Demonstrates the Brownian motion of each nanoparticle and the scattered light of the SOEC sample.

# NANOSIGHT

## SOEC2 EVs 2021-11-18 13-15-15



FTLA Concentration / Size graph for Experiment:  
SOEC2 EVs 2021-11-18 13-15-15



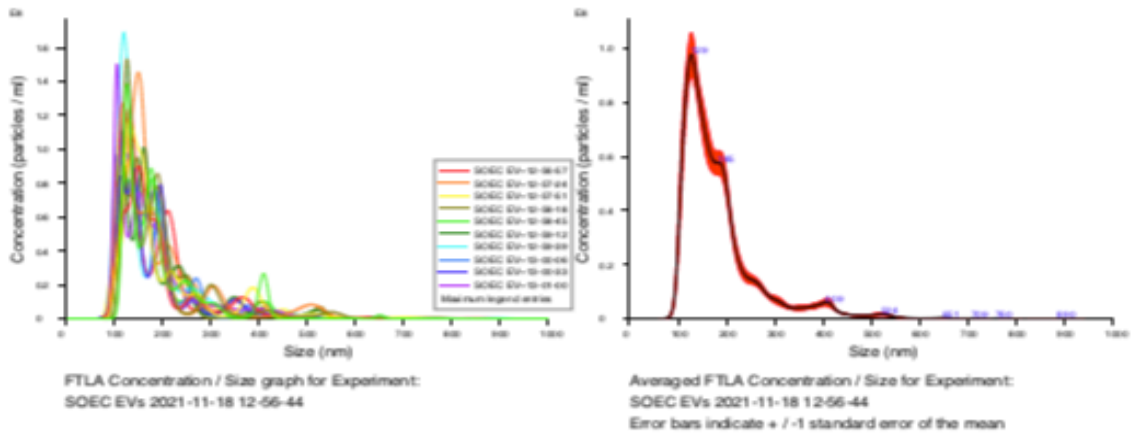
Averaged FTLA Concentration / Size for Experiment:  
SOEC2 EVs 2021-11-18 13-15-15  
Error bars indicate +/- 1 standard error of the mean

Included Files		Results	
SOEC2 EVs 2021-11-18 13-15-24, SOEC2 EVs 2021-11-18 13-15-54, SOEC2 EVs 2021-11-18 13-16-24, SOEC2 EVs 2021-11-18 13-16-53, SOEC2 EVs 2021-11-18 13-17-23, SOEC2 EVs 2021-11-18 13-17-52, SOEC2 EVs 2021-11-18 13-18-22, SOEC2 EVs 2021-11-18 13-18-51, SOEC2 EVs 2021-11-18 13-19-20, SOEC2 EVs 2021-11-18 13-19-49, SOEC2 EVs 2021-11-18 13-20-19, SOEC2 EVs 2021-11-18 13-20-51, Limited file list. Full list printed on Page 2.		Stats: Merged Data Mean: 200.0 nm Mode: 139.7 nm SD: 106.0 nm D10: 122.6 nm D50: 162.3 nm D90: 350.0 nm	
<b>Details</b> NTA Version: NTA 3.4 Build 3.4.4 Script Used: SOP Standard Measurement 01-15-15PM 18- Time Captured: 13:15:15 18/11/2021 Operator: Bathan Pre-treatment: Sample Name: SOEC2 EVs Diluent: 10 Remarks:		Stats: Mean +/- Standard Error Mean: <u>200.4 +/- 4.0 nm</u> Mode: 136.1 +/- 3.9 nm SD: 103.0 +/- 5.2 nm D10: 124.5 +/- 1.8 nm D50: 162.6 +/- 2.7 nm D90: 337.5 +/- 15.5 nm Concentration (Upgrade): <u>3.46e+09 +/- 1.14e+08 particles/ml</u> 50.3 +/- 1.6 particles/frame 55.5 +/- 1.4 centres/frame Concentration measurements may require some caution due to noise See summary file for more info	
<b>Capture Settings</b> Camera Type: sCMOS Laser Type: Blue488 Camera Level: 14 Slider Shutter: 1259 Slider Gain: 366 FPS: 25.0 Number of Frames: 499 Temperature: 23.3 °C Viscosity: (Water) 0.922 - 0.924 cP Dilution factor: 1 x 10e1			
<b>Analysis Settings</b> Detect Threshold: 4 Blur Size: Auto Max Jump Distance: Auto: 9.7 - 11.0 pix			

Figure 4.3: NTA analysis result of the SOEC-EVs concentration and mean size of single EV obtained by flushing method.

# NANOSIGHT

## SOEC EVs 2021-11-18 12-56-44



Included Files	Results
SOEC EVs 2021-11-18 12-56-57, SOEC EVs 2021-11-18 12-57-24, SOEC EVs 2021-11-18 12-57-51, SOEC EVs 2021-11-18 12-58-18, SOEC EVs 2021-11-18 12-58-45, SOEC EVs 2021-11-18 12-59-12, SOEC EVs 2021-11-18 12-59-39, SOEC EVs 2021-11-18 13-00-06, SOEC EVs 2021-11-18 13-00-33, SOEC EVs 2021-11-18 13-01-00, SOEC EVs 2021-11-18 13-01-27, SOEC EVs 2021-11-18 13-01-54, SOEC EVs Limited file list. Full list printed on Page 2.	Stat: Merged Data Mean: 184.0 nm Mode: 128.1 nm SD: 80.9 nm D10: 114.9 nm D50: 161.4 nm D90: 281.4 nm
<b>Details</b> NTA Version: NTA 3.4 Build 3.4.4 Script Used: SOP Standard Measurement 12-56-44PM 18- Time Captured: 12:56:44 18/11/2021 Operator: Bahuan Pre-treatment: Sample Name: SOEC EVs Diluent: 10 Remarks:	Stat: Mean $\pm$ Standard Error Mean: <u>184.2 <math>\pm</math> 1.7 nm</u> Mode: 133.9 $\pm$ 4.8 nm SD: 80.0 $\pm$ 2.6 nm D10: 115.9 $\pm$ 1.4 nm D50: 161.1 $\pm$ 1.8 nm D90: 288.8 $\pm$ 8.0 nm Concentration (Upgrade): <u>9.80e+09 <math>\pm</math> 2.11e+08 particles/ml</u> 120.5 $\pm$ 2.4 particles/frame 122.5 $\pm$ 2.0 centres/frame
<b>Capture Settings</b> Camera Type: sCMOS Laser Type: Blue488 Camera Level: 14 Slider Shutter: 1259 Slider Gain: 366 FPS: 25.0 Number of Frames: 499 Temperature: 22.6 - 22.7 °C Viscosity: (Water) 0.937 - 0.940 cP Dilution factor: 1 x 10e1	
<b>Analysis Settings</b> Detect Threshold: 8 Blur Size: Auto Max Jump Distance: Auto: 10.3 - 11.1 pix	

Figure 4.4: NTA analysis result of the SOEC-EVs concentration and mean size of single EV obtained by scraping method.

### 4.3. ASSESSMENT OF THE EMBRYO QUALITY

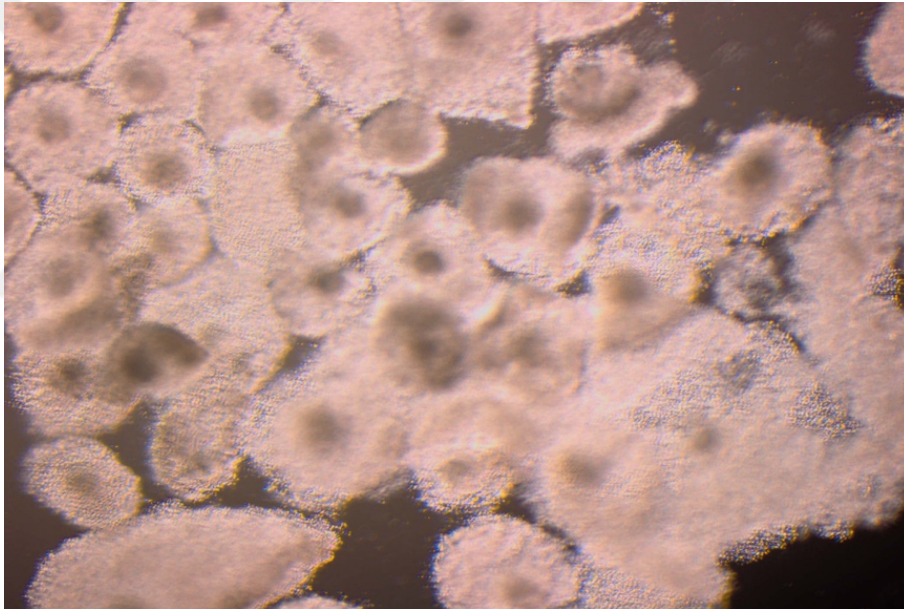
#### 4.3.1. Embryo development

Cleavage rates were registered on Day 2, and blastocyst yield on Day 8 after in vitro fertilization (Table 4.1).

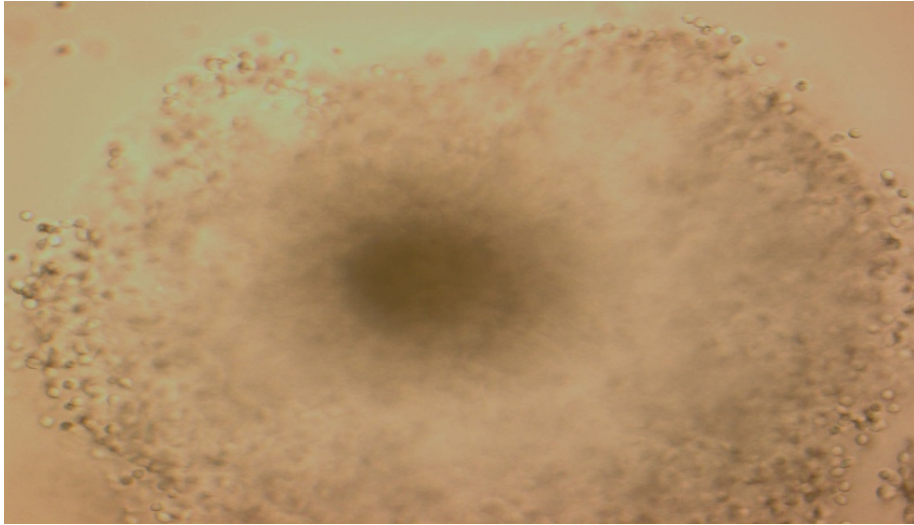
**Table 4.1:** Influence of SOEC EVs on embryo development in vitro.

Group	Dose of EVs (N.of EVs)	N. of presumptive embryos	N. of cleaved embryos (%)*	N. of blastocysts (%)**
Control	0	250	171 (68.40)	40 (23.39)
Low EV	1 $\mu$ L ( $9.8 \times 10^6$ )	249	161 (64.66)	54 (33.54)
Medium EV	5 $\mu$ L ( $49.0 \times 10^6$ )	250	165 (66.00)	48 (29.09)
High EV	10 $\mu$ L ( $98.0 \times 10^6$ )	257	153 (59.53)	41 (26.80)

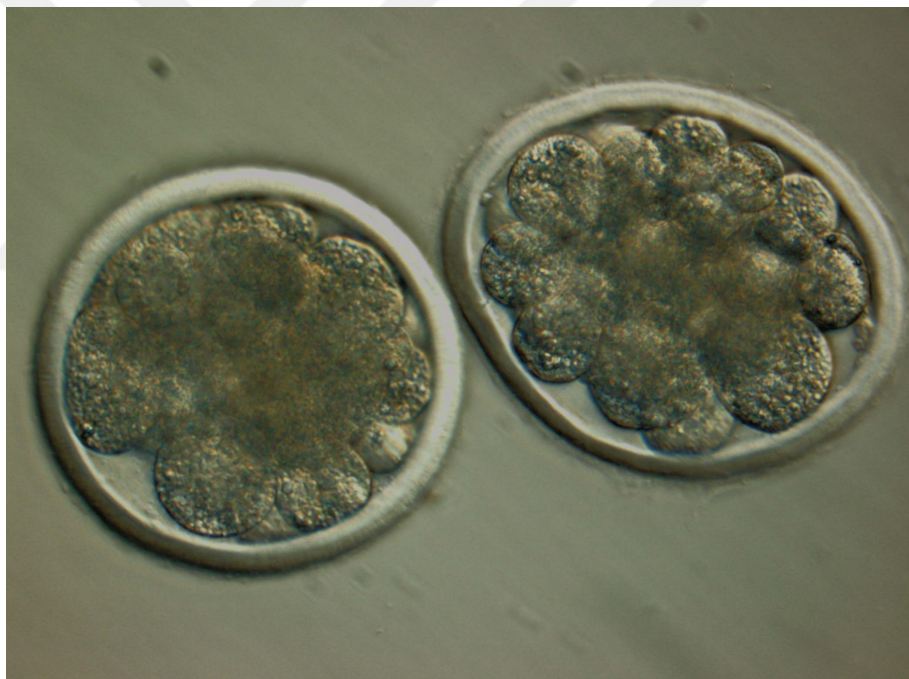
\*, \*\*: Differences between groups are the same statistically ( $p > 0.05$ ).



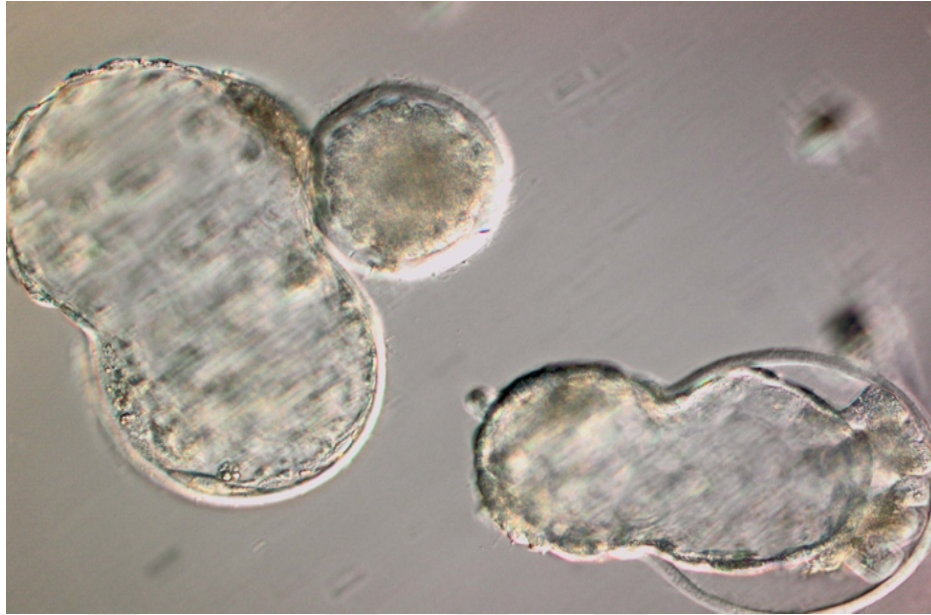
**Figure 4.5:** Cumulus expanded oocytes.



**Figure 4.6:** Cumulus expansion of an oocyte after maturation.



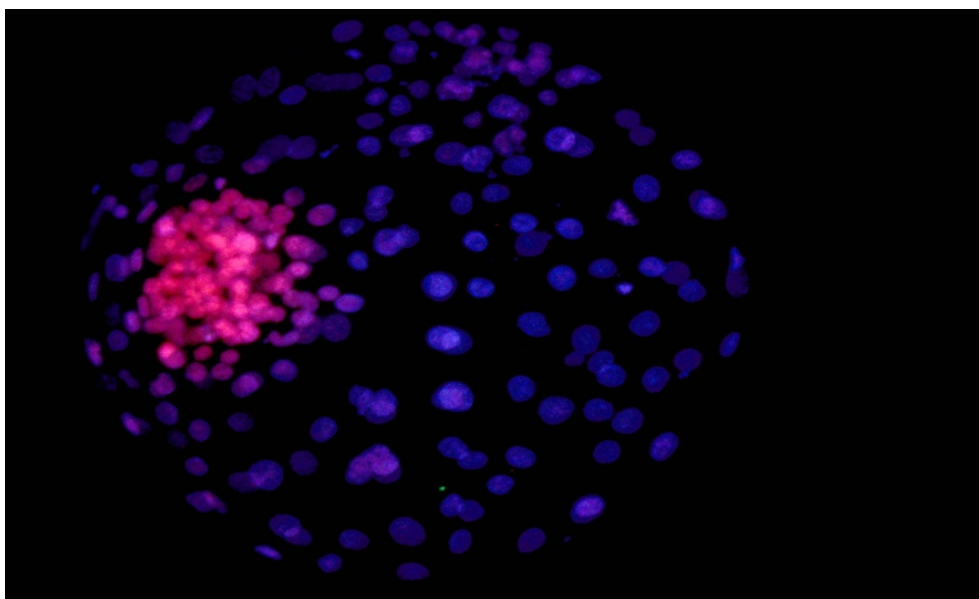
**Figure 4.7:** Cleavage stage embryos after in vitro culture.



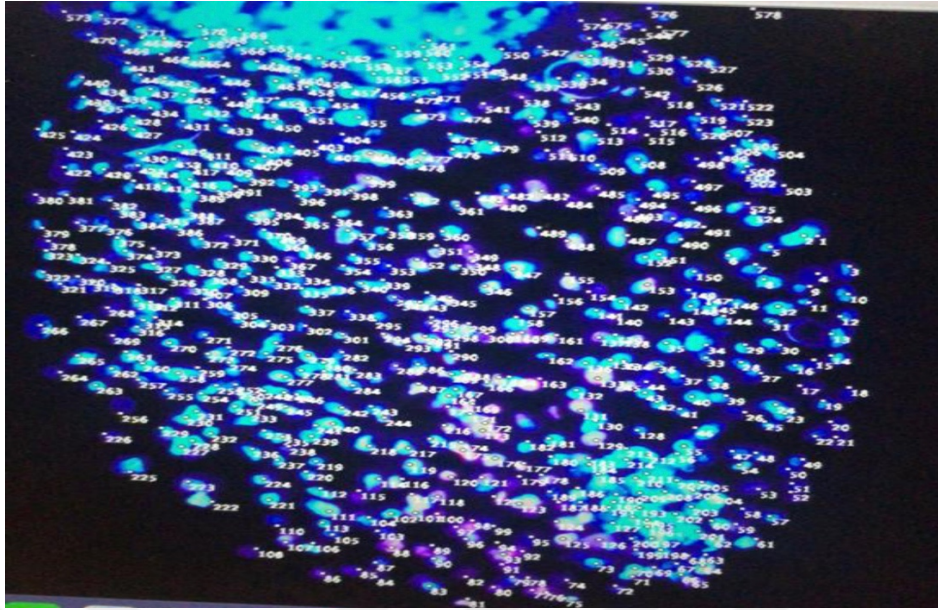
**Figure 4.8:** Hatching blastocysts after in vitro culture.

#### 4.3.2. Differential Staining of Blastocysts

Blastocysts on Day 8 in in vitro culture were used for differential staining collected from all groups, a control, low EV, medium EV, and high EV. In terms of total cell number, blastocysts grown in the presence of SOEC-derived EVs had a significantly higher total number, particularly low EV 298.25, medium EV 225.33 and high EV 279.38, and the control group had the lowest cell number of 142.75 (Table 4.2). The differences between the control group and EV present groups were important statistically ( $p < 0.05$ ).



**Figure 4.9:** A blastocyst after differential staining. Inner cell mass (red), trophoblast cells (blue).



**Figure 4.10:** Counting blastocyst cell number.

**Table 4.2:** Effect of SOEC-EVs on the blastocysts total cell number.

Groups	Dose of EVs (No. of EVs)	No. of Blastocysts	Mean of Total Cell Count $\pm$ SEM
Control Group	0	8	142.75 $\pm$ 11.93 <sup>c</sup>
Low EV	1 $\mu$ L (9.8 $\times$ 10 <sup>6</sup> )	9	298.25 $\pm$ 36.11 <sup>a</sup>
Medium EV	5 $\mu$ L (49.0 $\times$ 10 <sup>6</sup> )	8	225.33 $\pm$ 15.38 <sup>b</sup>
High EV	10 $\mu$ L (98.0 $\times$ 10 <sup>6</sup> )	8	279.38 $\pm$ 15.32 <sup>ab</sup>

a,b,c: Different groups with different letters in a column are important statistically ( $p < 0.05$ ).

## 5. DISCUSSION

This study illustrates that *in vitro* cultured sheep oviduct epithelial cells can release EVs into the environment and play a significant role in enhancing embryo development. Only a few investigations have studied the influences of oEVs on early embryo growth *in vitro*.

We designed an effective strategy to enhance the quality of the production of sheep embryos *in vitro* and demonstrated that the EVs isolated from the conditioned medium of culture SOEC-EVs improve embryo quality *in vitro* culture.

Firstly, we accomplished two different methodologies to achieve high reproducibility of a SOEC line from oviduct epithelial cells. The flushing method was collected by squeezing each oviduct and washing it with media. The scraping method was carried out by opening each oviduct longitudinally and then scratching the mucosa to collect the epithelial cells. The obtained cells were seeded, and after they reached 90% confluency, the present media was substituted with DMEM cell culture media supplemented with 10% FCS-depleted EVs. We used the FCS-depleted EVs to ensure that only cultured oviduct epithelial cells released the isolated EVs.

The cultured sheep oviduct epithelial cells (SOEC) conditioned media (CM) were collected to isolate the oEVs. The ultracentrifugation technique was used to isolate EVs from two samples, and the starting volumes were 27 mL in the scraping method and 10 mL in the flushing method. The isolated EVs characterized by Nano sight in both methods, the diameter and concentration of the flushing method were 200-350 nm,  $3.46 \times 10^9$  EVs/mL, and in the scraping method, their diameter and concentration were 184-281 nm,  $9.8 \times 10^9$  EVs/mL. In comparing the processes, the flushing method produced a low EV concentration.

Only, Lopera-Vasquez et al. [49] have confirmed the presence of EVs in ruminant oviductal fluid, both from *in vivo* and cultured epithelial cell lines conditioned medium, especially in bovines. Their isolated EVs were characterized by Nano sight as 150-200 nm, and the concentration was  $3 \times 10^5$  EVs/mL from 10 mL of BOEC-CM. Their produced volume was 10 mL ( $3 \times 10^5$  EVs/mL), similar to our flushing method conditioned media, whereas the concentration was significantly high in our medium ( $3.46 \times 10^9$  EVs/mL).

To date, just one study confirmed the presence of EVs in the sheep reproductive tract achieved by Burns et al. [16]. They isolated the exosomes and microvesicles from the uterine fluids of pregnant and cyclic ewes. They showed that those EVs carry specific proteins, miRNA, and mRNA.

Lopera-Vasquez et al. [49] confirmed that BOEC-derived EVs positively affect the quality of embryos produced in vitro. All these studies showed proof of interaction between the embryo and the reproductive tract through EVs during the pre-implantation period.

Co-condition media have been used to improve embryo development before the EVs. However, the co-condition medium can contain undesirable elements, which may have harmful effects on molecules, such as cell debris, metabolites, and apoptotic cells. Through EVs isolation, these unwanted molecules are removed and purified, so it seems healthier for the embryo to develop.

Lopera-Vasquez et al. [49] documented the investigation results that compared applying of co-culture and EVs on embryo culture. Their data showed the effects of co-culture and EVs on cleavage rate (89.4% vs 89.9%, respectively) and blastocyst rate (33.5% vs 43.4%, respectively).

We applied SOEC-EVs in various concentrations: Low EV group was 1 $\mu$ L ( $9.8 \times 10^6$ ), the medium EV 5 $\mu$ L ( $4.9 \times 10^6$ ), and high EV 10 $\mu$ L ( $98.0 \times 10^6$ ) to in vitro embryo culture media to investigate the effect on the cleavage and blastocyst rates and embryo quality (total number of embryonic cells). The results demonstrated that SOEC-EVs significantly affected in vitro cultured embryos compared to the control group. The control group had a reasonable cleavage rate of 69.78%; however, the high EV group was 59.05%, and the medium EV was 68.00% which was more acceptable than other groups. In terms of blastocyst rate, the low EV group produced an effective rate of 32.19% compared to other groups, particularly the control group, which was 21.02%. Similarly, the blastocyst total cell numbers were used to evaluate embryo development quality. In terms of total cell number, Day 8 blastocysts grown in the presence of SOEC-derived EVs had a significantly higher total cell (trophoblast + ICM) number ( $p < 0.05$ ). The low EVs group revealed significant total cell numbers of 298.25 cells, the high EV group 279.38 cells, and the medium EV group 225.33 cells. However, the control group demonstrated statistically low total cell numbers of 142.75 cells ( $p < 0.05$ ).

Oviduct epithelial-derived EVs have been shown to improve fertilization and embryo growth and reduce the incidence of polyspermy. Molecular cargo such as miRNAs, mRNAs, proteins, and lipids harbored by oEC-EVs can be shared with recipient cells.

The oviduct-derived extracellular vesicles positively affect embryos in different species, such as rodents, domestic and farm animals, reptiles, and humans. The conducted studies on oviduct epithelial cell secretion, such as EVs effects in vivo and in vitro (oEC), are few.

A few studies have recorded oEVs in some species, such as canine [44] and bovine [49], [6]. Our research was the first to investigate and identify ovine oEVs, particularly OC-EVs derived from sheep oviductal epithelial cells cultured in vitro.

Our results demonstrated that the cleavage rates were significantly high, 59.53-68.40%, when compared to other research, Öztürkler et al. [66] and Gardner et al. [30] had cleavage rates of 21.9-29.9% and 45%, respectively. Our studies documented a blastocyst rate of 23.4-33.5%, which is higher than other research, such as Öztürkler et al.[66] registered a blastocyst rate of 0.6-4.3%.

In terms of blastocyst total cell numbers, Gardner et al.[30] revealed about 123 cells, and our studies demonstrated blastocysts' mean of total cell count  $142.75 \pm 11.93$ - $298.25 \pm 36.11$ .

According to the results, we verified that SOEC-derived EVs, when supplemented with the embryo culture medium, positively affect cleavage, blastocyst rate, and total cell number.

The usage of EVs in in vitro embryo culture positively affects embryo quality, blastocyst rate, and total cell numbers. The suitable dose between the groups; a low EV dosage seemed to be an appropriate dose compared to others. EVs generally dissolve in PBS while isolated. We suggested that the PBS negatively affected embryos on their first days of incubation, which resulted in reducing the productivity of the effects of SOEC-derived EVs when applied in vitro culture medium was represented as 2% in low EV, 10% in medium EV, and high EV group 20% of the droplet volume (50  $\mu$ L). However, when the embryos adapted to the PBS, its effects disappeared, and the blastocyst rate increased either was no significant effect ( $p > 0.05$ ). The suggestions were verified by comparing the Lopera-Vasquez et al. [49] results were cleavage rate in the control group 88.8%, EVs group 89.6% (100% dilution EV), 88,6 (50% dilution EV), 86.2% (25% dilution of EV), and the blastocyst rate (day 8) in the control group 37.8%, EVs 41.2%, 43.4% and 41,2%, respectively.

We used SOEC-derived EVs to imitate the extracellular vesicle relationships between oviduct epithelial cells and embryos during the early development stages; the consequences verified that using SOEC-derived EVs in culture impacted the quality of in vitro-produced sheep embryos positively in terms of blastocyst rate and total cell numbers. The current research is the first study in which sheep oviduct epithelial cells were cultured, and from their

conditioned medium, EVs were isolated, characterized, and applied successfully to support embryo development and the quality of in vitro culture.

In the recent studies, the authors identified proteins (n=319) in EV cargos, from which 175 were in common, 97 were expressed in vivo and 47 were only in vitro [3,4,6]. Alminana et al. [7] identified the first oviduct EV proteins and compared them in vivo and in vitro.

Alminana et al. [4,5] analyzed the very first time of the molecular status of oEVs during the estrous cycle in bovine, demonstrating significant differences between post- and pre-ovulatory phases. Their results contribute to a sufficient knowledge of the role of oviductal EVs as a regulator of relations between gametes and/or embryos-female reproductive tract and the implications of such relationship for successful reproduction.

Lee and Saadeldin [44] characterized and examined OC-EVs' protein contents to determine their contribution to canine oocyte maturation. Lee et al. [45] verified that OC-EVs could benefit canine oocyte maturation and embryo development. Several analyses have been documented on the cargo of oEVs in some species; however, there needs to be more information on OC-EVs in other species.

This research accomplished the first definition of sheep epithelial cells derived-EVs, demonstrating the impact of SOEC-derived EVs as modulators of the embryo-oviduct relations. Moreover, our results indicate them as means to improve embryo development and quality under in vitro conditions.

## 6. CONCLUSION AND RECOMMENDATIONS

### 6.1. CONCLUSION

This study is the first research in which sheep oviductal epithelial cells have been cultured from a conditioned medium EVs isolated, characterized, and successfully applied in vitro embryo culture to improve the development quality. Our research provides the first definition of sheep epithelial cells derived- EVs, demonstrating that SOEC-derived EVs interact as modulators between the embryo–oviduct relations. Our results demonstrate the SOEC-EVs as means to improve embryo development and quality under in vitro conditions.

As a consequence, testing to mimic the extracellular associations between oviductal tissue and embryos, we demonstrate that the extracellular vesicles obtained from the SOEC culture media improve the embryo quality.

### 6.2. RECOMMENDATIONS

- The water used to prepare buffers and media must be at room temperature.
- The media must be preserved for a week at 4°C after preparation.
- Obtaining SOEC cell culture while scraping the mucosa, the epithelial cells can be contaminated with red blood cells. The red cell lysis buffer must be used to confirm they have not contaminated the cells' aliquot.
- To quit contamination, the organs must be washed with prewarmed physiological saline supplemented with antibiotics. Then moved to a beaker and soaked in warmed saline.
- Removing tissues around the oviduct could be done in the cabinet or beside the burner to reduce contamination.
- Before starting centrifuge steps, particularly with conditioned medium obtained by the flushing method must be filtered with 0.45 µm to remove the floating cells that may contaminate the EVs with intracellular vesicles of the bursting cells.
- The isolated EVs must be preserved at -20°C until to be used.
- Oocytes with evenly granulated cytoplasm and minimum 4 layers of cumulus cells must be selected for further maturation.

- Oocytes must be washed 2-3 times in oocyte washing medium before being transferred to IVM medium.
- After maturation, oocytes must be washed 2-3 times in HSOF medium before being transferred to the IVF medium.



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